Prevention of disease in ferrets fed an inactivated whole cell Campylobacter jejuni vaccine

Donald H. Burr a, David Rollins b, Lanfong H. Lee b, Dawn L. Patarrini a, Steven S. Walz b, Jing-Hui Tian c, John L. Pace c, A.L. Bourgeois b, Richard I. Walker c,

Received 10 November 2004; received in revised form 28 March 2005; accepted 29 March 2005

Abstract

Ferrets were used to demonstrate the potential of a killed whole cell vaccine prepared from Campylobacter jejuni to protect against disease. C. jejuni strain 81–176 was grown in BHI broth, formalin-fixed, and resuspended in PBS to a concentration of 10^10 cells per ml. This vaccine (CWC) or live organisms were delivered orally with a nasogastric tube into anesthetized animals treated to reduce gastric acidity and intestinal motility. When 5 × 10^9 CFU of the vaccine strain (Lior serotype 5) or one of two other serotypes, CGL-7 (Lior 4) or BT44 (Lior 9), was used to challenge the ferrets, all of the animals developed a mucoid diarrhea. If the animals had been challenged with 5 × 10^9 CFU of the homologous strain 1 month before challenge with 10^10 CFU, 80–100% protection against disease was seen. This protection was also obtained after an initial exposure to the 81–176 strain followed by challenge with either of the heterologous strains. CWC was used to see if protection demonstrated with the live organisms could be produced with the non-living preparation. When 10^9 cells of CWC was given as two doses 7 days apart with or without 25 µg of a coadministered mucosal adjuvant, LT R192G , only 40–60% of the animals were protected. If the regimen was changed to four doses given 48 h apart, 80% of the animals were free of diarrhea after subsequent challenge. Increasing the number of cells in the four dose regimen to 10^10 cells did not improve protection. Animals given four doses of 10^10 cells combined with LT R192G were subsequently challenged with 10^9 cells of the homologous strain or the heterologous strain CGL-7. The CWC protected against both strains. Serum IgG antibody titers determined by ELISA showed little increase following the CWC four dose vaccination regimen, compared to animals given one dose of the live organism. On subsequent challenge, however, both CWC vaccinated and live-challenged ferrets showed comparable antibody titer increases above those obtained following the initial challenge or vaccination. Western blots were used to show that the immunodominant antigen in vaccinated animals was a 45 kDa protein, while in ferrets challenged with live organisms the immunodominant antigen was a 62 kDa protein. These data show that the CWC can be used to protect against disease caused by Campylobacter. They also show that protection and serum IgG responses do not depend upon the use of the mucosal adjuvant and that cross protection among some of the major serotypes of Campylobacter responsible for human disease is possible.

1. Introduction

Campylobacter jejuni is now recognized as a leading cause of foodborne disease in the United States, as well as worldwide [1–4]. It is likely that an effective vaccine can be developed against disease caused by Campylobacter. Prospective epidemiological and human challenge studies suggest that protective immunity develops after a prior C. je-
Prevention of disease in ferrets fed an inactivated whole cell Campylobacter jejuni vaccine

Food and Drug Administration, 8301 Muirkirk Rd., Laurel, MD 20708

Naval Medical Research Center 503 Robert Grant Avenue Silver Spring, MD 20910-7500

Approved for public release, distribution unlimited
Campylobacter jejuni infection [4–6]. Human volunteers fed C. jejuni become infected and respond with the development of specific serum IgG, IgM, and IgA to Campylobacter [6]. In developed countries, a peak incidence of disease is seen in children below 1 year of age and in young adults [7–10]. In developing countries, where campylobacters are hyperendemic, symptomatic disease occurs in young children and persistent carriage occurs in adults without symptomatic disease [11,12]. It appears that a high level of endemic disease (i.e., hyperendemicity) results in the development of specific serum and secretory antibodies and less severe disease [13–15]. Following exposure, specific serum and secretory antibodies develop that provide strain-specific immunity and protection from disease caused by the homologous strain, although recolonization may occur [5,6].

Vaccines have not been developed for use against Campylobacter. Orally-administered inactivated whole cell Campylobacter vaccines offer a potentially useful approach towards immunization against C. jejuni. Physically, inactivated organisms are naturally-occurring microparticles that should enhance interactions between the antigens they carry and the mucosal lymphoid tissues. As vaccines, they are safe when given orally and inexpensive to produce and administer. Whole cells possess multiple antigens that can be particularly important to protection, particularly when protective antigens are not known.

When grouped according to a serotyping scheme based on heat labile antigens [16] only about a dozen of the over 100 Lior serotypes are associated with disease in humans. Strain 81–176 of C. jejuni was selected to make an inactivated whole cell vaccine since it is one of the clinically important serotypes, Lior 5. Further, this strain has been used in clinical challenge studies [5,6] and does not show mimicry of any gangliosides associated with Guillain Barre syndrome (GBS) (A.P. Moran, personal communication).

An important question is whether a single serotype, such as Lior 5, can protect against disease induced by other clinically important serotypes. Due in part to the difficulty in doing cross serotype protection studies in humans, as well as the problem of trying to be sure no challenge strains may induce GBS, an animal model is needed to show cross-strain protection against disease.

Colonization, rather than disease, models have been used to show that the CWC vaccine does protect against C. jejuni. Mice have been orally immunized with a three-dose primary series of particles of CWC (48 h intervals) at doses of $10^5$, $10^7$, or $10^9$ cells [17]. The vaccine was given to mice with or without the mucosal adjuvant consisting of the heat-labile enterotoxin of Escherichia coli (LT) [18]. These studies showed that the Campylobacter-specific intestinal IgA response was dependent on the use of LT, whereas serum immunoglobulin A responses were not. Colonization resistance was induced over a broad range of vaccine doses when LT was included. However, only the highest dose ($10^9$) of CWC alone gave comparable levels of protection against colonization. Both the advantaged and un advantaged formulations given at the intermediate dose ($10^5$) provided equivalent protection against systemic spread of challenge organisms.

Colonization has also been studied in the Removable Intestinal Tie Adult Rabbit Diarrhea, or RITARD, model for Campylobacter infection [19]. This model relies on a surgical procedure, in which the challenge organism is introduced into the bowel of immunized and nonimmunized rabbits to model intestinal colonization: no disease is produced in animals old enough to have completed colonization. Protection against colonization was seen when three doses of CWC were given by gavage at weekly intervals [20]. Protection was not obtained in this model if LT was not present. This protection against colonization was Lior serotype specific [16,20].

Ferrets are one of the few animals that develop a Campylobacter-induced diarrheal disease similar to that seen in humans [21–23]. Ferrets develop enterocolitis associated with natural C. jejuni infection and following experimental inoculation with pure cultures, develop mild to moderate diarrhea characterized by the presence of mucus, fecal leukocytes and blood in the stool. Diarrhea was more severe in younger (6–7 week old) animals than older (11–12 week old) animals, with more fluid stools often accompanied by anorexia for a day or two. Ferrets fed live ferret-derived strains of Campylobacter develop an immune response making these animals resistant to disease following a second challenge with the same strain [22]. For both adult and young ferrets, infection and disease occur without any prior manipulation of the animals.

The purpose of the present study was to use the ferret to provide evidence that protection against enteric disease caused by Campylobacter could be obtained in a natural host and that this protection might be relatively conserved. Protection against disease was found to be at least somewhat conserved among serotypes of Campylobacter. These studies showed, furthermore, that protection against disease was associated with vaccine dose and, in contrast to colonization, was not enhanced by use of adjuvant. Further, following vaccination with the killed preparation, a strong serum immune response was obtained in protected animals with a 45 kDa OMP as the immunodominant antigen.

2. Materials and methods

2.1. Bacterial culture

C. jejuni strain 81–176 (Lior serotype 5) was isolated during a 1981 foodborne outbreak. Strains CGL7 (Lior serotype 4) and BT44 (Lior serotype 9) were isolated during military field exercises in Thailand from the stools of patients with acute diarrhea. Frozen stocks were thawed, inoculated onto tryptic soy blood agar plates and incubated at 42 °C in polybags (Levin Bros Paper Co., Chicago, IL) with an atmosphere of 85%N₂–10%CO₂–5%O₂. Cells were first passed through Mueller Hinton motility agar to confirm colonies remained motile and then plated to Muller Hinton agar. After
The organism was grown to a concentration of $1 \times 10^7$ of $2.5 \times 10^7$ particles per ml. Preparation of whole cell vaccine

The CWC vaccine is a monovalent preparation made up of $2.5 \times 10^{10}$ particles of *C. jejuni* strain 81–176 per ml. The organism was grown to a concentration of $1 \times 10^9$ CFU/ml by overnight incubation at 37°C in brain heart infusion (Difco, Detroit, MI) plus 0.1% deoxycholate (Sigma, St. Louis, MO) at 37°C in a 10% CO₂ and 5% O₂ atmosphere. The broth culture was inoculated with 5 ml of saline rinse from an overnight blood agar plate culture per 400 ml of broth. At the time of harvest the culture was centrifuged and the bacteria resuspended in Hank’s Balanced Salt Solution to which formalin was added to a concentration of 0.025 M. After overnight incubation at room temperature, the inactivated bacteria were centrifuged again and concentrated by resuspension in phosphate buffered saline to a concentration of $2.5 \times 10^{10}$ particles per milliliter. The vaccine was stored at 4°C. Twenty-five micrograms of the adjuvant LT R192G [18] was used with some vaccine preparations.

2.3. Animal challenge

Six week old female ferrets were purchased (Marshall Farms, N.Y.) and were housed singly in polycarbonate guinea pig cages covered with a filter bonnet top and modified to contain immobilized feed and water pans. A piece of PVC pipe (2 in. o.d.) was added to each cage to provide a nesting area. Animals were provided with Marshall Farms Ferret Chow and reverse deionized water ad libitum and were allowed to acclimate to the housing for at least 48 h prior to being fed any vaccine and at least 1 week prior to being infected with live organisms. During this time animals were observed for any signs of distress and diarrhea. Prior to being shipped by Marshall Farms and during the acclimation period, rectal swabs were taken from each ferret and cultured for Campylobacter organisms. During this time animals were either left untreated or intragastrically fed PBS containing *C. jejuni* 81–176 whole cells were used as antigen to evaluate strain-specific immune response of the primary antibody in the ferret serum. For IgG immunoassay, horse

2.4. Study protocol

On the day of initial feeding, serum samples were taken from all animals and assayed to determine baseline serum anti-campylobacter titers. When animals were re-challenged, serum samples were taken on the day of challenge and 12 days post-challenge. In selected rechallenge studies serum samples were obtained 1 week post initial feeding as well. For primary feeding/immunization studies, ferrets were intragastrically dosed at 6–8 weeks of age with live *C. jejuni* strains or various CWC vaccine regimens. For secondary challenge, ferrets were intragastrically dosed at 11–12 weeks of age. Homologous challenge studies involved oral feeding, followed by oral rechallenge with the same Lior serotype, whereas heterologous challenge studies involved oral feeding and rechallenge with different Lior serotypes. Control animals were either left untreated or intragastrically fed PBS containing *E. coli* heat-labile toxin (LT B926G). Stools were graded on a 0–2+ scale: 0 = normal stool; 1+ = loose stool; 2+ = gross mucus diarrhea. Only animals with 2+ stools were considered to be positive for diarrhea. Initially attempts were made to include additional physiological parameters including body temperature, blood pressure, and fecal leukocytes. These measurements were difficult to obtain and produced inconsistent results. For this reason they were discontinued.
C. jejuni ferrets, groups of 7-week-old ferrets were fed the CWC vaccine (at a concentration of 10^9 cells), either with or without the addition of the adjuvant. Increasing the number of doses of the 10^9 CWC vaccine from two to four increased the level of protection to 100%. Again there was no difference between the protection with or without the adjuvant. The level of protection compared favorably to that obtained with a previous challenge with live bacteria. Since the four dose regimen with 10^9 particles of vaccine showed higher protection than two doses, ferrets were also given four oral doses of the vaccine at a concentration of 10^9 particles per dose. The level of protection was not improved with the higher concentration of vaccine.

3.3. Cross strain protection following oral immunization with CWC

Groups of ferrets were immunized orally with either a single feeding of 5 × 10^9 CFU of live C. jejuni strain 81–176 or four doses of 10^10 particles of CWC vaccine combined with 25 μg of LT\(\_\)R192G. Control animals were fed four doses

3. Results

3.1. Homologous and heterologous protection following challenge with live organisms

Ferrets were fed human clinical strains of C. jejuni (5 × 10^9 CFU) and subsequently challenged 1 month later with 5 × 10^10 CFU of the same Lior serotype or a different Lior serotype (Table 1). Homologous protection against signs of disease were observed when ferrets were fed either strain CGL7 (Lior 4), BT44 (Lior 9) or 81–176 (Lior 5) and then subsequently challenged with the homologous strain. Eighty to 100% protection was seen in all animals upon rechallenge. In contrast, none of the control animals remained free of disease. To determine the extent of heterologous protection that develops upon feeding Campylobacter to ferrets, groups of animals were first fed strain 81–176 then 1 month later chal-

Table 1 Protection against re-challenge with homologous and heterologous Lior serotypes following primary infection

<table>
<thead>
<tr>
<th>Primary feeding strain</th>
<th>Challenge strain</th>
<th>No. sick/no. tested</th>
<th>% protected</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>81–176</td>
<td>5/5</td>
<td>0</td>
</tr>
<tr>
<td>CGL7</td>
<td>81–176</td>
<td>0/6</td>
<td>100</td>
</tr>
<tr>
<td>BT44</td>
<td>81–176</td>
<td>0/6</td>
<td>100</td>
</tr>
<tr>
<td>CGL7</td>
<td>BT44</td>
<td>0/6</td>
<td>83</td>
</tr>
<tr>
<td>BT44</td>
<td>81–176</td>
<td>2/8</td>
<td>75</td>
</tr>
<tr>
<td>BT44</td>
<td>81–176</td>
<td>2/8</td>
<td>82</td>
</tr>
</tbody>
</table>

A formalin-inactivated whole cell vaccine (CWC) for C. jejuni, strain 81–176, was used to determine whether protection seen following live challenge could be duplicated with CWC. Groups of 7-week-old ferrets were fed the CWC vaccine (at a concentration of 10^9 cells), either with or without 25 μg of adjuvant. One week later the animals were fed a second dose. Control animals were either fed two doses of PBS along with 25 μg of LT\(\_\)R192G or a single live dose (at the time of the second dose of vaccine) of 5 × 10^9 CFU of C. jejuni strain 81–176. One month following the second vaccine dose, when the ferrets were now 12 weeks of age, the animals were challenged with approximately 5 × 10^10 CFU of the homologous strain. As shown in Table 2, at this concentration the level of protection was 40% with just two doses of the vaccine. Protection was not greatly improved (60%) with the addition of the adjuvant. Increasing the number of doses of the 10^9 CWC vaccine from two to four increased the level of protection to 80%. Again there was no difference between the protection with or without the adjuvant. The level of protection compared favorably to that obtained with a previous challenge with live bacteria. Since the four dose regimen with 10^9 particles of vaccine showed higher protection than two doses, ferrets were also given four oral doses of the vaccine at a concentration of 10^10 particles per dose. The level of protection was not improved with the higher concentration of vaccine.
Table 2
Comparison of oral dosing regimens with and without adjuvant (LTR192G) for protection and IgG response following delivery of CWC vaccine

<table>
<thead>
<tr>
<th>Immunization group</th>
<th>No. sick/no. tested</th>
<th>Percent protection</th>
<th>Median IgG reciprocal titer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>Pre-challenge</td>
<td>Post-challenge</td>
</tr>
<tr>
<td>PBS-LTR192G</td>
<td>17/19</td>
<td>11</td>
<td>125</td>
</tr>
<tr>
<td>One dose live strain 81–176</td>
<td>1/6</td>
<td>83</td>
<td>125</td>
</tr>
<tr>
<td>Two doses at 10^9 CWC</td>
<td>3/5</td>
<td>40</td>
<td>125</td>
</tr>
<tr>
<td></td>
<td>2/5</td>
<td>60</td>
<td>125</td>
</tr>
<tr>
<td>Four doses at 10^9 CWC</td>
<td>1/5</td>
<td>80</td>
<td>125</td>
</tr>
<tr>
<td>Four doses at 10^9 CWC+LTR192G</td>
<td>1/6</td>
<td>83</td>
<td>125</td>
</tr>
<tr>
<td>Four doses at 10^10 CWC</td>
<td>1/9</td>
<td>89</td>
<td>125</td>
</tr>
<tr>
<td>Four doses at 10^10 CWC+LTR192G</td>
<td>6/19</td>
<td>68</td>
<td>125</td>
</tr>
</tbody>
</table>

of 25 μg LTR192G alone. At 4 weeks post primary immunization, animals were challenged with 5 × 10^{10} CFU of either the homologous strain, 81–176 or the heterologous strain CGL7. Animals were then observed daily for signs of diarrhea (Table 3). Ferrets fed live 81–176 were protected against subsequent challenge with either 81–176 or the heterologous strain CGL7. The vaccine afforded demonstrable protection against live challenge with either the homologous or the heterologous strains tested. Control animals receiving only the LTR192G were not significantly protected against challenge with either strain of Campylobacter.

### 3.4. Immune responses to vaccination

Serum samples were collected on the day of primary feeding/immunization, on the day of secondary challenge, and 12–19 days post-challenge. The magnitude of the serologic response to live challenge following two or four doses of vaccine was compared to the serologic response generated by a group of ferrets which was subsequently reinfected with live bacteria. The median antibody titers in the vehicle and two dose groups following challenge were found to be comparable (3,125) among these groups. In contrast, all animals receiving four vaccine doses uniformly developed antibody titers of 78,125, which is significantly greater (p < 0.001) than the antibody titers generated following vaccination with the vehicle or two doses of vaccine (Table 2). The magnitude of the serologic response in the group receiving the four doses followed by live challenge was comparable to that of the previously infected group challenged with live organisms. The addition of the adjuvant to the vaccine had no apparent effect on the serum titers at the given dose. Although animals given CWC had titers after challenge that were comparable with ferrets given live challenge previously, their prechallenge IgG titers were not as high as those of previously challenged animals, but was slightly higher than the titers in unprotected animals.

The IgG response was also compared in ferrets orally immunized with either four doses of the CWC vaccine or a single dose of live C. jejuni then challenged with the homologous or heterologous strain of C. jejuni (Table 3). Although the levels are low compared to those in other experiments, the titers from the group of animals fed either the live strain 81–176 of C. jejuni or the four doses of vaccine followed by challenge with live 81–176 were again the same. Although lower than in the homologous challenge group, similar titers were also observed when the animals were immunized with either live 81–176 or CWC then challenged with the heterologous strain, CGL-7.

Immune responses in the ferrets were also evaluated by western blotting. Analyses were done on serum from animals prior to treatment, 1 week after the fourth vaccination, immediately prior to challenge, and 1 week after challenge. No responses were seen in the placebo group until after challenge with live organisms (Fig. 1). At this time a band was seen corresponding in location to that expected for flagellar antigen. The animals immunized with CWC developed bands in the region of the 45 kDa outer membrane protein of Campylobacter prior to challenge. Post challenge with live organisms, these animals also showed responses to Campy-

Table 3
Comparison of protection and IgG response to challenge with homologous and heterologous strains of Campylobacter

<table>
<thead>
<tr>
<th>Group</th>
<th>Challenge strain</th>
<th>No. sick/no. tested</th>
<th>Percent protected</th>
<th>Median IgG reciprocal titer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>Pre-challenge</td>
<td>Post-challenge</td>
<td></td>
</tr>
<tr>
<td>81–176</td>
<td>81–176</td>
<td>2/14</td>
<td>86</td>
<td>75</td>
</tr>
<tr>
<td></td>
<td>CGL-7</td>
<td>1/13</td>
<td>92</td>
<td>125</td>
</tr>
<tr>
<td>Four doses at 10^{10} CWC+LTR192G</td>
<td>81–176</td>
<td>7/16</td>
<td>56</td>
<td>125</td>
</tr>
<tr>
<td>Four doses at 10^{10} CWC+LTR192G</td>
<td>CGL-7</td>
<td>4/16</td>
<td>75</td>
<td>125</td>
</tr>
<tr>
<td>Four doses LTR192G</td>
<td>81–176</td>
<td>13/15</td>
<td>13</td>
<td>125</td>
</tr>
<tr>
<td>Four doses LTR192G</td>
<td>CGL-7</td>
<td>11/15</td>
<td>27</td>
<td>125</td>
</tr>
</tbody>
</table>
lobacter antigen in the 62 kDa region seen post challenge in the placebo group.

4. Discussion

Ferrets were used to show some conservation of protection against disease induced by Campylobacter, a factor which could indicate the possibility of making a vaccine for humans with one strain rather than many. The animals were given a primary oral challenge with C. jejuni strain 81–176 (Lior 5), CGL7 (Lior 4), or BT44 (Lior 9). Upon rechallenge approximately 1 month later, homologous protection was 84% (81–176), 76% (CGL7), and 67% (BT44). Heterologous challenge of ferrets originally infected with strain 81–176 resulted in 80% protection against strain CGL7 and 67% protection against strain BT44. These data indicated that strain 81–176, the strain chosen as the vaccine strain for use in human trials, protected against disease caused by two of the major serotypes responsible for human disease as well as these two heterologous strains protected against reinfection with themselves.

An inactivated Campylobacter whole cell was used to demonstrate the importance of antigen dose in protection of ferrets from disease. An oral four-dose regimen of CWC (10^9 or 10^10 cells/dose) delivered on Days 0, 3, 5, and 7 provided enhanced protection following oral homologous challenge compared to a two-dose CWC (10^9 cells/dose) regimen delivered at Days 0 and 14. The percent protection observed was 20% in PBS controls, 100% in previously infected ferrets, 50% in a two-dose CWC group, and 89% in a four-dose CWC group. These studies not only showed that protection was afforded through vaccination with sufficient inactivated whole cell vaccine, but they also showed that protection was obtained regardless of the inclusion of the adjuvant LTR192G.

It was not possible to conduct extensive immunological studies in the ferrets, but the studies conducted found that live infection induces a stronger serum IgG response upon primary exposure than does the vaccine. Upon subsequent challenge, however, vaccinated animals responded with as strong a serum antibody response as did the rechallenged animals. The immunodominant antigen in ferrets given the killed vaccine is the 45 kDa major OMP; in live-challenged animals the 62 kDa flagellin antigen is dominant. This difference may reflect loss of some flagella during the fixation process. The 45 kDa OMP [24] could be an important vaccine component and could contribute to cross serotype protection.

There is antigenic similarity between OMPs of different C. jejuni strains and OMP is also recognized by convalescent human sera [25].

The strong IgG antibody responses seen upon rechallenge of vaccinated or previously challenged animals may not be protective in themselves, but could indicate the magnitude of other unmeasured antibody responses following immunization. Anti-Campylobacter IgA antibodies in mucus taken from rabbits previously infected with C. jejuni were shown to trap the organisms and prevent attachment to underlying cells in vitro [26]. Whatever the exact nature of the protective immune responses associated with the CWC vaccine, it is clear that the vaccine offers protection which may be
relatively conserved among clinically important serotypes of Campylobacter.

Acknowledgement

The authors appreciate the excellent technical assistance of Carl Harding and Craig Zikan.

References

[15] Caldwell MB, Trust TJ, Ruiz-Palacios GM, Lopez-Vidal AB, Ramos A, Bojalil R. Immunogenicity and protec-