

Review

***Helicobacter pylori*: A Brief History of a Still Lacking Vaccine**

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Abstract: *Helicobacter pylori* colonizes the gastric mucosa of more than half of the human population worldwide. Soon after its discovery, the causative relationships between *H. pylori* infection and chronic atrophic gastritis, peptic ulcer and gastric mucosa-associated lymphoid tissue lymphoma were evidenced. Then, a significantly increased risk of developing gastric cancer was found to be associated with *H. pylori* infection. The efficacy of the treatment for *H. pylori*, based on a proton pump inhibitor plus antibiotics, has dropped below 80%, mainly due to antibiotic resistance. Vaccination would overcome antibiotic resistance and would lead to the eradication of this pathogen; however, in spite of almost twenty-five years of investigation on *H. pylori* vaccine candidates and good protective results obtained in animal models, no vaccine is currently licensed. This review focuses on the studies on the efficacy of those *H. pylori* vaccine candidates that underwent clinical trials. Efficacy trials have given unsatisfactory results, so far, with bacterial colonization remaining unaffected by vaccination. However, a vaccine able to counteract *H. pylori*-induced diseases, such as gastric cancer, even without providing sterilizing immunity, could be considered valuable.

Keywords: *Helicobacter pylori*; vaccine; animal models; efficacy

1. Introduction

The presence of bacteria in mammalian stomach has been reported since the late 19th century; nevertheless, only at the beginning of the 1980s did Barry Marshall and Robin Warren describe a bacterium isolated and cultured from human gastric biopsies [1], initially classified as *Campylobacter pylori* and, then, as *Helicobacter pylori* [2]. Soon after the discovery of *H. pylori*, its causative relationship with gastritis and peptic ulcer in humans was proven [3]. In 2005, Marshall and Warren received the Nobel Prize in Physiology or Medicine for their revolutionary discovery.

H. pylori is a curved or spiral-shaped, flagellated, microaerophilic, Gram-negative bacillus. It colonizes the gastric mucosa of more than 50% of human population, with much higher prevalence in developing than in developed countries [4,5], most probably as a consequence of different hygiene and living conditions.

H. pylori is generally acquired during childhood, within the family [6,7]. The colonization is long-lasting, commonly for the entire life. The most likely route of transmission is oral-oral and/or fecal-oral [8]. Moreover, since *H. pylori* was detected both in water and milk [9–11], these could represent possible further routes of transmission, although to date, there is not a formal demonstration of this hypothesis.

The *H. pylori* colonization remains asymptomatic in the majority of the subjects, but a subset of the *H. pylori*-infected population develops chronic gastritis, peptic ulcer or gastric mucosa-associated lymphoid tissue (MALT) lymphoma [3,12,13]. Moreover, *H. pylori* colonization was found associated with an increased risk of gastric carcinogenesis [14,15]: thus, the WHO has included *H. pylori* among the Category 1 carcinogens [16]. *H. pylori* is the only bacterium that has been clearly associated with the development of cancer to date [17]. In particular, *H. pylori* CagA (the product of cytotoxin-associated Gene A, *cagA*) has been shown to trigger many cancer-related signaling pathways *in vitro* and *in vivo*, as will be described more in detail below. Several studies have tried to define the possible association of *H. pylori* with other carcinomas: significant association was found with squamous cell laryngeal cancer and squamous cell cancer of the upper aerodigestive tract (excluding the esophagus), while for other carcinomas, to date, the results are controversial or the size of the studies too small to reach definitive conclusions [18]. *H. pylori* may also induce or worsen gastric autoimmunity through the activation of cytolytic CD4⁺ Th1 cells specific for *H. pylori* peptides cross-reactive with human H⁺, K⁺-ATPase: these cells infiltrate the gastric mucosa and may contribute to developing gastric atrophy [19,20].

The diagnosis of *H. pylori* infection in symptomatic subjects is generally followed by eradication therapy. The eradication causes the regression of *H. pylori*-induced peptic ulcer and MALT lymphoma [21] and may represent a tool for the reduction of gastric cancer incidence in populations at risk [22]. Current therapies against *H. pylori* are based on the use of one proton pump inhibitor plus two or more antibiotics for one–two weeks, with various approaches that also vary according to the geographic area [23–25]. Antibiotic-based therapy presents some drawbacks. If eradication is done at late stages of colonization, when pre-malignant lesions are already present, it could be too late for reverting the mechanisms of carcinogenesis already triggered [26]. The efficacy of the treatments has dropped even below 80% in some cases [27], mainly due to the increase of antibiotic resistance; thus, modifications in the combination and in the sequence of drug administration are continuously under

investigation [23,28]. Moreover, after treatment, recurrence and/or reinfection can occur [29], especially in developing countries [30].

Vaccination would represent a valid and cost-effective [31,32] alternative approach to overcome the problems with the antibiotic-based therapy.

2. Vaccination against *H. pylori* in Animal Models

Several animal models of infection with *H. pylori* have been described [33]; among them, the mouse model was the most exploited. These animal models allowed the production of a large body of data demonstrating the feasibility and the efficacy of vaccination against *H. pylori* [34,35].

Since *H. pylori* colonization occurs at the mucosal level, particular emphasis has been given to oral immunization, although other mucosal routes of immunization [36], as well as the parenteral route [35] or prime-boost regimens, have been considered [37–39]. Mucosal immunization requires the use of strong mucosal adjuvants, as proteins are poor immunogens when administered mucosally. Some of the strongest mucosal adjuvants presently known are bacterial toxins, such as cholera toxin (CT) and the *E. coli* heat-labile enterotoxin (LT), which, however, induce severe diarrhea in humans, seriously limiting their use; thus, non-toxic mutants of these molecules were developed to be used as mucosal adjuvants [40].

The feasibility of mucosal immunization was first demonstrated in mice immunized orally with bacterial lysates or inactivated whole-cell bacteria together with CT, LT or their non-toxic mutants [35], obtaining a high rate of protection against *H. pylori*. Presently, the use in humans of whole-cell based vaccines could be hampered by the presence of bacterial components that may induce unwanted responses, as, for instance, antigens that have homology with human ones. In particular, *H. pylori* expresses Lewis antigens identical to those occurring on the surface of gastric epithelial cells [41] and peptides cross-reactive with human H⁺, K⁺-ATPase [19,20]. Thus, the current research on an *H. pylori* vaccine is focused on recombinant protein antigens.

The efficacy of prophylactic and therapeutic immunization against *H. pylori* has been demonstrated for a variety of native or recombinant antigens, such as urease, heat shock proteins, VacA, CagA, HP-NAP, catalase [35], HpaA (*Helicobacter pylori* adhesin A) [42] and SOD (superoxide dismutase) [43]. Furthermore, DNA vaccines and different delivery systems, such as live *Salmonella* or poliovirus vectors, have been used in animals [44]. In most cases partial protection was observed, *i.e.*, gastric colonization was reduced, but not abolished, suggesting that higher efficacy could be achieved by combining more than one antigen. The most relevant non-clinical studies carried out with *H. pylori* antigens that subsequently were included in vaccines that underwent clinical trials are described in the next section of this review, dedicated to these antigens.

Recent studies attempted to identify further protective antigens in mice, such as Hp0410 (neuraminylactose-binding hemagglutinin HpaA homologue) [45], Tpx (thiol peroxidase) [46], outer membrane proteins [47] and alkyl hydroperoxide reductase [48].

Other studies attempted to use previously known protective antigens in novel forms (e.g., fusion proteins, portions encompassing defined epitopes, constructs epitope-based vaccines), demonstrating either therapeutic or prophylactic efficacy in mice [49–56].

All of these studies confirmed that protection against *H. pylori* can be achieved by vaccination in animal models; however, complete protection is rarely achieved, suggesting that the optimization of the antigen combination, adjuvant and route and regimen of immunization is still required. Moreover, efficacy in animals is not necessarily predictive of efficacy in humans; unfortunately, this seems to have happened so far with *H. pylori*, as highlighted in further sections of this review.

3. Non-Clinical Studies with the *H. pylori* Vaccine Candidates that Subsequently Underwent Clinical Trials

The studies in animals led to identifying some *H. pylori* factors relevant to the colonization and/or the disease, thus representing suitable vaccine targets. Some of these factors exerted protective efficacy when used as a vaccine in animal models. In the following paragraphs, the antigens that were included in vaccines that underwent clinical trials are described; the non-clinical studies that led to propose them as potential vaccine candidates in humans and thus to test them in clinical trials are also reported.

3.1. Urease

H. pylori colonizes a particularly harsh niche, due to the acidic gastric juices. *H. pylori* urease is required for bacterial colonization [57]; it catalyzes the conversion of urea to carbon dioxide and ammonia: carbon dioxide diffuses into the blood and is exhaled through the lungs, while ammonia forms ammonium hydroxide, which neutralizes the local acidity in favor of *H. pylori* survival. *H. pylori* urease consists of two moieties, UreA (27 kDa) and UreB (62 kDa), and constitutes up to 10% of the total bacterial protein content. The production of ammonia is able to produce histological damage in the gastric mucosa [58]. Moreover, urease may contribute to mucosal damage through local activation of inflammatory cells [59,60].

The first evidence of the protective efficacy exerted by urease immunization was provided in a mouse model of *H. felis* infection [61]: in this study, recombinant UreA or UreB vaccine was administered orally, and it was shown that both subunits were efficacious, though with different kinetics, in clearing the experimental colonization. Native, purified urease oral vaccine, including *E. coli* heat-labile enterotoxin (LT) as an adjuvant, was then assessed in a mouse model of *H. pylori* infection, and its protective efficacy was confirmed [62,63]. Other mucosal routes of vaccination (intranasal or rectal, besides oral) with recombinant urease were investigated, using LT as an adjuvant, achieving protection by all routes [64], as evaluated by the significant reduction of urease activity and bacterial load in vaccinated mice as compared with sham-immunized controls. In the same study, mucosal vaccination, and, in particular, the rectal route, was also found to induce specific mucosal IgA [64]. Systemic immunization with recombinant urease was then demonstrated to be protective in mice: urease was administered subcutaneously along with different systemic adjuvants, or orally with CT or LT, resulting in good protection with both routes [65]. Interestingly, this study also indicated that urease immunization achieved better protection if adjuvants that induced strong Th1/Th2 responses were used, in comparison with those inducing the Th2 response only.

The protective efficacy of urease was also assessed using systems of antigen delivery at the mucosal level by live, engineered *Salmonella*. The live attenuated *Salmonella typhimurium phoP^c* strain was engineered to express *H. pylori* urease and administered intranasally in mice; the specific Th1/Th2

response was elicited and significant protection against gastric colonization obtained [66]. A similar study was carried out using the UreA- and UreB-expressing attenuated *S. typhimurium* SL3261 strain, administered orally to mice: immunization elicited a specific humoral and mucosal antibody response and conferred complete protection [67].

The encouraging results obtained with urease in mice led to assessing its protective efficacy in non-human primates (reviewed in [68]). The immunogenicity of urease administered orally with LT was proven in squirrel monkeys (*Saimiri* species) [69], a model that, however, was found not suitable for a challenge study. Further studies were conducted in rhesus monkeys (*Macaca mulatta*), primates that naturally acquire *H. pylori* infection. In a first study, female, nine-month-old rhesus monkeys were selected on the basis of seronegativity to *H. pylori* sonicate and *H. pylori* urease, then orally immunized with five administrations of 8 mg of recombinant urease along with 25 µg of LT [70]. Vaccination elicited specific IgG and IgA in both serum and saliva. Ten months later, the number of individuals that had acquired *H. pylori* infection was significantly lower for the vaccinated group than for the control group: moreover, antral gastritis was lower in vaccinated than in control animals. A second study was conducted in *H. pylori*-infected rhesus monkeys, to assess the possible efficacy of therapeutic urease vaccination [71]. Animals received six oral administrations of 40 mg of recombinant urease plus 25 µg of LT: specific serum IgG and IgA, as well as salivary IgA, were induced by the vaccination, but no therapeutic efficacy was observed. This study was continued with the eradication of *H. pylori* by standard antimicrobial + omeprazole treatment, and then, monkeys received a further administration of vaccine: the subsequent experimental challenge with *H. pylori* showed that gastric colonization was significantly lower in vaccinated than in control animals, accompanied by a trend toward the reduction of gastric inflammation. This confirmed the feasibility of *H. pylori* prophylactic vaccination in primates. Another study in rhesus monkeys evaluated the efficacy of prime-boost vaccination strategy [72]. Animals were subjected to antimicrobial + omeprazole therapy, then received either four parenteral (intramuscular) vaccine administrations or one oral administration followed by three intramuscular boosts. The parenteral vaccine consisted of 100 µg of recombinant urease with either 100 µg of aluminum hydroxide (alum) or 600 µg of the synthetic glycolipid adjuvant *N*-[(2R,3R,4R,5S,6R)-3-[[[(2S)-2-amino-3-methylbutanoyl]amino]-4,5-dihydroxy-6-(hydroxymethyl)oxan-2-yl]-*N*-octadecyl dodecanamide (BAY-R 1005); the oral vaccine consisted of 4 mg of urease plus 100 µg of LT. After experimental *H. pylori* challenge, only animals vaccinated with the prime-boost regimen showed gastric colonization significantly lower than that of control animals that received placebo. A further study included *H. pylori*-negative rhesus monkeys that received either oral (four administrations of 4 mg of urease plus LT) or intramuscular vaccination with recombinant urease (four administrations of 100 µg of urease plus 600 µg of BAY R 1005) [73]. Serum IgG and salivary IgA were elicited by the vaccination; nevertheless, after experimental *H. pylori* challenge, no efficacy of vaccination against gastric colonization was observed. The lack of efficacy by parenteral vaccination observed in this study is consistent with a previous one [72]; as for the lack of efficacy of the oral vaccination, it must be noted that these results are not directly comparable with those of a previous study that found protective efficacy [72], as the dose of urease (4 vs. 40 mg) and the number of administrations (four vs. six) were quite different, and in addition, monkeys in the previous study were *H. pylori*-primed.

The results obtained with urease vaccination in non-human primates, although often resulting in only partial protection, provided indications on the antigen dose, adjuvant and regimen to be used in the subsequent trials conducted in humans.

3.2. Neutrophil-Activating Protein (HP-NAP)

HP-NAP consists of dodecamers of 17-kDa protein subunits [74,75]. It induces chemotaxis and both the direct and indirect activation of neutrophils and monocytes [76,77], with the production of oxygen radicals, and promotes neutrophil adhesion [78]. HP-NAP is now considered a crucial factor in driving the Th1 inflammation in *H. pylori* infection, due to its inflammatory and immunomodulatory properties [79].

The protective efficacy of HP-NAP was assessed in the mouse model [76]. Mice were orally immunized with recombinant HP-NAP along with LTK63, the non-toxic mutant of LT, as an adjuvant. Subsequent experimental challenge with *H. pylori* revealed protection against gastric colonization in the majority of vaccinated mice. No further reports on the protective efficacy of HP-NAP alone are available. HP-NAP was used in non-clinical studies in combination with other *H. pylori* antigens, as described in the last subsection of this section, “Multi-Component Vaccines”.

3.3. Vacuolating Cytotoxin (VacA)

In early studies, it was found that *H. pylori* culture supernatants were able to induce vacuolization on several epithelial cell lines [80]. Then, the cytotoxin responsible for this activity was purified and characterized [81]. VacA toxin is now among the best characterized *H. pylori* molecules. It is composed by hexa- or heptamers of 88-kDa subunits [82,83]. Each subunit consists of two moieties, p33 and p55, both required for exerting the toxicity: p55 is necessary for binding to target cells, while p33 is essential for the internalization of the toxin [84]. The VacA sequence is well conserved among different isolates, but with some allelic variations: in the signal (s) region (alleles s1a, s1b, s1c and s2), in the mid- (m) region (alleles m1 and m2), localized in the p55 domain, and in the intermediate (i) region (alleles i1 and i2), localized in the p33 domain [85,86]. The m1 allele associates most frequently with s1 allele: this s1m1 form of VacA is related to the most severe pathological outcomes, including gastric cancer [87].

Oral immunization with native purified VacA given along with LT as an adjuvant was shown to be protective in mice [62]. A further study confirmed the prophylactic protective efficacy of immunization with both native and recombinant VacA, administered intragastrically along with LTK63 [63]: in this study, it was also demonstrated that the whole VacA molecule was required to achieve protection, as the two VacA moieties individually expressed, purified and used as an immunogen were unable to elicit significant protection against experimental *H. pylori* challenge. In a study of therapeutic vaccination, animals were infected with *H. pylori* and then received intragastric immunization with recombinant VacA plus LTK63: a high rate of non-infected mice was observed in vaccinated mice, demonstrating the feasibility and the efficacy of therapeutic vaccination [88]. VacA was then used in non-clinical studies in combination with other *H. pylori* antigens, as described in the last subsection of this section, “Multi-Component Vaccines”.

3.4. Cytotoxin-Associated Gene (*cagA*)

Early studies on *H. pylori* virulence determined the classification of the strains as type I or type II, based on the presence or the absence, respectively, of the cytotoxin-associated gene pathogenicity island (*cag* PAI) in their genome. Type I constitutes the majority of *H. pylori* clinical isolates, associated with the more toxic s1 allele of VacA, and with the most severe outcome of the infection [89–91]. Type II strains are *cag* negative, most frequently associated with the less toxic s2m2 type of VacA, and induce mainly asymptomatic gastritis [92].

Cag PAI encompasses several genes, including *cagA*, which encodes the CagA protein [93,94], and the components of a type IV secretion system (T4SS) [94–97]. T4SS, through its component, CagL, binds the $\alpha 5\beta 1$ integrin on the host cell surface and translocates CagA, which is then phosphorylated by the host's enzymes [98,99]. CagA is also able to bind the host $\beta 1$ integrin or phosphatidylserine to mediate its own internalization [100,101]. The translocation and phosphorylation of CagA are followed by the induction of IL-8 production by epithelial cells, the activation of NF- κ B, the remodeling of the cytoskeleton, the formation of cellular pedestals and the induction of abnormal cell proliferation [93,94].

The CagA properties led to defining it as an oncoprotein [100,102], explaining its association with the most severe pathological outcome and giving suggestions on the early mechanisms of *H. pylori*-induced carcinogenesis. Besides the studies that linked CagA to the activation of oncogenic pathways, its effects on tumor suppressor pathways have been also reported [103–105], confirming the strong relationships between the infection with type I *H. pylori* strains and the risk of developing gastric cancer.

Recombinant CagA administered intragastrically to mice along with LTK63 was found to be protective against gastric colonization upon subsequent *H. pylori* experimental intragastric challenge [63,88]. It was also shown that a recombinant protein encompassing a short fragment of CagA that contains immunodominant epitopes in humans was ineffective in eliciting protection in the mouse model, suggesting that a full-length molecule should be used to maintain protective efficacy [63].

CagA was further used in non-clinical studies in combination with VacA and HP-NAP, as described in the last subsection of this section, “Multi-Component Vaccines”.

3.5. Multi-Component Vaccines

The combination of CagA, VacA and HP-NAP was assessed for its efficacy as a therapeutic vaccine [106] in the model of *H. pylori* experimental infection of beagle dog that was previously set up [107]. The dog model presents some advantages as compared with the murine one, in particular those related to the body size, which allows taking gastric biopsies repeatedly from the same animal, without sacrificing it, thus providing a more reliable time course of the evolution of both *H. pylori* colonization and gastric inflammation. Previous, unpublished results indicated that the combination of these antigens was protective as a prophylactic vaccine administered parenterally [106]. For the therapeutic study, dogs were infected by intragastric administration of *H. pylori* and then vaccinated intramuscularly with the CagA + VacA + HP-NAP recombinant proteins along with aluminum hydroxide as an adjuvant, giving three immunizations, either one week or one month apart. No side-effects

due to immunization were observed. In vaccinated dogs, the reduction of both *H. pylori* colonization and gastric inflammation was achieved as compared with the controls. Both regimens appeared similar in terms of *H. pylori* eradication, while the monthly regimen appeared slightly superior in reducing gastritis. A specific IgG response was elicited against each of the vaccine components.

4. Vaccination against *H. pylori* in Humans

Although a very large number of studies of *H. pylori* vaccination have been conducted in animals, achieving protective results, very few clinical studies have been carried out so far in humans, and no vaccines are currently licensed.

Several of the trials conducted focused on the use of the most abundant *H. pylori* antigen, *i.e.*, urease. In addition, with one exception, all trials were carried out using the mucosal route of immunization or delivery. The number of clinical trials with these mucosal vaccines has been limited also due to the still incomplete optimization of mucosal adjuvants for human use [108] and of their formulation. A model of experimental *H. pylori* infection in human volunteers has been set up; it is useful not only for studying the vaccine efficacy in humans, but also for better understanding *H. pylori* pathogenesis [109].

4.1. Inactivated Whole-Cell Vaccines

The efficacy of inactivated whole-cell vaccines against *H. pylori* has been shown in a large number of studies carried out in animals immunized either mucosally or parenterally [35]. Whole-cell vaccines offer the advantage of eliciting immune responses against a wide variety of antigens; however, they may include potentially dangerous components of the bacterium, such as those sharing homologies with the self-antigens and potentially able to induce autoimmune responses, *e.g.*, *H. pylori* lipopolysaccharide (LPS), which mimics human Lewis antigens [110], or, as already mentioned, *H. pylori* peptides cross-reactive with human H⁺, K⁺-ATPase [19,20].

A formalin-inactivated whole-cell *H. pylori* vaccine was evaluated in a phase I trial in both *H. pylori*-negative and *H. pylori*-positive subjects [111]. The vaccine, containing various amounts of bacterial cells, was given orally three times, on Days 0, 14 and 28, together with 25 µg of the LTR192G mutant of the LT toxin. The first part of the trial was an open-label, dose-response study in which *H. pylori*-infected or -uninfected individuals received 2.5×10^6 to 2.5×10^{10} inactivated bacterial cells together with the LT mutant. Vaccination elicited *H. pylori*-specific antibody responses only in subjects receiving the highest dose of the vaccine. A marginal increase of IgA and IgG titers was observed only in *H. pylori*-positive patients. The number of specific antibody-secreting cells induced by the vaccine remained negligible; however, some detectable responses were observed at the duodenal level in *H. pylori*-negative subjects [112]. While some antibody response was induced only in *H. pylori*-positive patients, proliferative responses of peripheral blood mononuclear cells and the production of IFN-γ were observed only in uninfected volunteers who had received the highest dose of the vaccine, following *in vitro* re-stimulation with a bacterial sonicate (and not with a purified antigen). The second part of the trial was a randomized, double-blind study in which *H. pylori*-positive individuals received either 2.5×10^{10} bacteria plus 25 µg of LTR192G or placebo plus 25 µg of LTR192G. Vaccinated subjects had significantly higher IgA antibody titers in the stools than subjects

receiving the placebo. The co-administration of the vaccine with the adjuvant influenced only marginally the serum antigen-specific IgA antibody response. Vaccination of *H. pylori*-infected patients did not achieve bacterial eradication, since in both parts of the trial, the [^{13}C]urea breath test remained positive up to 7.5 months after vaccination [111]. It is not known, however, whether vaccination affected the degree of *H. pylori* gastric colonization, since no microbiological data were reported in this study. Finally, diarrhea occurred in five out of 18 subjects vaccinated with the highest dose of bacteria plus the LTR192G mutant and in one out of three who received the LTR192G mutant. Conceivably, diarrhea was due to LTR192G, which retains partial toxic activity *in vitro* and *in vivo* [113].

4.2. Vaccination with Purified Recombinant Urease

In the first study conducted with recombinant urease as a vaccine, *H. pylori*-infected volunteers were orally immunized with high doses of recombinant urease (20, 60 or 180 mg once weekly for four weeks) together with 5 μg of LT [114]. Diarrhea was observed in 16 out of 24 of the vaccines, regardless of the dose of urease, thus conceivably due to LT toxicity. Volunteers receiving the highest doses of urease (60 or 180 mg) showed high levels of anti-urease IgA serum antibodies. In spite of a significant decrease of gastric colonization being observed, neither eradication nor a decrease of the gastric inflammation was achieved.

A subsequent trial attempted to reduce the side effects of the vaccine by decreasing the amount of LT administered [115]. In this study, 42 healthy *H. pylori*-negative subjects were immunized orally with 60 mg of recombinant urease either in soluble or acid-resistant, encapsulated form together with 2.5, 0.5 or 0.1 μg of LT. The vaccine was given on Days 1, 8, 28 and 57. The subjects who received the highest dose of LT showed a slightly better urease-specific antibody response and an increase of CD4^+ , CD45RO^+ and CD69^+ cells; however, diarrhea was evident in 50% of the subjects of this group. These data confirm, as already known from *in vitro* assays and animal studies, that LT toxicity, immunogenicity and adjuvanticity are dose-dependent and that a fine tuning is required in order to induce protective immune responses against the vaccine without causing unacceptable side effects, such as diarrhea.

Another attempt, made to circumvent the safety issues inherent to the use of the oral administration of wild-type LT, has been made by replacing the oral with the rectal route of vaccination, which had been previously successfully used in mice [64]. Furthermore, in this study, recombinant urease was used at a dose of 60 mg, administered as a rectal enema to 18 healthy, *H. pylori*-negative adults, together with either 5 or 25 μg of LT, given three times on Days 0, 14 and 28 [116]. A strong systemic antibody response to LT was detectable in the majority of the vaccines, mainly in the group of subjects receiving the lowest dose of LT (5 μg). Only a small minority of subjects developed systemic anti-urease IgG or IgA antibody responses. However, no anti-urease or anti-LT IgA antibodies were detectable in stool or in salivary samples. Finally, the urease-driven proliferative response and IFN- γ production were negligible.

All of these studies clearly show that oral immunization with recombinant antigens, urease in particular, requires the use of strong and safe mucosal adjuvants to be given at doses sufficiently high to exert their adjuvant effects, but still unable to induce unwanted effects, such as diarrhea. Both the

oral and the rectal routes require the development of appropriate formulations and regimens able to induce adequate protective immune responses to *H. pylori*.

4.3. *Salmonella*-Vectored Urease

Several clinical trials have been conducted so far using recombinant *Salmonella* strains as a live vaccine vector for *H. pylori* urease. Unfortunately, in marked contrast with the results previously obtained in mice, all of these attempts resulted in poor immunogenicity and efficacy. Nevertheless, the most recent trials provided some useful information relevant to the possible cell-mediated mechanisms of protections.

In the first study with *Salmonella*-vectored urease, one or two oral administrations of 10^{10} CFU of a *Salmonella enterica* serovar Typhi strain, attenuated by the deletion of the *phoP/phoQ* virulence regulon and expressing *H. pylori* urease, induced mucosal and systemic immune responses to *Salmonella* antigens, while no detectable responses to urease were observed, even after an oral booster dose of recombinant urease plus wild-type LT [117].

Slightly better results were reported in a subsequent study, in which six volunteers were immunized orally (5 to 8×10^7 CFU) with *S. enterica* serovar Typhimurium harboring the same *phoP/phoQ* deletion [118]. Only one of the six volunteers had detectable urease-specific IgA antibody secreting cells; two others had slight amounts of urease-specific antibodies produced *in vitro* by cultured peripheral blood mononuclear cells; two subjects had some specific serum IgA antibodies detectable by ELISA, but not by western blotting.

Another study was carried out in volunteers immunized orally with *S. enterica* serovar Typhi Ty21a expressing *H. pylori* urease. None of the nine vaccinated volunteers showed any detectable antibody response against urease, but five subjects developed cellular response [119]. Similar results were obtained in a further study, in which five out of nine vaccinated subjects that were pre-immunized with the carrier strain developed cellular, but not antibody, response against urease; however, in this study, vaccinated subjects that were not pre-immunized with the carrier strain did not develop any kind of specific immune response [120]. In one of the latest studies with *Salmonella*-vectored vaccine, which included the experimental challenge of volunteers, a small number of subjects cleared the infection, but protection was not related to vaccination, as it was similarly distributed between vaccines and controls [121]. In these subjects, a specific T-cell response was observed that could be helpful in understanding the mechanisms of protective response.

It appears that *Salmonella*-vectored *H. pylori* urease vaccines still require optimization, in particular in terms of the increase of urease expression [120]; also, co-expression of an adjuvant or immunostimulatory, as shown in mice [122], could increase the efficacy of this vaccine.

4.4. Multi-Component Vaccines

The only multi-component vaccine against *H. pylori* that underwent clinical trial so far consisted of recombinant VacA, CagA and HP-NAP. Differently from the *H. pylori* vaccines described above, this vaccine was administered parenterally rather than mucosally. The safety and the immunogenicity of this multicomponent vaccine were evaluated in human volunteers. *H. pylori*-uninfected individuals were immunized intramuscularly three times, following three different immunization regimes, with a

vaccine consisting of either 10 or 25 µg each of CagA, VacA and HP-NAP, plus aluminum hydroxide as an adjuvant [123]. This vaccine was extremely safe and highly immunogenic, inducing antibody and cellular responses to the three antigens. Months after the last immunization, most of the subjects had still detectable antibody responses to each of the three antigens. Interestingly, parenteral vaccination with VacA, CagA and HP-NAP induced very strong and sustained antigen-driven cellular proliferative responses and IFN-γ production, as well as strong and long-lasting immunological memory [123].

5. Reasons that May Limit the Development of Vaccines against *H. pylori*

No further results on clinical trials of *H. pylori* vaccines, and, in particular, of efficacy trials, have been disclosed in the recent literature. Presently, there is not any licensed anti-*H. pylori* vaccine.

There are several reasons, besides the disappointing results of the efficacy trials conducted with urease-based vaccines, that, currently, the development of a *H. pylori* vaccine seems to be discontinued (reviewed in [124,125]).

A major issue is the still incomplete knowledge of the mechanisms behind protective immunity against *H. pylori*. The host immune response against *H. pylori* will be not treated in the present review; several updated reviews are available that extensively describe the abundance of the immunomodulatory effects of *H. pylori* on the host immune system [124–132].

The majority of non-clinical studies resulted in a significant decrease of bacterial colonization rather than complete, sterilizing protection. This extent of efficacy against experimental infection in animals could be insufficient when translated to human infection. Moreover, ideally, an *H. pylori* vaccine would be both prophylactic and therapeutic, given the high rate of the currently infected population. Therefore, further research is needed to understand the protective mechanisms and to identify vaccine formulations able to prevent and cure the infection.

The current lack of immunological correlates of protection for *H. pylori* constitutes *per se* a severe drawback for the further development of a vaccine, as the vaccine efficacy could only be proven by appropriate phase III clinical trials. This would be considered by companies as too expensive and risky.

A further element that may limit the development of an *H. pylori* vaccine is represented by reports suggesting some beneficial roles of *H. pylori* colonization for the host, as, for instance, the reduction of the risk of developing allergic and chronic inflammatory disorders [133,134]. The beneficial effects would be exerted in the first part of the host's life, while detrimental effects start to appear over 50 years of age [133]. If, on the one hand, these observations represent an interesting scientific field that deserves further investigation, on the other hand, most of them still appear controversial [135]. Similarly, some epidemiological studies suggested the reduction of the risk of developing esophageal adenocarcinoma for *H. pylori*-infected subjects, but the results of other studies were in contrast with this hypothesis [18]. It is now evident that the highest risk of developing the most severe disease upon *H. pylori* infection is related to the combination of both particular pathogen characteristics (such as, for instance, the *cag* PAI presence) and host genetic background (such as specific polymorphisms of inflammation-related genes) [136,137]; although, it is clear that, when such a combination of *H. pylori* virulence factors with particular host susceptibility occurs, *H. pylori* infection can eventually lead to the development of gastric cancer, the feeling that it could also provide the host with some advantages could adversely affect compliance with a vaccination campaign.

6. Conclusions and Perspectives

Although vaccination against *H. pylori* was successful in animal models with various antigens and administration routes, the disclosed results of efficacy trials in humans have been disappointing so far, resulting in similar bacterial colonization for both vaccinated and placebo groups.

Rather than abandoning hope of having a vaccine against *H. pylori*, in the frame of all of the above considerations, an objective different from that of obtaining sterilizing immunity could be now proposed for *H. pylori* vaccination, which currently seems unlikely to be achieved. Since some *H. pylori* factors have been already well characterized and its particular dangerousness for the host has been proven, a vaccine specifically targeting those factors could be proposed: such a vaccine should be aimed at affecting *H. pylori*-induced disease rather than bacterial colonization. In other words, a vaccine against *H. pylori* able to prevent gastric cancer, even without providing sterilizing immunity, would be a valuable vaccine. This could be the case for CagA, whose relationships with *H. pylori*-induced carcinogenesis are now well documented [104].

The ongoing studies on *H. pylori* pathogenesis may also help in identifying novel vaccine targets. One promising aspect is the study of T-regulatory (Treg) cells. Treg cells increase in the gastric mucosa of *H. pylori*-infected subjects. This suggests the involvement of these cells in suppressing mucosal immune responses, therefore contributing to the infection persistence and to the modulation of *H. pylori*-induced gastritis [138–142]. A study in a neonatal mouse model suggested that, while in adults, the suppression of immune response to *H. pylori* infection may lead to gastric disease, in neonates, the same mechanism induces tolerance and may prevent the development of gastric disease and preneoplastic lesions [143]. This relatively recent finding of the involvement of Treg cells in the development of *H. pylori*-related gastric diseases may help to focus the studies also on the *H. pylori* factors that drive the Treg activation, as potential vaccine targets.

The research on novel possible vaccine candidates against *H. pylori* is still active, as well as studies on more efficacious adjuvants, regimens and routes of administration. Moreover, the identification of novel vaccine candidates may benefit from “reverse vaccinology” and “-omics” approaches [144,145], which can exploit the availability of the complete genome sequence of *H. pylori*.

Conflicts of Interest

The authors declare potential conflicts of interest, being Novartis Vaccines employees.

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