JOURNAL OF CLINICAL MICROBIOLOGY, Jan. 1985, p. 108–112
0095-1137/85/010108-05$02.00/0
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Vol. 21, No. 1

Antigenic Analysis of *Campylobacter* Flagellar Protein and Other Proteins

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Received 19 July 1984/Accepted 1 October 1984

Outer membrane proteins of *Campylobacter jejuni* and other campylobacter species were analyzed for their antigenic potentials by immunoblotting. Polypeptides were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred electrophoretically, and reacted with rabbit antisera to *C. jejuni*. Each *Campylobacter* species analyzed demonstrated a unique outer membrane protein antigenic profile; interspecies antigen sharing was observed to be compatible with the degree of DNA relatedness between the species. The most highly conserved outer membrane protein antigen was the flagellum (molecular weight, 62,000). An aflagellate mutant was found to be untypable with the heat-labile system, in contrast to its parental isolate. The immunogenic potentials of *C. jejuni* proteins were examined by immunoblot analysis of sera from infected humans. Sera of convalescent patients, reacted with their homologous *C. jejuni* isolates, recognized a variety of campylobacter proteins. The most consistent immunogen in human infection was the flagellar protein. Patient sera assayed by the immunoblot technique were easily distinguished from control sera, which did not recognize specific campylobacter antigens. These findings suggest that the campylobacter flagellar protein is an essential determinant of the heat-labile antigen typing scheme and is the dominant immunogen recognized during *C. jejuni* infections in humans.

Since the development of selective media for the culture of *Campylobacter jejuni* (18), this agent has been recognized as a common cause of diarrhea throughout the world and is the most frequently reported bacterial agent of enteritis in parts of Europe and North America (4, 10, 19). *C. jejuni* accounts for ca. 97% of cases of campylobacter gastroenteritis, and *Campylobacter coli* accounts for the remainder (11). *Campylobacter fetus* subsp. *fetus* causes abortion in cattle and sheep and is occasionally implicated in human bacteremia, especially in the compromised host (5, 8). In contrast, *C. fetus* subsp. *venerealis* is associated with sterility in cattle but is not considered to be pathogenic in humans (6). An additional species, *Campylobacter laridis*, formerly known as the nalidixic acid-resistant thermophilic campylobacter group, has been isolated commonly from seagulls but, again, is not known to cause disease in humans (2).

The epidemiology of campylobacter infections in humans is not well understood. The organism is known to be a pathogen in a wide range of animal species; consequently, a large natural reservoir exists (3, 6, 10). The mode of transmission and source of *C. jejuni* and other species will be understood clearly only when effective methods become available for typing isolates of these organisms. To this end, two serotyping systems have been developed for *C. jejuni*. Penner and Hennessy (17) used a passive hemagglutination technique based on soluble heat-stable antigens. This work indicates that many different serotypes exist but that three are most common among campylobacter isolates (17). Lior et al. have developed slide agglutination tests based on heat-labile protein antigens which have identified eight serogroups encountered most frequently (13). However, the molecular structures upon which each of these schemes is based have not yet been identified or characterized.

The objectives of the present study were to specify antigenic molecular structures and to identify which campylobacter polypeptides are recognized by the human immune system during infection. Elucidation of the human immune response to these infections will help in understanding the pathogenic mechanism(s) of campylobacters, which is largely unknown at this stage. Further, we wished to investigate the antigenic relatedness of different *Campylobacter* species and to develop an identification scheme for these species based upon their protein antigen profiles. This information may be useful in investigating the epidemiology, both reservoirs and transmission, of campylobacters.

MATERIALS AND METHODS

***Campylobacter* strains and growth conditions.** Seven isolates of *C. jejuni* were obtained from an epidemiological study of diarrhea undertaken at the University of Manitoba. These strains were isolated from stools on Skirrow medium (19). Four the the strains were Lior serotype 4. The other strains belonged to serotypes 5, 16, and 17. Stock cultures of *C. jejuni*, *C. fetus*, *C. coli*, *C. laridis*, and *C. fetus* subsp. *venerealis* used in this study are shown in Table 1. Once isolated in pure form, all strains were cultured on Columbia-base agar (Oxoid) supplemented with 10% defibrinated sheep blood. Growth conditions were 7% CO_2, 37°C, and 85% humidity for 48 h.

Identification of flagellate and aflagellate strains. Presence of flagella was monitored by picking isolated colonies of *C. jejuni* 81116 (16) onto a blood-free medium containing 0.5% agar without addition of hemin or reducing agent (20). Flagellate strains showed distinct swarming, and halos were observed, whereas the aflagellate mutants remained as a

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TABLE 1. Strains of campylobacter used in this study

University of Alberta no.	Original strain no.	Species	Source[a] (reference)
67	SD2	*C. jejuni*	HSC (20)
3	MK22	*C. jejuni*	HSC (20)
1	MK175	*C. jejuni*	HSC (20)
501	81116 Fla$^+$	*C. jejuni*	PHLS (16)
502	81116 Fla$^-$	*C. jejuni*	PHLS (16)
30	PC86	*C. coli*	HSC
100	1380	*C. coli*	NADC
417	C2633	*C. coli*	LCDC
424	CIP7077	*C. coli*	IP
60	ATCC 27374	*C. fetus* subsp. *fetus*	ATCC (20)
76	60(pMAK175)	*C. fetus* subsp. *fetus*	UA
419	BCV2751	*C. fetus* subsp. *fetus*	UA
63	PC19	*C. fetus* subsp. *venerealis*	OVC
423	CIP6829	*C. fetus* subsp. *venerealis*	IP
418	C3331	*C. laridis*	LCDC

[a] Abbreviations: HSC, The Hospital for Sick Children, Toronto, Canada; PHLS, Public Health Laboratory, Southampton, United Kingdom; LCDC, The Laboratory Centre for Disease Control, Ottawa, Canada; ATCC, The American Type Culture Collection; UA, The University of Alberta, Canada; NADC, National Animal Diseases Center, Ames, Iowa; IP, Institute Pasteur, France; and OVC, Ontario Veterinary College, University of Guelph, Canada.

discrete colony on the plate. These identifications were further confirmed by electron microscopy.

Sera. Human convalescent antisera corresponding to *C. jejuni* isolates from patients were obtained from patients with *C. jejuni* diarrhea. Rabbit antisera to *C. jejuni* strains were raised by injecting the rabbits subcutaneously with Freund adjuvant and intravenously with *C. jejuni* whole cell protein (1 mg/ml) prepared in saline by sonication (model W-220F; Heat Systems Ultrasonics Inc.). Thereafter, booster shots were given intravenously after 2 and 4 weeks, and the rabbits were bled during week 6.

Outer membranes. Campylobacter whole cells were suspended in 0.001 M sodium phosphate buffer (pH 7.0) and sonicated three times with the microtip setting at 2 for 30 s each. After sonication, the cellular debris was removed by centrifuging at 5,000 × *g* for 20 min. Collection of total membrane fraction was facilitated by centrifugation at 100,000 × *g* for 30 min. The inner membrane was digested with 2% Sarkosyl with gentle rocking for 30 min at room temperature as described by Filip et al. (7). The preparation was then centrifuged at 100,000 × *g* for 30 min, and the Sarkosyl insoluble membrane was collected for analytical studies.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Whole organisms or outer membranes were placed into a reducing buffer containing 5% (vol/vol) 2-mercaptoethanol, 10% (vol/vol) glycerol, 2% (wt/vol) sodium dodecyl sulfate, and 0.0625 M Tris base. Samples were boiled at 100°C for 3 min, and 10 μg of protein was applied to each lane. Vertical slab gel electrophoresis was performed by the method of Laemmli (12) by aligning proteins in a 4.5% stacking gel and separating them in a 12% sodium dodecyl sulfate-polyacrylamide gel. Electrophoresis was continued at a constant current of 8 mA overnight.

Immunoblotting. This sensitive and specific technique allows polypeptides which have first been resolved on polyacrylamide gels to be transferred electrophoretically to a solid matrix; their separation by molecular weight is maintained. The polypeptides retain antigenicity after transfer and, therefore, can be characterized serologically. The technique thus allows identification of specific polypeptide antigens within a complex protein mixture. Polypeptides were transferred from sodium dodecyl sulfate-polyacrylamide gels by electrophoresis in a Tris-glycine-methanol buffer as described by Towbin et al. (21). Electrophoresis was carried out at a constant current of 195 mA for 2 h, after which the blot was stained with Amido Schwartz and destained, and then the unsaturated antibody-binding sites were blocked with 5% ovalbumin. Appropriate antisera, used in a dilution of 1:100 unless otherwise indicated, were reacted with the blots for 16 h in 2% ovalbumin-Tris buffer. The blots were then washed and probed with ^{125}I-labeled protein A of *Staphylococcus aureus* (5×10^6 cpm per blot) for 2 h. After washing, the blots were air dried and autoradiographed with Kodak X-Omat AR film.

RESULTS

Protein antigens of *C. jejuni*. Total proteins of various *C. jejuni* strains were analyzed by immunoblotting against rabbit *C. jejuni* antiserum; at least 11 proteins were found to be antigenic (Fig. 1). Prominent antigens include the major outer membrane protein (MOMP), with a molecular weight of 40,000, as well as proteins with molecular weights of 72,000, 62,000, 50,000, and 29,000. *C. jejuni* antiserum recognizes a consistent pattern of protein antigens within that species, representing an identifiable pattern. There

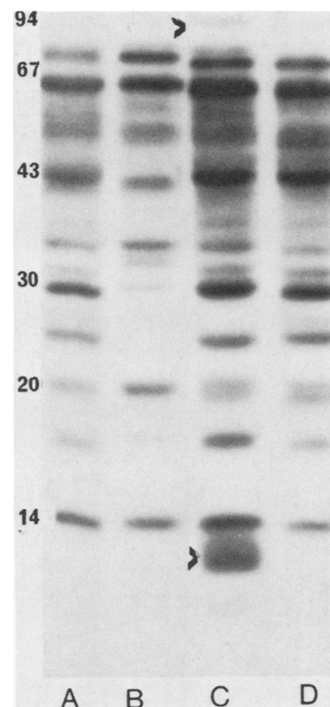

FIG. 1. Immunoblot autoradiogram of total proteins of four *C. jejuni* strains, which were reacted against rabbit antiserum to the strain in lane C. All isolates were originally from patients with campylobacter enteritis. Arrows indicate a 92K polypeptide and lipopolysaccharide detected only on the isolate homologous to the antiserum used in this assay. Molecular weight standards were phosphorylase b (94,000), albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), trypsin inhibitor (20,000), and α-lactalbumin (14,400) from Pharmacia Fine Chemicals.

appears to be very little variation among the strains of *C. jejuni* analyzed, with the exception of the strain homologous to the antiserum probe. It was noted that two additional antigens were recognized in the homologous *C. jejuni* strain only (Fig. 1, lane C), a 92,000-molecular-weight (92K) protein and lipopolysaccharide, as observed previously by Logan and Trust (15), thus identifying one or both of these structures as potential strain-specific antigens.

OMP antigens of *Campylobacter* species. The potentially antigenic nature of outer membrane proteins (OMP) of various *Campylobacter* species was analyzed by immunoblotting. These OMP, when reacted against rabbit *C. jejuni* antiserum, demonstrated consistent antigenic profiles that are unique for each species (Fig. 2). *C. jejuni* and *C. coli* showed the most antigenic relatedness, a result compatible with their close DNA homology (1, 9). Prominent protein antigens conserved between the two species have molecular weights of 62,000, 50,000, 40,000 (MOMP), and 29,000. *C. fetus*, however, shares only two antigens strongly with *C. jejuni* and *C. coli*, proteins with molecular weights of 62,000 and 50,000. *C. laridis*, which shares four protein antigens with *C. jejuni*, appears to be intermediately related to that species; the 62K protein, but not the MOMP, is a common antigen. Although only two *C. venerealis* strains were analyzed, they each displayed an unusual and unique antigenic profile, suggesting that *C. venerealis* is an antigenically heterologous group.

The MOMP is an important intraspecies antigen (Fig. 1) which is most strongly shared between the closely related species *C. jejuni* and *C. coli*. However, it is not otherwise a strong interspecies antigen, despite its predominance in the outer membrane. The most highly conserved protein antigen, shared by all *Campylobacter* species except *C. venerealis*, is a 62K polypeptide which was recognized by four different *C. jejuni* antisera raised in rabbits. This OMP, which has the highest ratio of antigenicity to molar representation, has been previously identified as the flagellar protein (16).

Characterization of the campylobacter flagellar protein. We have confirmed the 62K OMP as the flagellum by studying isogenic strains of *C. jejuni* 81116 which differed only by their ability to produce flagella. Immunoblot analysis of the parent strain and the aflagellate mutant strain demonstrated that the mutant lacks the 62K antigen when reacted with *C. jejuni* antiserum, which is present in the parent strain (Fig. 3). A revertant of the aflagellate mutant was isolated after growth for 3 to 4 days on blood-free medium. Moreover, the spontaneous revertant had once again acquired the 62K OMP.

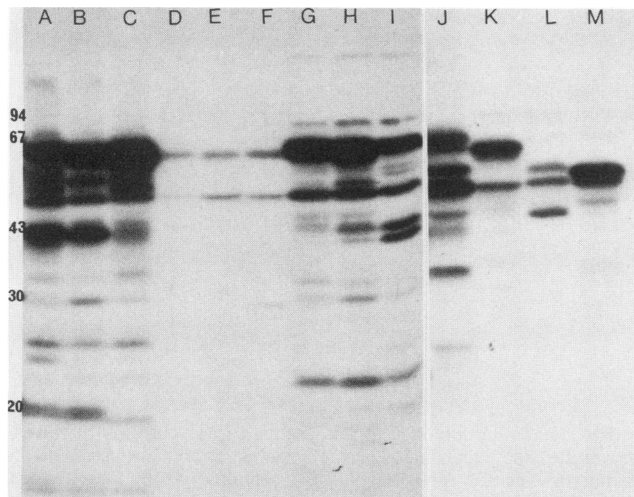

FIG. 2. Immunoblot autoradiogram showing OMP of various campylobacter species reacted with rabbit antiserum to *C. jejuni*. Strains analyzed were *C. jejuni* (lanes A through C), *C. fetus* (lanes D, E, F, and K), *C. coli* (lanes G through I), *C. laridis* (lane J), and *C. venerealis* (lanes L and M).

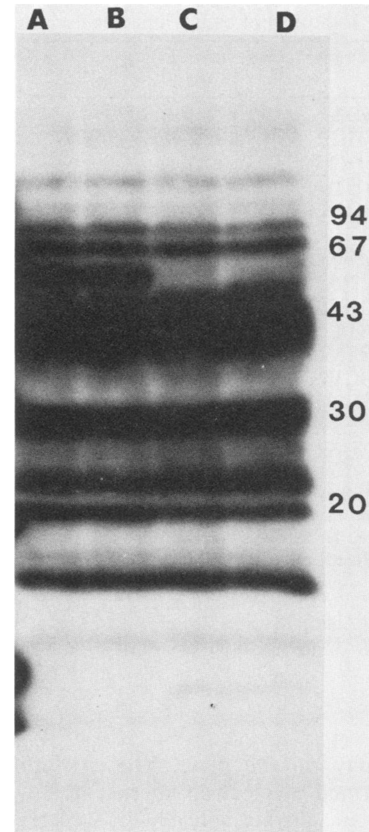

FIG. 3. Immunoblot analysis of a *C. jejuni* isolate (lanes A and B) and an aflagellate mutant of the same strain (lanes C and D), showing the absence of the 62K flagellar antigen in the mutant strain. The aflagellate mutant was isolated as outlined in the text.

C. jejuni 81116 wild type was identified as serotype 6 with the Lior system (13). However, the aflagellate mutant of 81116 reacted with the type 6 crude serum but not with the system adsorbed serum preparation (13). This information suggests that the flagellar protein is a key component of the heat-labile campylobacter serotyping system.

Campylobacter antigens recognized during human infection. To determine which proteins of *C. jejuni* are immunogenic, strains isolated from patients with campylobacter diarrhea were reacted against their homologous sera. Immunoblot analysis demonstrated that although a variety of protein antigens, including the MOMP, are recognized during infections, the predominant immunogen is the 62K flagellar protein (Fig. 4). Although all of the sera studied were collected during convalescence, they recognized a wide range of antigens, from only 3 to over 20 proteins. The flagellar immunogen was consistent to all infections, and the MOMP, in a weaker fashion, was observed in the majority of cases analyzed. Another antigen which was commonly but not invariably recognized by convalescent sera was lipopolysaccharide.

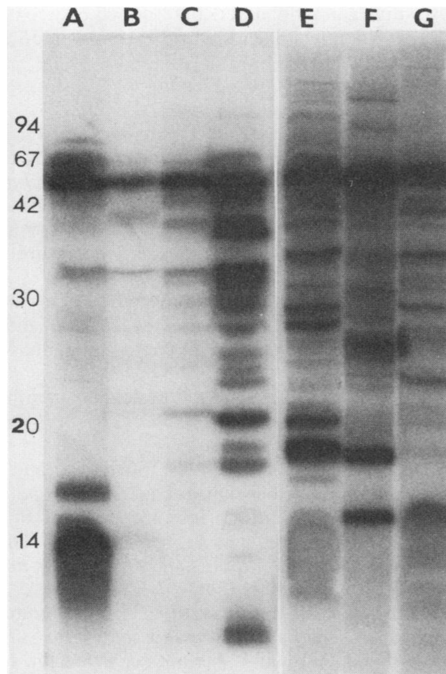

FIG. 4. Isolates, analyzed by immunoblotting, from patients with *C. jejuni* diarrhea. Strains were reacted with their homologous antisera, which were collected in the convalescent phase of illness. Biotypes and serotypes of these isolates are shown in Table 1. The 62,000 flagellar antigen is recognized by all patient sera.

Sera from well patients were also analyzed by immunoblot technique with a *C. jejuni* strain as antigen. Unless they were assayed at a very low dilution, the control sera did not recognize detectable antigen profiles (Fig. 5a). The sera were indistinguishable from one another but distinctly different from infected sera. Figure 5b shows the relative antibody strength of infected human sera (lane A), control human sera (lane B), and immunized rabbit antisera (lane C). Sera from well humans do not appear to recognize specific campylobacter antigens.

DISCUSSION

Our results demonstrate that many proteins, predominantly OMP, are antigenic when probed with antiserum of the same species. Each *Campylobacter* species possesses a unique OMP antigenic profile recognized by rabbit antisera; the species of the isolate can be readily determined from this profile. The antigen profiles show good agreement with previous campylobacter DNA relatedness studies (1, 9), which reveal that *C. jejuni* DNA is only ca. 10% homologous with *C. fetus* DNA but is ca. 30% concordant with *C. coli* DNA (depending upon the stringency of hybridization conditions).

The present studies confirm the findings of Logan and Trust (14) that the flagellum and MOMP are important antigens of *C. jejuni*. The flagellum, however, is the major interspecies antigen and is conserved among the *Campylobacter* species analyzed. This protein has a particularly high ratio of antigenicity to molar representation on the outer membrane. The 62K protein was confirmed as the flagellum by analysis of an aflagellar mutant and its revertant. The wild-type isolate was identified as type 6 by the Lior system, whereas the aflagellar mutant was untypable. We have recently produced additional aflagellar mutants of *C. jejuni* by UV light mutagenesis. These mutants, whose parental strains included serotypes 5, 6, and 7, were all untypable (W. M. Wenman, H. Lior, and D. E. Taylor, unpublished observations). Thus, the flagellar protein appears to be an essential determinant of the heat-labile antigen typing scheme (13). This finding is compatible with the high degree of conservation of the flagellar antigen.

We have also investigated the immune response of infected humans to *C. jejuni* proteins by immunoblot analysis. The sera from ill patients recognized a variety of campylobacter proteins, in contradistinction to control sera from well humans. The most consistent and dominant *C. jejuni* immunogen was the 62K flagellar protein, which was recognized by all of the infected sera.

The *C. jejuni* strains isolated from infected humans used in the present study were serotyped by the heat-labile antigen system (13). Logan and Trust have identified a 92.5K OMP which, they reported, conferred antigenic specificity in this typing scheme (14). We have observed a 92K protein antigen when probing clinical *C. jejuni* isolates with their homologous sera, but not as a consistent finding. The specific antigens detected in the heat-labile typing system may be a complex group of surface structures, including the flagellum, as well as a 92K protein. It is clear from our studies that the flagellar protein is a key determinant of the antigenic typing scheme developed by Lior et al. (13).

The present investigation has delineated an antigenic protein profile unique to each campylobacter species. The 62K flagellar protein is the most highly conserved antigen and is recognized by the heat-labile serotyping system (13). It may therefore be a useful tool in investigating the epide-

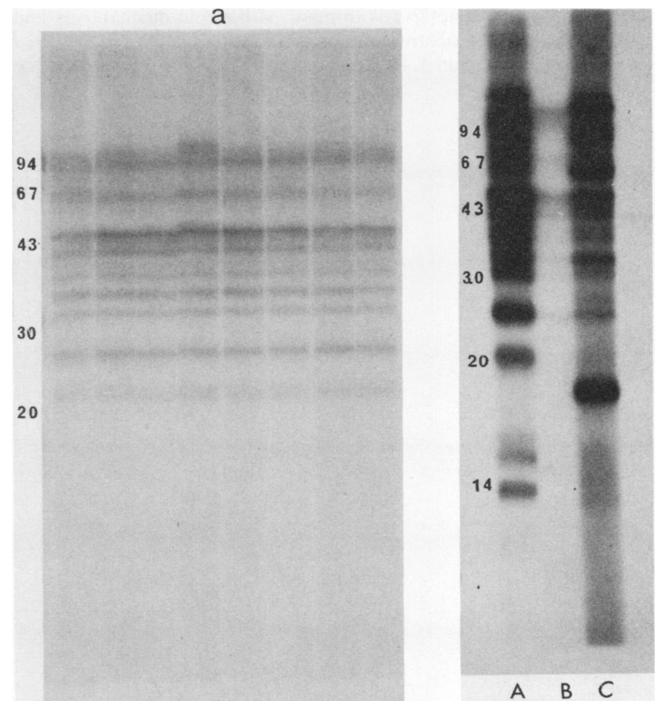

FIG. 5 (a) Immunoblot analysis of control human sera reacted against *C. jejuni*. Sera were assayed at 1:10 dilution, and the film was developed after 48 h of exposure. (b) Immunoblot autoradiograms showing sera from immunized rabbit (lane A), human control (lane B), and infected human (lane C); all reacted at 1:100 dilution against a *C. jejuni* isolate.

miology of campylobacter infections. Further, the flagellar protein is highly immunogenic in humans and thus demonstrates potential as a vaccine reagent. Studies which characterize the flagellum and its immunogenic potential more fully are currently in progress.

ACKNOWLEDGMENTS

We thank Doris Volz, Rosemary Garner, and Lai-King Ng for technical assistance, Heather Mitchell for secretarial assistance, and Richard Sherburne for photography. We thank the following people for providing strains: M. A. Karmali, J. Bryner, J. Prescott, G. Marien, and T. Williams.

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