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Human Antibody Response to Outer Membrane Proteins of *Campylobacter jejuni* During Infection

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Two techniques were used to isolate outer membrane proteins from *Campylobacter jejuni*, EDTA-lysozyme extraction and sodium-*N*-lauroylsarcosinate (Sarkosyl) solubilization. The protein profiles of the two preparations were similar, with a few additional bands in the EDTA-lysozyme preparations. The major outer membrane protein was 43,000 (43K) daltons, and there were 8 to 10 minor bands ranging from 92K to 14K daltons. There was no difference in the protein profile of a strain causing an infection (strain 17) and the resulting stool isolate (strain 17J). Sera collected before the infection and during the acute and convalescent stages were used with Western blotting and immunoautoradiographic techniques to determine the antigenicity of outer membrane proteins. A number of antigenic proteins were detected before the infection by their reaction with preinfection serum (61K, 51K, 43K, 40K, 34K, and 31K daltons), and three additional bands appeared during the infection when acute and convalescent sera were used (92K, 56K, and 19K daltons). Furthermore, an area of the gel at less than 14.4K daltons that did not stain with Coomassie brilliant blue became visible in the immune blots when the convalescent serum was used.

Although *Campylobacter jejuni* is one of the leading causes of human diarrhea (2, 20), very little is known of the mechanism of its pathogenicity. The organism can attach to gut epithelial cells, multiply, cause production of diarrhea, and, in some cases, invade the bloodstream (6). In many enteric pathogens, attachment is mediated by fimbriae, diarrhea is mediated by a specific toxin(s), and invasiveness is mediated by an unknown characteristic of the gram-negative cell envelope (4). *C. jejuni* cells are able to attach to both HeLa cells and intestinally derived tissue culture cells (Int 407); however, the presence of fimbriae has not been reported (14, 18). To date, all of the standard assays for toxin production have been negative with *C. jejuni*, including destruction of tissue culture cells and fluid accumulation in infant and suckling mice or ileal loops from various animals (14). *C. jejuni* is capable of invading tissue culture cells without causing disruption, even though invasiveness in the Sereny test has not been shown (14). In many bacteria, one or more of these virulence properties are encoded by plasmids (8). *C. jejuni* is known to harbor plasmids, although no plasmid has yet been correlated to pathogenicity (1, 3, 21).

An alternate mechanism of pathogenicity may reside in the outer membrane (OM), which is comprised of protein and lipopolysaccharide and serves as the interface between the pathogen and its host (7). In many bacteria the OM proteins play a role in attachment, invasion, serum resistance, chelation of iron, and resistance to phagocytosis (4). There has been one report characterizing the number and molecular weights of the OM proteins of several laboratory strains of *C. jejuni* and *Campylobacter coli* (13).

To determine whether these proteins act as antigens during an infection, the OM proteins of a strain that caused enteritis (strain 17) and the resulting stool isolate (strain 17J) were characterized by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The proteins that elicited an antibody response during the infection were identified by their reactions with preinfection, acute, and convalescent sera with the techniques of Western blotting and immunoautoradiography. Moreover, with these techniques it was possible to determine the variation in the host antibody response to particular OM proteins during the course of the infection.

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MATERIALS AND METHODS

Bacterial strains and cultural conditions. The three strains of *C. jejuni* used in this study were 17, 17J, and 16. Strains 17 and 16 are reference strains for the serotyping system of Penner et al. (19) and are unrelated on the basis of their thermostable antigens. Strain 17J is a stool isolate obtained from a laboratory worker who experienced an infection with serotype reference strain 17. The evidence that 17J was an in vivo-passaged strain 17 was obtained by using the techniques of passive hemagglutination and restriction enzyme analysis of chromosomal DNA (J. L. Penner, J. N. Hennessy, S. D. Mills, and W. C. Bradbury, J. Clin. Microbiol., in press).

Stock cultures were maintained at −70°C in 15% glycerol–1% Proteose Peptone no. 3 (Difco Laboratories, Detroit, Mich.). When required, strains were thawed slowly, plated on 5% citrated calf blood agar plates, and incubated for 48 h at 37°C in anaerobic jars. The jars were flushed with a gas mixture containing 5% O_2, 10% CO_2, and 85% N_2.

OM protein isolation. (i) EDTA-lysozyme extraction. The technique of Mizushima and Yamada (16) was used, with modifications. The cells from 50 blood agar plates were removed with 0.1 M Tris-hydrochloride buffer (pH 8.3) and washed once (8,000 × *g*, 10 min, 4°C). The cell pellet was suspended in 60 ml of buffer and placed on ice, and the reagents were added in the following order: 30 ml of 2 M sucrose, 1.2 ml of 1% EDTA (Na salt, pH 7.0), and 4.8 ml of 0.5% lysozyme (Sigma Chemical Co., St. Louis, Mo.). Upon addition of lysozyme, tubes were incubated for 45 min at 25°C. The spheroplasts were removed by centrifugation at 20,000 × *g* for 20 min (25°C), and the supernatant was centrifuged at high speed (100,000 × *g*, 1 h, 4°C) to pellet the

membranes. The pellet was suspended in 2.0 ml of 1% EDTA (Na salt, pH 7.0) and dialyzed overnight against several changes of 1% EDTA. After dialysis, the preparation was centrifuged at high speed (100,000 × *g*, 1 h, 4°C), and the pellet was suspended in 500 μl of 0.1 M Tris-hydrochloride (pH 8.3) buffer and frozen at −20°C until required.

(ii) Sodium-*N*-lauroylsarcosinate (Sarkosyl) solubilization. Cells were scraped from 50 blood agar plates and washed once in 10 mM sodium phosphate buffer, pH 7.4 (8,000 × *g*, 10 min, 4°C). After cells were suspended in buffer, DNase and RNase (1 μg/ml, final concentration; Sigma) were added, and the cells were sonicated on ice by six 30-s pulses. Whole cells were removed (8,000 × *g*, 10 min, 4°C), and the membrane suspension was washed once in buffer (100,000 × *g*, 1 h, 4°C). Membranes were suspended in buffer to a protein concentration of 10 mg/ml. The solubilization procedure was carried out by the method of Filip et al. (9) with a membrane protein/detergent ratio of 2:1 (milligrams/milliliters). Membranes were diluted to 1 mg of protein per ml and frozen at −20°C until required.

Released cellular components. Cellular components released during growth were isolated by a differential centrifugation technique similar to that of Logan and Trust (13). Whole cells were washed twice (see above), and the supernatants were pooled and centrifuged at 20,000 × *g* (20 min, 4°C) and then at 100,000 × *g* (1 h, 4°C). The pellet from the final centrifugation was diluted in sodium phosphate buffer (pH 7.4) and frozen at −20°C until required.

Chemical analysis. Membrane protein was analyzed by the technique of Markwell et al. (15) with lysozyme as the standard. Lactic acid dehydrogenase was assayed by the technique of King (11) with enzyme obtained from Sigma as a positive control.

SDS-PAGE. The OM proteins were separated on an SDS-polyacrylamide gel system by the method of Laemmli (12) with a stacking gel of 5% acrylamide and a separating gel of 11% acrylamide. With 10 wells per gel, 40 μg of protein was loaded per lane, and the proteins were electrophoresed at a constant current of 20 mA until the tracking dye had run 10 cm into the separating layer. The following standards of known molecular weights were run in each gel: lysozyme (14,400), soybean trypsin inhibitor (21,500), carbonic anhydrase (31,000), ovalbumin (45,000), bovine serum albumin (66,200), and phosphorylase *b* (92,500). A linear regression plot of the logarithm of the molecular weight versus electrophoretic mobility was constructed to estimate apparent molecular weights. Gels were stained with Coomassie brilliant blue R dye (Sigma) and destained in several changes of 20% methanol–10% acetic acid.

Western blot and immunoautoradiography. The Western blot technique of Burnette (5) was used in conjunction with the Bio-Rad Trans-Blot system (Bio-Rad Laboratories, Richmond, Calif.). After electrophoresis, gels were immediately transferred to nitrocellulose paper (Millipore Ltd., Mississauga, Ontario) at constant voltage (60 V) for 3 h in prechilled buffer. After transfer, blots were dried, wrapped in cellophane, and either used immediately for immunoautoradiography or stored overnight at 10°C in vacuo.

The immunoautoradiography technique was a modification of that of Towbin et al. (22) and Burnette (5). The nitrocellulose filter paper (blot) was soaked for 1 h at 40°C in 200 ml of 10 mM Tris-hydrochloride (pH 7.4) with 0.9% NaCl (buffer A) and 0.25% gelatin (Bio-Rad) (buffer B), and then for 16 h in 100 ml of buffer B containing the appropriate serum at a final dilution of 1/400. All preparations were agitated constantly. The following day, the blots were washed for 1 h in several changes of buffer A, for 1 h in buffer A with 0.05% Nonidet P-40 (Sigma), and again for 1 h in several changes of buffer A. After the washes, the blots were incubated in 100 ml of buffer B containing 2×10^5 to 5×10^5 cpm of ^{125}I-staphylococcal protein A (Amersham Radiochemicals, Oakville, Ontario) per ml for 1.5 h and then washed as before. The blots were dried, wrapped in cellophane, and exposed at −70°C (12 to 48 h) to Kodak AR X-ray film (Eastman Kodak Co., Rochester, N.Y.) with Cronex Xtra Life intensifying screens (E.I. du Pont de Nemours & Co., Inc., Wilmington, Del.).

RESULTS

OM proteins. Table 1 lists the molecular weights of the Coomassie brilliant blue-stained proteins from one of the strains studied (17J).

TABLE 1. Comparison of OM proteins prepared by EDTA-lysozyme and Sarkosyl techniques from *C. jejuni* strain 17J as detected by Coomassie brilliant blue (CBB) staining and immunoautoradiography with preinfection, acute, and convalescent sera[a]

CBB-stained proteins prepared with EDTA-lysozyme (× K daltons)	Sera			CBB-stained proteins prepared with Sarkosyl (× K daltons)	Sera		
	Preinfection	Acute	Convalescent		Preinfection	Acute	Convalescent
92				92		+	+
80				80			
75-76				75-76			
61	+	++	+++	61	+	++	+++
56			+	56		++	++
53-54				53-54			
51	+	++	++				
43	+	+	+	43	+	++	++
40	+	++	++	40	+	+	++
37-38							
36				36			
34	+	++	++	34	+	+	++
31	+	++	++				
22				22			
19			+	19			+
14							

[a] The proteins of strain 17 were the same. The number of pluses indicates the relative intensity of bands detected by immunoautoradiography.

The two techniques used to prepare OM proteins from strains of *C. jejuni* showed the same profile, with the exception of several additional proteins found in the EDTA-lysozyme preparations (Fig. 1A, lanes 4, 6, and 8). The presence of the extra protein bands due to contamination of the OM by the cytoplasmic membrane could not be ruled out, as the assays for lactic acid dehydrogenase, a cytoplasmic membrane protein, consistently showed 10 to 40% contamination of the EDTA-lysozyme prepared outer membranes, but none in those obtained with Sarkosyl.

The OM proteins of strains 17 and 17J were the same with either technique (Fig. 1A, lanes 4 through 7). When Sarkosyl extraction was used, there were two major bands at 61,000 (61K) and 43K daltons (Fig. 1A, arrows in lanes 5 and 7). The 43K-dalton band was also the major protein in the EDTA-lysozyme membranes (Fig. 1A, arrows in lanes 4 and 6). Although the 61K-dalton protein band was present, it was much reduced in intensity and could no longer be considered a major OM protein. The minor proteins that were observed are listed in Table 1. The protein profile of strain 16, a different serotype, was the same as those shown for 17 and 17J when the EDTA-lysozyme technique was used. By contrast, the Sarkosyl-insoluble proteins of strain 16 were quite different. A number of proteins of 80K, 56K, 53K-54K, 40K, and 34K daltons were absent, and one new band of 52K daltons appeared.

Released cellular components. The protein profiles of released cellular components as determined by SDS-PAGE (Fig. 1A, lanes 1 through 3) were similar for the three strains (16, 17, and 17J) and consisted of proteins of >100K, 100K, 92K, 61K, and 43K daltons. Variable amounts of bands ranging from 75K to 85K daltons were present, and occasionally bands of 54K, 56K, 58K, and 59K daltons were observed.

Immune blots. Each of the immune blots shown in Fig. 1B through D was processed at the same time, developed, and photographed under identical conditions to enable comparison of the relative antibody titers of the various sera.

The sera obtained before the infection reacted with low intensity to several of the proteins in the EDTA-lysozyme preparations of strains 17 and 17J (Fig. 1B, Table 1). This was also observed when sera obtained from persons who had no recent symptoms of diarrhea were reacted with the same OM proteins. The intensity of these bands increased in both the acute sera (5 days postinfection) and convalescent sera (21 days postinfection), which suggested a rise in the titer of serum antibodies specific to the OM proteins during the course of the infection (Fig. 1C and D). Two protein bands of 56K and 19K daltons became visible during the infection (Fig. 1D, triangles in lanes 4 through 7); occasionally a 37K-38K doublet and 36K- and 22K-dalton proteins were also observed (Fig. 1C and D). Results obtained from the immune blots of the Sarkosyl OM preparations were very similar to those with the EDTA-lysozyme preparations, with the exception of a 92K-dalton protein present with the convalescent serum (Fig. 1D, triangles in lanes 5 and 7; Table 1).

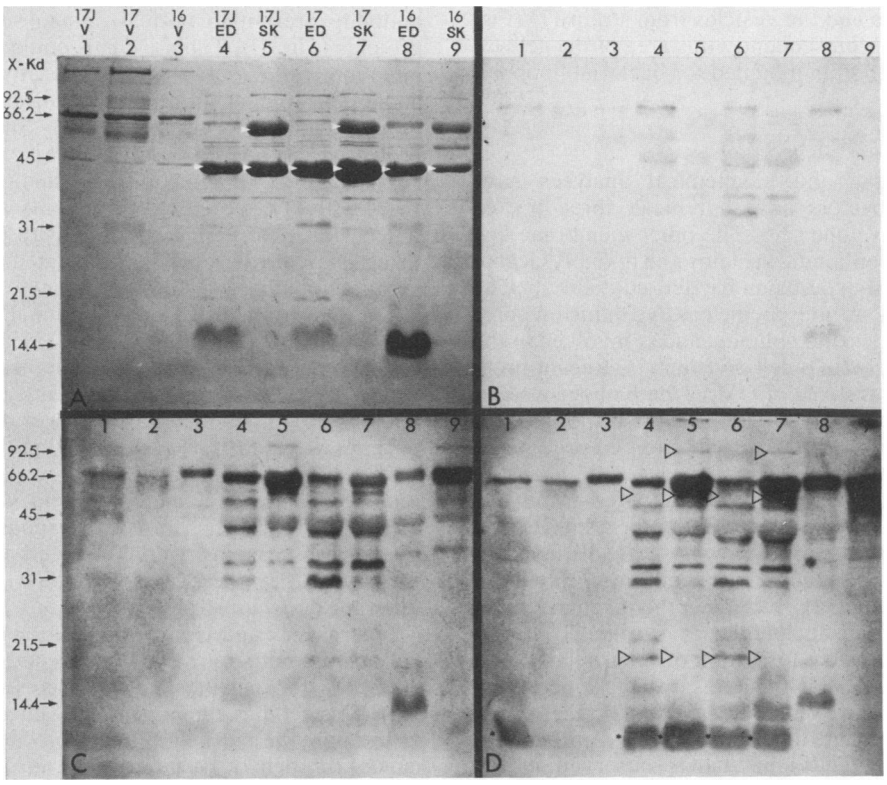

FIG. 1. Use of immunoautoradiography to detect antibodies in patient sera against the OM proteins and vesicles from three strains of *C. jejuni* (16, 17, and 17J). OM proteins were prepared by two separate techniques, EDTA-lysozyme extraction and Sarkosyl solubilization. Panels: A, Coomassie brilliant blue-stained SDS-PAGE gel; B, immune blot with serum obtained before the infection; C, immune blot with serum obtained in the acute phase of the infection (5 days postinfection); D, immune blot with serum obtained in the convalescent phase of the infection (21 days postinfection). Lanes: 1, 2, and 3, vesicle preparations from strains 17J, 17, and 16, respectively; 4, 6, and 8, EDTA-lysozyme OM preparations of strains 17J, 17, and 16, respectively; 5, 7, and 9, Sarkosyl-insoluble OM preparations of stains 17J, 17, and 16, respectively.

Bands were not observed on the X-ray film when the serum was replaced with an equal volume of buffer during incubation with the nitrocellulose sheets. The lack of nonspecific binding of the ^{125}I-staphylococcal protein A to the OM proteins and the failure of antibody to bind some OM and molecular weight marker proteins indicated that the technique was specific.

The OM proteins of a strain of *C. jejuni* (strain 16) unrelated to the infection were also assayed by immune blotting. When either the EDTA-lysozyme or Sarkosyl preparations were used, the preinfection sera bound to only three proteins (61K, 52K, and 43K daltons) (Fig. 1B). When acute and convalescent sera were used (Fig. 1C and D), a protein of 92K daltons was present in the Sarkosyl OM preparations, and a protein of 19K daltons appeared in the EDTA-lysozyme extracts. It was apparent that the three strains used in this study had proteins of similar molecular weight and antigenicity.

Immune blots were also performed with vesicle preparations from the three strains 16, 17, and 17J (Fig. 1B, C, and D, respectively). There were antibodies in the preinfection sera to the 61K-dalton protein in all strains, and they increased in intensity in the acute and convalescent sera. Occasionally, reactions to the 54K-, 43K-, and 58K- or 59K-dalton proteins were also observed.

An area of the gel at less than 14.4K daltons did not react with the preinfection and acute sera, although it did react very strongly with the convalescent sera against strains 17 and 17J, but not with that against strain 16 (Fig. 1D, asterisks in lanes 4 through 7). A similar reaction was also observed with convalescent sera and the vesicles from strain 17J (Fig. 1D, asterisk in lane 1). Subsequently this area of the gel was visualized with a silver stain modified for bacterial lipopolysaccharide (23).

DISCUSSION

Electron microscopic and biochemical analyses have shown that campylobacters have a typical, three-layered gram-negative cell envelope (20). The outer membrane is a phospholipid bilayer containing proteins and lipopolysaccharide that acts as a passive diffusion barrier, contains specific uptake systems as well as nonspecific passive diffusion pores and receptors for phages and colicins, and is involved in the processes of conjugation and cell division (7). Recent progress in understanding the role of OM in the pathogenesis of gram-negative bacterial infections prompted the present investigation of human antibody response to OM proteins of *C. jejuni* during infection.

The OM proteins of *C. jejuni* were prepared using two techniques, EDTA-lysozyme extraction and Sarkosyl solubilization. The detergent Sarkosyl was originally used to preferentially solubilize cytoplasmic membrane proteins of *Escherichia coli*, leaving OM proteins in the insoluble pellet (9). The EDTA-lysozyme technique, also originally developed for *E. coli*, involves the formation of spheroplasts with the concomitant release of OM proteins into the supernatant (16). There were differences in the OM protein profiles of the two preparations, although the similarity was, in general, quite remarkable. The additional bands observed in the EDTA-lysozyme preparations could be due to cytoplasmic membrane contamination, peptidoglycan-binding proteins released through the enzymatic activity of lysozyme or periplasmic space proteins. In addition, an increased amount of 61K-dalton protein was observed when the Sarkosyl technique was used. Therefore, the numbers and relative amounts of OM proteins observed, although fairly constant, differed depending on the preparation technique employed.

The protein profiles of strains 17 and 17J were compared to determine whether there were changes in the infecting strain (strain 17) as a result of its passage through the human host (strain 17J). Although there were no obvious differences in the protein profiles of the two organisms (strains 17 and 17J), this did not preclude the possibility that a virulence property acquired in the host could have been lost on the three or four laboratory subcultures required to isolate the organism from the stool and to prepare OM proteins. The results of this study were comparable to those reported previously (13) in which the major OM protein had a molecular mass of 45K daltons and in which approximately 8 to 10 minor protein bands ranging from 92K to 19K daltons were observed.

Gram-negative bacteria are known to release membrane fragments consisting of lipopolysaccharide, phospholipid, and proteins during growth (10, 17). The cellular components released by the three strains of *C. jejuni* used in the present study contained three OM proteins of 92K, 61K, and 43K daltons as well as several other proteins. Because these cellular components may contain proteins other than OM proteins, the differences between their profiles were not unexpected (Fig. 1A, lanes 1 through 3). Flagella were observed in these preparations, as determined by electron microscopy, and therefore it is likely that one of the protein bands in the SDS-PAGE profiles is flagellar protein.

With the application of the Western blotting technique the immune response of a human host to *C. jejuni* OM proteins was assessed. It was interesting to observe a low titer of antibody present in normal human serum to several OM proteins (Fig. 1B, Table 1). This could be due to a previous infection with *C. jejuni* or cross-reactivity of these proteins and OM proteins of other gram-negative organisms. Although this low level of antibody did not appear to be protective, as the sera used were taken from a person who later acquired the infection, this finding does not preclude the possibility of protective immunity at the level of the gut mucosa. An increase in the intensity of all bands with the acute and convalescent sera indicated a definite increase in serum antibody levels during the infection.

The appearance of three additional proteins (92K, 56K, and 19K daltons) in strains 17 and 17J is of particular interest because they only appeared in the acute and convalescent stages of the infection. When immune blots were performed with the preinfection sera at a lower dilution (1:100), these proteins were not present, indicating that they were in fact new and not simply present in low dilution. An antibody response to the 56K-dalton protein was not observed for strain 16. However, antibody responses to the 92K- and 19K-dalton proteins were observed for all three strains, indicating that there exist common antigens among the different *C. jejuni* strains.

In strains 17 and 17J, the area of the gel at less than 14.4K daltons did not stain with Coomassie brilliant blue, but was stained with a silver stain modified for lipopolysaccharide (23). This cellular component showed a very strong reaction in the immune blots with only convalescent sera (Fig. 1D, lanes 4 through 7). By contrast, strain 16 did not react in this area of the gel, which was not surprising as it was of a different antigenic grouping than strains 17 and 17J as determined on the basis of their thermostable antigens (19); moreover, this strain was not involved in the infection. In addition, this cellular material appeared to be released by strain 17J as it reacted very strongly with the convalescent serum (Fig. 1D, asterisk in lane 1), but not with strains 17

and 16. Thus it would appear that not only is human antibody produced against this cellular material, but also that there is an elaboration of the material during the course of the infection.

In summary, the application of immunoautoradiography revealed that several OM proteins (92K, 56K, and 19K daltons) and additional cellular material observed below the 14.4K-dalton area in SDS-PAGE were antigenic during human infection with *C. jejuni*.

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