Capsule Polysaccharide Conjugate Vaccine against Diarrheal Disease Caused by *Campylobacter jejuni*[†]

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The capsule polysaccharide (CPS) of *Campylobacter jejuni* is one of the few identified virulence determinants of this important human pathogen. Since CPS conjugate vaccines have been so effective against other mucosal pathogens, we evaluated this approach using CPSs from two strains of *C. jejuni*, 81-176 (HS23 and HS36 serotype complex) and CG8486 (HS4 serotype complex). The CPSs of 81-176 and CG8486 were independently linked to the carrier protein CRM₁₉₇ by reductive amination between an aldehyde(s), strategically created at the nonreducing end of each CPS, and accessible amines of CRM₁₉₇. In both cases, the CPS:CRM₁₉₇ ratio used was 2:1 by weight. Mass spectrometry and gel electrophoresis showed that on average, each glycoconjugate preparation contained, at least in part, two to five CPSs attached to one CRM₁₉₇. When administered subcutaneously to mice, these vaccines elicited robust immune responses and significantly reduced the disease following intranasal challenge with the homologous strains of *C. jejuni*. The CPS₈₁₋₁₇₆-CRM₁₉₇ vaccine also provided 100% protection against diarrhea in the New World monkey *Aotus nancymae* following orogastric challenge with *C. jejuni* 81-176.

Campylobacter jejuni, a member of the epsilon division of the *Proteobacteria*, is a major cause of bacterial diarrhea worldwide (16). In North America, the organism is among the leading bacterial causes of food-borne illness, with an estimated incidence of 25 to 50 per 100,000. In some developing countries, the incidence of *C. jejuni* diarrhea has been reported to be as high as 40,000/100,000 (12, 16, 42). Campylobacteriosis is also associated with a number of important sequelae, including reactive arthritis, Reiter's syndrome, irritable bowel syndrome, and Guillain-Barré syndrome (GBS) (40). GBS is thought to occur as a result of molecular mimicry of the outer lipooligosaccharide (LOS) regions of some strains of *C. jejuni* with human gangliosides (23, 60, 61). Thus, antibodies directed against LOS during infection can result in an autoimmune disease that affects peripheral nerves.

There are no licensed vaccines against *C. jejuni*, although several have undergone preclinical and clinical development (55). One of the main obstacles to vaccine development is the association of *C. jejuni* with GBS, a fact that precludes whole cell-based approaches and also poses safety concerns for human challenge studies. Although *C. jejuni* strain 81-176 has been fed to human volunteers (10; D. Tribble, personal communication), these studies predated the awareness of the association of *C. jejuni* with GBS and the knowledge that the LOS of this strain contained ganglioside mimics (19). A clinical isolate of *C. jejuni* lacking ganglioside mimicry has recently been characterized and is currently being used to develop a

human challenge model (45). Another hurdle in vaccine development is that *C. jejuni* has proven particularly recalcitrant to studies on molecular pathogenesis. Despite extensive study and numerous genome sequences (15, 21, 43, 45, 46), there are few defined virulence factors that could be targeted as subunit vaccines.

Early structural analyses by Aspinall and coworkers revealed the presence of polysaccharides (PSs) in several *Campylobacter* species that were not associated with an expected lipopolysaccharide (LPS) component but that were more akin to teichoic acid PSs and capsule PSs (CPSs) (1, 3, 5, 6). More recent genomic analysis provided further evidence that *C. jejuni* was indeed encapsulated (43), and subsequent structural studies confirmed that the *C. jejuni* PSs, previously designated as LPS O-chain PSs, were in fact CPSs. Thus, unlike most other gram-negative enteric pathogens, *C. jejuni* does not express an LPS (O-chain→core→lipid A), instead producing an LOS (core→lipid A) and a CPS (1). Additional genetic studies have indicated that *C. jejuni* CPS modulates the invasion of intestinal epithelial cells (7, 8) and serum resistance (8). Moreover, CPS is a component of the Penner or heat-stable (HS) serotyping scheme (8, 30). CPSs are unusual surface structures for enteric pathogens, but given the clinical success of CPS conjugate vaccines against other mucosal pathogens (14, 24, 32–35, 57), we hypothesized that a CPS conjugate vaccine would protect against diarrheal disease by *C. jejuni*. Here, we report on the synthesis of two prototype *C. jejuni* CPS conjugate vaccines based on the well-characterized strains 81-176 (HS23 and HS36) (10, 21) and CG8486 (HS4 complex) (46) (Fig. 1). The well-characterized diphtheria toxin mutant CRM₁₉₇ was used as the carrier protein (56). This 63-kDa protein is an approved carrier for licensed pneumococcal vaccines and has been shown to be safe and effective in numerous clinical trials (27, 48, 51). The *C. jejuni* CPS conjugate vaccines synthesized

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FIG. 1. Chemical structures and conjugation schemes to CRM₁₉₇ of *C. jejuni* strain 81-176 (A) and *C. jejuni* strain 8486 CPSs (B).

in this study were immunogenic in mice and reduced the disease following intranasal challenge (9) with the homologous strain of *C. jejuni*. We also show here that the 81-176 CPS conjugate vaccine is immunogenic and 100% protective against diarrheal disease in New World monkeys (26).

MATERIALS AND METHODS

Bacterial strains and growth conditions. *C. jejuni* strains 81-176 and CG8486 have been described previously (10, 21, 46). Bacterial cells for capsule extraction were grown in brain heart infusion medium at 37°C in a microaerophilic environment as described previously (11, 28). Growth of bacteria for mouse and monkey experiments has been described previously (9, 26).

Extraction and purification of CPSs. The extraction of the CPSs was achieved by stirring the cells in a hot water-phenol mixture for 2 h at 70°C, followed by overnight dialysis of the water layer, and then subjecting it to high-speed centrifugation to separate the insoluble LOS from the soluble CPS. The supernatant containing the CPS material was further purified through a size-exclusion column (Bio-Gel P-4), which, in each case, yielded a single carbohydrate fraction. The structures and purity of the already structurally defined CPSs of 81-176 and CG8486 were confirmed by ¹H and ³¹P nuclear magnetic resonance (NMR) and by gas chromatography-mass spectrometry (GC-MS) of the alditol acetate derivatives (monosaccharide analysis) (11, 28).

Conjugation of CPSs to CRM₁₉₇. Prior to each conjugation, the molecular weights of the CPSs and carrier protein, CRM₁₉₇, were confirmed by matrix-assisted laser desorption-time of flight MS (MALDI-TOF MS), which was carried out on a MALDI Micro MX mass spectrometer in the linear mode with an N₂ laser source (337 nm) and with positive ion detection. The samples were mixed with a sinapinic acid matrix, and 1 to 2 µl was deposited on a plate to dry (dry droplet method) and was then subjected to the spectrometer. In each case, the CPSs were activated (oxidized at the nonreducing end) with 0.04 M NaIO₄ in 0.1 M sodium acetate buffer (pH 4) at 4°C for 2 days. Following 2 days of dialysis against water, the activated CPS was freeze-dried and passed through a Bio-Gel P-2 column. The structural integrity of the activated CPS was analyzed by NMR, MALDI-TOF MS, and GC-MS of the alditol acetate derivatives. The

activated CPS was then solubilized in 0.1 M borate buffer (pH 9.0), to which CRM₁₉₇, obtained from Sigma-Aldrich, was added immediately. In all conjugate preparations, the CPS:CRM₁₉₇ ratio used was 2:1 by weight. After 1 min of stirring at room temperature, sodium cyanoborohydride (0.5 weight eq. to CPS) was added to the reaction mixture, which was then placed in a heating block and allowed to stir for 3 days at 37°C. Subsequently, the reaction mixture containing the glycoconjugate was dialyzed for 2 days against water and then freeze-dried. Each glycoconjugate was analyzed by MALDI-TOF MS and on 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels. For this study, four batches of CPS₈₁₋₁₇₆-CRM₁₉₇ and one batch of CPS₈₄₈₆-CRM₁₉₇ glycoconjugate vaccines were synthesized.

Animals. Female BALB/c mice, ages 6 to 8 weeks (Jackson Laboratory, Bar Harbor, ME), were housed in laminar flow cages for 8 to 12 days before use. Food and water were provided ad libitum. *A. nancyrae* nonhuman primates (of the captive-born Instituto Veterinario de Investigaciones Tropicales y de Altura [IVITA]) were purchased from IVITA, University of San Marcos, Lima, Peru. For the dose-ranging experiment, 10 male and 10 female animals were used (average age, 80.2 months; weight, 0.87 to 1.59 kg), and for the challenge experiment, an additional 25 animals were used (15 male, 10 female; average age, 43.6 months; weight, 0.9 to 1.8 kg). All animals were selected from the nonhuman primate pool at the Naval Medical Research Center Detachment (NMRC), Lima, Peru. The animals were stool culture negative for *Campylobacter* and had baseline immunoglobulin G (IgG) and IgA titers of <1:200 or <1:20, respectively, against glycine-extracted surface antigens (3 µg/ml) of *C. jejuni* 81-176 (9), as measured by enzyme-linked immunosorbent assay (ELISA). The animals were single housed in standard metal cages with nest boxes and perches. Their cage pans were cleaned daily, and the cages were sanitized every 2 weeks on an alternate schedule from the nest boxes to allow continuous scent marking. Ten pellets of a commercially formulated monkey diet (New World Primate Diet 8794N, Harlan Teklad, Madison, WI) were provided daily to each monkey. The diet was supplemented with a variety of fresh fruits and monkey biscuits purchased from IVITA. Distilled water was provided ad libitum. The animals were provided a reverse 12:12 h light-dark cycle. The Institutional Animal Care and Use committees of the Naval Medical Research Center (NMRC) and the NMRC approved the animal use for experimentation. The experiments were conducted according to the principles set forth in the *Guide for the Care and Use*

FIG. 2. MALDI-TOF MS of the CPS of *C. jejuni* strain CG8486 (the peak at m/z 3,300 is the doubly charged ion) (A), the CPS₈₄₈₆-CRM₁₉₇ conjugate (the peak at around m/z 35,000 is the doubly charged ion) (B), the CPS of *C. jejuni* strain 81-176 (the peak at m/z 2,900 is the doubly charged ion) (C), and the CPS₈₁₋₁₇₆-CRM₁₉₇ conjugate, in which only the doubly charged ion at m/z 34,180 is observed (D).

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Mouse vaccination and sample collection. Groups of 10 to 15 mice were injected subcutaneously in the scruff of the neck with 100 μ l of phosphate-buffered saline (PBS) alone (controls) or PBS containing the vaccine at the indicated dose. Tail blood was collected at the indicated time points, and serum was separated and stored at -30°C until assayed for CPS-specific IgM, IgG, and IgA levels by ELISA.

Intranasal challenge of mice with *C. jejuni*. Fourteen weeks following the last vaccination, the mice were intranasally challenged with 3×10^9 CFU of *C. jejuni* strain 81-176 or CG8486. The details of the challenge procedures are described elsewhere (9). Following the challenge, the animals were observed twice per day for 6 days and the following scores based on the severity of the illness were assigned: 0, apparently healthy; 1, ruffled fur; 2, ruffled fur plus a hunched back; and 3, dead. The data are presented as the daily mean illness indices of the group.

Immunization of *A. nancymae* with CPS₈₁₋₁₇₆-CRM₁₉₇ and blood sample collection. The animals were anesthetized with ketamine hydrochloride (10 mg/kg; Ketalar, Park-Davis) prior to immunization or blood draw. Lyophilized vaccine was resuspended in Ringer's solution and combined with alum (Alhydrogel; Brenntag Biosector, Denmark) and mixed for 30 min to 2 h at room temperature. The vaccine or PBS was administered to the animals subcutaneously.

Oral challenge of *A. nancymae* with 81-176. Challenge of *A. nancymae* with 81-176 has been described previously (26). Basically, monkeys were injected intramuscularly with ranitidine (1.5 mg/kg body weight; Zantac, GlaxoSmith-Kline) 90 min prior to *C. jejuni* inoculation. Thirty minutes prior to inoculation, the monkeys were anesthetized as described above and then administered 5.0 ml CeraVax I (CeraProducts, Jessup, MD) intragastrically (i.g.). CeraVax and *C. jejuni* preparations were administered through a 5 French/Charriere (1.7-mm by 41-cm feeding tube inserted orally). The animals were challenged with 6×10^{11} CFU in the dose-ranging study and with 5×10^{11} CFU in the immunization experiment. An episode of diarrhea was defined as the passing of at least one loose-to-watery stool on at least two consecutive days during the observation period (26).

Determination of *A. nancymae* colonization. Stools from monkeys that had been challenged with 81-176 were serially diluted in PBS and cultured daily on brucella agar containing cefoperazone, vancomycin, and amphotericin B (CVA; BD, Sparks, MD). The plates were incubated under microaerobic conditions at 42°C for 48 h.

Determination of immune responses by ELISA. The antigen wells of ELISA plates were coated with purified CPS (4 μ g/ml) from either 81-176 or CG8486, CRM₁₉₇ (1 μ g/ml), or bovine serum albumin (10 μ g/ml) in carbonate buffer (pH

9.6). For the mouse studies, peroxidase-conjugated goat anti-mouse IgM (μ chain; 0.2 μ g/ml), IgG (γ chain; 0.125 μ g/ml), or IgA (α chain; 0.25 μ g/ml) were used as detecting antibodies (Kirkegaard and Perry Laboratories, Gaithersburg, MD). The IgM, IgG, and IgA endpoint titers for individual mice were determined (reciprocal of the highest dilution showing a net optical density at 405 nm of 0.15 for IgA and IgG and 0.3 for IgM). IgM, IgG, and IgA titers from primates were determined using *Aotus*-specific reagents (Lampire, Pipersville, PA). The endpoint titers were $\log_e$ transformed and presented as the mean and standard error of the group.

Statistical analysis. Statistical comparisons of the $\log_e$ titers and illness indices were made using a repeated-measure analysis of variance with the study group as the between-subject factor and the sample collection time points as the repeated factor. Posthoc pairwise comparisons were made using a Tukey adjustment to control the type I error rate. All statistical analyses were performed using SAS v. 8.2 (Cary, NC).

RESULTS

Synthesis of CPS conjugate vaccines. Prior to conjugation, the CPSs were analyzed by MALDI-TOF MS, which revealed that the molecular masses of the CPSs were on average 6,500 Da for CPS₈₄₈₆ and 5,500 Da for CPS₈₁₋₁₇₆ (Fig. 2).

The CPS₈₁₋₁₇₆-CRM₁₉₇ and CPS₈₄₈₆-CRM₁₉₇ glycoconjugates were synthesized by the attachment of the CPS to CRM₁₉₇ by reductive amination (Fig. 1). In each CPS, the nonreducing monosaccharide was oxidized by periodate to yield aldehyde functionalities at the nonreducing end of the CPS, which served as the attachment point to CRM₁₉₇ (Fig. 1). In both cases, only the nonreducing terminus of each CPS was oxidized, due to the inaction of periodate in the inner regions (no vicinal hydroxyls are available) and to the presence of the lipid anchor at the reducing ends (13). The structural integrity of each activated (oxidized) CPS was analyzed by NMR spectroscopy, MALDI-TOF MS, and GC-MS of the alditol acetate derivatives. The characterization of a tri-*O*-acetyl 1- $[-^2\text{H}_1]$ glycerol unit in the oxidized CPSs was of particular interest, in that it afforded evidence that oxidation at the nonreducing ends of the CPSs had occurred.

FIG. 3. SDS-PAGE analysis of CPS conjugate vaccines. Samples were electrophoresed on 12.5% SDS-PAGE gels and stained with Gel Code Blue (Pierce). Lane 1, Precision Plus protein markers (Bio-Rad); lane 2, CPS₈₁₋₁₇₆-CRM₁₉₇; lane 3, CPS₈₄₈₆-CRM₁₉₇; lane 4, CRM₁₉₇. The masses of the protein standards are shown in kDa. Some high-molecular-weight material can be seen in the well of lane 3.

The MALDI-TOF MS spectra of the CPS conjugates yielded broad m/z ions in which the parent ion, on average, ranged from 68,000 to 80,000 Da (Fig. 2). Of particular note, the doubly charged ions, for which the m/z ranged between 33,000 and 36,000, were repeatedly shown as the dominant species and in some cases, especially those involving CPS₈₁₋₁₇₆-CRM₁₉₇ conjugates (Fig. 2), were the only species observed. The MALDI-TOF MS of the CRM₁₉₇ used here yielded a broad m/z ion between 54 and 57 kDa. These MS data suggested that the CPS conjugates were present, at least in part, as single-ended conjugates with two to five CPSs per CRM₁₉₇. SDS-PAGE analyses (Fig. 3) of the CPS conjugates also revealed the absence of free CRM₁₉₇ and that the masses of the CPS conjugate vaccines were consistent with those obtained by MALDI-TOF MS (Fig. 2). The scanning of higher m/z ranges (up to 200 kDa) in the MALDI-TOF MS did not reveal any significant m/z ion that would indicate the presence of CPS conjugates of high molecular weights. However, the absence of high m/z peaks did not preclude the possibility that CPS conjugates of higher molecular weights were present, and indeed, upon closer inspection, some high molecular weight material (>250 kDa) could be observed in some vaccine preparations that did not enter SDS-PAGE gels (Fig. 3, lane 3). This would imply that, in addition to the single-ended-form glycoconjugates described above, cross-linked glycoconjugates (lattice model) could also be present, in which a single CPS is attached to two CRM₁₉₇ proteins as a result of two aldehyde groups being present in the nonreducing end of the activated CPS. Within the limits of detection, no free CPSs were observed in the MALDI-TOF MS spectra of the CPS conjugates. The data obtained from the MALDI-TOF MS and the gel electrophoresis suggested that both CPS₈₁₋₁₇₆-CRM₁₉₇ and CPS₈₄₈₆-CRM₁₉₇ glycoconjugate vaccines contained, in the single-

FIG. 4. Kinetics of CPS₈₁₋₁₇₆-specific serum IgM and IgG in mice following immunization with CPS₈₁₋₁₇₆-CRM₁₉₇. Mice were immunized subcutaneously at 2-, 4-, or 6-week intervals with the indicated doses of vaccine. Animals were immunized so that the third and the final doses of each immunization regimen were delivered at the same day of the study, i.e., the initial doses for each immunization regimen were delivered at different times. The section to the left of the dotted line shows the immunization phase of the study, and the section to the right of the dotted line shows the time after the third vaccination, which was identical in all groups. Immediately before each vaccination, blood samples were collected to determine antigen-specific serum antibody levels. Therefore, data shown at the x axis marked "1" represent preimmunization IgM or IgG levels, data at "2" represent the results of a single vaccination, data at "3" are the levels achieved after two vaccinations, and data at "4" (to the right of the dotted line) are the results of the complete three-dose vaccination series. Symbols: triangles, 1 µg; squares, 5 µg; filled circles, 25 µg; open circles, PBS. Data are presented as the mean $\pm$ standard error for each group.

ended model, CRM₁₉₇ proteins that carried up to five CPS molecules each.

Immunogenicity of CPS₈₁₋₁₇₆-CRM₁₉₇ and CPS₈₄₈₆-CRM₁₉₇ in mice. Preliminary immunizations of mice with escalating doses of unconjugated CPS from 81-176 had indicated that the polysaccharide alone was poorly immunogenic and resulted in a T-cell-independent response (data not shown). The immunogenicity of both the CPS₈₁₋₁₇₆-CRM₁₉₇ and CPS₈₄₈₆-CRM₁₉₇ vaccines was tested in mice. Initially, several doses and regimens of the CPS₈₁₋₁₇₆-CRM₁₉₇ vaccine were compared. As shown in Fig. 4, doses of 1 µg, 5 µg, or 25 µg of CPS₈₁₋₁₇₆-CRM₁₉₇ delivered at 2-week intervals resulted in significantly higher IgM titers than PBS 4 weeks after the third dose, although titers in the 1-µg group declined significantly thereafter. Vaccination at 4-week and 6-week intervals showed similar IgM responses. Only the 25-µg dose (at 4- and 6-week intervals) demonstrated a significant increase in baseline serum IgM titers.

FIG. 5. Kinetics of CPS₈₄₈₆-CRM₁₉₇-specific IgG responses in mice following immunization with CPS₈₄₈₆-CRM₁₉₇. Mice were subcutaneously immunized at 4-week intervals with the indicated doses of the vaccine as described in the legend to Fig. 4. Symbols: triangles, 1 µg; squares, 5 µg; filled circles, 25 µg; open circles, PBS. Data are presented as the mean $\pm$ standard error for each group.

For all immunization regimens, the CPS₈₁₋₁₇₆-specific serum IgG titers increased with increasing doses and number of vaccinations; however, the kinetics varied among the three dosing regimens (Fig. 4). For the 1-µg dose given at 2-week intervals, there was no significant increase in IgG titer until 14 weeks after the third vaccination. In contrast, immunization with the 5- and 25-µg doses at 2-week intervals resulted in significantly increased IgG titers after the second and third immunizations. These titers remained elevated for the duration of the study (week 26).

The 4- and 6-week regimens resulted in a gradual increase in IgG titers after each vaccination. Peak titers for the 5-µg and 25-µg doses were detected 14 weeks after the third immunization, while the titers for the 1-µg dose peaked at either 4 (4-week regimen) or 8 (6-week regimen) weeks after the vaccination series. For the 4-week regimen (all dose levels), IgG titers persisted up to 20 to 26 weeks postvaccination. Although the IgG response rates varied somewhat by the immunization regimen, the 4- and 6-week vaccination regimens resulted in 100% seroconversion regardless of the dose (data not shown). Sera collected after the third vaccination from the group immunized at 4-week intervals were used to determine CPS-specific IgA. The peak IgA log_e titers for the PBS group (3.06 ± 0.7) were significantly lower than the titers for animals receiving 5 µg (5.72 ± 0.13) or 25 µg (5.05 ± 0.18) of CPS₈₁₋₁₇₆-CRM₁₉₇. An anti-CRM₁₉₇ IgG dose response was also evident across all regimens (see Fig. S1 in the supplemental material).

A 4-week regimen was used to determine the optimum dose of the CPS₈₄₈₆-CRM₁₉₇ vaccine. Groups of 10 mice were immunized with three doses of CPS₈₄₈₆-CRM₁₉₇ (either 1, 5, or 25 µg) or PBS. While immunization with 1 µg of CPS₈₄₈₆-CRM₁₉₇ failed to induce CPS₈₄₈₆-specific IgG, the animals immunized with 5 and 25 µg of the vaccine had high levels of antigen-specific serum IgG after the second vaccination (Fig. 5). The third vaccination significantly increased IgG titers for the animals receiving the 5-µg dose. The peak IgG titers in the 5-µg and 25-µg groups were significantly higher than those receiving 1 µg but were not significantly different from each

FIG. 6. Mean illness indices of mice challenged intranasally following immunization with either CPS₈₁₋₁₇₆-CRM₁₉₇ or CPS₈₄₈₆-CRM₁₉₇. Symbols: diamonds, PBS; squares, 5 µg; circles, 25 µg. Within immunization intervals, solid symbols denote a significant difference ($P < 0.05$) from PBS at the respective time after challenge and an asterisk indicates a significant difference from the 5-µg dose ($P < 0.05$).

other. After completing the immunization series, $\geq 90\%$ of the animals in the 5- and 25-µg groups seroconverted.

Mouse intranasal challenges with *C. jejuni*. To determine the ability of the CPS₈₁₋₁₇₆-CRM₁₉₇ vaccine to reduce illness following homologous challenge, animals immunized with 5- and 25-µg doses of CPS₈₁₋₁₇₆-CRM₁₉₇ or PBS were challenged intranasally (9) with *C. jejuni* strain 81-176 14 weeks following the last vaccination. The illness indices, calculated as described in Materials and Methods, are summarized in Fig. 6 and in Table S1 in the supplemental material. Irrespective of the immunization regimen or vaccine dose, within 2 days following challenge, all immunized animals showed a significant reduction in illness compared to the PBS recipients. The animals receiving the 25-µg dose in the 2- and 4-week regimens recovered faster than those immunized with 5 µg over the same regimen with a significant difference in the mean illness index on day 2.5 ($P < 0.05$).

The animals immunized with 5 or 25 µg of CPS₈₄₈₆-CRM₁₉₇ or PBS at 4-week intervals were challenged intranasally with *C. jejuni* strain CG8486. The illness indices are also summarized in Fig. 6. The vaccinated animals never reached the same level

FIG. 7. Kinetics of anti-CPS₈₁₋₁₇₆ IgG in *A. nancymaae*. The animals received three doses of vaccine at the times indicated. Symbols: diamonds, PBS; triangles, 1 µg; squares, 5 µg; circles, 25 µg. Statistically significant increases over baseline titers within a group are denoted with an asterisk, and significantly higher titers compared to those of the PBS group at a given time are denoted with a dagger.

of disease severity as the control animals. One day after challenge, the animals immunized with 25 µg showed significantly lower illness indices than the controls or the 5-µg group, although the mean illness index increased until day 3.5. Three days after challenge, the immunized animals (5- and 25-µg doses) showed significantly lower illness indices than the controls. The mean illness index remained significantly higher than the prechallenge levels until day 4.5 (25 µg) or 5.5 (5 µg). In contrast, 50% of the control animals remained ill for the 6-day observation period.

Dose finding study in *A. nancymaae*. Although encouraging, the mouse intranasal model may not predict protection against diarrheal disease. Since a diarrheal disease model for *C. jejuni* strain 81-176 has been described for the New World monkey *A. nancymaae* (26), we utilized this nonhuman primate model to evaluate the immunogenicity and protective efficacy of 1, 5, or 25 µg of CPS₈₁₋₁₇₆-CRM₁₉₇ adjuvanted with alum. Four groups of five animals were immunized subcutaneously with three doses of vaccine or PBS at 6-week intervals. There was a clear dose-related response in serum IgM (data not shown) and, as shown in Fig. 7, IgG titers after vaccination (peak geometric mean titer of 12.62 [PBS], 35.52 [1 µg], 145.75 [5 µg], and 456.00 [25 µg]). While only the 25-µg group demonstrated significantly higher IgG titers than the PBS controls (weeks 9, 12, 18, and 20), both the 5- and 25-µg groups demonstrated a significant increase from the baseline titers after dose 3 or dose 2, respectively. There were no significant differences in IgG titers between the 5- and 25-µg groups. Compared to those of the placebo recipients, there were no significant differences in the serum IgA titers for any dosing group (data not shown).

The animals were challenged with *C. jejuni* 81-176 9 weeks following the last immunization and were monitored for the development of diarrhea (Table 1). The attack rate for the animals immunized with PBS was 60% (3/5 animals), which is lower than that previously reported (26). There was a statisti-

cally significant trend (χ^2 test for trend, $P = 0.03$) toward protection with increasing vaccine dose (diarrhea attack rates were 40%, 20%, and 0% for the 1-, 5-, and 25-µg dose groups, respectively).

Immunization and challenge of *A. nancymaae*. Additional monkeys were immunized with three doses of either PBS or 25 µg of CPS₈₁₋₁₇₆-CRM₁₉₇ (batch A) (Table 2) with alum or PBS at 6-week intervals and challenged as described above. The diarrhea attack rate in the placebo group was 70% (7/10 animals) and 0% (0/9) for the vaccine group ($P = 0.003$), as shown in Table 2. One of the 10 animals in the vaccine group was excluded from the analysis due to diarrhea onset prior to inoculation, although significant protection is seen even with an intent-to-treat analysis including this animal ($P = 0.02$). An additional five animals were also immunized with a second batch of CPS₈₁₋₁₇₆-CRM₁₉₇ vaccine (batch B) to assess lot-to-lot reproducibility. All five of these animals were also protected ($P = 0.03$). All immunized animals were colonized with 81-176, and there was no significant difference in the duration of colonization among animals in any group.

DISCUSSION

Despite its importance as a human diarrheal pathogen and its strong association with GBS, reactive arthritis, and irritable bowel syndrome (23, 40, 60, 61), no vaccines are available against *C. jejuni*. Subunit vaccine approaches generally utilize surface antigens that play a role in virulence, but *C. jejuni* pathogenesis remains poorly understood, in spite of intensive study (47, 59). *C. jejuni* is novel among enteric pathogens, in that it expresses a CPS (30) that is one of the few defined virulence determinants (47, 59). Given the general success of CPS conjugate vaccines, we sought to determine if a similar approach would be successful against *C. jejuni*. The data presented here demonstrate that CPS conjugate vaccines from two strains of *C. jejuni* are immunogenic in two animal species and that the CPS₈₁₋₁₇₆-CRM₁₉₇ vaccine was 100% efficacious against diarrhea in *A. nancymaae*. Interestingly, the vaccine did not prevent *C. jejuni* intestinal colonization of the challenged animals, consistent with what has been reported in the majority of volunteer rechallenges (10; D. Tribble, unpublished data; B. Kirkpatrick, unpublished data).

Although there is both experimental and epidemiological evidence that immunity develops after *C. jejuni* infection (10, 55), there is limited understanding of the protective immune response. There are reports that secretory and serum IgA

TABLE 1. Effect of escalating doses of CPS₈₁₋₁₇₆-CRM₁₉₇ on the protective efficacy against *C. jejuni* 81-176 oral challenge in *A. nancymaae*

Vaccine and dose	Attack rate ^a (%) (no. of positive animals/total no.)	Median no. of days in duration (range)	Median day of onset (range)
PBS (placebo)	60 (3/5)	5 (5–10)	3 (1–4)
CPS ₈₁₋₁₇₆ -CRM ₁₉₇			
1 µg	40 (2/5)	2.5 (2–3)	4 (3–5)
5 µg	20 (1/5)	2	1
25 µg	0 (0/5)		

^a Cochran-Armitage chi-square test for trend, $P = 0.03$.

TABLE 2. Protective efficacy of three doses of 25 µg CPS₈₁₋₁₇₆-CRM₁₉₇ against diarrheal disease in *A. nancymae*

Vaccine and batch	Attack rate (%) (no. of positive animals/total no.)	Median no. of days in duration (range)	Median day of onset (range)	Efficacy (%) (two-sided 95% CI) ^b	Fisher's exact <i>P</i> value
PBS (placebo)	70 (7/10)	3 (2–5)	4 (1–11)		
CPS ₈₁₋₁₇₆ -CRM ₁₉₇ (25 µg)					
Batch A	0 (0/9) ^a			100 (27, 100)	0.003
Batch B	0 (0/5)			100 (–16, 100)	0.03

^a One animal was excluded from the analysis due to diarrhea onset prior to inoculation with 81-176. If this animal were included in the analysis, the statistical significance would remain (Fisher's exact *P* value, 0.02).

^b CI, confidence interval.

against crude acid-extracted surface antigens correlate with protection (55), but specific protective antigens have not been identified. Moreover, we are unaware of any studies that have examined the immune response to *C. jejuni* CPSs during human infection. Significant rises in serum IgA titers were observed in mice following immunization with both CPS conjugate vaccines, but surprisingly, there was not a similar increase in serum IgA titers following the immunization of nonhuman primates with CPS₈₁₋₁₇₆-CRM₁₉₇. Both IgA and IgG contribute to protection against pathogens at mucosal surfaces (50, 53), but the mechanism by which systemic IgG gains access to mucosal surfaces is not completely understood. The epithelial cell linings of humans and rodents express IgG neonatal Fc receptor, a major histocompatibility complex class I-related Fc receptor of IgG that mediates the transport of IgG across epithelial cells by transcytosis (18, 22, 58). Thus, anti-CPS IgG may have provided protection by crossing the epithelial barrier at the intestinal level via these receptors. Elucidation of the mechanism of immunity will clearly require additional study.

An effective CPS conjugate vaccine against *C. jejuni* will have to be multivalent, as are other licensed CPS conjugate vaccines. *C. jejuni* CPS is a component of the Penner serotyping scheme (8, 30), of which there are >40 serotypes (49). When the *kspM* gene, which encodes part of the CPS export machinery, was mutated in eight strains of *C. jejuni* from different HS serogroups, seven of eight of the mutants became untypable, indicating that the CPS contributed to serospecificity (30). However, in one case (HS6), the mutant retained the ability to be serotyped, indicating that the serodeterminant was something other than CPS (30). Early studies demonstrated that LOS contributes to serospecificity in some serotypes (25, 49), and recent chemical analyses have also revealed that some serotypes can differ by relative amounts of specific carbohydrate components in the CPS and/or modifications of individual sugars, including a phosphoramidate group (31, 38). For example, the differences between the CPSs of HS23 and HS36, which cross-react immunologically, relate to differences in phosphoramidate modification on the galactose moiety and the presence of one of four variant forms of heptose (2, 38). In contrast, one would not expect cross-reactivity between chemically distinct capsules such as those studied here (HS23/36 and HS4). Thus, there is a multiplicity of CPS structures (4, 6, 11, 20, 35, 37, 41, 54), but the number of CPS types that would be required in an effective, multivalent CPS conjugate vaccine against *C. jejuni* remains unknown. There are epidemiological data suggesting that most *C. jejuni* disease in a given geographical area is caused by a limited number of serotypes (17, 29, 36,

44, 52), and a recent study demonstrated that elderly patients are more likely to be infected with rare serotypes, suggesting that immunity develops against the more common CPS types in a given area (39). Thus, a final multivalent CPS conjugate vaccine against *C. jejuni* diarrhea may need to be tailored to include the prevalent CPS types found in different geographical areas.

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