INFECTION AND IMMUNITY, Nov. 1992, p. 4938–4944
0019-9567/92/114938-07$02.00/0

Vol. 60, No. 11

Human Immune Response to *Campylobacter jejuni* Proteins Expressed In Vivo

PINAKI PANIGRAHI,[1,2]* GENEVIEVE LOSONSKY,[1] LOUIS J. DETOLLA,[2,3] AND J. GLENN MORRIS, JR.[1]

Department of Medicine, Division of Geographic Medicine,[1] Division of Infectious Diseases,[3] and Department of Pathology,[2] University of Maryland School of Medicine, Baltimore, Maryland 21201

Received 24 June 1992/Accepted 18 August 1992

***Campylobacter jejuni* 81-176 grown in vivo in rabbit ileal loops expresses novel proteins that are not expressed under standard laboratory culture conditions. A new protein with a molecular mass of ca. 180 kDa is expressed at 14, 24, and 48 h of infection. Three other proteins, with molecular masses of ca. 66, 43, and 35 kDa, are overexpressed during different phases of infection. Expression of these proteins stops immediately during the first passage in laboratory media, and they do not elicit a human immune response. Two other proteins, with molecular masses of ca. 84 and 47 kDa, expressed 48 h after infection can be identified by using convalescent sera from human volunteers who were immune to *C. jejuni* infection upon rechallenge; these proteins were not visualized on sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels by Coomassie blue staining or silver staining. Antibodies to the 84- and 47-kDa proteins are of the immunoglobulin G class. Both preinfection and convalescent human sera react strongly to the *C. jejuni* flagellin (a 58-kDa protein), suggesting the presence of cross-reactive antibodies to this protein in healthy humans. Major outer membrane protein and flagella may play a role in providing protection against *C. jejuni* disease, but our data suggest that there are other proteins expressed only during in vivo growth of the organism that elicit a strong immune response in human *C. jejuni* infections.**

Campylobacter jejuni is one of the most frequently isolated bacterial pathogens in diarrheal diseases throughout the world (4, 6, 9, 14, 40). In developing countries, *C. jejuni* is endemic, the highest infection rates being reported among young children (9). Children over the age of two years in such areas are often asymptomatic carriers (9, 14, 17). In developed countries (presumably because of a higher degree of sanitation), reinfection is not common and *C. jejuni* infection presents with a more severe form of inflammatory diarrhea, often with fecal leukocytes, blood, and mucus (40).

Humoral immune responses have been described following *C. jejuni* infection in humans (6, 7, 9, 17, 24–26, 28, 29, 31, 32) and in several animal models, including rabbits (11), mice (33), hamsters (2, 19), and monkeys (37). In immunoblot analysis, several immunogenic proteins have been described by different investigators (13, 28, 41). A secretory mucosal immunoglobulin A (IgA) response has also been documented in human infections (41) and in experiments with rabbits (10, 24). Flagella of *C. jejuni* are immunogenic, play a role in colonization (2, 12, 33, 35), and provide partial protection against strains having the same flagellar type (1, 2). Though there are ample suggestions in the literature that other proteins in *C. jejuni* provide protection against disease, no nonflagellar protective antigen has been identified in this widely distributed enteric pathogen.

In a human volunteer study conducted in our institution, specific serum IgG, IgM, and IgA responses were seen in volunteers who had diarrhea after ingestion of *C. jejuni*. None of the volunteers who received a second challenge with the same strain became ill, indicating that protective immunity was elicited after a primary infection (5). There have been reports regarding change in the expression of proteins and also in the virulence of several organisms, including *C. jejuni*, *Vibrio cholerae*, and *Mycoplasma dispar*, by modification of growth conditions in vivo and in vitro (3, 15, 20, 21). In this paper, we describe the reaction of the human volunteer immune sera to proteins from *C. jejuni* grown under standard microaerophilic conditions and also to novel proteins from *C. jejuni* that were expressed in vivo in rabbit ileal loops.

MATERIALS AND METHODS

Bacterial strain and human volunteer sera. *C. jejuni* 81-176 (Penner serotype 23/36) was isolated from a nine-year-old girl in a diarrhea outbreak in Minnesota in which raw milk consumption was implicated as the source of *C. jejuni* infection (23). Strains were recovered from frozen stocks and grown on Columbia agar (Remel, Lenexa, Kans.) in anaerobic jars under a microaerophilic atmosphere produced by CampyPak II gas-generating envelopes (BBL Microbiology Systems, Cockeysville, Md.) at 37°C. Plates were incubated for 14, 24, and 48 h. For all experiments with a medium-grown organism, *C. jejuni* was obtained after 48 h of growth. Serum samples were from a previously reported human volunteer study conducted by Black et al. (5). In that study, 39 volunteers were initially challenged with 10^6 to 10^9 CFU of *C. jejuni* 81-176. Overall, 18 (46%) of these volunteers became ill, illness being defined as the presence of diarrhea (total of >200 ml of liquid stool) or fever (temperature >30°C), but all the volunteers were colonized with *C. jejuni*. Seven of these volunteers rechallenged with 10^8 to 10^9 CFU of *C. jejuni* one month after their primary infection were immune to *C. jejuni* disease. Serum samples were obtained from these volunteers before the initial challenge, on days 7, 21, and 28 after the initial challenge, and after the rechallenge. Antibody titers were measured by enzyme-

* Corresponding author.

linked immunosorbent assay (ELISA) using an acid extract of *C. jejuni* surface proteins as the antigen. Details of the methods and chronology of the antibody response for different groups of volunteers have been described previously (5). In the present study, for each volunteer we compared the immune reaction of serum obtained before primary infection (preimmune) with the immune reaction of post-rechallenge serum having the highest antibody titer by ELISA.

In vivo modification of *C. jejuni* proteins in the rabbit ileal loop. Ileal loops were prepared in New Zealand White rabbits by standard protocols (21) with some modifications. The ileum was flushed thoroughly with warm sterile saline three times, and two 15-cm loops were made. A 10-cm control loop was maintained between the two test loops. Care was taken not to include any vasculature in the tie which could result in occlusion in the arterial blood supply or in venous stasis. Test loops were inoculated by using a 26 gauge needle with *C. jejuni* 81-176 in 2 ml of phosphate-buffered saline (PBS) by using challenge doses of 10^9 and 10^{11} CFU per loop. Control loops received sterile PBS. Rabbits were sacrificed after 14, 24, and 48 h, and the contents of the loops collected by washing the mucosa in 25 ml of chilled PBS. Epithelial aggregates were removed by centrifugation of the samples at 3,000 × *g* two times. Bacteria were then pelleted at 10,000 × *g* for 10 min and resuspended in PBS. Swabs from the three loops were collected separately for each rabbit and plated for growth under aerobic and microaerophilic conditions, and portions of the bacteria were processed for outer membrane protein (OMP) extraction and analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

OMP extraction. *C. jejuni* grown on Columbia agar under microaerophilic conditions and the in vivo-modified *C. jejuni* from the ileal loops were subjected to lithium chloride-lithium acetate extraction (21, 30). Bacteria were suspended in buffer containing 0.2 M lithium chloride and 0.1 M lithium acetate. The pH was adjusted to 6.0 with acetic acid. Membrane vesicles were generated by shaking the cell suspension at 250 rpm at 45°C for 2 h in flasks containing 3-mm glass beads. Whole cells and debris were removed by centrifugation at 10,000 and 25,000 × *g* for 10 min. Outer membrane vesicles were washed once and were pelleted by centrifugation at 100,000 × *g* for 1 h in Tris buffer (10 mM Tris, 100 mM NaCl [pH 8.0]). The final pellet was suspended in sample buffer and processed for analysis by SDS-PAGE (24).

Preparation of flagellin. *C. jejuni* flagellin was prepared by the method of Nachamkin and Hart (31). Bacteria grown on Columbia agar were harvested in PBS, sonicated on ice, and centrifuged at 10,000 × *g* for 15 min to remove intact bacteria. The supernatant was centrifuged at 100,000 × *g* for 1 h at 4°C. The pellet was suspended in distilled water adjusted to pH 2.0 and was held at 4°C for 15 min. Insoluble material was removed by centrifugation again at 100,000 × *g* for 1 h. The supernatant was adjusted to pH 7.0 with NaOH and incubated for 30 min at 4°C to reassociate flagellin. Flagellin was further concentrated by lyophilization.

PAGE. Proteins from *C. jejuni* grown under standard conditions and from *C. jejuni* modified in the ileal loops were analyzed by PAGE. Samples were run in 10% polyacrylamide gels under reducing conditions by the standard procedure of Laemmli (24). Gels were stained with Coomassie blue stain and silver stain (Bio-Rad) to visualize the protein bands.

Immunoblot analysis. Whole-cell proteins of medium-grown and in vivo-modified *C. jejuni* with prestained molecular mass markers were run on SDS–10% polyacrylamide gels and transferred to nitrocellulose membranes. Membranes with the transferred proteins were blocked with 5% nonfat dry milk (BLOTTO) for 1 h at 37°C. After being washed in PBS–0.1% Tween 20 three times, individual strips were cut from the membrane and reacted with appropriate sera. The incubation was performed at 37°C for 1 h by using the human serum at a dilution of 1:50 made in 5% BLOTTO. Strips were washed three times in PBS-Tween and then incubated with alkaline phosphatase-conjugated anti-human IgG (Fab specific) (Sigma, St. Louis, Mo.) at a dilution of 1:1,000 (and at a dilution of 1:500 for IgA and IgM conjugates) (Kirkegaard and Perry Labs, Gaithersburg, Md.) for 1 h at 37°C with shaking. Strips were thoroughly washed three times and reacted with 5-bromo-4-chloro-3-indolylphosphate toluidinium–Nitro Blue Tetrazolium substrate (Sigma). The substrate buffer consisted of 0.1 M Tris, 100 mM sodium chloride, and 5 mM $MgCl_2$ at pH 9.5. The final steps were carried out at room temperature, and the reaction was stopped by adding an excess amount of water at the time of optimal color development.

RESULTS

In vivo modification of *C. jejuni* proteins in rabbit ileal loop. There was minimal fluid accumulation (1 to 2 ml) in all the loops. On gross examination, a thin smear of clotted blood was seen to cover the mucosal surface of each loop. *C. jejuni* from duplicate test loops from all three rabbits was confirmed by growth under microaerophilic conditions, typical morphology by Gram staining, and biochemical analysis (oxidase, catalase, and hippurate tests). SDS-PAGE analysis of the *C. jejuni* obtained from the experimental loops was performed, and the result was compared with the protein pattern of medium-grown (grown on Columbia agar for 48 h) organisms. The protein profiles of *C. jejuni* obtained after 14, 24, and 48 h of growth in vitro were identical by PAGE analysis, and for all experiments, medium-grown (48 h) organisms were used. During all phases of in vivo infection, a new protein with a molecular mass of ca. 180 kDa was expressed that was absent in medium-grown *C. jejuni*. In the 14-h-postinfection sample, a distinct protein band of 66 kDa was overexpressed compared with medium-grown organisms. At 24 h postinfection, the same protein continued to be overexpressed. However, at 48 h in rabbit no. 3, the intensity of this protein band (66 kDa) was reduced and two strong bands with molecular masses of 43 and 35 kDa were visualized (Fig. 1 and 2). Contents from the duplicate test loops in each rabbit had identical protein profiles. Contents from the PBS control loops processed in the same manner did not show any bands. There was growth of no other organisms from the loops under aerobic conditions on blood agar. There was no growth from PBS control loops in aerobic or anaerobic culture.

Flagellins of the medium-grown and in vivo-modified samples appeared as faint bands in the gels (Fig. 1 and 2). Since there was in vivo expression of a 66-kDa protein (close to the molecular mass of flagellin of many *C. jejuni* strains), we wanted to confirm that this was not flagellar overexpression in our strain 81-176. The purified flagellin of strain 81-176 ran with a distinctly lower molecular mass at 58 kDa (Fig. 2).

OMPs of *C. jejuni* 81-176. Outer membranes extracted from *C. jejuni* grown under microaerophilic conditions on Columbia agar and from the in vivo-modified *C. jejuni* were compared by SDS-PAGE. The 84- and 47-kDa proteins

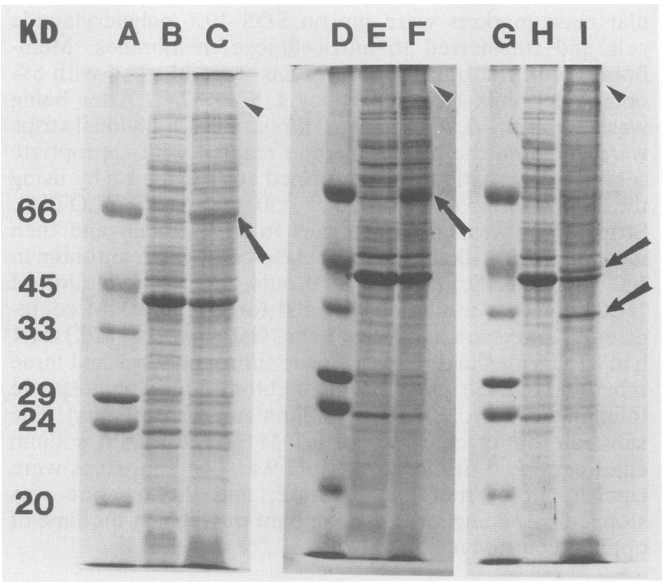

FIG. 1. SDS-PAGE (Coomassie blue stained) protein profiles of *C. jejuni* grown in vitro and in vivo in rabbit ileal loops. Lanes: B, E, and H, protein profiles of medium-grown *C. jejuni* 81-176; C, in vivo-modified *C. jejuni* proteins (14 h, 10^{11} CFU) (note the newly expressed 66-kDa protein [arrow]); F, in vivo-modified *C. jejuni* proteins (24 h, 10^9 CFU) with a pattern similar to that in lane C; I, in vivo-modified *C. jejuni* proteins (48 h, 10^9 CFU). Note the disappearance of the 66-kDa band and overexpression of two other proteins (44 and 35 kDa) (arrows) during this phase of infection. There is expression of the 180-kDa protein in the in vivo-modified proteins during all phases of infection in lanes C, F, and I (arrowheads). Lanes A, D, and G, molecular mass markers (in kilodaltons).

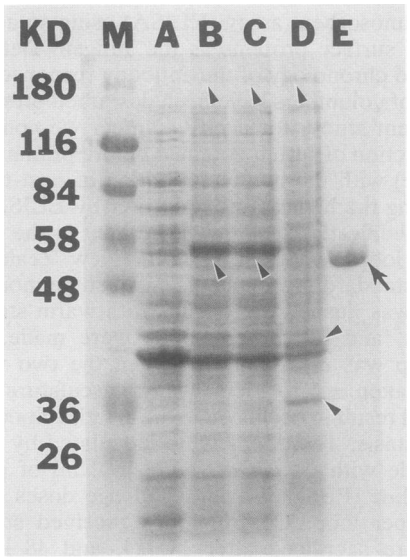

FIG. 2. SDS-PAGE (Coomassie blue stained) protein profiles of *C. jejuni* grown in vitro and in vivo and of the purified flagellin. Lanes: A, protein profiles of medium-grown *C. jejuni* 81-176; B, 14-h in vivo-modified *C. jejuni* proteins; C, 24-h in vivo-modified *C. jejuni* proteins; D, 48-h in vivo-modified *C. jejuni* proteins; E, purified flagellin of *C. jejuni* 81-176 (arrow). In vivo-expressed novel proteins are shown by arrowheads in lanes B, C, and D. Lane M, molecular mass markers (in kilodaltons).

could not be visualized in the OMP preparation of the in vivo-modified *C. jejuni*, even after silver staining. The 42-kDa major OMP (MOMP) appeared in both samples (data not shown).

Immunoblotting pattern of *C. jejuni* proteins with sera from volunteers. Medium-grown (48 h on Columbia agar) and in vivo-modified *C. jejuni* (14, 24, and 48 h) proteins were analyzed along with the purified flagellin and the prestained molecular mass markers. A Coomassie blue-stained gel of these proteins is shown in Fig. 2. Gels were run in the same format as in Fig. 2 and were immunoblotted with various antisera and reacted with anti-IgG conjugates. Convalescent serum from the immune volunteer no. 1 reacted with the flagellin (58 kDa) and MOMP (42 kDa) in all the protein samples, including the medium-grown organism. However, the serum reacted strongly with two other novel proteins of the 48-h modified sample from the ileal loops at 84 and 47 kDa (Fig. 3). The preinfection serum from the same volunteer reacted with the flagellin only (Fig. 4). On immunoblotting with serum from the same volunteer, the OMP extract did not show any reaction (data not shown). Since the two novel proteins recognized in the whole-cell *C. jejuni* extract were expressed only after 48 h of incubation, sera from all the other volunteers were tested against the modified proteins obtained at this time point. Sera from volunteers 2, 3, and 4 showed an identical reaction to the flagellin and the 84- and 47-kDa proteins (Fig. 5). Three other volunteers (volunteers 5, 6, and 7) showed a reaction to the flagellin only and not to the 84- or 47-kDa protein. However, analysis of the previously determined (5) serum ELISA titers (conducted by using group-specific surface proteins as the antigen) revealed that sera from two of these volunteers (volunteers 5 and 6) had very low preinfection titers and had a minimal increase after rechallenge (Table 1). One of these volunteers had one episode of liquid stool (67 ml, which did not meet the

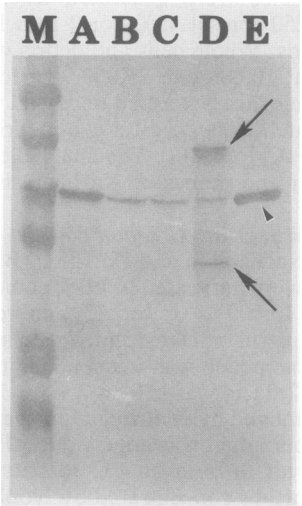

FIG. 3. Immunoblot pattern of *C. jejuni* proteins reacted with convalescent immune human volunteer (volunteer 1) serum (1:50) and anti-human IgG (Fab specific). The *C. jejuni* proteins are in the same format as in Fig. 2 for easy comparison. Lanes: A, protein profiles of medium-grown *C. jejuni* 81-176; B, 14-h in vivo-modified *C. jejuni* proteins; C, 24-h in vivo-modified *C. jejuni* proteins; D, 48-h in vivo-modified *C. jejuni* proteins; E, purified flagellin of *C. jejuni* 81-176 (arrowhead). Note the strong reaction with two proteins of ca. 84 and 47 kDa in lane D (arrows). The MOMP gives a mild reaction in lanes A to D. Lane M, molecular mass markers.

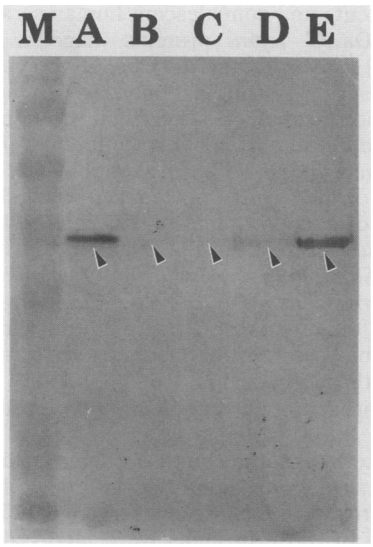

FIG. 4. Immunoblot pattern of *C. jejuni* proteins reacted with preinfection human volunteer (volunteer 1) serum (1:50) and anti-human IgG (Fab specific). The *C. jejuni* proteins are in the same format as in Fig. 2. Lanes: A, protein profiles of medium-grown *C. jejuni* 81-176; B, 14-h in vivo-modified *C. jejuni* proteins; C, 24-h in vivo-modified *C. jejuni* proteins; D, 48-h in vivo-modified *C. jejuni* proteins; E, purified flagellin of *C. jejuni* 81-176. The flagellin protein reacts in all the lanes (arrowheads), showing a stronger reaction with the medium-grown *C. jejuni* proteins in lane A and with the purified flagellin in lane E. Lane M, molecular mass markers.

criterion for diarrhea in the volunteer study). Serum from the third volunteer who did not show any response to the 84- and 47-kDa proteins (volunteer 7) had a moderately high preinfection titer and evoked a low immune response upon rechallenge (Table 1). All four volunteers showing reaction to the two novel proteins (volunteers 1, 2, 3, and 4) presented a good immune response with a clear increase over preinfection titers (Table 1).

TABLE 1. Serum antibody response to *C. jejuni*[a]

Volunteer no.	Preinfection titer	Titer on postrechallenge day[b]:		
		7	21	28
1	0.14	0.63	0.57	0.40
2	0.12	0.45	0.45	0.41
3	0.11	0.53	0.64	0.75
4	0.19	0.43	0.48	0.54
5	0.03	0.22	ND	0.17
6[c]	0.04	0.20	ND	0.17
7	0.36	0.60	0.44	0.50

[a] Antibody titers were measured by ELISA using an acid extract of *C. jejuni* surface proteins as the antigen and an alkaline phosphatase-labeled goat anti-human IgG conjugate. Titers were expressed as the optical density at 405 nm.
[b] Rechallenge was done 1 month after primary challenge. ND, not done.
[c] Volunteer 6 had one episode of liquid stool (67 ml) after rechallenge.

To determine whether there were any non-IgG antibodies to the novel proteins in the volunteers' sera, similar immunoblotting was performed by using the serum of volunteer 1, with anti-IgA and anti-IgM conjugates. There was no reaction with the novel (84- or 47-kDa) proteins. Preinfection and convalescent sera showed a similar reaction to the flagellin and a weak reaction to the MOMP (Fig. 6), confirming that antibodies to the 84- and 47-kDa *C. jejuni* novel proteins described in four immune volunteers were of the IgG class.

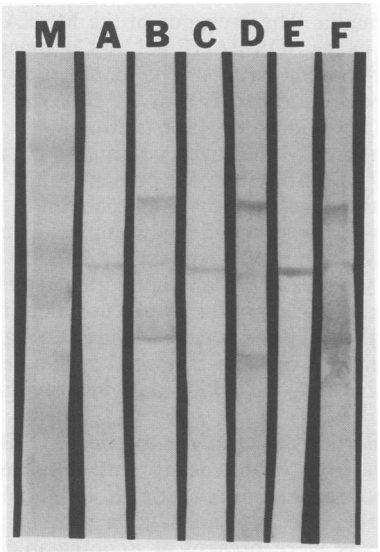

FIG. 5. Immunoblot pattern of 48-h in vivo-modified *C. jejuni* proteins reacted with preinfection and postchallenge sera from human volunteers (volunteers 2, 3, and 4) (1:50) and anti-human IgG (Fab specific). Lanes A, C, and E were reacted with preinfection sera from volunteers 2, 3, and 4, respectively. Lanes B, D, and F were reacted with postchallenge immune sera from the corresponding volunteers. Note the reaction of the sera in lanes B, D, and F with the 84- and 27-kDa proteins. Lane M, molecular mass markers.

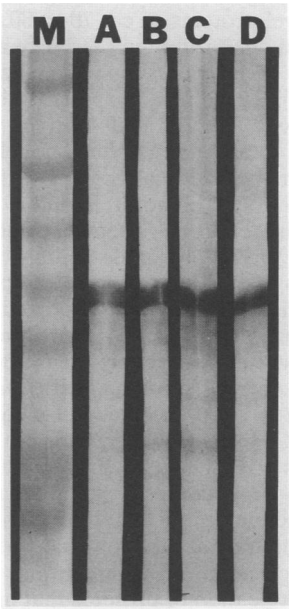

FIG. 6. Immunoblot pattern of 48 h in vivo-modified *C. jejuni* proteins reacted with preinfection and postchallenge human volunteer serum (volunteer 1) (1:50) and anti-human IgA and anti-human IgM. Lanes: A, reacted with preinfection serum and anti-human IgA; B, reacted with postchallenge immune serum and anti-human IgA; C, reacted with preinfection serum and anti-human IgM; D, reacted with postchallenge immune serum and anti-human IgM. There is reaction of only the flagellin protein in all the lanes. M, molecular mass markers.

DISCUSSION

Expression of bacterial virulence factors may be dependent on appropriate environmental stimuli. Many of the standard in vitro culture conditions may not be suitable for expression of these factors (18, 38). Jonson et al. have reported expression of surface proteins of *V. cholerae* in the rabbit small intestine which were not produced during growth in culture media (21). Recently, Almeida and Rosenbusch have described induction of a capsule-like material by *M. dispar* during in vitro growth with cultured bovine cells as well as during in vivo growth in calves (3). Two proteins with molecular masses of 98 and 94 kDa in *Campylobacter fetus* were not expressed upon several passages in vitro but were regained after a single animal passage (16). These proteins have been shown to be extremely important in resistance to serum bactericidal activity and resistance to phagocytosis. The virulence of *C. jejuni* has been reported to be increased by passing it through chicken embryos (15).

We wanted to grow *C. jejuni* in rabbit ileal loops to simulate an in vivo intestinal milieu comparable to that which might be found during human infection. In the ileal loops, expression of a new protein of ca. 180 kDa was seen by 14 h postinfection and persisted until 48 h. There was expression of other proteins of ca. 66, 43, and 35 kDa during different phases of infection. The 66-kDa protein was overexpressed during 14 and 24 h infection compared with the distinct overexpression of the 43- and 35-kDa proteins only during 48 h. These proteins were visualized in SDS-PAGE gels by Coomassie blue staining. Modified silver staining did not reveal any new proteins other than these four. The observation that expression of these proteins ceases immediately upon passaging in laboratory media suggests that expression is directly mediated by environmental conditions. Very recently, Konkel and Cieplak reported de novo synthesis of novel proteins of ca. 70 to 80, 45 to 48, 37, and 12 to 14 kDa and one protein in the range above 150 kDa by different strains of *C. jejuni* upon their adherence to tissue culture cells (22). These results appear to be similar to what we have observed in our ileal loop in vivo system and suggest that the expression of these novel proteins may be controlled in part by bacterial contact with enterocytes.

Systemic and local humoral immune response against campylobacter infection has been reported both in humans (4, 7, 26) and in the available animal models (10, 27). Circulating campylobacter-specific antibodies of the IgG, IgM, and IgA classes have been reported during the acute and convalescent phases of *C. jejuni* infection (5, 28, 31). Blaser et al. have reported complement-dependent bactericidal activity of normal serum against *C. jejuni* (8). According to Pennie et al., the complement-mediated bactericidal activity against *C. jejuni* is only 0.2 $\log_{10}$ in nonimmune sera, which increases to 1.8 $\log_{10}$ killing by homologous acute sera. This strain-dependent $\log_{10}$ killing reaches a titer of 3.7 when convalescent serum is used (36). Though elevation of anti-*C. jejuni* immunoglobulin titers has been documented by ELISA in a large number of human and animal infections (40), actual identification of proteins that may be involved in imparting such immunity has been done in only a few studies (28, 32, 41).

We initially evaluated the reaction of volunteer sera to medium-grown *C. jejuni* proteins. Immunoblotting revealed reaction of the immune sera to *C. jejuni* flagellin (ca. 58 kDa) and the MOMP (ca. 42 kDa) and a mild reaction with protein(s) in the range of ca. 14 to 30 kDa. Nachamkin and Hart, as well as other investigators, have described the reaction of acute and convalescent human sera with 66- and 43- to 46-kDa *C. jejuni* proteins (flagellin and MOMP, respectively) and with the 12-kDa lipopolysaccharide by immunoblotting (31). Interestingly, we found an equally strong reaction to the 58-kDa flagellin protein in both pre- and postinfection sera from our volunteers. *C. jejuni* flagellin genes *flaA* and *flaB* have been cloned and sequenced, and the analysis of the flagellin protein sequence shows that the amino- and carboxy-terminal regions are highly similar to those of many other bacterial flagellins, including *Escherichia coli*, *Salmonella typhimurium*, *Treponema pallidum*, *Serratia marcescens*, and *Borrelia burgdorferi* (34). This may explain the cross-reactivity of antiflagellum antibodies expected to be present from infections and natural exposure of adults to these organisms.

In a second series of experiments, we used the in vivo-modified *C. jejuni* proteins as an antigen to examine the reaction pattern with the same sera from volunteers. Since we had observed expression of several new proteins during the in vivo growth of this organism, we wanted to determine whether any of those proteins were involved in providing protective immunity to the volunteers. On immunoblot analysis, none of the novel *C. jejuni* proteins (180, 66, 43, and 35 kDa) reacted with either preinfection or postchallenge immune sera. However, two distinct bands appeared at ca. 84 and 47 kDa in the 48-h in vivo-modified sample when convalescent sera from four of seven volunteers were used. The appearance of these bands suggests that these two proteins were newly expressed in the ileal loop in vivo at 48 h and that they were absent in the medium-grown organism or during 14 or 24 h of in vivo growth of the organism. On Coomassie blue staining or silver staining, these differences were not discernible, suggesting that the proteins were present in very small amounts. A similar event has been described very recently; Jonson et al. observed the expression of mannose-sensitive hemagglutinin (MSHA) pili by El Tor vibrio strains only in the rabbit ileal loops and in the stool of human patients but not in the organisms grown in laboratory media. The presence of the MSHA proteins was not evident in the SDS-PAGE gel by Coomassie blue staining or silver staining but could be identified by immunoblotting the in vivo-modified El Tor *V. cholerae* proteins with monoclonal antibodies to MSHA (20).

Sera from three of the seven volunteers did not react with the 84- or 47-kDa protein in our present study. They showed reaction to the flagellin and a mild reaction to the MOMP in preinfection and postrechallenge samples. These volunteers showed a weak immune response by ELISA. It is possible that though the sera of these volunteers had antibodies to the 84- and 47-kDa proteins, they were not identified under our experimental conditions because of the small amount. Alternatively, humoral or local intestinal antibodies to other *C. jejuni* proteins may be taking part in providing protection against diarrhea.

Immunoblotting of our in vivo-modified OMP extract with immune sera from volunteers (preinfection and postrechallenge) did not reveal any reaction. It is difficult at this point to say whether the 84- and 47-kDa proteins were OMPs. These proteins were present at undetectable levels in the whole-cell extract after staining with the Coomassie blue or silver stain and became apparent only after immunoblotting with convalescent sera. Additional loss of proteins during OMP extraction could have rendered the immunoblot technique insensitive for the detection of these proteins; alternatively, they may not be OMPs. The 58-kDa flagellin of strain 81-176 appeared as a faint band in the medium-grown organ-

isms. Expression of flagellin in vivo in the ileal loops was reduced even further, as indicated in the immunoblots. However, the reaction pattern with volunteer sera suggests that the *C. jejuni* flagellin is consistently immunogenic during human infections.

Our results show that there is expression of novel proteins by *C. jejuni* 81-176 during in vivo growth of the organism in the rabbit intestine. Because of the very small amount, identification of some of these proteins is possible only by utilizing immunoblotting with appropriate antisera. Two such novel proteins described here elicit a strong serum IgG response in immune volunteers. Their precise role in providing protection against *C. jejuni* disease and the mechanisms involved in such a phenomenon need detailed structural and functional analysis of the 84- and 47-kDa proteins.

ACKNOWLEDGMENTS

We thank Kyle C. Stump, Robert G. Russell, and S. Srinivas in the Division of Veterinary Resources, University of Maryland, Baltimore, for their advice and help and for assistance in conducting the animal studies.

P. Panigrahi was supported in part by USPHS grant T32-AI 07376.

REFERENCES

1. **Abimiku, A., and J. M. Dolby.** 1988. Cross-protection of infant mice against intestinal colonization by *Campylobacter jejuni*: importance of heat-labile serotyping (Lior) antigens. J. Med. Microbiol. **26**:265–268.
2. **Aguero-Rosenfeld, M. E., X.-H. Yang, and I. Nachamkin.** 1990. Infection of adult Syrian hamsters with flagellar variants of *Campylobacter jejuni*. Infect. Immun. **58**:2214–2219.
3. **Almeida, R. A., and R. F. Rosenbusch.** 1991. Capsulelike surface material of *Mycoplasma dispar* induced by in vitro growth in culture with bovine cells is antigenically related to similar structures expressed in vivo. Infect. Immun. **59**:3119–3125.
4. **Ani, E. A., T. Takahashi, and R. A. O. Shonekan.** 1988. *Campylobacter jejuni* antibodies in Nigerian children. J. Clin. Microbiol. **26**:605–606.
5. **Black, R. E., M. M. Levine, M. L. Clements, T. P. Hughes, and M. J. Blaser.** 1988. Experimental *Campylobacter jejuni* infection in humans. J. Infect. Dis. **157**:472–479.
6. **Blaser, M. J., R. E. Black, D. J. Duncan, and J. Amer.** 1985. *Campylobacter jejuni*-specific serum antibodies are elevated in healthy Bangladeshi children. J. Clin. Microbiol. **21**:164–167.
7. **Blaser, M. J., and D. J. Duncan.** 1984. Human serum antibody response to *Campylobacter jejuni* infection as measured in an enzyme-linked immunosorbent assay. Infect. Immun. **44**:292–298.
8. **Blaser, M. J., P. Smith, and P. F. Kohler.** 1985. Susceptibility of Campylobacter isolates to the bactericidal activity of human serum. J. Infect. Dis. **151**:227–235.
9. **Blaser, M. J., D. N. Taylor, and P. Echeverria.** 1986. Immune response to *Campylobacter jejuni* in a rural community in Thailand. J. Infect. Dis. **153**:249–254.
10. **Burr, D. H., M. B. Caldwell, A. L. Bourgeois, H. R. Morgan, R. Wistar, Jr., and R. I. Walker.** 1988. Mucosal and systemic immunity to *Campylobacter jejuni* in rabbits after gastric inoculation. Infect. Immun. **56**:99–105.
11. **Caldwell, M. B., R. I. Walker, S. P. Stuart, and J. E. Rogers.** 1983. Simple adult rabbit model for *Campylobacter* enteritis. Infect. Immun. **42**:1176–1182.
12. **Dolby, J. M., and D. G. Newell.** 1986. The protection of infant mice from colonization with *Campylobacter jejuni* by vaccination of the dams. J. Hyg. Camb. **96**:143–151.
13. **Dunn, B. E., M. J. Blaser, and E. L. Snyder.** 1987. Two-dimensional gel electrophoresis and immunoblotting of *Campylobacter* outer membrane proteins. Infect. Immun. **55**:1564–1572.
14. **Echeverria, P., D. N. Taylor, U. Lexosomboon, M. Bhalbulaya, N. R. Blacklow, K. Tamura, and R. Sakazaki.** 1989. Case control study of endemic diarrheal disease in Thai children. J. Infect. Dis. **159**:543–548.
15. **Field, L. H., V. L. Headley, J. L. Underwood, S. M. Payne, and L. J. Berry.** 1986. The chicken embryo as a model for campylobacter invasion: comparative virulence of human isolates of *Campylobacter jejuni* and *Campylobacter* coli. Infect. Immun. **54**:118–125.
16. **Fujimoto, S., A. Umeda, A. Takade, K. Murata, and K. Amako.** 1989. Hexagonal surface layer of *Campylobacter fetus* isolated from humans. Infect. Immun. **57**:2563–2565.
17. **Glass, R. I., B. J. Stoll, M. I. Huq, M. J. Strulens, M. J. Blaser, and A. K. M. G. Kibriya.** 1983. Epidemiologic and clinical features of endemic *Campylobacter jejuni* infection in Bangladesh. J. Infect. Dis. **148**:292–296.
18. **Hall, R. H., P. A. Vial, J. B. Kaper, J. J. Mekalanos, and M. M. Levine.** 1988. Morphological studies on fimbriae expressed by *Vibrio cholerae* O1. Microb. Pathog. **4**:257–265.
19. **Humphrey, C. D., D. M. Montag, and F. E. Pittman.** 1985. Experimental infection of hamsters with *Campylobacter jejuni*. J. Infect. Dis. **151**:485–493.
20. **Jonson, G., J. Holmgren, and A.-M. Svennerholm.** 1992. Expression of toxin-coregulated pilus in *Vibrio cholerae* in vitro and in vivo, abstr. B-400, p. 92. Abstr. 92nd Annu. Meet. Am. Soc. Microbiol. 1992. American Society for Microbiology, Washington, D.C.
21. **Jonson, G., A.-M. Svennerholm, and J. Holmgren.** 1989. *Vibrio cholerae* expresses cell surface antigens during intestinal infection which are not expressed during in vitro culture. Infect. Immun. **57**:1809–1815.
22. **Konkel, M. E., and W. Cieplak, Jr.** 1992. Altered synthetic response of *Campylobacter jejuni* to co-cultivation with human epithelial cells promotes internalization, abstr. B-216, p. 62. Abstr. 92nd Annu. Meet. Am. Soc. Microbiol. 1992. American Society for Microbiology, Washington, D.C.
23. **Korlath, J. A., M. T. Osterholm, L. A. Judy, J. C. Forfang, and R. A. Robinson.** 1985. A point-source outbreak of campylobacteriosis associated with consumption of raw milk. J. Infect. Dis. **152**:592–596.
24. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
25. **Lane, E. M., R. A. Batchelor, A. L. Bourgeois, D. H. Burr, and J. G. Olson.** 1987. Urine and faecal IgA response during naturally acquired infection with *Campylobacter jejuni*. Lancet **ii**:1141.
26. **Martin, P. M. V., J. Mathiot, J. Ipero, M. Kirimat, A. J. Georges, and M.-C. Georges-Coubot.** 1989. Immune response to *Campylobacter jejuni* and *Campylobacter coli* in a cohort of children from birth to 2 years of age. Infect. Immun. **57**:2542–2546.
27. **McSweegan, E., D. H. Burr, and R. I. Walker.** 1987. Intestinal mucus gel and secretory antibody are barriers to *Campylobacter jejuni* adherence to INT-407 cells. Infect. Immun. **55**:1431–1435.
28. **Mills, S. D., and W. C. Bradbury.** 1984. Human antibody response to outer membrane proteins of *Campylobacter jejuni* during infections. Infect. Immun. **43**:739–743.
29. **Mizuno, S.-I., S. Maki, T. Honda, T. Miwatani, and K. Arita.** 1985. Appearance in patients' sera of antibodies against purified antigen of *Campylobacter jejuni* and its relation to the bacterium-excreting period. J. Infect. Dis. **151**:742.
30. **Morris, J. G., Jr., A. C. Wright, L. M. Simpson, P. K. Wood, D. E. Johnson, and J. D. Oliver.** 1987. Virulence of *Vibrio vulnificus*: association with utilization of transferrin-bound iron, and lack of correlation with levels of cytotoxin or protease production. FEMS Microbiol. Lett. **40**:55–59.
31. **Nachamkin, I., and A. M. Hart.** 1985. Western blot analysis of the human antibody response to *Campylobacter jejuni* cellular antigens during gastrointestinal infection. J. Clin. Microbiol. **21**:33–38.
32. **Nachamkin, I., and X.-H. Yang.** 1989. Human antibody response *Campylobacter jejuni* flagellin protein and a synthetic N-terminal flagellin peptide. J. Clin. Microbiol. **27**:2195–2198.
33. **Newell, D. G.** 1986. Monoclonal antibodies directed against the

flagella of *Campylobacter jejuni*: cross-reacting and serotypic specificity and potential use in diagnosis. J. Hyg. Camb. **96**:377–384.

34. **Nuijten, P. J. M., F. J. A. M. van Asten, W. Gaastra, and B. A. M. Van der Zeist.** 1990. Structural and functional analysis of two *Campylobacter jejuni* flagellin genes. J. Biol. Chem. **265**:17798–17804.
35. **Pavlovskis, O. R., D. M. Rollins, R. L. Haberberger, Jr., A. E. Green, L. Habash, S. Strocko, and R. I. Waler.** 1991. Significance of flagella in colonization resistance of rabbits immunized with *Campylobacter* spp. Infect. Immun. **59**:2259–2264.
36. **Pennie, R. A., R. D. Pearson, L. J. Barrett, H. Lior, and G. L. Guerrant.** 1986. Susceptibility of *Campylobacter jejuni* to strain-specific bactericidal activity in sera of infected patients. Infect. Immun. **52**:702–706.
37. **Russell, R. G., M. J. Blaser, J. I. Sarmiento, and J. Fox.** 1989. Experimental *Campylobacter jejuni* infection in *Macaca nemestrina*. Infect. Immun. **57**:1438–1444.
38. **Siddique, A. B., and S. Q. Akhtar.** 1991. Study on the pathogenicity of *Campylobacter jejuni* by modifying the medium. J. Trop. Med. Hyg. **94**:175–179.
39. **Ueki, Y. A., A. Umeda, S. Fijimoto, M. Mitsuyama, and K. Amako.** 1987. Protection of *C. jejuni* infection in suckling mice by anti-flagellin antibodies. Microbiol. Immunol. **31**:1161–1171.
40. **Walker, R. I., M. B. Caldwell, E. C. Lee, P. Guerry, T. J. Trust, and G. M. Ruiz-Palacios.** 1986. Pathophysiology of *Campylobacter* enteritis. Microbiol. Rev. **50**:81–94.
41. **Winsor, D. K., Jr., J. J. Mathewson, and H. L. DuPont.** 1986. Western blot analysis of intestinal secretory immunoglobulin A response to *Campylobacter jejuni* antigens in patients with naturally acquired *Campylobacter* enteritis. Gastroenterol. **90**: 1217–1222.