Discussion

In this work the cleavage event affecting the GNA2132 antigen was characterized.

GNA2132 is a surface-exposed lipoprotein identified by the reverse vaccinology approach during the development of a novel recombinant-based vaccine against *N. meningitidis*. It is able to evoke a strong bactericidal antibody response (Pizza et al., 2000; Giuliani et al., 2006) and for this reason has been included in the multi-component vaccine against MenB (5CMBV).

In this study we show that GNA2132 binds heparin and heparan sulphate when passed trough a heparin column and that the Arg-rich region determines the specific electrostatic interaction with these ligands. Our results demonstrate unequivocally the essential role the argine-rich site in the interaction of GNA2132 with heparin. In fact the binding was abolished when we delete the arginine rich region in the GNA2132dRR-his protein or mutate each single Arginine residues with an Alanine in GNA2132mRR-his demonstrating that the presence of the arginine rich-region is crucial for the GNA2132-GAGs interaction. Moreover, it's important to notice that this Arginine-rich motif is highly conserved between *N. meningitidis* strains, suggesting a key functional role in the pathogen.

Heparin binding by microbes was recently reported by Duensing as a novel strategy in microbial pathogenesis that complements the classical virulence strategy. This mechanisms enables different bacterial species that bind heparin and heparan sulfate to recruit structurally and functionally diverse bioactive host proteins, including adhesive glycoproteins (fibronectin and vitronectin), inflammatory and immunomodulatory mediators and grown factors (Duensing at al 1999). This allows the formation of a stable molecular bridge between heparin binding proteins present on the surfaces of most pathogens and mammalian heparin binding proteins. Consequently we can speculate that the GNA2132 protein could be function as a bridge between the bacterium and the host to permit an interaction with human cells. It is reported for the human pathogen *Neisseria gonorrhoeae* the binding with vitronectin without the involvement of a specific gonococcal vitronectin receptor. In this event, vitronectin binding is indirect and mediated through heparin or functionally related sulfated polysaccharides. These molecules form a stable

molecular bridge between the bacterial surface adhesin OpaA and vitronectin, both of which are heparin binding proteins.

Moreover, adhesive proteins have been implicated as intermediates in bacterial interactions with host cell integrin receptors, resulting in bacterial colonization and invasion of epithelial cells (Ljungh et al 1996). From our ongoing studies we showed that the GNA2132 antigen is able to interact with epithelial cells: *in vivo* this property could be due to the capability of the GNA2132 protein to bind the heparan sulfate exposed on epithelial cells. Glycosaminoglycan-binding microbial proteins may mediate adhesion of bacteria to eukaryotic cells, which may be a primary mechanism in mucosal infections (Wadstrom 1999), and are also involved in secondary effects such as adhesion to cerebral endothelia in cerebral malaria or to synovial membranes in arthritis caused by *Borrelia burgdorferi* ().

Endogenous heparin is an intracellular polysaccharide produced in human mast cells and stored in intracellular granules from which is released in the epithelial and sub-epithelial environment during the inflammatory response to infection. In an other way the affinity of GNA2132 to heparin could influence the early stages of meningococcal infection having a role during the colonization of the nasopharyngeal epithelium. It is known that heparin is able to bind several complement proteins (Sahu 1993) contributing to immobilization of complement components that are no longer capable of effectively killing bacteria. For example, C4b, in particular, has been shown to bind heparin effectively (Blom2002): this complement protein subunit can be captured on the surface of several pathogens, including *Neisseria gonorrhoeae, Bordetella pertussis* and *Escherichia coli*. The establishment of the GNA2132-heparin complex on the meningococcus cell surface could led to the heparin-mediated immobilization of complement components, contributing to bacterial resistence to serum and to increase its pathogenicity.

The GNA2132 antigen is a target of the meningococcal NalP (Neisseria autotransporter lipoprotein, also described as AspA). We showed that on the surface of meningococcus the NalP protease cleaves the GNA2132 antigen releasing a C-terminal fragment. By N- terminal sequencing and mass spectrometry we demonstrated that this released fragment posses the highly conserved Arginine-rich region.

NalP is reported to cleave surface exposed Neisseria virulence factors such as the IgA1 protease, the App adhesion (van Ulsen 2006), AusI (van Ulsen 2006) and MspA (Turner et al 2006). Beside van ULsen and coworker hypothesized that NalP cleavage could contribute to

the virulence of N. meningitidis, the relevance of NalP-mediated modulation on autotransporter secretion for neisserial virulence remains to be investigated.

Looking at a sequence alignment among different *N. meningitidis* strains (Pizza et al., 2000), the released fragment of GNA2132 corresponds to the most conserved part of the protein, while the most variable portion which is the N-terminal one remains associated to meningococcal outer membrane.

From previous studies performed in our laboratory we know that the GNA2132 protein is cleaved also by the human Lactoferrin. Human Lactoferrin belongs to the serine protease class and on *Haemophilus influenzae* acts cleaving and inactivating two surface exposed virulence factors (IgA protease and Hap) (REFs). The action of human Lactoferrin on GNA2132 generates a C-terminal fragment without the Arg-rich region.

From this we can summarize that the GNA2132 antigen is a target of two proteases: the meningococcal NalP and the human Lactoferrin, but the two proteases have a different specificity cleaving the antigen at two different sites. NalP cuts proximal to the Arg-rich region, while human Lf cuts distal this region. On the basis of these data it has been possible to propose a model of action of the two proteases on GNA2132 Neisseria surface-exposed antigen, which is schematically shown in the follow picture.

Considering the different nature of the two proteases involved in the GNA2132 processing there are several point of view to understand the double cleavage event on GNA2132.

Starting of meningococcus a possible interpretation might be that if the functional portion of the protein is localized at the C-terminal region and the action of NalP protease mediates its release in the melieu in order to favorite its activity. The released fragment might interact with the host assuming an interesting role in the pathogenesis of bacterium. In this way the N-terminal portion would be like an arm necessary for the right exposure of the protein and its cross-talk with NalP. NalP protease might also act on the full length protein in order to inactivate its function through the release of a fragment. Finally, NalP might also act unmasking a function in the N-terminal region of GNA2132 that is inactive when the protein is exposed as full-length. We have proven the cleavage of GNA2132 in *in vitro* growth conditions where we found that it is partial and some GNA2132 remains in the full length form. It is possible that in other conditions (e.g. during colonization or infection) NalP cleaves the protein with another kinetic.

Concerning the host protease, the lactoferrin is present in all human and in the nasopharinx play a major role in mucosal defense. At the first stay of meningococcal pathogenesis and carriage the human Lf could cleave GNA2132 releasing the more conserved fragment of the protein that might be the more known epitope for the immune system. This event might trigger the human immune response against bacterial infection. In the contrary this event might favor the bacterium that without its more known portion could result unrecognizable by the host immune system. Moreover in this scenario meningococcus might have the more active portion, the arginine rich-region, of the GNA2132 antigen more exposed to carry out its function.

Based on our results we can speculate that N.meningitidis use the GNA2132 exposed on its surface to interact with the host thought different ways: the contact with human lactoferrin lead to the protein cleavage and the binding to heparin and to heparan sulfate can be an useful tool to increase the bacteria pathogenicity. We can conclude that for these reasons the GNA2132 is an important virulence factor for meningococcus which might be forced to change with the NalP cleavage the nature of this protein in order to adapt itself in the delicate equilibrium between bacterium and its host.