# Chaperonin (MSP63) complexes induce bactericidal and opsonophagocytic cross-reactive antibodies

Juan Marzoa[1], Sandra Sánchez[1], Charlotte Brookes[2], Stephen Taylor[2], Andrew Gorringe[2], M. Teresa Criado[1] and **Carlos M. Ferreirós**[1]

[1]University of Santiago de Compostela, Departamento de Microbiología y Parasitología, Facultad de Farmacia, Campus Sur, Universidad de Santiago de Compostela, 1782 Santiago de Compostela, Spain.
[2]Health Protection Agency, Centre for Emergency Preparedness and Response, Porton Down, Salisbury, UK.

**email:** carlos.ferreiros@usc.es

Alteration of the native structure of antigens can lead to the loss of protective epitopes. Our previous results showed that separation of the meningococcal outer membrane proteins in native conditions revealed the existence of protein complexes that could be relevant for the development of new vaccine formulations. The aim of this work was to analyse the immunogenic characteristics of a highly conserved 700 kDa chaperonin complex (CxChap) detected and purified by using high resolution clear native electrophoresis. Analysis of the anti-CxChap serum by Western-blotting revealed the presence of antibodies against the MSP63 but also against the macrophage infectivity potentiator-like protein (MIP), which is coopurified with the chaperonin complex. Antibodies raised by immunisation with CxChap chaperonin complex show bactericidal and opsonophagocytic activity.

**Keywords:** *Neisseria meningitidis* complexes, MSP63, MIP,hrCNE, bactericidal activity, opsonophagocytosis.

## Introduction

The association of outer membrane proteins to form complexes may produce epitopes that contribute to the effective immune response elicited by outer membrane vesicle vaccines.

Methods to identify intact multi-protein complexes can help to achieve more effective formulations but their application to OMVs can be difficult due to the hydrophobicity of membrane proteins. Our previous studies of the meningococcal porin complexes using blue native polyacrylamide gel electrophoresis (BNE) (1) allowed the detection of a 700 kDa major complex. Analysis by LC/MS-MS revealed that it is a homomeric association of meningococcal stress protein MSP63 units, a protein that was found to be expressed and immunogenic during natural meningococcal infection (2).

A new technique named high resolution clear native electrophoresis (hrCNE) (3), in which the resolution of complexes attained in BNE is combined with excellent preservation of the structure and function of complexes. The technique removes the interferences produced by the Coomassie dye, resulting in an excellent separation of membrane complexes in native conditions, making it optimal for functional proteomic analysis.

This technique allowed the purification of the CxChap complex which was used to raise specific serum in mice (anti-chapCx). This study analyses the immune response generated by CxChap complex by Western blotting, bactericidal and opsonophagocytic assays against the homologous (H44/76) and a heterologous *N. meningitidis* strain (NZ98/254).

## Material and Methods

### 1. Strains and culture conditions

*Neisseria meningitidis* H44/76 and its homologous mutant lacking PorA were kindly provided by Dr. Ian Feavers (National Institute for Biological Standards and Control, Great Britain). Strain NZ98/245 was kindly provided by Dr D.Martin (Institute of Environmental Science and Research, Porirua, New Zealand). All cultures were performed under iron restriction in Mueller-Hinton broth with addition of 100 $\mu$M Desferal.

### 2. 2-D hrCNE/SDS-PAGE

hrCNE was carried out following a modification of the protocol described by Wittig *et al.* (3). Complexes were separated in 5-15% native polyacrylamide gradient gels in a Mini-Protean 3 Cell® (Bio-Rad). OMVs were solubilised by adding 50 mM Bis-Tris HCl and 1M 6-aminohexanoic acid (pH 7.0) and 10% (w/v) DDM at a 4.5:1 DDM/protein final ratio.

Power was set to 50 V constant voltage for the first hour and 100 V constant voltage for about six hours. Gels were stained Coomassie blue G-250. The CxChap complex from a mutant lacking PorA was purified from the gel by electroelution and then used to obtain an anti-Cxchap serum in mice. Bidimensional analyses to identify the components of membrane complexes were done using SDS-PAGE in the second dimension. Lanes cut from hrCNE gels were incubated in SDS sample buffer for 10 min at 95°C and placed on top of 12% polyacrylamide gels for separation.

### 3. Image analysis and protein identification

The analysis of complexes in 1-D separations and proteins in 2-D maps, and calculation of their molecular weights were carried out using the Quantity One® and PD Quest® software (BioRad Chemicals S.A., Spain). Components of the complex resolved by 1-D hrCNE were identified by LC/MS-MS

### 4. Western blotting

Proteins from OMVs were transferred from 12% SDS-PAGE gels to PVDF membranes using a Bio-Rad Mini-Trans Blot Electrophoretic Transfer Cell (Bio-Rad Chemical S.A., Spain) according to the manufacturer's instructions. Anti-CxChap serum was used at 1/1 000 working dilution.

### 5. Bactericidal assays (SBA)

SBA was performed by using baby rabbit serum (CEDARLANE, Canada) as complement source. SBA titres were expressed as the reciprocal of the final serum dilution yielding =50% killing at 60 min.

### 6.Opsonophagocytic assay (OPA)

Opsonophagocytic assays were performed against the H44/76 and NZ98/254 strains using flow cytometry (4), except that Trypan Blue was added immediately before flow cytometric analysis to quench the fluorescence of non-internalised fluorescent bacteria.

## Results and Discussion

The 2-D electrophoretic analysis of OMVs using hrCNE in the first dimension and SDS-PAGE in the second allowed to obtain proteomic maps in which, theoretically, the spots aligned vertically correspond to the individual proteins forming each complex. OMVs complexes from the strain H44/76 are shown in Figure. 1A.

The CxChap complex was purified using OMVs from a mutant lacking PorA to avoid contamination with this protein, as this produced horizontal streaking in native electrophoresis when the wild type H44/76 was used. Although the characterisation of the purified complex by SDS-PAGE and silver stain (Figure 1B) showed a highly pure complex formed by a 63 kDa protein

**Table 1.** Results from serum bactericidal and opsonophagocytic assays

| Serum | SBA (titer) | | OPA (FIR) | |
|---|---|---|---|---|
| | H44/76 | NZ98/254 | H44/76 | NZ98/254 |
| non immune serum | <4 | <4 | 1.7 | 1.7 |
| anti-OMVs | 16384 | <4 | 8.6 | 9.67 |
| anti-OMVs PortA- | <4 | <4 | 8.9 | 10.59 |
| anti-chapCx | 64 | <4 | 4.01 | 11.35 |

(MSP63) alone, Western-blots (Figure 1C) showed that anti-CxChap serum reacted with the MSP63 but, surprisingly, also with a 33 kDa protein not detected previously by LC-MS/MS in BNE. LC-MS/MS identification of the purified complex also revealed the presence of a homologue of the macrophage infectivity potentiator (MIP). MSP63 forms two superimposed heptameric rings in other bacteria (5), which is consistent with the molecular mass of the CxChap. Although chaperonins are theoretically cytoplasmic, they have been found associated with the outer membrane in some species (6). This is consistent with the presence of chaperonin complexes in the outer membrane of *N. meningitidis* and with the need for OM proteins to be correctly folded after transportation. It is also consistent with meningococcal chaperonins being immunogenic during infection, suggesting that they are expressed at the cell surface (7).

The MIP is considered an important virulence factor involved in the initiation of the macrophage infection in *Legionella pneumophila* and *Neisseria gonorrhoeae*. Our results showed that this protein is highly immunogenic in *N. meningitidis* even when it is present at low concentration. It is not clear if the MSP63 and the MIP are components from the same complex or if both proteins are present in two different complexes with a similar mobility in hrCNE electrophoresis. The cross reactivity observed with the heterologous strain is in agreement with the high sequence homology of both proteins among *N. meningitidis* strains and between *N.meningitidis* and *N.gonorrhoeae* strains.

Results from serum bactericidal (SBA) and opsonophagocityc (OPA) assays are shown in Table 1.

It can be seen that the anti-CxChap serum is bactericidal against the homologous strain and mediates opsonophagocytosis against both the homologous and the heterologous strains. Further studies using knock-out mutants are being carried out to determine which of both proteins is the responsible of the bactericidal and/or opsonophagocytic antibodies in the anti-CxChap serum and, if both proteins are

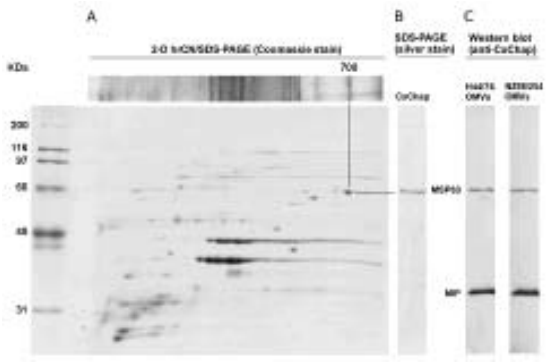


**Figure 1.** (A) 2-D hrCN/SDS-PAGE of OMVs from strain H44/76; (B) Silver stained SDS-PAGE of purified CxChap; (C) Western blots of OMVs from strains H44/76 and NZ98/254 separated by SDS-PAGE and revealed with anti-CxChap.

components from the same complex. Although the implication of the *N. gonorrhoeae* MIP in pathogenicity is clear (8), it's role in the pathogenesis of *N. meningitidis* disease is unknown and should be the subject of further investigation.

## Acknowledgments

This work was supported by grants PI050178 from the Fondo de Investigación Sanitaria (FIS, Ministerio de Sanidad y Consumo), and 2007/XAD63 from the Xunta de Galicia Spain.

## References

1. Marzoa J, Abel A, Sanchez S, Chan H, Feavers I, Criado MT, et al. Analysis of outer membrane porin complexes of *Neisseria meningitidis* in wild-type and specific knock-out mutant strains. Proteomics 2009 Feb 9(3):648-56.
2. Pannekoek Y, Schuurman IG, Dankert J, van Putten JP. Immunogenicity of the meningococcal stress protein MSP63 during natural infection. Clin Exp Immunol 1993 Sep 93(3):377-81.
3. Wittig I, Karas M, Schägger H. High resolution clear native electrophoresis for in-gel functional assays and fluorescence studies of membrane protein complexes. Mol Cell Proteomics 2007 Jul 6(7):1215-25.
4. Findlow J, Taylor S, Aase A, Horton R, Heyderman R, Southern J, et al. Comparison and correlation of *Neisseria meningitidis* serogroup B immunologic assay results and human antibody responses following three doses of the Norwegian meningococcal outer membrane vesicles vaccine MenBvac. Infect Immun 2006 Aug 74(8):4557-65.
5. Arakere G, Kessel M, Nguyen N, Frasch CE. Characterization of a stress protein from group B *Neisseria meningitidis*. J Bacteriol 1993 Jun 175(11):3664-8.
6. Qu J, Mayer C, Behrens S, Holst O, et al. The trimeric periplasmic chaperone Skp of *Escherichia coli* forms 1:1 complexes with outer membrane proteins via hydrophobic and electrostatic interactions. J Mol Biol 2007 Nov 374(1):91-105.
7. Pannekoek Y, Dankert J, van Putten JP. Construction of recombinant neisserial Hsp60 proteins and mapping of antigenic domains. Mol Microbiol 1995 Jan 15(2):277-85.
8. Leuzzi R, Serino L, Scarselli M, Savino S, Fontana MR, Monaci E, et al. Ng-MIP, a surface exposed lipoprotein of *Neisseria gonorrhoeae*, has a peptidyl-prolyl cis/trans isomerase (PPIase) activity and is involved in persistence in macrophages. Mol Microbiol 2005 Nov 58(3):669-81.