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Attachment of the outer membrane lipoprotein (OprI) of *Pseudomonas aeruginosa* to the mucosal surfaces of the respiratory and digestive tract of chickens

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Summary The development of mucosal vaccines requires antigen delivery and adjuvant systems that can efficiently help in presenting vaccine antigens to the mucosal immune system. The outer membrane lipoprotein I (OprI) of *Pseudomonas aeruginosa* seems to possess both the quality to induce a non-specific immune response (adjuvant effect through its lipid tail) as well as the quality to facilitate uptake of the vaccine antigen by interacting with Toll-like receptor 2/4 (TLR2/4) on antigen-presenting cells (APC) and epithelial cells (adhesion effect). Here, we show for the first time the adhesion of OprI to epithelial cells of the trachea and small intestine of chickens. Adhesion could be seen on cryosections after *in vitro* as well as after *in vivo* incubation of the trachea and intestine. This proves the value of OprI as a fusion partner in mucosal protein vaccine development, which is especially important for poultry where mass vaccination is only possible by the respiratory or oral route.

Introduction

Vaccination continues to be one of the most important management tools for controlling infectious diseases in livestock. However, conventional vaccine delivery technologies are frequently based on (intramuscular) injection. While this parenteral route of immunization has proven to be effective, it often lacks stimulation of local immunity at the site where most pathogens enter the body: the mucosal surface. The most effective way to induce mucosal immunity is to administer a vaccine directly to the mucosa [1,2]. In addition to this local immunity, mucosal vaccination offers several other advantages such as needle-free administration, reduced side

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effects, the potential for unlimited boosting without the need of trained personnel and the possibility of mass vaccination [3]. Nevertheless, under normal circumstances, soluble proteins which are not associated with replicating microorganisms do not provoke strong immune responses [4,5]. Therefore a way to enhance the potency of mucosal protein vaccines should be further investigated. Several factors are known to increase the immunogenicity of mucosal antigens. First, antigens encapsulated in polymers, or liposomes, are more immunogenic as they can reach the MALT in higher concentrations [6]. Secondly, the immunogenicity of protein vaccines can be enhanced by increasing the adhesive capacity at the site of the mucosa (by a non-particulated or receptor-specific mucosal adjuvant) [7,8]. Finally, the success of a vaccine is dependant on its ability to activate the innate immune system. The presence of an adjuvant in a vaccine can greatly increase the innate immune response to the vaccine antigen by augmenting the activities of dendritic cells, lymphocytes and macrophages [8].

The innate immune system recognizes certain bacterial components, namely, pathogen associated molecular patterns (PAMPs). As a consequence immunocompetent cells are activated, which leads to an increased expression of co-stimulating molecules and cytokines [6,9,10]. Therefore, the incorporation of such a bacterial component in a mucosal vaccine could improve the immune response considerably. In addition, the recognition and presentation of mucosal antigens can be further improved by conferring them the ability to bind to immunocompetent cells in the mucosa-associated lymphoid tissue (MALT) [7,11].

OprI, the outer membrane lipoprotein I of *Pseudomonas aeruginosa*, has proven to be a good carrier molecule in vaccination studies in mice, pigs and humans. The lipid tail elicits immune responses because of its attachment to TLR2/4 on host immune cells [12]. The results of the vaccination experiments reveal that OprI acts as an immunomodulatory element to induce a long-term cellular and humoral immune response [13—15]. To assess the potential of OprI fusion constructs for mucosal vaccination of poultry, the attachment of OprI to the mucosa of the respiratory and intestinal tract of chickens was examined.

Material and methods

Bacterial strains and plasmids

JM109 *E. coli* cells [e14-(McrA-) recA1 endA1 gyrA96 thi-1 hsdR17(rK-mK+) supE44 relA1 D(lac-proAB) [F' traD36 proAB lacI^{q}ZDM15]] (Stratagene) were transformed with the vector pVUB3, containing the outer membrane lipoprotein (OprI) of *Pseudomonas aeruginosa* (VUB-VIB, Brussels, Belgium) [16].

Expression and purification of OprI

Starter cultures of JM109 transformed with pVUB3, in LB broth containing ampicillin were incubated at 37 °C at 250 rpm until turbid after which they were stored at 4 °C. The following day, 300 ml Luria Bertani (LB) medium containing 100 μg ampicillin/ml and 1% glucose were inoculated with the starter culture (1/20) and incubated at 37 °C while shaking at 250 rpm to an OD_{600} of 0.6. Expression was induced by adding isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 1 mM. After 4 h incubation at 30 °C, cells were harvested by centrifugation at 6000 rpm for 15 min and the bacterial pellet was stored at −20 °C until further use. As a negative control, the same protocol was performed with non-transformed JM109 *E. coli* cells.

To purify the OprI containing fraction, the outer membrane proteins were isolated. Briefly, the pellet from 1 l of culture was resuspended in 25 ml GTE buffer (50 mM glucose, 25 mM Tris—HCl pH 8.0 and 10 mM EDTA) into which lysozyme (Sigma) was added to a concentration of 4 mg/ml. The resuspended cells were incubated on ice for 30 min and 25 ml of 2% *N*-lauroylsarcosine (Promega) and 1 ml of 2 mM protease inhibitor cocktail mix VII (Sigma) were added. Incubation was continued at 4 °C while sonicating until the solution was clear and lost its viscosity. The outer membrane proteins were pelleted at 28.000 rpm for 2 h at 4 °C. The pellet was resuspended overnight at 4 °C in 2 ml buffer (100 mM NaCl, 0.2% SDS and 20 mM Tris—HCl pH 8.0). The resuspended outer membrane (OM) fraction was stored at 4 °C until further use. Before use, the fraction was centrifuged for 10 min at 6000 rpm to remove any precipitates. The supernatants were analyzed on 12.5% SDS-PAGE gels and immunoblotted using the anti-OprI monoclonal antibody (MAb) QB2 (VUB-VIB) [17].

Attachment of OprI to cryosections of the trachea and small intestine of chickens

Cryosections of the trachea and small intestine (frozen in Methocel, 2 μm, fixed with acetone and stored at −80 °C) were placed at room temperature to defrost. To block non-specific binding, the sections were incubated with 5% BSA in PBS at 37 °C for 1 h, followed by 2 × 5 min washing in PBS. The sections were then incubated with 100 μl of OM fraction (500 μg/ml) for 30 min at 37 °C and washed 3 times with PBS. Thereafter, the sections were incubated with the primary antibody QB2 (1:100 in blocking buffer) for 30 min at 37 °C, washed 3 times for 5 min in PBS and incubated with FITC-labeled rabbit anti-mouse IgG (DAKO Diagnostics, 1:30 in blocking buffer) for 30 min at 37 °C. After washing, the sections were counterstained for 10 min at RT with 0.03% methyl green (Sigma). After mounting with 50% glycerol in PBS, the tissue samples were examined for fluorescence by microscopy.

In vitro attachment of OprI to the trachea and intestine of chickens

One centimeter fragments of trachea and small intestine were removed and placed in a Petri dish with PBS + 1% penicillin/streptomycin (Invitrogen) to rinse the lumen. Next, the fragments were placed in a sterile tube with 1 ml TOC medium (MEM + 1% penicillin/streptomycin/glutamine + 2% HEPES buffer) and incubated at 37 °C while shaking at 250 rpm until further use. To inoculate the fragments, the medium was removed, one end of the trachea/intestine was closed and incubated with 100 μl of OM fraction (500 μg/ml) for 30 min, after which the trachea/intestine was rinsed with sterile PBS. Next, the fragment was submerged in Methocel (2%, Sigma), snap-frozen and stored at −80 °C until fur-