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New Approaches for *Helicobacter* Vaccine Development – Difficulties and Progress

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Abstract

Despite the enormous progress in understanding the process of bacterial pathogenesis and interactions of pathogens with eucaryotic cells the infectious diseases still remain the main cause of human premature deaths. It is now recognized that *Helicobacter pylori* infects about half of the world's population. Based on results of clinical studies the World Health Organization has assigned *H. pylori* as a class I carcinogens. The review presents new achievements aimed at construction efficient and safe anti-*Helicobacter* vaccine. We discuss the new global technologies such as immunoproteomics employed for selecting new candidates for vaccine construction as well as new vaccine delivery systems. The review presents also our knowledge concerning *H. pylori* interaction with immune system which might facilitate modulation of the host immune system by specific adjuvant included into vaccine

Key words: *Helicobacter pylori*, adjuvant, delivery system, immunoproteomics, vaccine

Introduction

Helicobacter pylori has been an object of intense scientific studies, since its isolation from human stomach biopsies (Marshall and Warren, 1984). In the course of studies, it has been classified as a Epsilon-Proteobacteria and determined to colonize the gastric mucosa of humans. At present, *H. pylori* is recognized as a causative agent of chronic inflammation, chronic gastritis and peptic ulceration, and is considered to be a risk factor in the development of mucosa-associated lymphoid tissue lymphoma and adenocarcinoma of the stomach. Over half of the world's population (87% of Polish population) is infected with *H. pylori*, with the highest rates in developing countries (Marshall, 2003). Based on results of clinical studies, the World Health Organization has categorized *H. pylori* as a class I carcinogen. Although *H. pylori* infections are widespread, the disease develops only in a subset of infected humans. Considerable amount of gathered evidences indicate that the bacterial genotype is an important factor determining the type of induced pathology. Moreover, the nature and severity of the disease depend on both-host characteristics and environmental factors. Epidemiological studies have revealed a correlation between the age of infected individuals and the type of pathology (peptic ulcer or cancer) developed in consequence of infection (Blaser and Atherton, 2004; Hatakeyama and Brzozowski, 2006).

The 2005 Nobel Prize in Physiology or Medicine was awarded to B.J. Marshall and J.B. Warren for their studies on "the bacterium *Helicobacter pylori* and its role in gastritis and peptic ulcer diseases".

Main *H. pylori* virulence factors

Many virulence factors produced either by all or some *H. pylori* clinical isolates are involved in the development of disease symptoms. Among them, most extensively studied are various adhesins – responsible for bacterial adhesion to gastric mucosal cells, urease which neutralizes the acidic environment of the stomach, CagA which influences the host cell signal-transduction pathways, VacA – a vacuolating toxin that also modulates the activity of immune cells and NapA – a neutrophil-activating protein.

The *cagA* gene, encoding a highly immunogenic protein of an apparent molecular weight of 120–140 kDa, is located within the PAI pathogenicity island

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present only in genomes of certain *H. pylori* strains. The Cag protein, after injection into eukaryotic cells by the type IV transport system apparatus, associates with the plasma membrane and undergoes tyrosine phosphorylation by the host Scr kinase. Phosphorylated CagA interacts with many signal-transduction pathways operating within epithelial cells. These interactions result in alteration of cell morphology as well as proliferation and spreading. CagA is a highly polymorphic protein. Its polymorphism is mainly confined to the C-terminal region and depends on the number of EPIYA phosphorylation motifs as well as the amino acid sequence of the flanking regions (Western *vs.* East Asian strains). The *H. pylori* type IV transport system is also involved in stimulating the IL-8 production by epithelial cells through activation of the NF-kB transcription factor, but does not involve the CagA protein. More recent data indicate that the function of effector molecules, which are able to interact with the Nod receptors, is performed by the soluble parts of the bacterial peptidoglycan (Blaser and Atherton, 2004; Bourzac and Guillemin, 2005; Naumann, 2005; Radosz-Komoniewska *et al.*, 2005; Viala *et al.*, 2004).

The VacA toxin, classified to the autotransporter protein family, is an oligomeric protein, which undergoes extensive processing. The mature 88 kDa toxin is cleaved proteolytically into two subunits: P55 and P33. Both subunits are necessary for toxin activity. P55 is responsible for recognizing receptors on the surface of eukaryotic cells, whereas P33 is involved in pore formation. VacA is a multifunctional protein, which, apart from inducing cell vacuolization – that requires also the activity of many host cell protein, contributes to cell apoptosis in a mitochondrial-dependent manner. Additionally, the protein modulates the functions of immune cells and influences the activity of T lymphocytes, neutrophils, macrophages and mast cells. Depending of the target cells, the activity of VacA can have either an immunosuppressive or immunostimulatory effect (Cover and Blanke, 2005; Fischer *et al.*, 2004).

Although only 50% of *H. pylori s*trains are toxigenic, all contain the *vacA* gene. Yet, the *vacA*-encoding nucleotide sequences are extremely variable. This variability influences the activity of the toxin and is confined mainly to nucleotide sequences encoding signal peptides and the middle part of the protein. The most frequently isolated strains from patients suffering from severe disease symptoms are characterized by the s1/m1 *vacA* genotype (Cover and Blanke, 2005).

Urease is another important *H. pylori* virulence factor, which facilitates pathogen colonization of the gastric mucosa. The enzyme catalyzes production of ammonia and sodium dioxide from urea, which, in result, decreases the pH in the stomach. Ammonia also contributes to development of disease symptoms (Radosz-Komoniewska *et al.*, 2005).

Many proteins involved in postranlational modification of virulence factors also influence *H. pylori* pathogenicity. Some extracytoplasmic proteins of gram-negative pathogens which contain two or more cysteine residues gain their proper structure as a result of an insertion of disulphide bridges. The process is facilitated by a Dsb (disulfide bond) family of the redox proteins. It has been established that members of the Dsb family are essential for correct folding or assembly of a number of pathogenic determinants (Łasica and Jagusztyn-Krynicka, 2007). We identified and characterized a new subfamily of disulfide oxidoreductases encoded by a gene denoted *dsbI* (*jhp0542* gene of *H. pylori* J99, *hp0595* of *H. pylori* 26695 strain and *cj0017c* of *C. jejuni* strain NCTC11168). HP0595 and Cj0017c belong to the DsbI protein family, paralogous to the DsbB family (Raczko *et.al.*, 2005). The inactivation of the *dsbI* gene in *H. pylori* led to the accumulation of proteins with free thiol groups in the periplasmic space. It was documented that mutation in *dsbI* almost eliminates the ability of *H. pylori* to colonize mice intestinal tract, supporting the importance of disulfide bond formation process for infection (Godlewska *et.al.,* 2006).

Main issues concerning *H. pylori* vaccination need to be resolved

The mechanism of *H. pylori* pathogenesis and details regarding the interaction between this bacterium and the human immune system are still far from being understood. Many important issues await clarification. First, a decision should be made concerning protective antigen(s), the route of immunization as well as the antigen delivery system. Recently published experimental data questions the role of antibodies and indicates that T cells might play an important role in protection against *H. pylori.* In all likelihood, both – Th1– and Th2-dependent branches of specific immune responses are involved in the process. It cannot be excluded that stimulation of different immune responses is required for obtaining effective therapeutic or prophylactic vaccines (Aebischer *et al.*, 2005; Chmiela and Michetti, 2006; Prinz *et al.*, 2003). This raises the question concerning the type of adjuvant that should be included into the subunit recombinant vaccine regiment. Differently modified LT and CT toxins or CpG ODN and aluminum salts have been added to the tested vaccine prototypes against *H. pylori*. Two of them, alum and CpGs, were approved for use in humans. The evaluation of the vaccine efficacy *in vivo* demands also a reliable animal infection model. Most commonly, preclinical immunological studies

are performed in mice. Although preclinical studies have proven several therapeutic and prophylactic vaccine prototypes to be efficient in a murine infection model, they were ineffective when administrated to humans (Aebischer *et al.*, 2005; Prinz *et al.*, 2003).

When the paper by Lagergren (1999) was published, another doubt appeared. The authors have documented that the acid reflux in the esophagus is a risk factor for adenocarcinoma. Thus, the question arose whether there is a relationship between a recently observed, in developed countries, decrease of *H. pylori* infections resulting from commonly applied antibiotic therapy, and the increase in the number of diagnosed esophagus adenocarcinoma incidences (Lagergren *et al.*, 1999).

H. pylori infections are mostly acquired during childhood and persist for years. Yet, the target population for therapeutic and prophylactic immunization against *H. pylori* should, most likely, be different. In developed countries antibiotic therapy is recommended for individuals who suffer from clinical symptoms. As development of *H. pylori*-induced pathology and disease symptoms normally lasts for years, the target population for therapeutic vaccines ought to consist of adults mainly. Contrarily, prophylactic vaccines should be preferably administrated to preschool and school-age children, which raises some ethical considerations. Results from experiments conducted on neonatal mice with alum-adjuvanted vaccine suggested that this goal is feasible (Minoura *et al.*, 2003).

Classical whole-cell inactivated vaccines

Vaccines constitute the most cost-effective tool for prophylaxis of infectious diseases. Vaccination against *H. pylori* is believed to be more effective for individual patients than antibiotic therapy as well as decrease the number of infections at the population level and, in result, lead to pathogen eradication.

Active vaccines can be divided into three main categories: killed vaccines, attenuated vaccines and subunit vaccines. Formalin-killed *H. pylori* cells were orally administrated to infected and uninfected volunteers together with a mucosal adjuvant – a mutated form of the *E. coli* heat-labile toxin, known to stimulate both Th1 and Th2 immune responses. The vaccine induced production of specific antigen-secreting cells, but did not eradicate the pre-existing infection. Despite several positive results obtained during trials, especially in the case of uninfected volunteers, the enormous genetic diversity of clinical *H. pylori* isolates is a substantial obstacle in developing a whole-killed cells vaccine. Additionally, the tested vaccine induced among participants many serious side effects, *e.g.* diarrhea or fever (Kotloff *et al.*, 2001; Losonsky *et al.*, 2003).

Another possibility was demonstrated by Smythies *et.al* (2005) who evaluated the prophylactic potential of the *H. pylori* "ghost". Bacterial "ghosts" are cell envelopes of Gram-negative bacteria devoid of cytoplasmic content as a result of controlled cell lysis by the PhiX174 gene product cloned into the bacterial genome. This strategy was shown to give partial protection in an animal model; yet, requires certain improvements and further validation.

Subunit *Helicobacter* vaccines – antigen selection

Conventional methods used to identify vaccine candidates are based on the analysis of known pathogenic virulence factors in regards to their immunogenicity. Development of genetic engineering methods facilitated the construction of genomic libraries and allowed their screening with specific antibodies taken from patients or immunized animals. A current approach in identifying new, potential, subunit vaccine candidates is reinforced by the recent progress in sequencing of bacterial genomes. Genomes of two *H. pylori* strains – 26695 and J99 – have been sequenced recently (Alm *et al.*, 1999; Tomb *et al.*, 1997). Analysis conducted *in silico* as well as comparison of the genomic content of numerous clinical *H. pylori* isolates using the microarray technology revealed that their genomes possess 1111–1281 common genes (Gressmann *et al.*, 2005; Salama *et al.*, 2000). Among them, some will probably be considered as candidates for vaccine development. Common molecular techniques, *e.g.* microarray technology or proteomics, allow evaluating the diversity of bacterial genomes, analyzing sets of genes expressed *in vivo* as well as screening of all pathogen antigens and evaluating their application as potential vaccine candidates. It was documented that protective antigens represent only a small fraction of all antigens. In the case of *H. pylori*, only 10 antigens out of 400 examined revealed protection in preclinical experiments (Ferrero and Labigne, 2001). Abundant, surface-located, conserved and seroreactive antigens might be the most promising candidates in constructing an efficacious vaccine (Bumann *et al.*, 2004; Sabarth *et al.*, 2002). So far, only a few *H. pylori* antigens, selected by conventional methodologies, were extensively evaluated for their protective potential (CagA, VacA, HspA, NapA, catalase and urease subunit A and B). Although all of these antigens are immunogenic and some were protective in animal models (mice or gerbils), none of them fully protected humans. CagA and VacA, included in many vaccine

prototypes, are not conserved and are highly polymorphic. The *cagA* gene is part of a pathogenicity island which is missing, fully or partially, in some clinical isolates. The *vacA* gene is present in all genomes but is expressed at different levels. Nonetheless, type I *H. pylori* strains, expressing both CagA and VacA, are the causative agents of the most dangerous pathologies. So far, the majority of analyzed subunit vaccine candidates contained one or two antigens. However, recent studies indicate that including more antigens might increase vaccine efficacy. Additionally, the latest studies point to an enormous genetic diversity of *H. pylori* clinical isolates. Thus, comparison of different strains at the protein level seems to be of great importance (Aebischer *et al.*, 2005).

Immunoproteomics is a novel strategy combining standard proteomics with immunological screening and is currently the method of choice for identifying new antigens of diagnostic and protective values. It is proposed that highly specific antigens could be used as biomarkers of different pathologies induced by *H. pylori* infections, whereas novel, highly immunogenic, conserved, abundant and surface-located proteins could facilitate construction of an efficient anti-*Helicobacter* vaccine.

Although proteomics are considered useful in evaluating the total protein content in bacterial cells and in studying protein-protein interactions, they display some significant limitations. One of the major problems is the fact that low-abundant and hydrophobic membrane proteins are undetectable by standard methods.

More than 1800 *H. pylori* proteins from the total cell extract have bee resolved by 2-D electrophoresis by Baumann *et al.* and 384 out of them, being products of 290 genes have been identified (Bumann *et al.*, 2001b). Many studies have been conducted to analyze different *H. pylori* subproteomes – whole sets of surface-located or secreted proteins (Baik *et al.*, 2004; Bumann *et al.*, 2002). Backert *et al.* has compared subproteomes of soluble and structure-bound proteins of *H. pylori* 26695 and observed that some proteins known as extracytoplasmic, *e.g.* NapA or GGT, were detected also in the cytoplasm (Backert *et al.*, 2005). Proteomes of *H. pylori* growing under different conditions *in vitro* have been evaluated (Chuang *et al.*, 2005; Slonczewski *et al.*, 2000); yet, our knowledge about sets of proteins produced *in vivo* is still limited.

The *Helicobacter* sp. immunoproteome has been evaluated in many studies. The majority of analyses was performed with proteins isolated from sequenced strains (mainly the 26695 strain) resolved by 2-D electrophoresis and blotted with sera taken from *H. pylori*-infected patients, exhibiting different pathologies (Haas *et al.*, 2002; Krah *et al.*, 2003). In the course of numerous studies several antigens were detected; proving the reliability of the proteomics approach. In fact, each study led to the identification of new immunogenic proteins of potential prophylactic value. Some of them, including HP410 (putative neuraminyl-lactose-binding hemagglutinin homologue) or HP0231 (DsbA and DsbC homologue) were protective in the murine infection model (Sabarth *et al.*, 2002). On the other hand, *H. pylori* membrane proteins described earlier were found to be highly immunogenic, yet were not detected in any immunoproteomic analysis (Lpp or HpaA) (Bumann *et al.*, 2004). Data available on the *H. pylori* subproteome allowed Bumann *et al.* to carry out a comparative and comprehensive analysis that resulted in a list of 15 antigens-candidates for vaccine development (Bumann *et al.*, 2004). Recently, Mini *et al.* (2006) has indicated that identification of antigen(s) of high prophylactic value will still require many immunoproteomic studies, in which, not only sera taken from patients showing different pathologies, but also proteins derived from many *H. pylori* clinical isolates should be used.

Bacterial cells (attenuated pathogenic bacteria and lactic acid bacteria) and viruses as carriers for *H. pylori* antigens

Most pathogenic microorganisms are either restricted to mucosal membranes or need to cross them to achieve their proper infectious niche. For years it was considered that due to the apparent compartmentalization of the mucosal and systemic immune systems, vaccines administrated parenterally are less effective in protection against mucosal pathogens than mucosal immunization. However, more recent data indicates that the protective mechanism can be also stimulated parenterally. The effect seems to be dependent on the antigen delivery system and type of adjuvant used for immunization. Moreover, it should be noticed that although *H. pylori* colonizes gastric mucosa, vaccine prototypes administrated parenterally were also evaluated.

Delivery of vaccine antigens *via* the mucosal route can be carried out using different strategies. The best examined strategy used both attenuated and commensal microorganisms as bacterial carriers (Kochi *et al.*, 2003). Delivery of vaccine antigens by live bacterial cells has resulted in elucidation of both mucosal and systematic immune responses. Several attenuated *Salmonella* strains have been exploited as delivery systems for *H. pylori* antigens, mainly for urease subunits A and B (Angelakopoulos and Hohmann, 2000; Bumann *et al.*, 2001a). The antigens were expressed either as cytoplasmic- or surface-located proteins. Rhiozo *et al*. showed that exposure of UreA on the

surface of *Salmonella* cells by employing part of the *E. coli* adhesin – AIDA-1, greatly reduced the level of *H. pylori* colonization compared to cytoplasm-located UreA (Rizos *et al.*, 2003).

Recently Smythies documented that the genetically engineered polio virus can be employed as a carrier of *H. pylori* antigens (UreB) (Smythies *et al.*, 2005). Vaccination of mice with a replicon construct resulted in clearance of established *H. pylori* infection in 73% of animals compared to 31% of mice immunized with the vector alone.

A few attempts have been also undertaken to evaluate lactic acid bacteria potential as carriers for *H. pylori* antigens (Corthesy *et al.*, 2005; Hanniffy *et al.*, 2004).

DNA *vs.* antigen vaccination

DNA vaccination offers an attractive novel approach aimed at eradicating *H. pylori* infections or at least decreasing the number of severe disease cases. Several attempts have been undertaken to estimate the strategy potential of developing a vaccine against *H. pylori.* Plasmid DNA containing urease-, catalase- or heat-shock protein-encoding genes as well as genes encoding immunogenic proteins that derived from *H. pylori* genomic library was administered to mice by different routes (intramuscularly, subcutaneously or intranasally) (Dzwonek *et al.*, 2004; Hatzifoti *et al.*, 2006; Xu *et al.*, 2007). In some experiments, decrease in the bacterial load in the stomach and induction of the humoral immune response were noticed. Vaccination also resulted in an up-regulation of the IL-10 level, whereas detection of β-defensin in the stomach indicated that immunization modulates both innate and adaptive immune responses (Hatzifoti *et al.*, 2006).

Attempts to employ attenuated *Salmonella* strains for anti-*Helicobacter* DNA vaccination have been also undertaken. *Salmonella* is an enteroinvasive pathogen which can target plasmid DNA, carrying heterologous genes cloned under the control of eukaryotic promoters, to antigen-presenting cells (APC), specifically to dendritic cells (DC) – the major target cells processing the antigen. Expression of foreign antigens results in antigen presentation by class I MHC and stimulation of both – Th1 and Th2, T lymphocytes (Garmory *et al.*, 2002; Moll, 2004). *Salmonella* expressing *H. pylori hpaA* and *napA* genes cloned into eucaryotic expression vectors showed high immunogenicity when evaluated in murine model (Sun *et al.*, 2006; Xu *et al.*, 2005). Hatzifoti (Hatzifoti *et al.*, 2006) using urease B DNA vaccine pointed out the role of innate immune response in reducing *H. pylori* colonization of the murine gastric mucosa.

Human trials

The safety and immunogenicity of some vaccines have been tested in several clinical trials. Yet, none of the vaccines against *H. pylori* has progressed beyond phase 1 of clinical trails. Trials have been conducted with a whole-cell killed vaccine and recombinant *H. pylori* proteins (urease, CagA, VacA, NapA) administrated orally or parenterally (with or without adjuvant). The prophylactic potential of the recombinant *Salmonella enterica* sv. Typhi/Typhimurium strains, expressing *H. pylori* antigens, has also been analyzed. Malfertheiner *et al.*, 2002 obtained promising results in evaluating the immunogenicity of a combined vaccine consisting of three antigens (NapA, CagA and VacA). Intramuscular administration of the vaccine to human volunteers, using alum as an adjuvant, was highly immunogenic. Nonetheless, its efficacy still needs to be determined (Aebischer *et al.*, 2005; Kabir, 2007).

Recently Graham *et al.* (2004) developed a human challenge model of *H. pylori* infection which is regarded as a significant step for vaccine development. The model has served in evaluating the efficacy of the recombinant *Salmonella* Ty21a strain expressing the *H. pylori* urease A and B subunits. For the first time, oral administration of a vaccine prototype to human volunteers conferred protection, proving that development of a human *H. pylori* vaccine is a challenging but feasible goal (Kabir, 2007).

Conclusions

We are still a long way from developing a therapeutic and/or prophylactic vaccine against *H. pylori* for humans. Dr B. Marshall at the Keio Medical Price Symposium in 2002 said: “It is likely that to be at least ten more years before we can see a useful vaccine for *H. pylori*.” At present, five years later, we are almost sure that he was perfectly right. The development of an efficacious vaccine requires better understanding of the mechanism of protection and induction of postimmunization gastritis observed in the mouse infection model. Recently developed strategies allowing analysis of the transcriptome and proteome of eukaryotic cells are promising technologies in studying these issues, especially the *Helicobacter pylori* effect on the intracellular signal transduction in epithelial and immune cells. Immunoproteomics should enable screening a larger number of clinical isolates aimed at identifying new, conserved and seroreactive proteins. In the light of the recent studies, one can also expect that an efficacious vaccine for *H. pylori* should consist of various formulas administrated as a primary immunization and a booster. Such goal could be achieved using both antigens and DNA vaccination.

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