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

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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 countereacting 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.
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 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 ≥ 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

<table>
<thead>
<tr>
<th>Immunization Group</th>
<th>rUreB</th>
<th>Adjuvant</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>None</td>
<td>None</td>
<td>0</td>
</tr>
<tr>
<td>Intragastric</td>
<td>160 μg</td>
<td>CpG 40 μg</td>
<td>200 μl</td>
</tr>
<tr>
<td>Intrarectal</td>
<td>80 μg</td>
<td>CpG 20 μg</td>
<td>100 μl</td>
</tr>
<tr>
<td>Intranasal</td>
<td>40 μg</td>
<td>CpG 10 μg</td>
<td>50 μl</td>
</tr>
<tr>
<td>Intramuscular (rUreB + CpG) *</td>
<td>80 μg</td>
<td>CpG 20 μg</td>
<td>100 μl</td>
</tr>
<tr>
<td>rUreB alone *</td>
<td>100 μg</td>
<td>None</td>
<td>100 μl</td>
</tr>
<tr>
<td>rUreB + alum *</td>
<td>50 μg</td>
<td>alum 50 μl</td>
<td>100 μl</td>
</tr>
<tr>
<td>rUreB + Freund’s *</td>
<td>25 μg</td>
<td>Freund’s 25 μl</td>
<td>50 μl</td>
</tr>
</tbody>
</table>

*: rUreB and adjuvant administered by intramuscular injection.
The quest for a vaccine against \textit{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. \textit{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 route as compared to other mucosas has been described for \textit{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 \textit{Helicobacter} antigens in animal models and in general found an enhanced antibody response...
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.

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CONFLICTS OF INTEREST

None.

REFERENCES