

Research article

## THE IMMUNOGENICITY OF THE LIPOSOME-ASSOCIATED OUTER MEMBRANE PROTEINS (OMPs) OF *Moraxella catarrhalis*

DARIA AUGUSTYNIAK<sup>1\*</sup>, JÓZEF MLECZKO<sup>1</sup> and JAN GUTOWICZ<sup>2</sup>

<sup>1</sup>Laboratory of Immunology, <sup>2</sup>Department of Physico-Chemistry of Microorganisms, Institute of Genetics and Microbiology, University of Wrocław, Przybyszewskiego 63/77, 51-148 Wrocław, Poland

**Abstract:** The outer membrane proteins (OMPs) are the most immunogenic and attractive of the *Moraxella catarrhalis* vaccine antigens that may induce the protective immune response. The aim of this study was to determine the effectiveness of two types of OMP-associated phosphatidylcholine (PC) liposomal formulations (OMPs-PC, PC-OMPs) and of Zwittergent-based proteomicelles (OMPs-Z) in potentiating an anti-OMP systemic immune response in mice. The immunogenicities of the above preparations were evaluated by assessing serum anti-OMP IgG and IgA reactivity in the post-immunized mouse antisera using ELISA and Western blotting. Additionally, the cross-reactivity of the most effective anti-OMP response was determined using heterologous sera from both humans and mice. Both the proteoliposomes and the proteomicelles showed high immunogenic properties and did not elicit any distinct quantitative differences in the antibody titer or qualitative differences in the pattern of the mouse antisera. The post-immunized mouse antisera predominantly recognized a ~60-kDa OMP of *M. catarrhalis*. That protein was also found to be a highly cross-reactive antigen interacting with a panel of pooled mouse antisera produced by immunization either with whole cells or the purified OMPs of heterologous *M. catarrhalis* strains. Furthermore, normal sera

---

\* Author for correspondence. E-mail: [august@microb.uni.wroc.pl](mailto:august@microb.uni.wroc.pl), tel: (+48) 71 3756 296

Abbreviations used: AFM – atomic force microscopy; BSA – bovine serum albumin; EDTA – ethylenediaminetetracetic acid; ELISA – enzyme-linked immunosorbent assay; HRP – horseradish peroxidase; LOS – lipooligosaccharide; OMPs – outer membrane proteins; OMPs-PC and PC-OMPs – the studied proteoliposomes; OMPs-Z – Zwittergent-based micelles of OMPs; OPD – *o*-phenylenediamine; PBS – phosphate buffered saline; PC – phosphatidylcholine; TPBS – PBS supplemented with Tween 20

collected from healthy children were found to be preferentially reactive with the 60-kDa OMP. The serum-specific IgG, IgA and IgM were respectively detected via immunoblotting in 90%, 85% and 30% of heterologous human sera. This similar immunogenic effectiveness of both OMP-associated liposomal formulations could contribute to the practical use of such formulations in the future in human vaccination. Moreover, the highly cross-reactive 60-kDa OMP seems to be an important antigenic marker of *M. catarrhalis*, and, as it is responsible for the induction of an antibody-mediated and long-lasting immune response, studying it may partially aid us in understanding the relatively low degree of pathogenicity of the bacterium in immunocompetent individuals.

**Key words:** *Moraxella catarrhalis*, Outer membrane proteins, Proteoliposomes, Proteomicelles, Anti-OMP antibodies, Cross-reactivity, Zwittergent

## INTRODUCTION

The development of an effective vaccine against *Moraxella catarrhalis* infection is of particular importance, as this bacterium is a significant human respiratory tract pathogen. *M. catarrhalis* is the third most common etiological factor (after *S. pneumoniae* and non-typeable *H. influenzae*) of *otitis media* in children, accounting for 15-20% of cases [1]. It is also the major bacterial pathogen responsible for acute exacerbations in patients with chronic obstructive pulmonary disease [2-4]. Furthermore, it frequently causes sinusitis, and occasionally pneumonia and rarely endocarditis or meningitis [5]. It was reported that the number of antibiotic-resistant strains of *M. catarrhalis* had increased significantly over the past few decades [6]. *M. catarrhalis* colonization is an active process in children and adults. It is accompanied by a steady turnover of microorganisms in the nasopharynx, with the elimination of one strain followed by colonization with another [1, 7-9]. *M. catarrhalis* colonization is more frequent than can be determined by surface culture, because the organism resides both within and beneath the epithelium in human pharyngeal lymphoid tissue, and it invades host cells [10]. The molecular pathogenesis of *M. catarrhalis* infection is not currently fully understood, although there is an increasingly large body of knowledge on which antigens confer protective immunity against *M. catarrhalis* infection. Importantly, there is no vaccine to prevent *M. catarrhalis* infection [11]. Peptide-based vaccine candidates against *M. catarrhalis* are currently under investigation.

With its lack of capsular polysaccharides and the rather weak immunogenicity of its lipooligosaccharide (LOS), the most immunogenic structures of *M. catarrhalis* participating in bacterial virulence and conferring protective immunity are its outer membrane proteins (OMPs) [1]. Being highly conservative and exposed on the surface of the bacterial cell, they are important targets for the host immune response. Moreover, some of them showed cross-reactivity with common antigenic determinants of *Neisseria meningitidis* or *Candida albicans*, suggesting that colonization by *M. catarrhalis* strains could

contribute to the acquisition of an effective natural immunization against meningococci or yeast-like fungi [12, 13].

Two of the major issues in the development of safe and effective vaccines derived from outer membrane antigens are the presence of toxic LOS in vaccines based on membrane vesicles, and the poor immunogenicity of the isolated and purified protein antigens. However, the immunogenic properties of such membrane antigens are very often influenced by the adjuvanticity of LOS. The strong adjuvanticity of LOS in *N. meningitidis* was evidenced by the greatly reduced immunogenicity of the outer membrane components or whole cells of meningococcal LOS-deficient mutants [14]. By contrast, the rather weak adjuvantic properties of LOS were observed in *M. catarrhalis* based on the similar bactericidal activity of antibodies elicited against both a *M. catarrhalis* mutant that completely lacked the LOS structure and its parental strain [10]. Additionally, although the bacterial endotoxin is an undesirable component of a potential vaccine, this cellular component, together with the phospholipids of the inner leaflet of the outer membrane, enable membrane proteins to be in the relevant lipid moiety and to maintain a native or native-like conformation [15, 16]. Purified or recombinant bacterial outer membrane proteins can be refolded to their native conformation by incorporation into micelles using a suitable detergent [17] or by incorporation into artificial liposomal membranes [18, 19]. For many years, liposomes have been used as models in vaccine studies to study lipid membranes that provide a native-like environment for membrane proteins. Another important application of liposomes is their immunoadjuvant potency, which confers an effective ability to recognize liposomal antigens on antigen-presenting cells, which in turn triggers both a humoral and cell-mediated immune response [20-22]. They have numerous merits, such as their biocompatibility, biodegradability, lack of toxicity and formulation facility, and their alteration of the pharmacokinetic and pharmacodynamic properties of encapsulated material (reduced elimination, increased circulation life time) [23-25]. This study aimed to determine the immunogenic properties of *Moraxella catarrhalis* OMPs incorporated into phosphatidylcholine liposomes, and to compare the cross-reactivity of the most effective anti-OMP response in the human and mouse systems *in vitro*.

## MATERIALS AND METHODS

### Materials

Soya bean L- $\alpha$ -phosphatidylcholine and 4-chloro-1-naphthol were obtained from Sigma-Aldrich (Germany), and Zwittergent 3-14 detergent and *o*-Phenylenediamine dihydrochloride tablets (OPD) were from Calbiochem (Germany). The conjugated antibodies were purchased as follows: rabbit anti-human IgG, IgA or IgM conjugated to horseradish peroxidase (HRP) from DAKO (Denmark); and goat HRP-conjugated, isotype-specific anti-mouse immunoglobulins from Sigma-Aldrich (Germany). In some experiments,

HRP-conjugated affinity pure goat anti-mouse IgG Fc $\gamma$  antibodies (Jackson Immunoresearch) were used. The other reagents and suppliers were: BHI (brain/heart infusion broth; from BIOCORP); BSA (from SERVA); 96-well MaxiSorp microtiter plates (from NUNC); Immobilon P (from Sigma); and Sephadex<sup>®</sup> G-300 (from Sigma-Aldrich).

#### **Bacterial strains and growth conditions**

The clinical isolates of *Moraxella catarrhalis* designated Mc1, Mc5, Mc6, Mc8 were taken from our collection. These strains had been isolated from the upper respiratory tract (oropharynx) and were obtained from Laboratory of Bacteriology of the Silesian Centre of Pediatrics in Wrocław, Poland. The final identification was made using a standardized commercial identification system API-NH (bioMerieux, France). The bacteria were cultured on Blood Agar (OXOID) containing 5% sterile sheep blood, or grown in brain/heart infusion broth (BHI) at 37°C with 5% CO<sub>2</sub>. The strains were stored in BHI containing 16% glycerol at -70°C.

#### **Isolation of *Moraxella catarrhalis* outer membrane proteins (OMPs)**

*Moraxella catarrhalis* was routinely cultured at 37°C BHI until it reached its stationary phase. The outer membrane proteins of *M. catarrhalis* were obtained using the zwitterionic detergent Zwittergent 3-14 according to a slightly modified version of the method described in [24]. Briefly, the harvested bacteria from 200 ml of culture were suspended in 5 ml of 1M sodium acetate buffer containing 1 mM  $\beta$ -mercaptoethanol, pH 4.0). To this suspension, a 45-ml volume of a solution of 0.5 M CaCl<sub>2</sub> containing 5% Zwittergent was added and stirred for 1 h at room temperature. The nucleic acids were precipitated by adding 12.5 ml of cold absolute ethanol and subsequently centrifuging the solution (17,000  $\times$  g, 10 min., 4°C). The pellet was discarded and the proteins remaining in the supernatant were precipitated by adding 187 ml of cold ethanol, and collected by centrifugation (17,000  $\times$  g, 20 min., 4°C). The pellet was air dried and then resuspended in 10 ml of Z buffer (0.05% Zwittergent, 50 mM Tris, 10 mM EDTA; pH 8.0). This mixture was stirred for 1 h at room temperature and centrifuged at 12,000  $\times$  g for 10 min. at 4°C, and the soluble fraction containing OMPs was retained. The OMPs were divided into aliquots and stored at -70°C. The OMP concentration was determined using Bradford reagent (Sigma) or the BCA protein assay kit (Pierce). The appropriate protein profile was confirmed after SDS-PAGE and gel staining with Coomassie (GelCode blue stain reagent; Pierce). Thus-prepared OMPs in Z buffer at an initial protein concentration of 0.5 mg/ml were used in formulations with liposomes.

#### **Preparation of the OMP-associated liposomes**

In this study, we used one Zwittergent-based micelle preparation of OMPs (OMPs-Z) and two types of OMP-associated liposome formulations, with

proteins associated with the preformed lipid vesicles (PC-OMPs) or incorporated into the lipid bilayer during the vesiculation process (OMPs-PC). The liposomes were prepared as follows. Initially, L- $\alpha$ -phosphatidylcholine was dissolved in chloroform in a round-bottomed flask. After the evaporation of the organic solvent under a vacuum, a thin film of dry lipids (with 50 mg of total lipids) was hydrated by adding 1 ml of PBS buffer (10 mM phosphate buffer containing 0.9% (w/v) NaCl; pH 7.4). During hydration, the liposomal suspension was vigorously vortexed at 20°C (the temperature above the phase transition of PC). To prepare the OMP-associated liposomes, a standard reconstitution at a lipid-to-protein ratio of 100 (w/w) was performed. To obtain PC-OMPs, OMPs were added to preformed liposomes, and to obtain OMPs-PC, OMPs were simultaneously mixed with lipids. Proteoliposomes were prepared by vigorous agitation for 30 min at 37°C. To obtain size-reduced and uniform proteoliposomes, the mixtures of OMPs-PC and PC-OMPs were extruded in a nine-time passage through polycarbonate filters with 400-nm pores (Nucleopore, Whatman) using a liposome minicalibrator (MARKER, Poland). To remove non-associated proteins from the OMP-coupled liposomal solution, the standard gel filtration method was used. The liposomal solution was applied directly on top of a Sephadex<sup>®</sup> G-300 gel filtration column (1 x 27 cm, Sephadex<sup>®</sup> G-300 medium) and eluted with PBS buffer containing 0.02% sodium azide at a flow rate of about 0.8 ml/min, at 4°C. The opalescent liposome fractions containing OMPs were collected, centrifuged (100,000 g, 1.5 h, 4°C) and resuspended in PBS at a protein concentration of 5 mg/ml. All of the OMPs-liposome preparations were stored in aliquots at -20°C until used. To determine which OMPs were associated with liposomes, standard 12% SDS-PAGE electrophoresis was performed. For immunization, we used the proteoliposomes in PBS buffer and the OMPs-Z in Z buffer at final protein concentrations of 100  $\mu$ g/ml.

#### **AFM measurements**

Dr Barwiński (Institute of Experimental Physics, University of Wrocław, Poland) performed the visualization of the liposomes and reconstituted liposome-protein complexes using an atomic force microscope (NanoScope E, Digital Instruments, USA). All the AFM observations were obtained with the contact mode using a V-shaped cantilever which was 200  $\mu$ m long and had a spring constant of 0.15 N/m. Glass plates, previously washed with a detergent solution, rinsed with distilled water and air dried were used as a solid support for the visualized object. Drops of the sample were placed on the surface of the glass plate and incubated for 30 min at 4°C before image recording. During visualization, the cantilever was immersed in the water solution of the sample drop. The Scanning Probe Microscopic Software (WSxM, Nanotec Electronica, Spain) was used for image processing.

**Immunization of animals**

8- to 10-week old female BALB/c mice were used for the immunization under a protocol approved by the Institutional Animal Care and Use Committee (Wrocław, No. 58/04). Mice of approximately equal weight were divided into groups of 5 to 7 animals and immunized intraperitoneally with the relevant antigen in a 0.1 ml dose per animal, three times, at two-week intervals (on days 0, 14 and 28). In the studies of OMP formulations, the immunizing antigens were: a) OMPs-PC reconstituted in PBS buffer; b) PC-OMPs reconstituted in PBS buffer; c) OMPs in Z buffer; and d) as a control, PC in PBS buffer. The OMPs in the liposomal formulations were from strain Mc1 at a concentration of 100 µg/ml (10 µg/dose). In the studies of cross-reactivity, the immunizing antigens were OMPs-Z (10 µg/dose) or heat-inactivated bacteria ( $5 \times 10^7$  cfu/dose) of strains: Mc1, Mc5, Mc6 and Mc8. The immunization schedule was identical to that above. In the two weeks after the last immunization, the mice were terminally bled through their retro-orbital sinus. The individual mouse sera were divided into aliquots, and a part of them in each group were pooled, divided and stored at -70°C until required.

**Collection of human sera**

Blood samples were obtained from 20 healthy children aged 10 to 15 years. The sera were collected, divided into aliquots and stored at -70°C until required. Two convalescent sera from children with *otitis media* caused by *M. catarrhalis* (confirmed by positive growth of *M. catarrhalis* ear exudates) were provided by Dr. Basiewicz-Worsztynowicz (Department and Clinics of Pediatrics, Immunology and Rheumatology of Developmental Age, Medical University, Wrocław, Poland). All the relevant ethical concerns (permission for the studies from the Bioethical Committee of the Medical University of Wrocław; No. 139/2000) were respected for this study, and agreement was obtained from the parents of the children enrolled.

**SDS-PAGE and electrotransfer**

Protein samples (20 µg per lane) were subjected to SDS-polyacrylamide gel electrophoresis in 12% separating gels. Fermentas SDS-PAGE molecular weight (MW) standards (10-170 kDa) were used. The separated proteins were electrotransferred to nitrocellulose-Immobilon P (Sigma) via semidry blotting at 150 mA for 2 h with a blotting buffer (0.025 M Tris, 0.192 M glycine and 20% methanol; pH 8.4). The transfer efficiency was checked using Ponceau S stain (Sigma).

**Detection of the immune response**

*Immunoblotting.* To detect human-specific antibodies, nitrocellulose strips with bounded OMPs were blocked overnight in PBS (pH 7.4) containing 1% BSA. The strips were then incubated with 4 ml of different normal human sera, diluted 1:100 (for IgG) or 1:50 (for IgA, IgM) with TPBS (0.05% Tween 20 in PBS) containing 1% BSA, for 1 h at 37°C and for 0.5 h at room temperature with

gentle shaking. The strips were rinsed four times in TPBS and incubated similarly at 37°C with HRP-conjugated rabbit anti-human IgG, IgA or IgM antibodies (DAKO) diluted 1:1,000 (for IgG) or 1:500 (for IgA and IgM) in TPBS containing 1% BSA. After four final washes in TPBS, the strips were added to a solution of freshly prepared color-producing substrate (20 mg 4-chloro-1-naphtol (Sigma), 4 ml methanol, 16 ml PBS, 20 µl 30% H<sub>2</sub>O<sub>2</sub>) for 30 min. To detect mouse antibodies, post-immunized pooled antisera produced by immunization with liposomal formulation were appropriately diluted with TPBS-BSA. Next, the strips were rinsed with TPBS and incubated with HRP-conjugated goat anti-mouse IgG, IgA or IgM antibodies (Sigma) respectively diluted 1:5,000, 1:1,000 and 1:1,000. In the cross-reaction experiments, strips with OMPs were incubated with homologous or heterologous pooled mouse antisera produced by immunization either with whole bacteria or isolated OMPs that were respectively diluted 1:100 and 1:1,000. HRP-conjugated affinity pure goat anti-mouse IgG Fcγ antibodies (Jackson Immunoresearch) were diluted 1:4,000. The remaining steps of the immunoblotting were the same as those above.

*ELISA.* Flat-bottom 96-well MaxiSorp microtiter plates (NUNC) were coated for 2 h at 37°C and overnight at 4°C with 100 µl per well of 3 µg/ml OMPs in 0.05 M carbonate/bicarbonate buffer (pH 9.6). The unoccupied sites on the plate were blocked with 300 µl of 1% BSA in PBS (pH 7.4) for 2 h at 37°C and then incubated again for 2 h at 37°C with two-fold serial dilutions of the mouse antisera in TPBS (PBS with 0.05% Tween 20) buffer containing 1% BSA (100 µl per well). After washing with TPBS, the antigen-antibody reaction was assessed using HRP-conjugated, isotype-specific anti-mouse immunoglobulins (Sigma) diluted 1:50,000 for IgG, 1:10,000 for IgA, and 1:5,000 for IgM. At that step, the plate was incubated for 2 h at 37°C. After washing, 100 µl of substrate solution containing 5 mg OPD and 20 µl H<sub>2</sub>O<sub>2</sub> in 10 ml citric buffer (pH 4.9) was added to each well, and the plate was incubated for 20 min at room temperature in the dark. The enzymatic reaction was stopped with 50 µl 1 M H<sub>2</sub>SO<sub>4</sub>. The optical density was read at 490 nm with a Dynatech 5000 photometric reader. The antibody endpoint titer was defined as the reciprocal value of the highest antiserum dilution giving an absorbance of 0.1 extrapolated from a linear portion of the serum titration curve. The results are presented as the geometric mean titre.

#### **Statistical analysis**

Analysis of variance and one-way ANOVA tests were used to check the differences between data sets. The normal distributions of the variables were determined with Shapiro-Wilk's test. Differences were considered significant at  $P \leq 0.05$ . Statistical analyses were performed using StatSoft software (STATISTICA 8).

## RESULTS

### OMP patterns

OMPs of four clinical isolates of *Moraxella catarrhalis*, designated Mc1, Mc5, Mc6 and Mc8, were prepared using a zwitterionic detergent (Zwittergent 3.14) procedure based on the previously described method, with final OMP preparations free from other cellular contaminations [26]. The OMP patterns of the *M. catarrhalis* strains obtained by SDS-PAGE and stained with coomassie revealed several major bands with molecular masses of approximately 90, 84, 60, 50, 43, 39, 28, 27, 25 and 17 kDa; they were all described previously [26]. A proteinogram of the isolated OMPs is presented in Fig. 1.

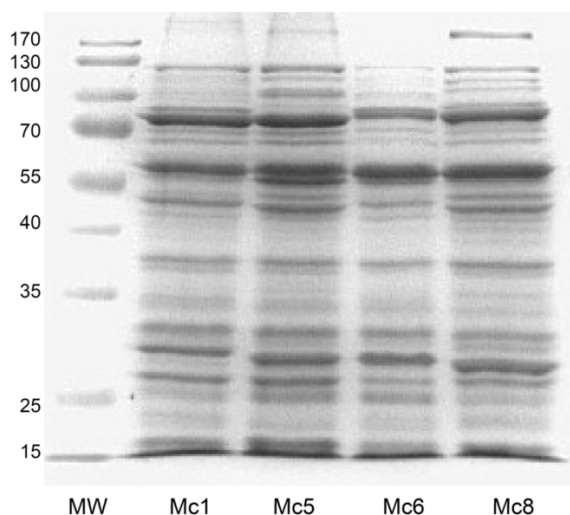


Fig. 1. A coomassie blue-stained 12% SDS-PAGE electrophoresis gel illustrating the OMP patterns of four clinical *Moraxella catarrhalis* isolates. The positions of the molecular mass standards (in kDa) are indicated on the left.

### Characterization of OMP-liposome formulations

In this study, we used one Zwittergent-based micelle preparation of OMPs (OMPs-Z) and two PC liposome-associated OMP formulations involving the association of the proteins with preformed lipid vesicles (PC-OMPs) or the incorporation of the proteins into the lipid bilayer during the vesiculation process (OMPs-PC). The two liposomal formulations were used to verify if their content and structure, and thus the immunogenic properties of obtained proteoliposome complexes, markedly depend on the formation procedure. The initially prepared proteoliposomes were next calibrated to obtain a more homogeneous system of vesicles using an extrusion procedure.

An atomic force microscopy analysis of the proteoliposomes showed that the vesicles had different shapes, from oblong to spherical, and that most of them had a tendency towards aggregation. Those features of proteoliposomes could



not be used to differentiate OMPs-PC from PC-OMPs (data not shown). This data also indicated that incorporating OMPs into the studied liposomes did not significantly affect the average diameter of the latter (Tab. 1). The association of OMPs with liposomes was confirmed via SDS-PAGE. This showed that within the initial panel of OMPs, incorporation was effective only for proteins with molecular masses of approximately 60 and 66 kDa. No differences were detected between OMPs-PC and PC-OMPs in terms of the contents of 60 and 66-kDa proteins (Fig. 2) as determined by densitometric analysis.

Tab. 1. The sizes of the liposomal formulations, as determined with AFM measurements.

Formulation	Sizes (nm)*
PC-OMPs	406.9 (386.6 - 427.1)
OMPs-PC	399.3 (373.1 - 425.5)
PC (empty)	383.3 (355.4 - 411.1)

\*The mean diameters with 95% confidence intervals; the intervals are given in the parenthesis.

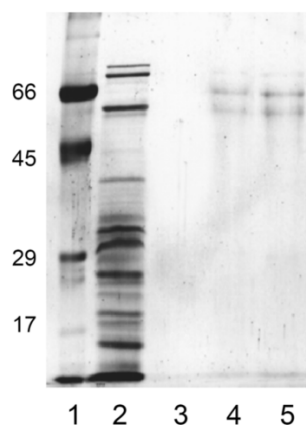


Fig. 2. The incorporation of OMPs from isolate Mc1 into PC liposomes, as identified by SDS-PAGE electrophoresis in a 12% gel. Lane 1, molecular mass standard; lane 2, OMPs; lane 3, empty; lane 4, OMPs-PC; lane 5, PC-OMPs. The positions of the molecular mass standards (in kDa) are indicated on the left.

#### **The mouse antibody response to OMP-liposome and OMPs-Z formulations**

To verify the properties of the OMPs in their native conformations, the OMPs incorporated into an artificial membrane-like environment (OMPs-PC; PC-OMPs) or comparatively into micelles by solubilization with the zwitterionic detergent Zwittergent 3.14 (OMPs-Z) were used in mouse immunization. Groups of 5 to 7 mice were immunized intraperitoneally with three doses each of one of the OMP preparations at 10 µg/per dose.

The adjuvanticity and immunogenicity of the above preparations were evaluated by detecting the reactivity of the post-immunized mouse antisera with the purified OMPs from the homologous strain in ELISA and Western blotting.

We included the non-immunized sera and sera after immunization with PC (empty) liposomes as a negative control. The specific IgG, IgA and IgM antibodies were determined with anti-mouse immunoglobulin isotype-specific conjugates. All the tested antisera from mice immunized with OMPs-PC, PC-OMPs and OMPs-Z preparations reacted against homologous OMPs. The liposomal formulations (OMPs-PC and PC-OMPs) and the micelle preparation (OMPs-Z) induced similar high titers of anti-OMPs IgG antibodies (Tab. 2). The OMPs-PC and PC-OMPs produced IgG titers that were respectively only 20% and 7% higher than the titer elicited by OMPs-Z. Thus, there were only marginal differences in the IgG anti-OMPs response between the two liposome-OMP-coupled formulations and OMPs-Z.

Tab. 2. The ELISA reactivity of the serum antibody response raised against OMPs preparations\*.

Formulation	Reciprocal geometric mean ELISA titer measured against OMPs		
	IgG	IgA	IgM
OMPs-Z	44,749 <sup>#</sup>	1,315	365
OMPs-PC	52,023	200	691
PC-OMPs	47,374	400	564
PC (empty)	< 100	< 100	< 100
Non-immunized serum	< 100	< 100	< 100

\*BALBc mice were immunized intraperitoneally with 100  $\mu$ l of each of the OMP preparations, each containing 10  $\mu$ g of OMPs. Non-immunized and post-immunized pooled antisera were tested against OMPs from the homologous *Moraxella catarrhalis* strain using the ELISA method. The data is presented as the geometric mean titers from groups of 5 to 7 mice. <sup>#</sup>There was no statistically significant difference when compared to OMPs-PC and PC-OMPs.

The IgA systemic antibody responses that were raised against OMPs following OMPs-PC, PC-OMPs and OMP-Z immunization were very weak. In terms of anti-OMP IgA in the post-immunized antisera, a 14-fold increase for OMPs-Z, a 2-fold increase for OMPs-PC and a 4-fold increase for PC-OMPs were observed in the titer, as compared to the response of PC-immunized and non-immunized mice. Interestingly, immunization with the OMPs-Z preparation induced a 7-fold and 3.5-fold higher IgA anti-OMPs response than that from OMPs-PC and PC-OMPs, respectively.

The reactivity of the post-immunization mouse sera with OMPs of *M. catarrhalis* was also evaluated in immunoblot assays. Two groups of pooled mouse antisera were tested in two independent experiments. Fig. 3 shows the results of two classic immunoblots with purified OMPs illustrating the dominating reactivity of IgG with the ~60-kDa protein, independently of the

antigen formulation that was used for immunization. An assessment of specific anti-OMPs IgA revealed a similar pattern of immunoreactivity for OMPs-Z and PC-OMPs. The serum IgA antibodies predominantly recognized the 60-kDa OMP. Immunization with OMPs-PC yielded no immunoreactivity between the OMPs and mouse antisera (data not shown). Only weak reactivity was seen between this protein and the IgM antibodies (data not shown). Fig. 3 also shows the presence of antibodies to other minor unidentified protein bands.

#### **An analysis of the cross-reactive mouse antibodies to the OMPs of *Moraxella catarrhalis***

To determine the cross-reactive antibodies against the 60-kDa OMP, an immunoblotting procedure was performed. We used homologous and heterologous bacterial OMPs to probe pooled mouse antisera from animals from each group that had been immunized with various OMPs-Z preparations or with whole bacteria from 4 different strains: Mc1, Mc5, Mc6 and Mc8.

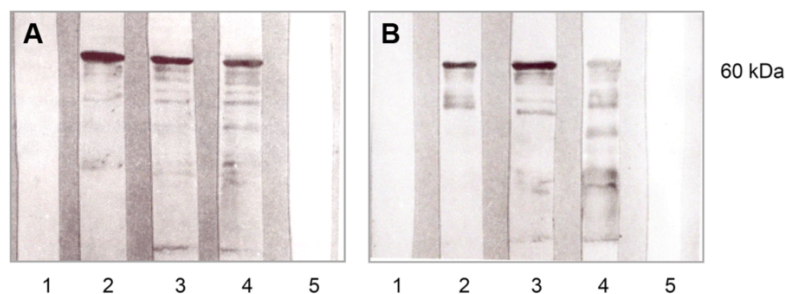


Fig. 3. The results of two independent immunization experiments (A and B): immunoblot analyses of *M. catarrhalis* (Mc 1) outer membrane proteins (OMPs) probed with a panel of pooled polyclonal antisera containing 5 to 7 mouse antisera per option. Lane 1, non-immunized mice; lane 2, mice immunized with OMPs-Z; lane 3, mice immunized with PC-OMPs; lane 4, mice immunized with OMPs-PC; lane 5, mice immunized with PC. HRP-conjugated goat antibodies specific against mouse IgG (1:5,000) were used in the immunodetection. The predominating immunoreactivity of the ~60-kDa protein is indicated.

As presented in Tab. 3, all the tested post-immunized sera reacted strongly with the 60-kDa OMP from the heterologous *M. catarrhalis* strains. Interestingly, that 60-kDa protein formed the only prominent immunoreactive band following incubation with most of the anti-Mc antisera. In reaction with the anti-OMPs antisera, it also formed the most prominent, but not the only band with specific antibodies. The ability of the mouse antisera produced by immunization either with whole *M. catarrhalis* or purified OMPs to bind to the 60-kDa protein of another strain in the blot confirmed the very strong serologic stability and antigenic conservation of the ~60-kDa OMP.

Tab. 3. The reactivities of a panel of pooled mouse antisera with ~60-kDa OMPs from homologous and heterologous strains of *M. catarrhalis*.

Antisera	OMPMc1	OMPMc5	OMPMc6	OMPMc8
anti-OMPMc1	+++	+	+++	+++
anti-OMPMc5	+++	+++	+++	+++
anti-OMPMc6	+++	++	+++	+++
anti-OMPMc8	+++	+++	+++	+++
anti-Mc1	+++	+++	+++	+++
anti-Mc5	+++	+++	+++	+++
anti-Mc6	++	++	+++	+++
anti-Mc8	+++	+++	++	+++
control	-	-	-	-

In the immunoblotting procedures, antisera from mice immunized with bacterial OMPs at 10 µg/dose (anti-OMPMc sera) were diluted 1:1000, whereas antisera from mice immunized with the whole bacteria at  $5 \times 10^7$  cfu/dose (anti-Mc sera) were diluted 1:100. The control serum was from the non-immunized group.

+++ : strong reactivity; ++ : medium-level reactivity; + : weak reactivity; - : no reactivity

#### **An analysis of cross-reactive human antibodies to the OMPs of *Moraxella catarrhalis***

The human antibody response against OMPs was assessed using the immunoblot procedure. To evaluate the antigenic properties of the 60-kDa OMP in the human system, the OMPs previously separated with SDS-PAGE were incubated with a panel of 100-fold diluted heterologous normal human sera obtained from 20 healthy children. Simultaneously, the reactivities of homologous serum and two additional heterologous sera from children that had just recovered from otitis media caused by *M. catarrhalis* were evaluated. The typical immunoreactivities of the anti-OMPs IgG, IgA and IgM are presented in Fig. 4 and Tab. 4.

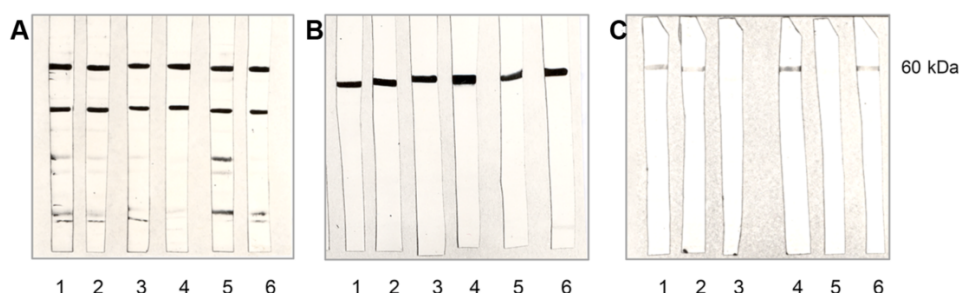


Fig. 4. Representative immunoblots of OMPs from the *M. catarrhalis* strain Mc1 incubated with various sera from children (1:100 dilution). Lane 1, homologous serum from a child (NHS<sub>H</sub>); lane 2, heterologous serum from a child with otitis media (NHS<sub>C1</sub>) whose otitis exudate yielded *M. catarrhalis* in culture; lanes 3-6, heterologous sera from children. The sera were tested for the presence of IgG (A) and IgA (B), both of which showed high reactivity, and IgM (C), which showed weak reactivity of the antibodies specific for OMPs. The dominant immunoreactivity of ~60 kDa OMP is indicated.

Tab. 4. Reactivity of normal human serum (NHS) with a 60-kDa OMP assessed via immunoblotting.

The reactivities of serum immunoglobulins with the 60-kDa OMP from the Mc1 strain			
	IgG	IgA	IgM
NHS <sub>1</sub>	+++	+++	+
NHS <sub>2</sub>	+++	+++	+
NHS <sub>3</sub>	+++	+++	-
NHS <sub>4</sub>	+++	+	-
NHS <sub>5</sub>	+++	+++	+
NHS <sub>6</sub>	+++	+++	+
NHS <sub>7</sub>	+++	+++	+
NHS <sub>8</sub>	+++	+	-
NHS <sub>9</sub>	+	+	-
NHS <sub>10</sub>	+++	+++	-
NHS <sub>11</sub>	+++	+++	-
NHS <sub>12</sub>	+++	+++	-
NHS <sub>13</sub>	+++	+++	-
NHS <sub>14</sub>	+++	+++	-
NHS <sub>16</sub>	+++	+++	-
NHS <sub>17</sub>	+++	+++	-
NHS <sub>18</sub>	-	-	-
NHS <sub>19</sub>	+++	+++	+
NHS <sub>20</sub>	-	-	-
NHS <sub>H</sub>	+++	+++	+
NHS <sub>C1</sub>	+++	+++	++
NHS <sub>C2</sub>	+++	+++	+

Normal human sera (NHS<sub>1-20</sub>) were collected from healthy children. NHS<sub>H</sub>: homologous serum. NHS<sub>C1</sub> and NHS<sub>C2</sub>: sera from convalescent children who had recently recovered from *M. catarrhalis* otitis media. All the sera were diluted 1:100 before the immunoblotting procedures. +++: strong reactivity; ++: medium level reactivity; +: weak reactivity; -: no reactivity

All three tested izotypes mainly recognized the 60-kDa OMP. However, with respect to IgG, the presence of antibodies against other protein bands was observed. The IgG antibodies directed against the 60-kDa OMP were detected in 90% (18/20) of the children's sera. Similarly, the IgA anti-60-kDa OMP were detected in 85% (17/20) of the children's sera. Most of the sera demonstrated strong reactivities of formed immune complexes. Immunoreactivity between the 60-kDa OMP and IgM was very weak. Only 30% (6/20) of the sera reacted, and it was a weak positive. It has been noted that the 60-kDa OMP of *M. catarrhalis* was the only antigen reacting with the IgA and IgM antibodies. In experiments in which immunoblots were incubated with unadsorbed serum and serum that had been previously adsorbed with *M. catarrhalis*, the corresponding band was only observed when the unadsorbed sera were used, confirming that specific IgG

or IgA antibodies reacted with /the epitope of the 60-kDa antigen that was expressed on the surface of the intact bacterium (data not shown).

The recognition of the 60-kDa OMP by IgG, IgA and, to a much lesser extent, IgM antibodies from heterologous sera from healthy children confirmed the high degree of serological stability against that protein in the human system.

## DISCUSSION

The purpose of this study was to determine the effectiveness of PC liposomes in potentiating a systemic immune response to *Moraxella catarrhalis* outer membrane proteins. The additional aim was to compare the cross-reactivity of the most effective anti-OMP response in the human and mouse systems *in vitro*.

The bacterial outer membrane proteins of *M. catarrhalis* are able to induce an effective immune response in humans [1]. However, it has been well established that OMPs in general possess strong immunogenic properties only when they have a native or native-like conformation [27]. To obtain this, the first step of our study relied on the association of the solubilized OMPs with neutral PC liposomes or with micelles composed of the zwitterionic detergent Zwittergent 3.14. As the reconstitution of the protein into the lipid bilayer occurs via two possible mechanisms, either insertion of the protein into the preformed lipid vesicles or incorporation of the protein into the lipid bilayer during the vesiculation process [28], we used the two procedures in our study. SDS-PAGE electrophoresis of OMPs-PC and PC-OMPs complexes showed an identical pattern of separated protein bands which referred to ~60 and 66 kDa (Fig. 2). This result suggests that the identified OMPs may possess a higher affinity leading to stronger interactions with lipids in comparison with other OMPs. Thus, they can be more easily and selectively conjugated to the PC lipid bilayer in way that is independent of the used formation procedure. The reasons for such an unequal association of proteins with liposomes are not known. An explanation may come from the physicochemical properties of phospholipid vesicles, such as lipid content, size and charge, or from the fact that incorporated proteins are the most prominent bands in the purified outer membranes of *M. catarrhalis* [29]. Moreover, we did not find any considerable differences in the sizes of the studied proteoliposomes based on AFM picture analyses (Tab. 1). Thus, we can speculate on the existence of the same type of structure in both proteoliposome complexes. To determine comparatively the immunogenicity of the free antigens, a zwitterionic preparation of *M. catarrhalis* OMPs was used. Zwittergent 3.14 is relatively mild, non-denaturing detergent with a relatively high critical micellar concentration. Additionally, it was reported that including zwitterionic detergent during protein solubilization and purification can restore the antibody-binding capacity of membrane proteins [30]. A good example can be the fully denaturated class 1 meningococcal outer membrane protein that, in the presence of the zwitterionic detergent 3.14, without added lipids, recovered a native-like conformation and elicited antibody reactivity with epitopes

presented on the surface of meningococci [31]. We tested liposomal vesicles of relatively small diameter because of their documented advantages such as longest half-life, prolonged circulation lifetime and highest safety for humans [23]. We did not use any additional adjuvants in our formulations such as aluminium hydroxide (Alum) or Freund's complete adjuvant because there have been reports of their influence on the augmentation of the non-specific immune response to antigens or on the balance between antibody-mediated and cell-mediated immunity [32, 33].

Next, we performed immunization experiments to investigate the immunogenic activity of the prepared OMP formulations in the mouse system. The efficiency of the reconstituted proteoliposomes (OMPs-PC, PC-OMPs) and proteomicelles (OMPs-Z) was analyzed by measuring their antigenic properties using enzyme-coupled methods such as ELISA and Western Blot. The studied OMP formulations exhibited a comparable and high degree of immunogenicity as determined by the high IgG antibody ELISA titers in mouse antisera (Tab. 2). Both liposomal formulations, OMPs-PC and PC-OMPs, showed quantitatively similar immunogenic properties in the induction of an anti-OMPs IgG response, indicating that the way of OMP reconstitution in PC liposomes did not determine their adjuvantic properties under our conditions. Moreover, this indicates that just the presence of the lipid (PC) is crucial for maintaining the appropriate conformational state of the studied proteins. Additionally, the immune activity of the OMP-liposome formulations was also similar to that of the OMPs-Z proteomicelles, suggesting that to maintain an active immunogenic structure, the studied proteins do not need the lipid bilayer but only amphiphilic neutral compounds rich in hydrophobic groups. Both the liposomal formulations and OMPs-Z proteomicelles did not show any distinct quality differences in the antibody pattern of mouse antisera in Western blot analysis. It was demonstrated that immunological complexes were formed preferentially between serum IgG or IgA and the 60-kDa OMP (Fig. 3). The lack of a potentiating effect of the studied liposomal formulations in comparison with free antigens on the systemic immune response following immunization was not surprising based on previous studies [34-36]. Therefore, our findings as well as those of others demonstrate that the adjuvantic properties of liposomes strictly depend on their lipid composition and may be leveled in the presence of neutral phospholipids such as phosphatidylcholine.

Because the immunogenic differences between the two liposome-OMP-coupled formulations and OMPs-Z in homologous serum systems were not significant, we decided to evaluate the reactivity of free OMPs with heterologous sera from mice and healthy humans, to compare the cross-reactivity of the most effective anti-OMPs response. It was found that the ~60-kDa antigen that was selectively incorporated into the liposomal formulation was preferentially involved in cross-reactions with specific antibodies. Its reactivity was detected both with a panel of pooled post-immunized heterologous mouse sera (Tab. 3) and with 20 heterologous sera derived from immunocompetent healthy children (Tab. 4, Fig. 4).

We also confirmed that adult sera and the sera from children suffering from recurrent respiratory tract infections interacted primarily with the 60-kDa OMP (data not shown). The highly cross-reactive 60-kDa OMP seems to be an important antigenic marker of *M. catarrhalis* responsible for the induction of an antibody-mediated and long-lasting immune response. This might have its implications in term of protective activity against different strains of *M. catarrhalis*. An analogous 38-kDa protein marker for the Enterobacteriaceae family was recently reported on [37]. The next stage of our investigations aims to assess the functional activity of such heterologous antibodies using a complement-dependent bactericidal assay and an opsonophagocytic assay, and blocking of bacterial adherence to mucosal surfaces. The cross-reactivities of other *M. catarrhalis* OMPs, especially the ~77- and ~32-kDa antigens, have been reported [12]. The results indicating a strong normal human serum reactivity to ~60-kDa OMP are consistent with data reporting that, following natural colonization or infection, specific antibodies develop to a common antigen with an apparent molecular mass of ~60 kDa, designated as OMP CD [38-40]. It has been documented that the OMP CD of *Moraxella catarrhalis* is: 1) highly conserved among strains of *M. catarrhalis* of diverse clinical, geographic and epidemiological origins [41, 42]; 2) abundantly expressed on the bacterial surface [43]; 3) the target for IgG antibodies from human convalescent sera exhibiting bactericidal activity [44]; 4) an important antigen eliciting a mucosal IgA immune response in children with respiratory infections [45] as well as in healthy adults [46]; 5) an adhesin for the human lung cell [47]; 6) responsible for the enhancement of pulmonary clearance of *M. catarrhalis* in a mouse challenge model [48]; 7) a safe and effective carrier for *M. catarrhalis* detoxified LOS-based conjugates [49].

This study has extended these observations by showing that: i) the 60-kDa OMP is most effectively incorporated into liposomal formulations composed of phosphatidylcholine; and ii) the 60-kDa OMP induces the dominant systemic cross-reactivity both in the human and mouse systems. The similar immune properties of OMPs associated with both liposomal formulations in comparison to zwittergent-based proteomicelles indicate that to maintain immunogenic properties *in vitro*, the studied proteins do not need the lipid bilayer but only the presence of amphiphilic neutral compounds rich in hydrophobic groups. This 60-kDa protein seems to be an important cross-reactive antigenic marker of *Moraxella catarrhalis* that may partially bring us to an understanding of the relatively low degree of pathogenicity of this bacterium in immunocompetent individuals. Such an effect may be the result of naturally acquired immunity following repeated exposure to different strains of *M. catarrhalis*. Its selective association with PC lipids in liposomal formulations could be important in the future for human vaccine application. However, whether the studied proteoliposomes can induce protection against infection is not yet known. An evaluation of the protective potency of the elicited immune response using a relevant animal challenge model is needed.



**Acknowledgements.** We wish to thank Dr. Bogdan Barwiński (Institute of Experimental Physics, University of Wrocław, Poland) for generating and analyzing the AFM images, and Dr. Barbara Basiewicz-Worsztynowicz (Department and Clinics of Pediatrics, Immunology and Rheumatology of Developmental Age, Medical University, Wrocław, Poland) for providing us with the serum samples from healthy children. This study was supported by University of Wrocław research grants.

## REFERENCES

1. Karaulus, R. and Campagnari, A. *Moraxella catarrhalis*: a review of an important human mucosal pathogen. **Microb. Infect.** 2 (2000) 547-559.
2. Murphy, T.F., Brauer, A.L., Aebi, Ch. and Sethi, S. Identification of surface antigens of *Moraxella catarrhalis* as targets of human serum antibody responses in chronic obstructive pulmonary disease. **Infect. Immun.** 73 (2005) 3471-3478.
3. Murphy, T.F., Brauer, A.L., Grant, B.J. and Sethi, S. *Moraxella catarrhalis* in chronic obstructive pulmonary disease: burden of disease and immune response. **Am. J. Respir. Crit. Care Med.** 172 (2005) 195-199.
4. Sethi, S., Sethi, R., Eschberger, K., Lobbins, P., Cai, X., Grant, B.J. and Murphy, T.F. Airway bacterial concentrations and exacerbations of chronic obstructive pulmonary disease. **Am. J. Respir. Crit. Care Med.** 176 (2007) 356-361.
5. Verduin, C.M. Hol, C. Fleer, A. van Dijk, H. and van Belkum, A. *Moraxella catarrhalis*: from emerging to established pathogen. **Clin. Microb. Rev.** 15 (2002) 125-144.
6. Bandak, S.M., Turnak, M.R., Allen, B.S., Bolzon, L.D., Preston, D.A., Bouchillon, S.K. and Hoban, D.J. Antibiotic susceptibility among recent clinical isolates of *Haemophilus influenzae* and *Moraxella catarrhalis* from fifteen countries. **Eur. J. Clin. Microbiol. Infect. Dis.** 20 (2001) 55-60.
7. Klingman, K.L., Pye, A., Murphy, T.F. and Hill, S.L. Dynamics of respiratory tract colonization by *Branhamella catarrhalis* in bronchiectasis. **Am. J. Respir. Crit. Care Med.** 152 (1995) 1072-1078.
8. Faden, H., Duffy, L., Wasielewski, R., Wolf, J., Krystofik, D. and Tung, Y. Relationship between nasopharyngeal colonization and the development of otitis media in children. **J. Infect. Dis.** 175 (1997) 1440-1445.
9. Yokota, S., Harimaya, A., Sato, K., Somekawa, Y., Himi, T. and Fujii, N. Colonization and turnover of *Streptococcus pneumoniae*, *Haemophilus influenzae*, and *Moraxella catarrhalis* in otitis-prone children. **Microbiol. Immunol.** 51 (2007) 223-230.
10. Heiniger, N., Spaniol, V., Troller, R., Vischer, M. and Aebi, C. A reservoir of *Moraxella catarrhalis* in human pharyngeal lymphoid tissue. **J. Infect. Dis.** 196 (2007) 1080-1087.

11. Peng, D., Hong, W., Choudhury, B., Carlson, R.W. and Gu, X.X. *Moraxella catarrhalis* bacterium without endotoxin, a potential vaccine candidate. **Infect. Immun.** 73 (2005) 7569-7577.
12. Troncoso, G., Sánchez, S., Criado, M.T. and Ferreirós, C. Analysis of *Moraxella catarrhalis* outer membrane antigens cross-reactive with *Neisseria meningitidis* and *Neisseria lactamica*. **FEMS Immunol. Med. Microbiol.** 40 (2004) 89-94.
13. Mleczo, J., Augustyniak, D. and Jankowski, A. Efficiency of oral immunization of mice with *Candida albicans* and *Moraxella catarrhalis* heat-killed cells and cross reactivity of induced antibodies. **Centr. Eur. J. Immunol.** 32 (2007) 185-188.
14. Steeghs, L., Kuipers, B., Hamstra, H.J., Kersten, G., Van Alphen, L. and Van der Ley, P. Immunogenicity of outer membrane proteins in a lipopolysaccharide –deficient mutant of *Neisseria meningitidis*: influence of adjuvants on the immune response. **Infect. Immun.** 67 (1999) 4988-4993.
15. Schweizer, M., Hindennach, I., Garten, W. and Henning, U. Major proteins of the *Escherichia coli* outer cell envelope membrane. Interaction of protein II\* with lipopolysaccharide. **Eur. J. Biochem.** 82 (1978) 211-217.
16. Bogdanov, M. and Dowhan, W. Lipid-assisted protein folding. **J. Biol. Chem.** 274 (1999) 36827-36830.
17. Qi, H.L., Tai, J.Y. and Blake, M.S. Expression of large amounts of neisserial porin proteins in *Escherichia coli* and refolding of the proteins into native trimers. **Infect. Immun.** 62 (1994) 2432-2439.
18. Wetzler, L.M., Blake, M.S. and Gotschlich, E.C. Characterization and specificity of antibodies to protein I of *Neisseria gonorrhoeae* produced by injection with various protein I-adjuvant preparation. **J. Exp. Med.** 168 (1988) 1883-1897.
19. Jolley, K.A., Appleby, L., Wright, J.C., Christodoulides, M. and Heckels, J. Immunization with recombinant Opc outer membrane protein from *Neisseria meningitidis*: Influence of sequence variation and levels of expression on the bactericidal immune response against meningococci. **Infect. Immun.** 69 (2001) 3809-3916.
20. Frézard, F. Liposomes: from biophysics to the design of peptide vaccines. **Braz. J. Med. Biol. Res.** 32 (1999) 181-189.
21. Altin, J.G. and Parish, Ch.R. Liposomal vaccines-targeting the delivery of antigen. **Methods** 40 (2006) 39-52.
22. Uchida, T. and Taneichi, M. Clinical application of surface-linked liposomal antigens. **Mini Rev. Med. Chem.** 8 (2008) 184-192.
23. Parmar, M.M., Edwards, K. and Madden, T.D. Incorporation of bacterial membrane proteins into liposomes: factors influencing protein reconstitution. **Biochim. Biophys. Acta** 1421 (1999) 77-90.
24. Stebelska, K., Wyrozumska, P., Gubernator, J. and Sikorski A.F. Highly fusogenic cationic liposomes transiently permeabilize the plasma membrane of HeLa cells. **Cell. Mol. Biol. Lett.** 12 (2007) 35-39.

25. Gutowicz, J. and Terlecki, G. The association of glycolytic enzymes with cellular and model membranes. **Cell. Mol. Biol. Lett.** 8 (2003) 667-680.
26. Murphy, T.F. and Bartos, L.C. Surface-exposed and antigenically conserved determinants of outer membrane proteins of *Branhamella catarrhalis*. **Infect. Immun.** 57 (1989) 2938-2941.
27. Rigaud, J-L. and Lévy, D. Reconstitution of membrane proteins into liposomes. in: **Methods in Enzymol. Liposomes, Part B.** 372 Elsevier Academic Press, Co. Elseviere Inc., 2003,65-86.
28. Rigaud, J.L., Paternoster, M.T. and Bluza, A. Mechanisms of membrane protein insertion into liposomes during reconstitution procedures involving the use of detergents.2. Incorporation of the light-driven proton pump bacteriorhodopsin. **Biochemistry** 27 (1988) 2677-2688.
29. Sarwar, J., Campagnari, A.A., Kirkham, C. and Murphy, T. Characterization of an antigenically conserved heat-modifiable major outer membrane protein of *Branhamella catarrhalis*. **Infect. Immun.** 60 (1992) 804-809.
30. Mandrell, R.E. and Zollinger, W.D. Use of a zwitterionic detergent for the restoration of the antibody-binding capacity of electroblotted meningococcal outer membrane proteins. **J. Immunol. Met.** 67 (1984) 1-11.
31. Idänpään-Heikkilä, I., Wahlström, E., Mutttilainen, S., Nurminen, M., Käyhty, H., Sarvas, M. and Mäkelä, P.H. Immunization with meningococcal class 1 outer membrane protein produced in *Bacillus subtilis* and reconstituted in the presence of Zwittergent or Triton X-100. **Vaccine** 14 (1996) 886-891.
32. Shahum, E. and Thérien, H.M. Effect of liposomal antigens on the priming and activation of the immune system by dendritic cells. **Int. Immunopharmacol.** 2 (2002) 591-601.
33. Ignatius, R., Mahnke, K., Rivera, M., Hong, K., Isdell, F., Steinman, R.M., Pope, M. and Stamatatos, L. Presentation of proteins encapsulated in sterically stabilized liposomes by dendritic cells initiates CD8(+) T-cell response in vivo. **Blood** 96 (2000) 3505-3513.
34. Alving, C.R. Immunologic aspects of liposomes: presentation and processing of liposomal protein and phospholipid antigens. **Biochim. Biophys. Acta** 1113 (1992) 307-322.
35. Wright, J.C., Williams, J.N., Christodoulides, M. and Haeckels, J.E. Immunization with the recombinant PorB membrane protein induces bactericidal immune response against *Neisseria meningitidis*. **Infect. Immune.** 70 (2002) 4028-4034.
36. Sprott, G.D., Dicaire, C.J., Gurnani, K., Deschatelets, L.A. and Krishnan, L. Liposome adjuvants prepared from the total polar lipids of *Haloferax volcanii*, *Planococcus spp.* and *Bacillus firmus* differ in ability to elicit and sustain immune responses. **Vaccine** 22 (2004) 2154-2162.
37. Witkowska, D., Masłowska, E., Staniszevska, M., Szosto, B., Jankowski, A. and Gamian, A. Enterobacterial 38-kDa outer membrane protein is an age-dependent molecular marker of innate immunity and immunoglobulin

- deficiency as results from its reactivity with IgG and IgA antibody. **FEMS Immunol. Med. Microbiol.** 48 (2006) 205-214.
38. Christensen, J.J. *Moraxella (Branhamella) catarrhalis*: clinical, microbiological and immunological features in lower respiratory tract infections. **APMIS** 107S (1999) 1-36.
  39. Mathers, K., Leinonen, M. and Goldblatt, D. Anibody response to outer membrane proteins of *Moraxella catarrhalis* in children with otitis media. **Pediatr. Infect. Dis. J.** 18 (1999) 982-988.
  40. Murphy, T.F., Kirkham, C., Liu, D.F. and Sethi, S. Human immune response to outer membrane protein CD of *Moraxella catarrhalis* in adults with chronic obstructive pulmonary disease. **Infect. Immun.** 71 (2003) 1288-1294.
  41. Murphy, T.F., Kirkham, C. and Lesse, A.J. The major heat modifiable outer membrane protein CD is highly conserved among strains of *Branhamella catarrhalis*. **Mol. Microbiol.** 10 (1993) 87-97.
  42. Hsiao, C.B., Sethi, S. and Murphy, T.F. Outer membrane protein CD of *Branhamella catarrhalis*: sequence conservation in strains recovered from the human respiratory tract. **Microb. Pathog.** 19 (1995) 215-225.
  43. McMichael, J. Vaccines for *Moraxella catarrhalis*. **Vaccine** 19 (2001) 101-107.
  44. Yang-Ping, Y., Myers, L.E., McGuinness, U., Chong, P., Kwok, Y., Klein, M.H. and Harkness, R.E. The major outer membrane protein, CD, extracted from *Moraxella (Branhamella) catarrhalis* is a potential vaccine antigen that induces bactericidal antibodies. **FEMS Immunol. Med. Microbiol.** 17 (1997) 187-199.
  45. Meier, P.S., Freiburghaus, S., Martin, A., Heiniger, N., Troller, R. and Aebi, C. Mucosal immune response to specific outer membrane proteins of *Moraxella catarrhalis* in young children. **Pediatr. Infect. Dis. J.** 22 (2003) 256-262.
  46. Meier, P.S., Heiniger, N., Troller, R. and Aebi, C. Salivary antibodies directed against outer membrane proteins of *Moraxella catarrhalis* in healthy adults. **Infect. Immun.** 71 (2003) 6793-6798.
  47. Holm, M.M., Vanlerberg, S.L., Foley, I.M., Sledjeski, D.D. and Lafontaine, E.R. The *Moraxella catarrhalis* porin-like outer membrane protein CD is an adhesion for human lung cells. **Infect. Immun.** 72 (2004)1906-1913.
  48. Liu, D.F., McMichael, J.C. and Baker, S.M. *Moraxella catarrhalis* outer membrane protein CD elicits antibodies that inhibit CD binding to human mucin and enhance pulmonary clearance of *M.catarrhalis* in a mouse model. **Infect. Immun.** 75 (2007) 2818-2825.
  49. Hu, W.G., Berry, J., Chen, J. and Gum, X-X. Exploration of *Moraxella catarrhalis* outer membrane proteins, CD and UspA, as new carriers for lipooligosaccharide-based conjugates. **FEMS Immunol. Med. Microbiol.** 41 (2004)109-115.