Open Access

Immunogenicity of Recombinant *Helicobacter pylori* Urease B Administered by Various Routes and with Different Adjuvants‡

Rodolfo E Bégué[1,2,*] and Alyson Moll[2]

[1]*Department of Pediatrics, Louisiana State University Health Sciences Center and* [2]*Research Institute for Children, New Orleans, Louisiana*

Abstract: Because of the high prevalence of *Helicobacter pylori* infection and the morbidity and mortality associated to the disease, development of a preventive vaccine has become a priority. To this goal, we produced recombinant *H. pylori* urease B (rUreB) and tested its immunogenicity in BALB/c mice when administered as 3 doses (week 0, 4 and 6) by either parenteral (intramuscular) or mucosal routes (intragastric, intranasal, intrarectal) and with the use of various adjuvants (none, CpG, alum or Freund's). The intramuscular route was more immunogenic than any mucosal route; of the mucosas, only intranasal induced modest levels of serum IgG. All adjuvants improved the seroresponse to plain rUreB and, of them, Freund's and alum were equally good and better than CpG ODN 1826. Stool IgA was barely detected by any immunization strategy.

Key Words: Bacterial vaccine, mucosal vaccine, helicobacter pylori, alum.

INTRODUCTION

Helicobacter pylori is one of the most common chronic bacterial infections affecting at least half of the world's population. Of the infected individuals, approximately 10% and 1% will develop peptic ulcer disease or gastric cancer, respectively, translating in marked morbidity and mortality [1]. The potential value of antibiotic treatment for this infection is undermined by the enormous number of people that would need to be treated, the frequent occurrence of re-infection and the emergence of antibiotic resistance [2]. Hence, the development of a protective vaccine has become a priority.

Choosing what could be the right immunogen to be included in a vaccine is not obvious, though, since the natural course of *H. pylori* infection is one of persistence despite a strong immune response by the host [3]. Still, the fact that a post-infection immune response is not able to clear the infection does not necessarily negate the possibility that pre-infection immunity may prevent acquisition of a new infection. In fact, experimental animal data suggest that oral administration of *Helicobacter* specific antibodies may be effective to prevent [4] as well as to treat *Helicobacter* infection [5]. A number of researchers are working in the development of a vaccine to prevent *H. pylori* infection, and of the various candidate antigens, the most promising is the B subunit of the urease protein (urease B) [6]. The choice of urease as a target for immunization is based on the facts that this protein is exposed to the surface of the cell membrane, it frequently elicits an immune response [7], and its activity (likely by counteracting the gastric acidity) is crucial for the survival of this bacterium, as shown by the finding that urease-deficient *H. pylori* mutants fail to colonize the gastric mucosa [8]. Urease is a protein complex and, of its various components, the B subunit is most important for immunogenicity. Monoclonal antibodies with the most anti-urease activity *in vitro* have been mapped to epitopes in urease B [9] and immunization of mice with purified urease B has resulted in better immunogenicity and protection as compared to the use of urease A [10].

Consequently, our group has prepared and purified recombinant urease B (rUreB) expressed in an *Escherichia coli* system and a DNA vaccine based on the whole *ureB* gene. We previously reported that when administered parenterally rUreB was highly immunogenic while *ureB* was not [11, 12]. As a follow-up study to better define the potential value of rUreB as a candidate vaccine against *H. pylori* we proceeded to compare the immunogenicity of parenterally administered to mucosally administered rUreB and the effect of different adjuvants.

MATERIALS AND METHODOLOGY

Animal experimentation protocol was reviewed, approved and supervised by the Institutional Animal Care and Use Committee of the Research Institute for Children, Children's Hospital, New Orleans, LA. SPF BALB/c mice were purchased from a commercial vendor (Harlan Sprague Dawley, Indianapolis, IN), housed in individually ventilated HEPA filtered cages, 5 animals per cage, fed regular chow and water and handled under BSL-2 conditions. Animals were tested baseline (blood and stool) to confirm they were free of *Helicobacter spp* infection and all animals in each cage received the same immunization regimen.

We have previously described the making and characterization of rUreB [12]. Briefly, the full length *ureB* gene was amplified using *H. pylori* genomic DNA (ATCC 43504D) as template, cloned into the SalI site of the pQE9 vector

*Address correspondence to this author at the Children's Hospital, 200 Henry Clay Avenue, New Orleans, LA 70118; E-mail: rbegue@lsuhsc.edu

‡The work is partially funded by NIH NIH "Public Access Policy".

1875-0354/09 2009 Bentham Open

(Qiagen) and used to transfom XL10Gold *E. coli* cells. Protein expression was induced with isopropylthio-β-galactoside (IPTG), purified by affinity to the $(His)_6$-tag (Ni-NTA Superflow Column, Qiagen), concentrated to 1 μg/μl in phosphate buffer saline and filter-sterilized.

Immunization

Six-week old mice (5 per group) were immunized three times at 0, 4 and 6 weeks. Two simultaneous experiments were performed. One to compare rUreB by different routes (intramuscular, intragastric, intrarectal, and intranasal) and another experiment to compare different adjuvants added to intramuscular rUreB. Intramuscular administration used a 23G needle injection in the rear legs; intragastric administration was done by gavage with an olive-tip 20G feeding needle (Fine Science Tools, Inc) with antacid solution (2% sodium bicarbonate), intrarectal inoculation was done by insertion of olive tip needle into rectum and slow instillation, and intranasal inoculation used a 10-μl pipette tip and slow dripping of solution into alternating nostrils. For intrarectal and intranasal (but not intragastric) inoculation mice were under light anesthesia with 3% vol/vol isoflurane. Adjuvants used were CpG ODN 1826 (5' – TCC ATG ACG TTC CTG ACG TT – 3'), 2% aluminum hydroxide (alum) and Freund's adjuvant (Complete for first dose and Incomplete for subsequent doses). Doses and volumes are shown in Table **1**.

Immunogenicity

Four weeks after the 3rd dose of immunization blood and stool were obtained from each mouse to determine anti-ureaseB immunoglobulin (Ig) G and IgA antibodies. Blood (100 μl) was obtained by puncture of the saphenous vein and serum separated by centrifugation. Stool (2 pellets) were dissolved by vortexing in 100 μl sterile phosphate buffer saline, incubated for 15 minutes at room temperature, centrifuged and the supernatant collected. Anti-urease B specific antibodies were determined by an enzyme-linked immunosorbent assay (ELISA) as previously described by our group and using rUreB expressed in a yeast system (*Saccharomyces cerevisiae*) as capture antigen and isotype-specific antibodies (IgG and IgA) [12]. Serum was initially tested at 1:50 and stool at 1:10 dilution. Reactive specimens were then serially diluted 2-fold and the titer determined as the reciprocal of the last dilution with an OD $\geq$ 5 times the negative specimens (unimmunized mice). The Geometric Mean Titer (GMT) was then calculated for each immunization group.

Statistics

GMT values between immunization groups were compared by calculating the non-parametric Mann-Whitney U test. For the purpose of GMT calculation, specimens non-reactive at the initial screening dilution, 1:50 for serum and 1:10 for fecal specimens, were assigned a value of 25 and 5, respectively.

RESULTS

The immunogenicity of rUreB for each vaccination group, expressed as the GMT (and range of values) is shown in Table **2**.

In the first experiment, we compared the immunogenicity of rUreB administered by different routes; the results for this experiment include the control and the first 4 immunization groups shown in Table **2**. As expected the control group showed no antibodies, a validation of our ELISA test. Of the various routes, intramuscular administration of rUreB elicited the highest levels of antibodies ($p<0.01$, as compared to control and the three mucosal groups); mostly detected in serum and mainly of the IgG-subtype. Of the mucosas, only intranasal administration resulted in some level of antibodies ($p<0.01$, as compared to control and the two other mucosal groups), but still the levels were significantly lower than by the intramuscular route and they were mostly systemic IgG and not gastrointestinal IgA. The intragastric and intrarectal administration failed to elicit any detectable antibody.

In the second experiment we further studied parenteral rUreB and evaluated the effect of different adjuvants in its immunogenicity. The data for this second experiment include the control and the last 4 groups of Table **2**. As noted, rUreB with no adjuvant was by itself modestly immunogenic ($p<0.01$, as compared to the control group)

Table 1. Immunization Groups Included in the Study, Indicating the Amount of Immunogen and Adjuvant Utilized and the Total Volume Administered

Immunization Group	rUreB	Adjuvant	Volume
Control	None	None	0
Intragastric	160 μg	CpG 40 μg	200 μl
Intrarectal	80 μg	CpG 20 μg	100 μl
Intranasal	40 μg	CpG 10 μg	50 μl
Intramuscular (rUreB + CpG) *	80 μg	CpG 20 μg	100 μl
rUreB alone *	100 μg	None	100 μl
rUreB + alum *	50 μg	alum 50 μl	100 μl
rUreB + Freund's *	25 μg	Freund's 25 μl	50 μl

*: rUreB and adjuvant administered by intramuscular injection.

Table 2. Immunogenicity of rUreB for the Different Groups (5 Animals Per Group), and Expressed as the Geometric Mean Titer (Range)

Immunization Group	Serum IgG	Stool IgA	Stool IgG
Control	<50 (<50)	<10 (<10)	<10 (<10)
Intragastric	<50 (<50)	<10 (<10)	<10 (<10)
Intrarectal	<50 (<50)	<10 (<10)	<10 (<10)
Intranasal	6,400 [b] (1,600-51,200)	<10 (<10-10)	12 (<10-80)
Intramuscular (rUreB * + CpG)	540,470 [a, d] (204,800-1,638,400)	<10 (<10-10)	23 (10-40)
rUreB * alone	86,108 [c] (25,600-409,600)	<10 (<10-40)	14 (<10-80)
rUreB * + alum	2,483,350 [e] (1,638,400-3,276,800)	61 [f] (40-160)	160 [f] (80-640)
rUreB * + Freund's	2,483,350 [e] (819,200-26,214,400)	160 [f] (<10-1,280)	368 [f] (20-2,560)

[a]: p<0.01, as compared to control and the three mucosal groups; [b]: p<0.01, as compared to control and the two other mucosal groups; [c]: p<0.01, as compared to the control group; [d]: p<0.01, as compared to rUreB alone; [e]: p=0.02 for both, as compared to the rUreB + CpG group; [f]: p=0.03 for both, as compared to all other groups.

and the immunogenicity was increased by CpG ODN 1826 (about 6-fold; p<0.01, as compared to rUreB alone) and markedly so by either alum or Freund's adjuvant (about 30-fold for each; p=0.02 for both, as compared to the rUreB + CpG group). These two latter adjuvants were also the only strategies that resulted in modest increase in stool antibodies (p=0.03 for both, as compared to all other groups).

DISCUSSION

The quest for a vaccine against *H. pylori* remains elusive. Since the best known antigen is urease B, we started by producing recombinant urease B (rUreB) and testing its immunogenicity, as a pre-requisite for its potential use as a vaccine. *H. pylori* is a gastrointestinal pathogen that does not invade the mucosa, hence a vaccine that induces local gastrointestinal antibodies, especially of the IgA sub-class, may be more relevant for protection against this infection than a vaccine that elicits mainly systemic serum IgG antibodies. Of the various routes, intramuscular administration of rUreB was the one eliciting the highest levels of antibodies. Still, the antibodies were mostly detected in serum and very little in stool; and in the stool they were mainly of IgG-subtype (maybe transudate from serum IgG) and not IgA. The poor performance of the intragastric group was particularly disappointing since this route would be ideal for administration of a vaccine because of ease of delivery and because *H. pylori* infection is acquired by ingestion. It should be noted, though, that the doses administered by each route were not the same (see Table **1**) due to limitations in the maximum allowable volume for each route. Yet, even though intragastric administration received the highest dose, still it did not elicit any detectable immune response. It is likely that, despite the use of an antacid, the protein does not survive the harsh environment of the stomach and upper gastrointestinal tract and either needs to be protected (e.g, by encapsulation) [13] or accompanied by better adjuvants. We elected to use CpG ODN as adjuvant for this experiment because it has been reported safe for the different mucosas that we were testing. We did not use cholera toxin, even though it is considered the best adjuvant for mucosal immunization, because, due to its toxicity, it has no application in human immunization. The lower gastrointestinal tract is known to have prominent lymphoid tissue and avoids the harsh conditions of the upper gastrointestinal tract and hence has been suggested as an alternative immunization route [14]. Our data, however, suggest that for rUreB the intrarectal is not a good route since no antibodies were elicited. Administration of the dose was problematic, though, and spills happened frequently so we are not sure some mice received the intended dose. Intranasal was the only mucosal route that resulted in some level of antibodies. This better performance of the intranasal route as compared to other mucosas has been described for *H. pylori* as well as other immunogens [15, 16]. Still, the titers obtained by intranasal immunization were about 10-fold lower than by the intramuscular route (even though the dose was only 2-fold lower) and they were mostly systemic IgG and not gastrointestinal IgA. The lower level of IgA as compared to IgG may partly be a technical artifact, though, since specimen collection and measurement of intestinal IgA is difficult and not standardized; and the predominance of IgG may not be bad necessarily since, at least for other intestinal infections, the presence of serum IgG correlates with protection [17]. Our conclusion from this experiment is that, until better mucosal adjuvants are developed, rUreB is more promising by intramuscular than by mucosal administration; and that, of the mucosas, intranasal administration may have some role in immunization.

We then proceeded to further study parenteral rUreB and for the second experiment we evaluated the effect of different adjuvants in its immunogenicity. We tested Freund's adjuvant as the standard parenteral adjuvant (but again with no human application), the safer (but not approved for human use) CpG ODN 1826 and alum (the only adjuvant currently approved for use in human immunization in the US). We were especially pleased by the results shown with alum. Since the safety of alum in humans is well documented and it is used in routine immunization, even for infants, it is the obvious adjuvant to use with parenteral rUreB when it is time to test in humans. Few other groups have evaluated alum as an adjuvant to *Helicobacter* antigens in animal models and in general found an enhanced antibody

response when used with *Helicobacter* lysate [18-21], urease [22-24], rUreB [25], and other *H. pylori* antigens (CagA, VacA and NAP) [26], but its role in protection has been uncertain. Recently, Malfertheiner *et al.* reported good tolerability and immunogenicity of a vaccine consisting of *H. pylori* VacA, CagA and NAP proteins with aluminum hydroxide administered to 57 human volunteers; protective efficacy was not evaluated [27].

So, our experiments support rUreB in combination with alum and administered by the intramuscular route as a promising approach for *H. pylori* immunization, at least from the immunogenicity point of view. While a higher antibody level would be desirable, it should be noted, though, that for *H. pylori* there is not an established level that would correlate with protection. Future studies will correlate antibody levels with protection and try to determine the optimal dose and schedule.

ACKNOWLEDGEMENTS

The present study was supported by grants from the National Institutes of Health, Bethesda, MD (R03CA128048) and the Research Institute for Children, New Orleans, LA.

This work was presented in part at the Pediatric Academic Societies' Annual Meeting, Toronto, Canada, May 5-8, 2007 (Abstract #: 752013).

CONFLICTS OF INTEREST

None.

REFERENCES

[1] Suerbaum, S.; Michetti, P. *Helicobacter pylori* infection. *N. Engl. J. Med.*, **2002**, *347* (15), 1175-1186.

[2] Meyer, J.M.; Silliman, N.P.; Wang, W.; Siepman, N.Y.; Sugg, J.E.; Morris, D.; Zhang, J.; Bhattacharyya, H.; King, E.C.; Hopkins, R.J. Risk factors for *Helicobacter pylori* resistance in the United States: the surveillance of *H. pylori* antimicrobial resistance partnership (SHARP) study, 1993-1999. *Ann. Intern. Med.*, **2002**, *136* (1), 13-24.

[3] Blanchard, T.G.; Czinn, S.J. Immunology of *Helicobacter pylori* and prospects for a vaccine. *Gastroenterol. Clin. NA*, **2000**, *29* (3), 671-685.

[4] Czinn, S.J.; Cai, A.; Nedrud, J.G. Protection of germ-free mice from infection by *Helicobacter felis* after active oral or passive IgA immunization. *Vaccine*, **1993**, *11* (6), 637-642.

[5] Casswall, T.H.; Nilsson, H.O.; Björck, L.; Sjöstedt, S. Xu, L.; Nord, C.E.; Borén, T. Wadström, T. Bovine anti-*Helicobacter pylori* antibodies for oral immunotherapy Scand. *J. Gastroenterol.*, **2002**, *37* (12), 1380-1385.

[6] Czinn, S.J.; Nedrud, J.G. Oral immunization against *Helicobacter pylori*. *Infect. Immun.*, **1991**, *59* (7), 2359-2363.

[7] Futagami, S.; Takahashi, H.; Norose, Y.; Kobayashi, M. Systemic and local immune responses against *Helicobacter pylori* urease in patients with chronic gastritis: distinct IgA and IgG productive sites. *Gut*, **1998**, *43* (2), 168-175.

[8] Eaton, K.A.; Brooks, C.L.; Morgan, D.R.; Krakowka, S. Essential role of urease in pathogenesis of gastritis induced by *Helicobacter pylori* in gnotobiotic piglets. *Infect. Immun.*, **1991**, *59* (7), 2470-2475.

[9] Hirota, K.; Nagata, K.; Norose, Y.; Futagami, S.; Nakagawa, Y.; Senpuku, H.; Kobayashi, M.; Takahashi, H. Identification of an antigenic epitope in *Helicobacter pylori* urease that induces neutralizing antibody production. *Infect. Immun.*, **2001**, *69* (11), 6597-6603.

[10] Ferrero, R.L.; Thiberge, J.M.; Huerre, M.; Labigne, A. Recombinant antigens prepared from urease subunits of *Helicobacter spp*: evidence of protection in a mouse model of gastric infection. *Infect. Immun.*, **1994**, *62* (11), 4981-4989.

[11] Zavala-Spinetti, L.; Breslin, M.B.; Correa, H.; Bégué, R.E. Construction and evaluation of a *Helicobacter pylori* urease B DNA vaccine. *Helicobacter*, **2006**, *11* (6), 517-522.

[12] Bégué, R.E.; Cruz, A.R.; Ramgoolam, A.; Breslin, M.B. Immunogenicity of *Helicobacter pylori* urease B protein and DNA vaccines in a mouse model. *J. Ped. Gastroenterol. Nutr.*, **2007**, *45* (4), 493-496.

[13] Kim, S.Y.; Doh, H.J.; Ahn, J.S.; Ha, Y.J.; Jang, M.H.; Chung, S.I.; Park, H.J. Induction of mucosal and systemic immune response by oral immunization with *H. pylori* lysates encapsulated in poly (D,L-lactide-co-glycolide) microparticles. *Vaccine*, **1999**, *17* (6), 607-616.

[14] Nakase, H.; Okazaki, K.; Tabata, Y.; Uchida, K.; Uose, S.; Ohana, M.; Nishi, T.; Watanabe, T.; Matsuura, M.; Hisatsune, H.; Matsumura, K.; Itoh, T.; Kawanami, C.; Chiba, T. Rectal immunization with antigen-containing microspheres induces stronger Th2 responses than oral immunization: a new method of vaccination. *Vaccine*, **2001**, *20* (3-4), 377-384.

[15] Shi, T.; Liu, W.-Z.; Gao, F.; Shi, G.-Y.; Xiao, S.-D. Intranasal CpG-oligodeoxynucleotide is a potent adjuvant of vaccine against *Helicobacter pylori*, and T helper I type response and interferon-γ correlate with the protection. *Helicobacter*, **2005**, *10* (1), 71-79.

[16] McCluskie, M.J.; Davies, H.L. Oral, intrarectal and intranasal immunizations using CpG and non-CpG oligodeoxynucleotides as adjuvants. *Vaccine*, **2000**, *19* (4-5), 413-422.

[17] Mantis, N.J.; McGuinnes, C.R. Protection of the intestinal epithelium from the direct effects of a shiga-like enterotoxin is conferred by anti-toxin serum IgG. *FASEB J.*, **2008**, *22* (4), 320.12.

[18] Gottwein, J.M.; Blanchard, T.G.; Targoni, O.S.; Eisenberg, J.C.; Zagorski, B.M.; Redline, R.W.; Nedrud, J.G.; Tary-Lehmann, M.; Lehmann, P.V.; Czinn, S.J. Protective anti-*Helicobacter* immunity is induced with aluminum hydroxide or complete Freund's adjuvant by systemic immunization. *J. Infect. Dis.*, **2001**, *184* (3), 308-314.

[19] Minoura, T.; Kato, S.; Otsu, S.; Fujioka, T.; Iinuma, K.; Nishizono, A. Childhood *Helicobacter pylori* infection in a murine model: maternal transmission and eradication by systemic immunization using bacterial antigen-aluminum hydroxide. *Clin. Exp. Immunol.*, **2003**, *134* (1), 32-37.

[20] Nyström, J.; Raghavan, S.; Svennerholm, A.M. Mucosal immune responses are related to reduction of bacterial colonization in the stomach after therapeutic *Helicobacter pylori* immunization in mice. *Microbes Infect.*, **2006**, *8* (2), 442-449.

[21] Maeda, K.; Yamashiro, T.; Minoura, T.; Fujioka, T.; Nasu, M.; Nishizono, A. Evaluation of therapeutic efficacy of adjuvant *Helicobacter pylori* whole cell sonicate in mice with chronic *H. pylori* infection. *Microbiol Immunol.*, **2002**, *46* (9), 613-620.

[22] Lee, C.K.; Soike, K.; Giannasca, P.; Hill, J.; Weltzin, R.; Kleanthous, H.; Blanchard, J.; Monath, T.P. Immunization of rhesus monkeys with a mucosal prime, parenteral boost strategy protects against infection with *Helicobacter pylori*. *Vaccine*, **1999**, *17* (23-24), 3072-3082.

[23] Weltzin, R.; Guy, B.; Thomas, W.D, Jr.; Giannasca, P.J.; Monath, T.P. Parenteral adjuvant activities of *Escherichia coli* heat-labile toxin and its B subunit for immunization of mice against gastric *Helicobacter pylori* infection. *Infect. Immun.*, **2000**, *68* (5), 2775-2782.

[24] Londoño-Arcila, P.; Freeman, D.; Kleanthous, H.; O'Dowd, A.M.; Lewis, S.; Turner, A.K.; Rees, E.M.; Tibbitts, T.J.; Greenwood, J.; Monath, T.P.; Darsley, M.J. Attenuated *Salmonella enterica* serovar Typhi expressing urease effectively immunizes mice against *Helicobacter pylori* challenge as part of a heterologous mucosal priming-parenteral boosting vaccination regimen. *Infect. Immun.*, **2002**, *70* (9), 5096-5106.

[25] Moschos, S.A.; Bramwell, V.W.; Somavarapu, S.; Alpar, H.O. Modulating the adjuvanticity by co-administration of muramyl di-peptide (MDP) or Quil-A. *Vaccine*, **2006**, *24* (8), 1081-1086.

[26] Rossi, G.; Ruggiero, P.; Peppoloni, S.; Pancotto, L.; Fortuna, D.; Lauretti, L.; Volpini, G.; Mancianti, S.; Corazza, M.; Taccini, E.; Di Pisa, F.; Rappuoli, R.; Del Giudice, G. Therapeutic vaccination against *Helicobacter pylori* in the beagle dog experimental model: safety, immunogenicity, and efficacy. *Infect. Immun.*, **2004**, *72* (6), 3252-3259.

[27] Malfertheiner, P.; Schultze, V.; Rosenkranz, B.; Kaufmann, S.H.E.; Ulrichs, T.; Novicki, D.; Norelli, F.; Contorni, M.; Peppoloni, S.; Berti, D.; Tornese, D.; Ganju, J.; Palla, E.; Rappuoli, R.; Scharschmidt, B.F.; Del Giudice, G. Safety and immunogenicity of an intramuscular *Helicobacter pylori* vaccine in noninfected volunteers. *A phase I study. Gastroenterology*, **2008**, *135* (3), 787-795.

Received: November 10, 2008 Revised: March 27, 2009 Accepted: March 30, 2009

© Bégué and Moll; Licensee *Bentham Open*.

This is an open access article licensed under the terms of the Creative Commons Attribution Non-Commercial License (http://creativecommons.org/licenses/by-nc/3.0/) which permits unrestricted, non-commercial use, distribution and reproduction in any medium, provided the work is properly cited.