RESEARCH ARTICLE

Oral Immunization with Cholera Toxin Provides Protection against *Campylobacter jejuni* in an Adult Mouse Intestinal Colonization Model

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ABSTRACT Immunity to *Campylobacter jejuni*, a major diarrheal pathogen, is largely Penner serotype specific. For broad protection, a vaccine should be based on a common antigen(s) present in all strains. In our previous study (M. J. Albert, S. Haridas, D. Steer, G. S. Dhaunsi, A. I. Smith, and B. Adler, Infect. Immun. 75:3070–3073, 2007), we demonstrated that antibody to cholera toxin (CT) cross-reacted with the major outer membrane proteins (MOMPs) of all *Campylobacter jejuni* strains tested. In the current study, we investigated whether immunization with CT protects against intestinal colonization by *C. jejuni* in an adult mouse model and whether the nontoxic subunit of CT (CT-B) is the portion mediating cross-reaction. Mice were orally immunized with CT and later challenged with *C. jejuni* strains (48, 75, and 111) of different serotypes. Control animals were immunized with phosphate-buffered saline. Fecal shedding of challenge organisms was studied daily for 9 days. Serum and fecal antibody responses were studied by enzyme-linked immunosorbent assay (ELISA) and immunoblotting. The cross-reactivity of rabbit CT-B antibody to MOMP was studied by immunoblotting. The reactivity of 21 overlapping 30-mer oligopeptides (based on MOMP's sequence) against rabbit CT antibody was tested by ELISA. Test animals produced antibodies to CT and MMP in serum and feces and showed resistance to colonization, the vaccine efficacies being 49% (for strain 48), 37% (for strain 75), and 34% (for strain 111) (P, ≤0.05 to ≤0.001). One peptide corresponding to a variable region of MOMP showed significant reactivity. CT-B antibody cross-reacted with MOMP. Since CT-B is a component of oral cholera vaccines, it might be possible to control *C. jejuni* diarrhea with these vaccines.

IMPORTANCE *Campylobacter jejuni* is a major cause of diarrhea worldwide. Patients who recover from *C. jejuni* diarrhea develop immunity to the infecting serotype and remain susceptible to infection with other serotypes. A vaccine based on a common protective antigen(s) present in all *C. jejuni* serotypes is expected to provide broad protection. In our previous study, we showed that antibody to cholera toxin (CT) reacted with the major outer membrane proteins (MOMPs) from different strains of *C. jejuni*. We assumed that the B subunit of the toxin (CT-B), which is nontoxic and a component of licensed oral cholera vaccines, might be the component that cross-reacts with MOMP. In the current study, we showed that orally immunizing mice with CT protected them against colonization upon challenge with different serotypes of *C. jejuni*. We also showed that CT-B is the component mediating cross-reaction. Therefore, it might be possible to use cholera vaccines to prevent *C. jejuni* diarrhea. This could result in significant savings in vaccine development and treatment of the disease.

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*C*ampylobacter jejuni poses a serious threat to our food security, since it is a food-borne pathogen. It is a major cause of diarrhea worldwide (1–3). The infection also results in serious sequelae, such as Guillain-Barré syndrome, Reiter's syndrome, reactive arthritis, and irritable bowel syndrome (4). The economic costs of treating *C. jejuni* diarrhea and its complications are considerable (5). Therefore, for many Western governments, food agencies, militaries, and the World Health Organization, prevention of campylobacteriosis is a priority (6, 7). One possible tool for the prevention of campylobacteriosis is vaccination, as immunity develops after infection (8–10). However, immunity in humans seems to be (Penner) serotype specific (11, 12). There are numerous Penner serotypes of *C. jejuni*, which are based on capsular polysaccharide antigens (13). Therefore, for broad protection, vaccines based on common protective antigens need to be developed.

In our previous study, we demonstrated that antibody to cholera toxin (CT) cross-reacts with the major outer membrane protein (MOMP) (PorA) from *C. jejuni* (14). Therefore, CT should be investigated as a possible vaccine. However, CT is the virulence factor of *Vibrio cholerae*, which mediates watery diarrhea (15). It is likely that the B subunit of CT (CT-B) is responsible for cross-reactivity with *C. jejuni* (14). CT-B is nontoxic and is a component of the licensed oral human cholera vaccines (16). Therefore, the
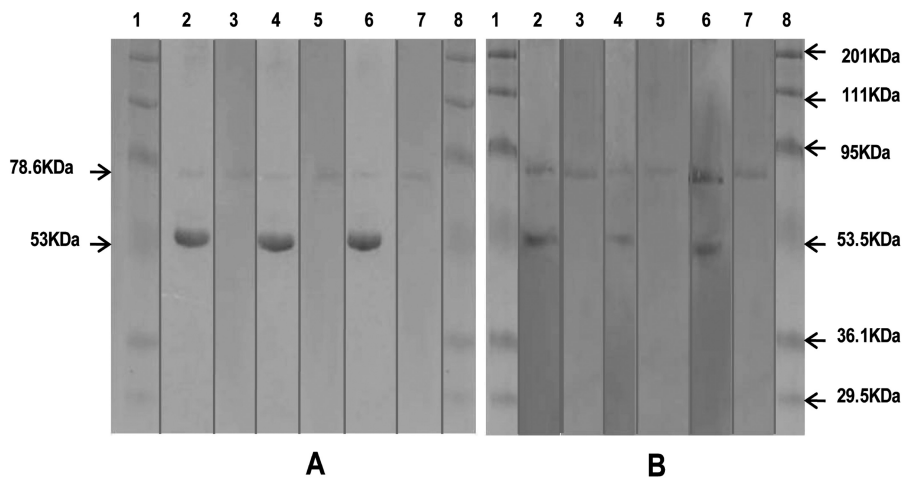
FIG 1 Immunoblots of enriched MOMPs from *C. jejuni* strains with antibody to CT (A) and antibody to CT-B (B). SDS-12% PAGE-separated MOMPs were transferred to a nitrocellulose membrane and probed with relevant rabbit antibodies. In both panels, lanes 2 to 3, 4 to 5, and 6 to 7 had MOMPs from *C. jejuni* strains 48, 75, and 111, respectively. Lanes 2, 4, and 6 in panel A were probed with anti-CT antibody, lanes 2, 4, and 6 in panel B were probed with anti-CT-B antibody, and lanes 3, 5, and 7 in both panels were probed with normal rabbit serum. The exposure times for development of bands in the lanes reacted with normal rabbit serum, anti-CT antibody, and anti-CT-B antibody were 60 s, 60 s, and 70 s, respectively. Lanes 1 and 8 in both panels have molecular mass markers, which are marked by arrowheads on the right side. The relevant proteins in the samples are marked by arrowheads on the left side.

possibility exists that human campylobacteriosis can be prevented by the existing cholera vaccines.

Widely available, inexpensive animal models of *C. jejuni* diarrhea are lacking. Oral feeding of adult mice with *C. jejuni* results in intestinal colonization, with chronic shedding of the organism, but the shedding is without diarrhea. Levels of shedding of the organism, determined by serial culture of stool specimens, vary. There is no tissue damage or severe inflammation. All levels of the intestine are colonized, and bloodstream infection occurs soon after oral feeding, with seeding of visceral organs, such as the liver. In response to feeding, the animals produce serum and intestinal antibodies to the vaccine (17–19). Therefore, the adult mouse colonization model has been used for evaluation of *C. jejuni* candidate vaccines by several investigators (18, 20, 21).

Prior to testing cholera vaccines against *C. jejuni* diarrhea, we evaluated CT as a vaccine against colonization by different Penner serotypes of *C. jejuni* in the adult mouse intestinal colonization model. We also studied whether CT-B is responsible for cross-reaction with the MOMP of *C. jejuni*. An attempt was made to identify by peptide mapping the epitope(s) on MOMP mediating cross-reaction.

RESULTS

Cross-reactivity of *C. jejuni* strains with CT. Western blots showing the cross-reactions among CT, CT-B, and the three enriched MOMPs of *C. jejuni* strains 48, 75, and 111 are shown in Fig. 1. Rabbit antibody to CT generated two bands, a specific 53-kDa band and a nonspecific 78.6-kDa band, with exposure to all three of the *C. jejuni* strains 48, 75, and 111. When normal rabbit serum was used, only the nonspecific band was visible (Fig. 1A). The same patterns of reactions were seen when rabbit antibody to CT-B and normal rabbit serum were used (Fig. 1B).

Fecal excretion of *C. jejuni* strains. The quantities of *C. jejuni* organisms excreted daily for 9 days by CT-immunized and control mice are shown in Fig. 2. Four oral CT doses were given at weekly intervals. The vaccinated mice excreted smaller amounts of *C. je-*

juni than control mice fed phosphate-buffered saline (PBS). The amounts of bacteria excreted varied on different days. Although there was a trend of decreasing excretion for strains 75 and 111 for the follow-up period, excretion increased for strain 48. For all three strains, on many days of the 9-day excretion period studied, the differences between results for vaccinated and control mice achieved statistical significance. The mean counts for the total 9-day duration were also significantly lower in vaccinated mice than in control mice for all three challenge strains.

Colonizations of *C. jejuni* strains and vaccine efficacies. The colonization rates of the three *C. jejuni* strains and the efficacy of the CT vaccine are shown in Fig. 3. In general, the colonization rates were lower in vaccinated mice than in control mice for each of the 9 days of observation. On certain days, the differences were statistically significant. The mean rates of colonization for the total 9-day period were significantly lower in vaccinated mice than in control mice for all three challenge strains.

The mean efficacies of the CT vaccine for the 9-day observation period for the three strains were 49% for *C. jejuni* 48, 37% for *C. jejuni* 75, and 34% for *C. jejuni* 111, all of which were highly significant.

Antibody responses to CT and *C. jejuni* MOMP. Sera and feces were collected prior to vaccination and 1 week after the last vaccine dose for antibody estimation. Postvaccination titers of serum antibody to the two antigens were significantly higher than prevaccination titers of antibody in mice vaccinated with CT. Antibodies to CT were of both the IgG and IgA isotypes. However, only IgM isotype antibodies to MOMP were present in postvaccination sera. When paired sera and feces from three randomly selected immunized mice were tested by immunoblotting for MOMP, postimmunization samples showed antibody responses to MOMP (data not shown). The immune response was stronger to CT than to MOMP (Table 1). There were no antibody responses to CT and MOMP in the preimmune and postimmunization sera of control mice fed PBS (data not shown). Antibody responses to both antigens in feces are given in Table 2. There were
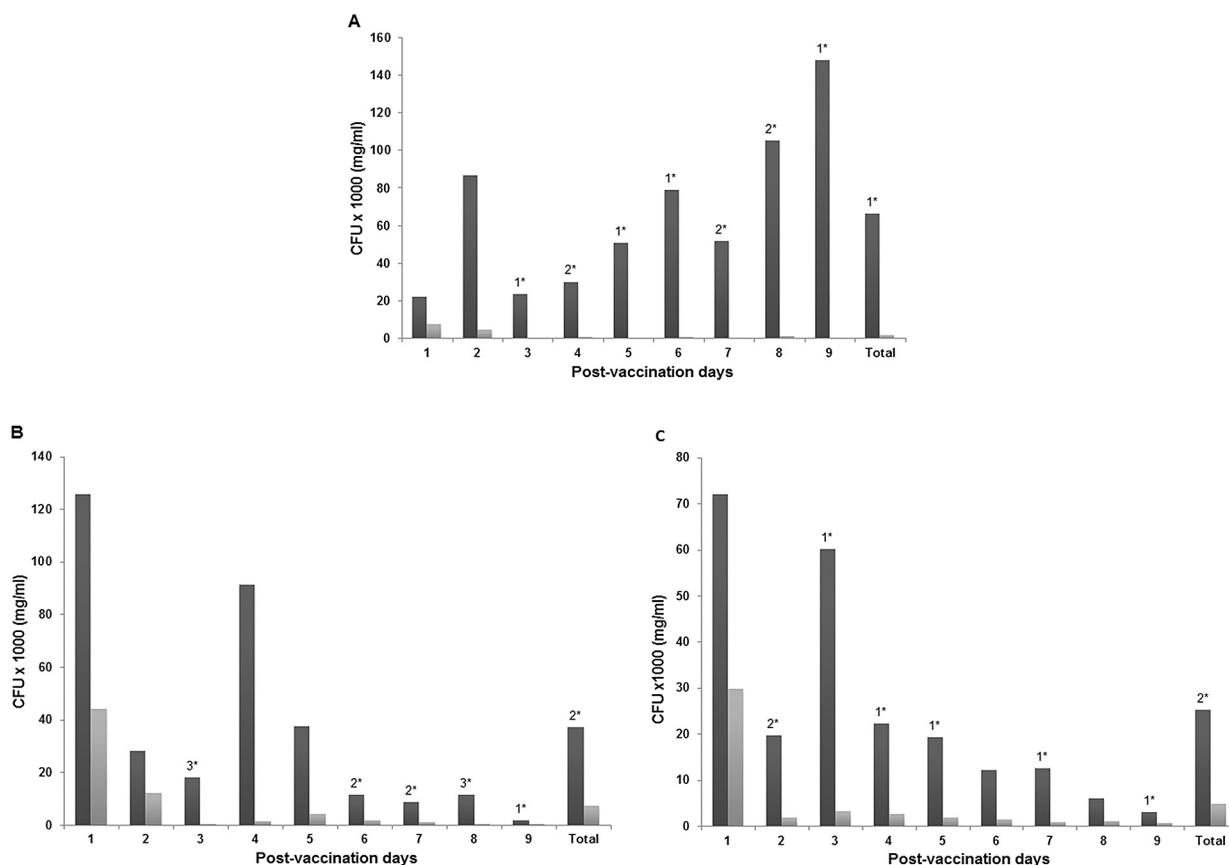
FIG 2 Excretion of challenge *C. jejuni* organisms by mice orally vaccinated with CT (*n* = 9) (light bars) or control mice fed PBS (*n* = 9) (dark bars). Levels of excretion of organisms for each of the postvaccination days (*x* axis) are given. "Total" represents the mean count for all 9 days. On the *y* axis, numbers of CFU of organisms (in mg of stool per ml) are given. Wherever there is a statistically significant difference in the levels of excretion of organism between vaccinated and control mice, appropriate *P* values are placed on top of the bars. 1*, $P \leq 0.05$; 2*, $P \leq 0.01$; 3*, $P \leq 0.001$. (A, B, and C) Data for *C. jejuni* 48, 75, and 111, respectively.

significantly higher responses to both antigens after vaccination with CT. The fecal antibody response to CT was higher than that to MOMP. There were no fecal antibody responses to CT and MOMP in control mice fed PBS (data not shown).

Reactivities of synthetic peptides with antibody to CT. This study was done to find out the epitope(s) on MOMP of *C. jejuni* that cross-reacts with CT. The antigens in enzyme-linked immunosorbent assays (ELISAs) were 30-mer oligopeptides that corresponded to the sequence of MOMP from *C. jejuni* strain NCTC 11168. The ratios of optical density (OD) readings of serum from a postimmunization rabbit (an animal immunized with CT) to its preimmune serum OD after reactivity with the synthetic 30-mer peptides varied from 0.96 for peptide 17 (P17) to 2.15 for P16. P16 was the only peptide that had a ratio greater than 2.0. Therefore, P16 was taken as the peptide that has cross-reactivity with CT (Table 3). The ratio for control MOMP was 0.488, and that for CT was 14.44. The amino acid identity of P16 with CT-B (from the classical *Vibrio cholerae* O1 strain) was 23.3%, while several other nonreactive peptides had even higher homologies with CT-B (Table 3).

DISCUSSION

As expected, the three *C. jejuni* challenge strains used in the current study reacted with antibody to CT. We suspected that the B

subunit of CT might have cross-reacted with MOMP of *C. jejuni*. This was indeed the case.

Even though CT is toxic to mice, they tolerate up to 20 μg of CT when it is given orally (22). We adjusted the dose such that it resulted in a good immune response and no death of animals. Mice immunized with CT shed significantly smaller amounts of challenge strains of *C. jejuni* than control mice fed PBS. As found in other studies, the levels of excretion of the challenge organism varied (17, 19, 23). It is possible that the shed organisms are not uniformly distributed in fecal pellets, as only a few pellets were sampled. Also, as strain 48 showed a trend of increasing excretion, it suggests that this strain is a robust colonizer of mouse intestine. The fact that certain vaccinated animals excreted challenge organisms, albeit at a lower level than control animals, suggests that immunity is partial, which allows some degree of colonization and multiplication of challenge organisms. It is possible that better immunity can be achieved by increasing the vaccine dose. The classical method of measuring protection is by determining colonization resistance (20, 23). Immunized mice showed colonization resistance to all three challenge organisms. This resistance was significant overall for the 9-day period and on certain days during the 9-day period.

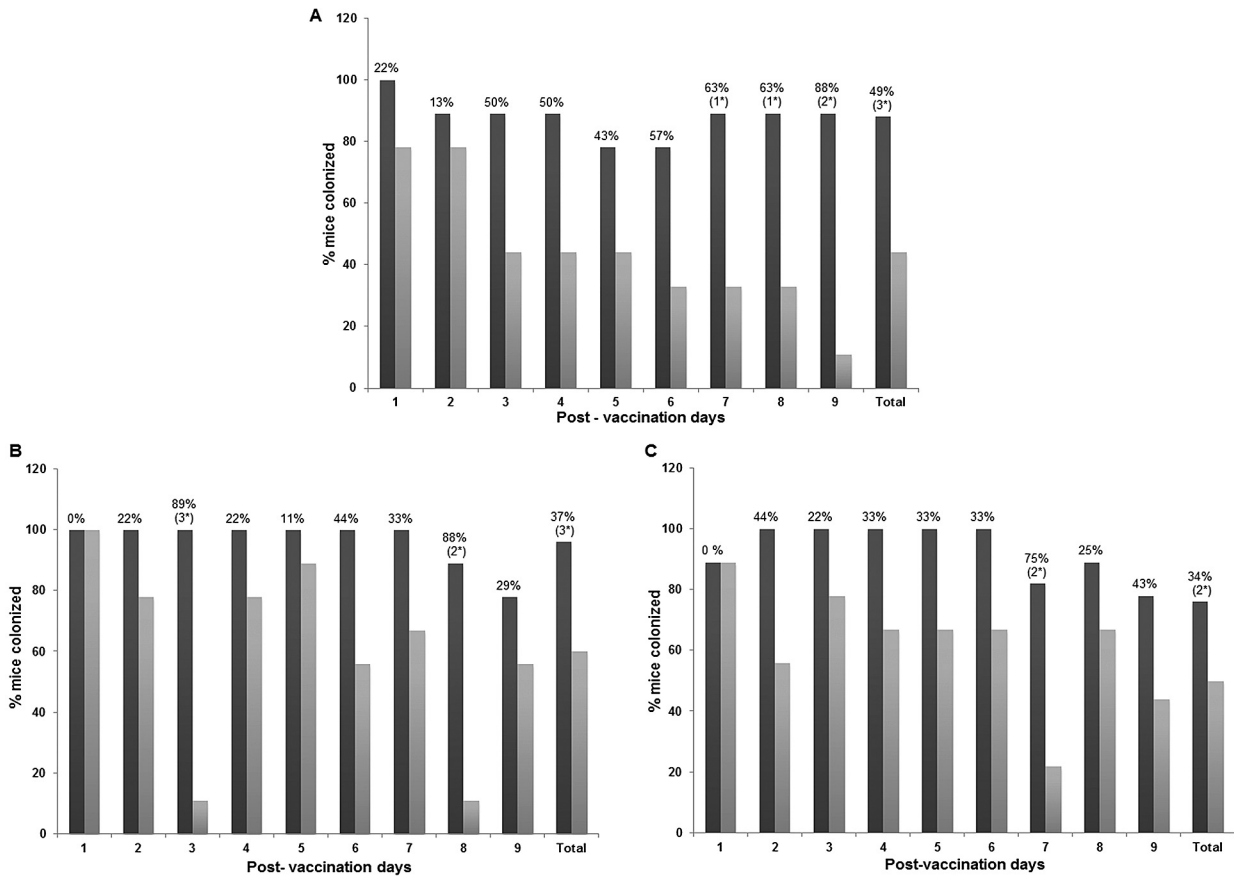The immunized mice produced robust serum antibody re-

FIG 3 Vaccine efficacy in mice orally vaccinated with CT ($n = 9$) compared to that in control mice fed PBS ($n = 9$). Levels of colonization of the challenge organism for each of the postvaccination days (x axis) are given. "Total" represents the mean colonization for all 9 days. On the y axis, percentages of mice colonized are given. Light-gray bars, vaccinated mice; dark-gray bars, control mice. On top of the bars, the percentage of mice showing vaccine efficacy is presented. Wherever vaccine efficacy is significant, P values are given within parentheses on top of the bars. 1*, $P \leq 0.05$; 2*, $P \leq 0.01$; 3, $P \leq 0.001$. (A, B, and C) Data for *C. jejuni* 48, 75, and 111, respectively.

sponses (IgG and IgA isotypes) and fecal antibody responses (IgA isotype) to CT. As expected, the antibody responses to MOMP of *C. jejuni* in response to CT vaccination were weaker. Surprisingly, the serum antibody response to MOMP was limited to the IgM class. The reason for this is not clear. In immune responses to certain antigens, the antibody response is segregated to certain isotypes of immunoglobulin (24); however, we might have seen responses to other isotypes had we extended the study to include isotype switching.

Again, as expected, postimmunization mouse sera reacted with MOMP in immunoblots. This suggests that the cross-reactive CT antibody reacts with MOMP of *C. jejuni*, providing resistance. It is likely that intestinal antibody might mediate resistance to colonization with *C. jejuni*.

Different antigens have been evaluated as possible vaccine candidates using different animal models (reviewed in reference 25). The vaccines evaluated in the mouse model are relevant for discussion. These included killed whole cells (18), flagellin fused to maltose-binding protein of *Escherichia coli* (26), periplasmic binding protein (27), secreted flagellum proteins (28), conjugated capsular polysaccharides (29), an adherence protein expressed on and delivered by an attenuated *Salmonella enterica* serovar Typhimurium strain (30), and recombinant MOMP (20). The mouse models were intranasal or intestinal colonization models. Vaccines were delivered intranasally, orally, or parenterally. The vaccines provided either no protection or various degrees of protection. Further studies with some of these promising candidate vaccines are awaited.

TABLE 1 Mean titers of pre- and postvaccination serum antibody to CT and *C. jejuni* MOMP in mice[a]

| Serum | Titer (SD) of indicated antibody to CT | | Titer (SD) of IgM antibody to MOMP |
	IgG	IgA	
Prevaccination	<200 (0.0) A	<200 (0.0) B	<200 (0.0) C
Postvaccination	22,755.6 (5,644.3) A	1,8311.1 (11,001.4) B	533.3 (264.6) C

[a] The number of mice was 9. The same letter is placed next to values that indicate a significant difference in titers by the Mann-Whitney test, as follows: A, $P = 0.0001$; B, $P = 0.0001$; and C, $P = 0.001$.

TABLE 2 Mean pre- and postvaccination titers of fecal IgA antibody to CT and *C. jejuni* MOMP/total IgA in mice[a]

| Fecal IgA | Titer of antibody/total IgA in μg/mg of stool (SD) to: | |
	CT	MOMP
Prevaccination	16.08 (5.03) A	0.23 (0.30) B
Postvaccination	441.19 (310.90) A	1.05 (0.49) B

[a] The number of mice was 9. The same letter is placed next to values that indicate a significant difference in titers by the Mann-Whitney test, as follows: A, $P = 0.0001$, and B, $P = 0.002$.

Synthetic overlapping peptides based on the amino acid sequence of MOMP of *C. jejuni* strain NCTC 11168 was reacted with CT antibody to identify the cross-reacting epitope(s). Only one peptide, P16, was reactive. Comparison of sequences of peptides with that of CT-B revealed that many nonreactive peptides had higher identities with CT-B than with P16. This suggested that the three-dimensional structure of the protein might determine the epitope for antibody binding to MOMP. Immunization with P16 and then challenge studies need to be carried out to evaluate the importance of this peptide.

The MOMP of *C. jejuni* comprises 18 β-strands that form an antiparallel β-barrel, with short turns at the bottom of the barrel facing the periplasm and long loops at the top of the barrel facing the external surface of the outer membrane. The β-strands form the conserved regions, and the external loops form the variable regions. Surprisingly, most of the amino acids in P16 were in variable external loop 7 (L7) (31). However, in L7, many amino acids are conserved among strains, suggesting that a sequence of a small number of amino acids may provide an antibody-binding epitope across all strains of *C. jejuni*. Our study indicated that CT-B is the subunit of CT responsible for cross-reaction with MOMP of *C. jejuni*. It is interesting that in our previous study, immunization with a recombinant MOMP provided heterologous protection against intestinal colonization (20). Since CT-B is a component of licensed cholera vaccines (16), there exists the possibility that *C. jejuni* diarrhea can be controlled by immunization with cholera vaccines. This possibility should be explored by immunization with CT-B and cholera vaccines. As all clinically important species of *Campylobacter* react with CT antibody (32), it is possible to control all *Campylobacter* species diarrheas with cholera vaccines. There will be significant economic benefits if we can use the existing cholera vaccines, as this will obviate the need for developing new vaccines against *Campylobacter* spp.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Three *C. jejuni* strains, 48 (Penner serotype O:19), 75 (serotype O:3), and 111 (serotype O:1,44), isolated from diarrheal patients in Kuwait were used as challenge strains as previously reported (20). The organisms were grown on *Campylobacter*-selective agar consisting of agar base (Oxoid, Basingstoke, Hampshire, England) supplemented with 7% defibrinated horse blood, growth supplement, and selective supplement (all from Oxoid). The culture was incubated at 42°C for 48 h microaerobically in a water-jacketed incubator (Nuaire, United States Autoflow, Plymouth, MN). The organisms were confirmed by cultural characteristics and by molecular methods. The three serotypes had unique fingerprints by *flaA* restriction fragment length polymorphism (RFLP) analysis, as described previously (20).

Animals. Studies were carried out using *Campylobacter*-free BALB/c female mice (6 to 8 weeks old) obtained from the Animal Resources Center, Health Sciences Centre, Kuwait University, and housed separately for 1 week prior to experimentation. The animals were fed a standard laboratory chow (Special Diet Food Services, Ltd., Essex, United Kingdom) (20).

Preparation of bacterial lysate. Bacterial lysates were prepared after growing the organism on campylobacter agar. Bacterial cells from plates were harvested in 50 ml of sterile distilled water. The culture was centrifuged at 5,000 × *g* for 30 min at 4°C in a Beckman centrifuge (Beckman,

TABLE 3 Sequence identities of synthetic peptides with CT-B and their ELISA reactivities with rabbit CT antiserum

Peptide	Peptide sequence[a]	% identity with CT-B	ELISA reactivity ratio[b]
P1	1-MKLVKLSLVAALAAGAFSAA<u>NATPLEEAIK</u>-30	26.7	1.37
P2	21-<u>NATPLEEAIK</u>DVDVSGVLRY<u>RYDTGNFDKN</u>-50	26.7	1.14
P3	41-<u>RYDTGNFDKN</u>FVNNSNLNNS<u>KQDHKYRAQV</u>-70	10.0	1.23
P4	61-<u>KQDHKYRAQV</u>NFSAAIADNF<u>KAFVQFDYNA</u>-90	23.3	1.06
P5	81-<u>KAFVQFDYNA</u>ADGGYGANGI<u>KNDQKGLFVR</u>-110	16.7	1.21
P6	101-<u>KNDQKGLFVR</u>QLYLTYTNED<u>VATSVIAGKQ</u>-130	26.7	1.15
P7	121-<u>VATSVIAGKQ</u>QLNLIWTDNAI<u>DGLVGTGVK</u>-150	10.0	1.26
P8	141-<u>IDGLVGTGVK</u>VVNNSIDGLT<u>LAAFAVDSFM</u>-170	26.7	1.30
P9	161-<u>LAAFAVDSFM</u>AAEQGADLLE<u>HSNISTTSNQ</u>-190	13.3	1.21
P10	181-<u>HSNISTTSNQ</u>APFKVDSVGN<u>LYGAAAVGSY</u>-210	30.0	0.96
P11	201-<u>LYGAAAVGSY</u>DLAGGQFNPQ<u>LWLAYWDQVA</u>-230	33.3	1.06
P12	221-<u>LWLAYWDQVA</u>FFYAVDAAYS<u>TTIFDGINWT</u>-250	23.3	1.10
P13	241-<u>TTIFDGINWT</u>LEGAYLGNSL<u>DSELDDKTHA</u>-270	20.0	1.15
P14	261-<u>DSELDDKTHA</u>NGNLFALKGS<u>IEVNGWDASL</u>-290	33.3	1.06
P15	281-<u>IEVNGWDASL</u>GGLYYGDKEK<u>ASTVVIEDQG</u>-310	20.0	1.23
P16	301-<u>ASTVVIEDQG</u>NLGSLLAGEE<u>IFYTTGSRLN</u>-330	23.3	2.15
P17	321-<u>IFYTTGSRLN</u>GDTGRNIFGY<u>VTGGYTFNET</u>-350	30.0	0.96
P18	341-<u>VTGGYTFNET</u>VRVGADFVYG<u>GTKTEAANHL</u>-370	20.0	0.99
P19	361-<u>GTKTEAANHL</u>GGGKKLEAVA<u>RVDYKYSPKL</u>-390	20.0	1.09
P20	381-<u>RVDYKYSPKL</u>NFSAFYSYVN<u>LDQGVNTNES</u>-410	16.7	1.13
P21	401-<u>LDQGVNTNES</u>ADHSTVRLQALYKF-424	13.3	1.06

[a] The amino acids are denoted by single-letter designation. The positions of amino acids at the beginning and end of peptides are numbered; amino acids that overlap adjacent peptides are underlined. Numbering is based on the sequence of the major outer membrane protein (MOMP) of *C. jejuni* NCTC 11168.
[b] The OD reading at a wavelength of 405 nm for postimmunization CT antiserum was divided by that for preimmune serum.

Fullerton, CA). The pellet was resuspended in 50 ml of phosphate-buffered saline (PBS; pH 7.2) and lysed by sonication (Labsonic; B. Braun, PA) at 0.7 cycle for 20 min. The sonicate was centrifuged at $100,000 \times g$ for 1 h at 4°C in an L8-70 ultracentrifuge (Beckman). The pellet was suspended in sterile distilled water and stored at −20°C until use. Protein concentration was measured using a NanoDrop 8000 spectrophotometer (Thermo Scientific, Waltham, MA).

Preparation of enriched MOMP. The MOMPs of *C. jejuni* 48, 75, and 111 were enriched by the Sarkosyl method (20). Briefly, the organism was grown on blood agar at 42°C for 48 h in a microaerobic atmosphere. Bacterial cells were disrupted by sonication and centrifuged at $5,000 \times g$ to remove whole cells. The supernatant was centrifuged at $100,000 \times g$ for 1 h at 4°C in an L8-70 ultracentrifuge (Beckman). The pellet was treated with sodium lauryl sarcosinate. The Sarkosyl-insoluble portion was used as the enriched MOMP.

Cross-reactivity of CT and CT-B with MOMP of *C. jejuni*. The cross-reactivities of CT and CT-B with MOMPs of *C. jejuni* strains 48, 75, and 111 were studied by Western blotting. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with a 12% separating gel according to the method of Laemmli (33). The nitro-cellulose membrane with transferred proteins was reacted with rabbit anti-CT antibody, rabbit anti-CT-B antibody (both from Sigma), or a normal rabbit serum control, as appropriate, all diluted to 1:2,000 in PBS with 0.05% Tween 20 (PBST) and 5.0% bovine serum albumin (BSA) (PBSTB). The secondary antibody, peroxidase-conjugated anti-rabbit immunoglobulins (Sigma) diluted to 1:50,000 in PBSTB, was added, after which the membrane was developed with enhanced-chemiluminescence Western blotting detection reagents according to the instructions of the manufacturer (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, United Kingdom).

In some instances, immunoblotting was done using pre- and postimmunization sera and feces from mice (see the section below for vaccination). The antigen used was MOMP from strain 48. The mouse sera were used at a dilution of 1:100, and the feces were used at a dilution of 1:20. The anti-mouse immunoglobulin peroxidase conjugates used were of the correct IgM isotype [F(ab′)$_2$ raised in goats] (Jackson ImmunoResearch Laboratories, Burlington, Ontario, Canada), diluted to 1:5,000 in PBSTB for serum antibody detection, and of the IgA isotype (Kirkegaard & Perry Laboratories, Gaithersburg, MD), diluted to 1:500 for fecal antibody detection.

Vaccination. Vaccination of mice was carried out as described by Baqar et al. (23). Briefly, BALB/c mice were orally vaccinated four times at weekly intervals with 10 μg (first week), 15 μg (second week), 15 μg (third week), and 15 μg (fourth week) of CT mixed in 200 μl of 0.1 M NaHCO$_3$ (pH 8.6). Vaccine was administered by using a sterile stainless steel, curved, ball-tip feeding needle (20 gauge, 1.5 inches long; Popper & Sons, Inc., New Hyde Park, NY). Mice immunized with PBS (pH 7.2) alone served as controls. There were 9 test mice and 9 control mice for challenge with each *C. jejuni* challenge strain.

Collection of feces and blood for antibody estimation. Fecal pellets and blood were collected from each mouse a day prior to vaccination and 1 week after the last vaccine dose. About 100 to 120 mg of fresh fecal pellets was collected and stored at −70°C until studied.

An aliquot of 50 μl of tail blood was mixed with 950 μl of PBS (pH 7.2) containing 0.1% Tween 20. After one cycle of freezing and thawing, the sample was centrifuged at $400 \times g$ for 15 min (Spectrafuge 24-D; Labnet, NJ) and the supernatant was stored at −70°C until studied.

Challenge of vaccinated mice. Mice were orally challenged with bacterial cultures (strains 48, 75, and 111 separately) 1 week after the last vaccination dose, immediately after fecal and blood collection. First, the gastric acidity was neutralized with two doses (0.5 ml each) of a 5% sodium bicarbonate (pH 8.5) solution given by oral gavage at an interval of 15 min. Then, 0.5 ml of the challenge organism in PBS (pH 7.2) containing 1×10^9 CFU per ml prepared from a 48-h bacterial culture grown on

campylobacter-selective agar was given orally. The oral feeding was done with the help of the feeding needle (20).

Fecal shedding of the challenge organism was monitored daily for 9 days. Feces from individual mice (100 to 120 mg per mouse) were collected and diluted to 10% with PBS (pH 7.2), followed by 5-fold serial dilutions. An aliquot of 50 μl of each dilution was plated in duplicate onto campylobacter agar. After 48 h of incubation, *C. jejuni* colonies were counted and expressed as numbers of CFU per mg of feces. The strain identity was confirmed by *flaA* RFLP analysis of random colonies (20).

Processing of fecal pellets for IgA antibody. IgA antibody was extracted by adding 20 μl of IgA extraction buffer per mg of feces as described by Baqar et al. (28).

Measurement of total IgA in fecal extract. The total IgA in fecal pellets was determined by ELISA (28). For all ELISAs in this study, MaxiSorp plates (Nunc, Rochester, NY) were used. The wells of a microtiter plate were coated with goat anti-mouse IgA (Kirkegaard & Perry Laboratories) in carbonate buffer (pH 9.6) and incubated at 37°C for 1 h, followed by 24 h of incubation at 4°C. The wells were washed three times with PBST and blocked with PBSTB at 37°C for 2 h.

Various concentrations (3 to 300 ng/ml) of purified mouse IgA (Bethyl Laboratories, Montgomery, TX) or serial doubling dilutions (starting with 1:100) of fecal extracts (from preimmunized and postimmunized mice) in PBST with 1.0% BSA (PBSTb) were added to the wells, followed by incubation at 37°C for 2 h. The wells were washed three times with PBST and then incubated with horseradish peroxidase-conjugated goat anti-mouse IgA antibody (Kirkegaard & Perry Laboratories) diluted 1:5,000 in PBSTb at 37°C for 2 h. The wells were washed three times with PBST. The substrate, 2,2′-azino-di-(3-ethyl-benzthiazoline sulfonate) (ABTS; Sigma, St. Louis, MO) was added, and after 30 min of incubation at 37°C, the optical density (OD) at 405 nm was measured using a PowerWave microplate spectrophotometer (BioTek Instruments Inc., Potton, Bedfordshire, United Kingdom). The amount of IgA present in a fecal pellet was determined by interpolation of OD on a standard curve constructed with OD readings of known quantities of purified mouse IgA.

Measurement of the CT antibody response in feces. CT-specific IgA antibody was measured in mouse fecal pellets by ELISA. The wells of a microtiter plate were coated with 1 μg/ml of CT (Sigma) in carbonate buffer at 4°C for 24 h. The wells were washed three times with PBST and blocked with PBSTB at 37°C for 1 h. Serial doubling dilutions (starting with 1:20) of fecal samples in PBSTb were added to the wells, followed by incubation at 37°C for 1 h. The wells were washed three times with PBST and then incubated with horseradish peroxidase-conjugated goat anti-mouse IgA antibody (Kirkegaard & Perry Laboratories) diluted 1:5,000 in PBSTb at 37°C for 1 h. The wells were washed three times with PBST. The substrate, ABTS (Sigma), was added, and after 30 min of incubation at 37°C, the OD at 405 nm was measured. Endpoint titers were expressed as the reciprocal of the dilution giving an absorbance value ≥2 standard deviations above the background absorbance in wells containing feces from normal mice (23). The specific IgA antibody titer was divided by the total IgA concentration (in μg/mg) in the feces of each mouse to adjust for variation among mice (34).

Measurement of the CT antibody response in serum. CT-specific antibodies (of the IgG or IgA isotype) were measured in blood samples by ELISA using the modified method of Baqar et al. (23). The wells of a microtiter plate were coated with 1 μg/ml of CT (Sigma) in carbonate buffer at 4°C for 24 h. The wells were washed three times with PBST and blocked with PBSTB at 37°C for 1 h. Serial doubling dilutions of lysed-blood samples (starting at 1:200) in PBSTb were added to the wells, followed by incubation at 37°C for 1 h. The wells were washed three times with PBST and then incubated with horseradish peroxidase-conjugated goat anti-mouse secondary antibodies (of the IgA or IgG isotype) (Kirkegaard & Perry Laboratories) diluted to 1:5,000 in PBSTb at 37°C for 1 h. The wells were washed three times with PBST. The substrate, ABTS (Sigma), was added, and after 30 min of incubation at 37°C, the OD at 405 nm was measured. Endpoint titers were expressed as the reciprocal of the

dilution giving an absorbance value ≥2 standard deviations above the background absorbance in wells containing lysed-blood samples from normal mice (23).

Measurement of the MOMP antibody response in serum. An initial screening suggested that MOMPs from all three challenge strains of *C. jejuni* were equally reactive against sera from immunized mice. Therefore, responses of antibodies (of the IgG, IgA, or IgM isotype) to MOMP from *C. jejuni* 48 only was studied. The wells of a microtiter plate were coated with 10 μg/ml of MOMP from *C. jejuni* 48 in carbonate buffer at 4°C for 24 h. The other steps were as described above except that the starting dilution of mouse serum was 1:100. The endpoint titers were determined as described above for the CT antibody.

Measurement of the MOMP IgA antibody response in feces. *C. jejuni* MOMP-specific antibody in the feces of mice was measured by ELISA using the modified method of Baqar et al. (23). MOMP from *C. jejuni* strain 48 was used as the antigen for measurement of antibody. The wells of a microtiter plate were coated as described above and reacted with serial doubling dilutions of fecal extracts (with a starting dilution of 1:20). Horseradish peroxidase-conjugated goat anti-mouse secondary antibody (of the IgA isotype) (Kirkegaard & Perry Laboratories) diluted 1:1,000 was used. Endpoint titers were expressed as the reciprocal of the dilution giving an absorbance value ≥2 standard deviations above the background absorbance in wells containing feces from normal mice (23). The specific IgA antibody titer was divided by the total IgA concentration (in μg/mg) in the feces of each mouse to adjust for variation among mice, as described above.

Vaccine efficacy. Vaccine efficacy was calculated as described previously (20).

Preparation of antibody to cholera toxin in rabbits. Two young adult rabbits weighing about 2 kg were used for immunization. Immunizations were carried out by the subcutaneous route in the area between the shoulder blades. Four doses were administered, with an interval of 3 weeks between the doses. The primary dose consisted of 25 μg of CT (Sigma) dissolved in 250 μl of PBS (pH 7.2) and mixed with 250 μl of Freund's complete adjuvant (Sigma). The booster doses consisted of the same amount of CT but were mixed with Freund's incomplete adjuvant (Sigma). The animals were bled 3 weeks after the last dose.

Peptide mapping. Overlapping 30-mer peptides (Thermo, Fisher Scientific, Ulm, Germany) based on the complete MOMP (PorA) sequence of *C. jejuni* NCTC 11168 (36) were used for epitope mapping. The 21 peptides (Table 3) were dissolved in PBS (pH 7.2) to a concentration of 1 mg/ml. Microtiter plates were coated with 10 μg/ml of each peptide in carbonate buffer and incubated at 4°C for 24 h. The wells were washed with PBST and blocked with 5% milk powder (Regilait, Saint-Martin-Belle-Roche, France) in PBST. Pre- and postimmunization rabbit sera from the rabbit immunized with CT described above (diluted to 1:1,000 in PBST with 1% milk powder [PBSTM]) were added to the wells in triplicate, followed by incubation at 37°C for 1 h. After being washed, the wells were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody (Jackson Immuno Research Laboratories, Suffolk, United Kingdom) diluted to 1:1,000 in PBSTM at 37°C for 1 h. Afterwards, the substrate (ABTS) (Sigma) was added and the OD at 405 nm was measured. A ratio of the OD reading of postimmunization serum to the OD reading of preimmune serum (corresponding to a peptide) that was ≥2.0 was considered a significant reactivity (35).

Statistical analysis. Statistical analysis was done by using SPSS version 17 (SPSS, Chicago, IL). Comparison of rates of mice free from colonization with *C. jejuni* was done by the Fisher exact test. The comparisons of endpoint antibody titers and fecal *C. jejuni* colony counts were done by the Mann-Whitney test. Values differing with a P of ≤0.05 were considered statistically significant.

ACKNOWLEDGMENTS

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