Western Blot Analysis of the Human Antibody Response to *Campylobacter jejuni* Cellular Antigens During Gastrointestinal Infection

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Western blot analysis was used to identify antigenic components of *Campylobacter jejuni* whole cells and outer membranes that elicit antibody responses in patients with campylobacter enteritis. Acute- and convalescent-phase sera from eight patients were analyzed for antibody activity against their homologous infecting strains and heterologous clinical isolates. Whole-cell and Sarkosyl-insoluble membrane components were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred electrophoretically to nitrocellulose paper for immunoblotting experiments. After the separated components were probed with patient sera, antibody binding was detected by autoradiography with ^{125}I-protein A. Using this method, we detected several immunogenic components in whole cells and outer membranes. In the acute-phase response of some patients to infection, two to three components with approximate molecular weights of 66,000 (p66), 43,000 to 46,000 (major outer membrane protein), and 12,000 (p12) were detected in immunoblots. Convalescent-phase sera showed a more broad array of antibody binding to cell components. p66, shown to be campylobacter flagellin, was the major immunodominant component in almost all sera tested, however, p66 was not a major protein in Coomassie blue-stained gels. The major outer-membrane protein also bound to antibody, but with less intensity than p66. In general, the antibody specificity of patient sera was not limited to the homologous infecting strain, and antibodies cross-reacted with most components in heterologous strains. A low-molecular-weight component, identified as lipopolysaccharide with a modified silver stain, showed serological specificity for some patient sera. The results of this study showed that the antibody response of patients with campylobacter enteritis to *C. jejuni* antigens is variable. Flagellin appeared to be the major immunodominant component during infection.

Campylobacter jejuni is now a well-recognized cause of bacterial gastroenteritis (3). Although much information has been collected on the clinical and epidemiological aspects of this disease, little is known about the mechanism of pathogenicity by which *C. jejuni* causes disease.

Patients with campylobacter enteritis produce high titers of serum antibodies in response to this organism (6, 8). The mechanism by which these antibodies are elicited is not known, but it may be an invasion of the intestinal mucosa that provides the antigenic stimulus. Clinical and histopathological evidence supports an invasive mechanism of disease (3, 5, 10, 16), although invasiveness may not be the only pathogenic mechanism (17).

Several recent studies which partially characterize the nature of animal and human immune responses to *C. jejuni* outer-membrane protein antigens have been reported by Mills and Bradbury (14) and Blaser et al. (2). Mills and Bradbury (14) showed that sera from an infected laboratory worker reacted with a number of proteins in the *C. jejuni* outer membrane, including the major outer-membrane protein (MOMP) (1, 12, 13). Using rabbit and human convalescent-phase sera, Blaser et al. (2) also showed that the MOMP was antigenic. In both studies, a limited number of human sera were tested, and reference strains were used to characterize the antibody responses in the sera tested.

A basic premise in immunology is that no two host immune responses are alike. Although previous studies have shown that antibodies react with campylobacter membrane

antigens, does each patient show the same kind of response to campylobacter membrane antigens? Understanding the nature of the array of host immune responses to *C. jejuni* cellular components is important in developing strategies for immune protection. The purpose of this study, therefore, was to study individual host antibody responses to homologous infecting strains. Thus, acute- and convalescent-phase sera from each patient were tested against their homologous strains as well as against heterologous clinical isolates to better understand possible variations in the host immune response to campylobacter infection.

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MATERIALS AND METHODS

Bacterial strains. The *C. jejuni* strains used in this study were obtained from adult patients with diarrhea seen at the Hospital of the University of Pennsylvania, Philadelphia. Isolates were obtained from stool samples upon primary isolation on brucella agar with 10% sheep blood and five antimicrobial agents (BBL Microbiology Systems, Cockeysville, Md.). Strains were frozen in aliquots of tryptic soy broth containing 10% glycerol at −70°C. *C. jejuni* was identified by standard criteria (7). *Campylobacter coli* was obtained from R. M. Smibert, Virginia Polytechnic Institute and State University, Blacksburg. For all studies, bacteria were taken from frozen stocks and passaged minimally in

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vitro. All bacteria were grown on sheep blood agar at 42°C in an atmosphere of 5% O_2–10% CO_2–85% N_2 for 48 h.

Patient sera. Sera were collected from each patient during the acute and convalescent phases of infection. Acute-phase sera were collected 3 to 10 days after the onset of acute symptoms. Two of the patients received erythromycin therapy, and this did not affect the antibody responses detected in this study, as compared with the antibody responses of patients who did not receive therapy. All blood samples were collected in Vacutainers (Becton Dickinson and Co., Paramus, N.J.) without anticoagulant and allowed to clot for 1 h at room temperature, and sera were then frozen in 0.2-ml aliquots at −70°C.

Preparation of cell components. Whole cells for analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were harvested from sheep blood agar after 48 h of growth at 42°C. Whole cells were suspended in distilled water at an optical density of 2.0 at 540 nm. This material was diluted 1:1 with SDS-PAGE solubilizing buffer. MOMP-enriched membranes were prepared as Sarkosyl-insoluble membranes by the methods of Blaser et al. (1) and Logan and Trust (12). Purified flagellin was prepared by the method of Logan and Trust (13) (see below).

SDS-PAGE. Membrane fractions and whole-cell proteins were analyzed by SDS-PAGE by the system of Laemmli (9) with a 4% stacking gel and a 12.5% running gel. Proteins were solubilized in SDS solubilizing solution containing 2-mercaptoethanol, boiled for 5 min, and applied to the gel at ca. 25 μg per lane. Proteins were electrophoresed by using a Bio-Rad slab gel apparatus (Bio-Rad Laboratories, Calif.) at a constant current of 15 mA per gel until the tracking dye entered the resolving gel and at 20 mA per gel through the running gel. Proteins were visualized with Coomassie blue stain. Unlabeled molecular weight markers were obtained from Bio-Rad. ^{14}C-labeled molecular weight markers (New England Nuclear Corp., Boston, Mass.) were used for immunoblotting experiments. Lipopolysaccharide (LPS) was identified in gels by the silver staining method described by Tsai and Frasch (18).

Flagellin was purified by the method of Logan and Trust with some modifications (13). Briefly, cells were harvested in 20 to 30 ml of distilled water and sonicated. Cells were removed by centrifugation at 10,000 × g for 1 h at 4°C, washed in distilled water, and removed by centrifugation at 10,000 × g for 1 h. The supernatants were pooled and centrifuged at 100,000 × g for 1 h at 4°C. The pellet was suspended in distilled water and centrifuged at 10,000 × g to remove debris, and the supernatant was centrifuged at 10,000 × g for 1 h. The pellet was resuspended in distilled water, adjusted to pH 2.0, and held at 0°C for 15 min. Insoluble material at pH 2.0 was removed by centrifugation at 100,000 × g for 1 h, and the supernatant was adjusted to pH 7.0 with NaOH and incubated for 30 min at 0°C to reassociate flagellin. Flagellin was concentrated by lyophilization.

Western blots. After electrophoresis, proteins were immediately transferred from slab gels to nitrocellulose paper (BA85; Schleicher & Schuell Inc., Keene, N.H.) by a modification of the method described by Burnette (4). The electroblotting was carried out at 12 V overnight at 4°C with a Bio-Rad Transblot apparatus and 30 mM phosphate buffer (pH 6.5), in accordance with the manufacturer's instructions. After transfer, the nitrocellulose paper was incubated with 10 mM Tris–0.9% NaCl (pH 7.4) containing 3% bovine serum albumin for 2 h at 37°C. The nitrocellulose paper was then incubated with either acute- or convalescent-phase sera

diluted 1:100 to 1:250 in 1% BSA buffer (50 ml) for 2 h at room temperature. The nitrocellulose paper was washed three times with 1% BSA buffer and incubated with ^{125}I-protein A (1 μCi per blot in 50 ml of buffer; specific activity, 90 μCi/μg; New England Nuclear) for 2 h at room temperature. The blots were washed, dried, and exposed for 14 to 48 h at −70°C to Kodak X-Omat AR film with a Cronex Lightning-Plus Xtralife intensifying screen (E. I. duPont de Nemours & Co., Inc., Wilmington, Del.).

Serotyping. Isolates were serotyped by the methods of Penner and Hennessy (15) and Lior et al. (11). The former method detects a heat-stable antigen by passive hemagglutination, and the latter method detects a heat-labile antigen by slide agglutination.

RESULTS

SDS-PAGE. Whole-cell and Sarkosyl-insoluble membrane protein profiles stained with Coomassie blue are shown in Fig. 1. Many components were apparent in whole cells and ranged in molecular weight from greater than 100,000 to less than 12,000 in this gel system. The MOMP had an approximate molecular weight of 43,000 to 46,000 among strains used in this study. This component corresponds to the MOMP described by other investigators (1, 2, 12, 13). The MOMP was also apparent in Sarkosyl-insoluble membranes, as shown in lane 2, with few other components present. The MOMP was shown in previous studies to be a surface-exposed protein (1, 12, 13). LPS was detected in both whole cells and Sarkosyl-extracted outer membranes with a modified silver strain, as shown in lanes 5 and 6, and had a molecular weight of less than 12,000.
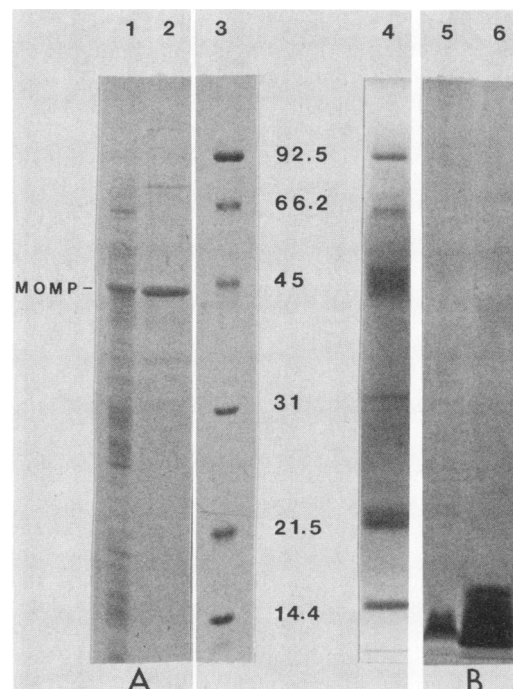

FIG. 1. SDS-PAGE of whole-cell and outer-membrane components of *C. jejuni*. (A) Coomassie blue stain of whole cells (lane 1) and Sarkosyl-insoluble outer membranes (lane 2). Molecular weight markers (×10³) are shown in lanes 3 and 4. (B) Modified silver strain of the LPS of whole cells (lane 5) and outer membranes (lane 6).
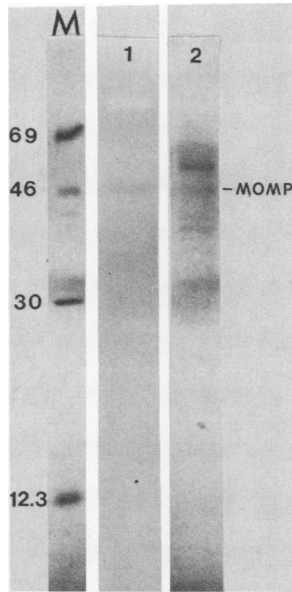
FIG. 2. Western blot analysis of sera from a noninfected individual (lane 1) and covalescent sera from a patient with diarrhea that was culture negative for *C. jejuni* (lane 2). ^{14}C-labeled molecular weight markers ($\times 10^3$) are shown in lane M.

Western blot analysis of controls. When ^{125}I-protein A alone was used, no binding of label to whole cells or outer membranes was detected. Sera from noninfected, healthy individuals (three tested) showed antibody activity to a component corresponding to the MOMP. Acute- and convalescent-phase sera from a patient with diarrhea that was culture negative for *C. jejuni* showed a broader range of

reaction with numerous cell components. Figure 2 shows representative patterns exhibited by sera from a noninfected individual (lane 1) and convalescent-phase sera from a patient with diarrhea that was culture negative for *C. jejuni* (lane 2). The patterns of acute- and convalescent-phase sera from this patient were identical. In both control sera, antibodies cross-reacted with the 13 heterologous strains tested in this study. In contrast to results for patients with campylobacter enteritis, the antibody reaction was notably diffuse and much weaker, with no predominant components showing antibody binding. Sera did not react with low-molecular-weight components such as LPS.

Patient sera. Acute- and convalescent-phase sera from eight patients were tested in this study. Although each serum sample tested was unique in that no two sera produced completely identical profiles, three general patterns of antibody-binding activity were observed, of which three are shown. Figure 3 shows the antibody-binding pattern of acute- and convalescent-phase sera from patient 3. In acute-phase sera (Fig. 3A), two components were detected when whole-cell and outer-membrane proteins were probed with antibody. One component, designated p66, had an approximate molecular weight of 66,000 which varied slightly among different clinical strains. The second component had an approximate molecular weight of 43,000 to 46,000 and corresponded to MOMP. Cross-reactivity of antibodies in these sera was seen with the five strains shown in Fig. 3. Convalescent-phase sera (Fig. 3B) reacted more strongly with both p66 and MOMP in both whole cells and outer membranes. Reactivity to low-molecular-weight components, including LPS, was not detected in these sera. Convalescent-phase sera cross-reacted with all five strains shown on the western blot. No correlation was observed with the serotypes of these five strains, although the isolates in lanes 3, 4, 9, and 10 were of the same serotype (Penner 1, Lior 2). The
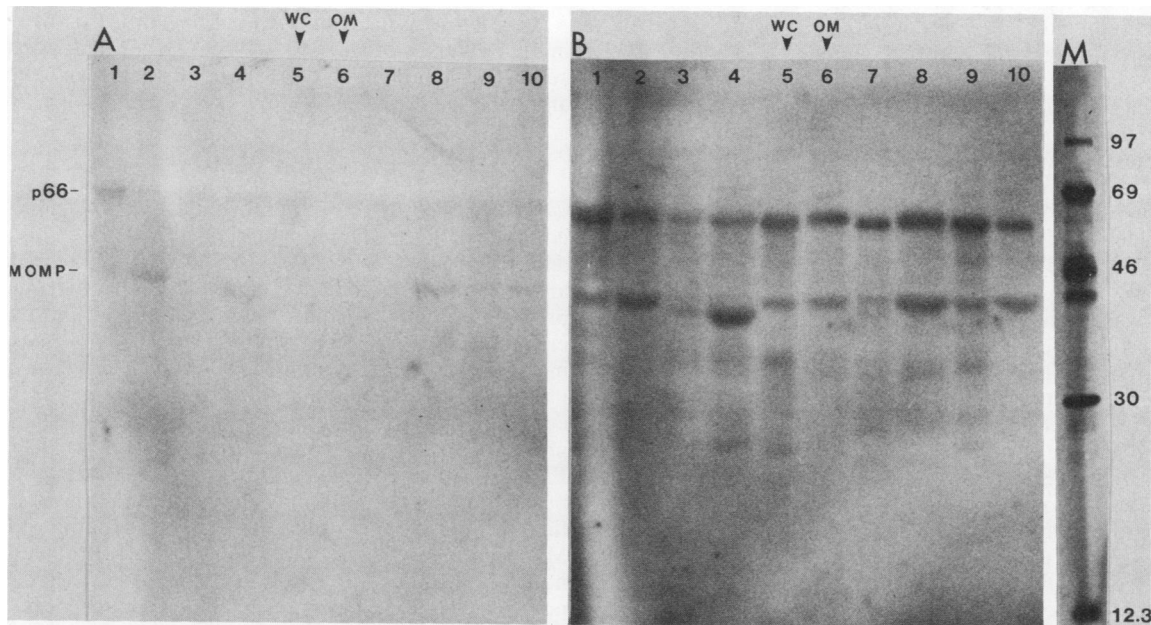

FIG. 3. Western blot analysis of sera from patient 3 against whole-cell (WC) and outer-membrane (OM) proteins. WC and OM proteins from five strains are shown as pairs on these western blots. Lanes 5 and 6 contain WC and OM proteins from the homologous infecting strain. Lanes 1, 3, 7, and 9 contain WC proteins from heterologous clinical isolates; lanes 2, 4, 8, and 10 contain OM proteins from the corresponding heterologous clinical isolates. Lanes 3, 4, 9, and 10 contain two different clinical isolates of the same serotype (Penner 1, Lior 2). The other three strains had unique serotype antigens: lanes 1 and 2 (Penner 1,2w, Lior 2); lanes 7 and 8 (Penner 5−,5+, Lior [not tested]); and lanes 5 and 6 (Penner 4, Lior 1,17,24). (A) Acute-phase sera; (B) convalescent-phase sera. Molecular weight markers ($\times 10^3$) are shown in lane M.
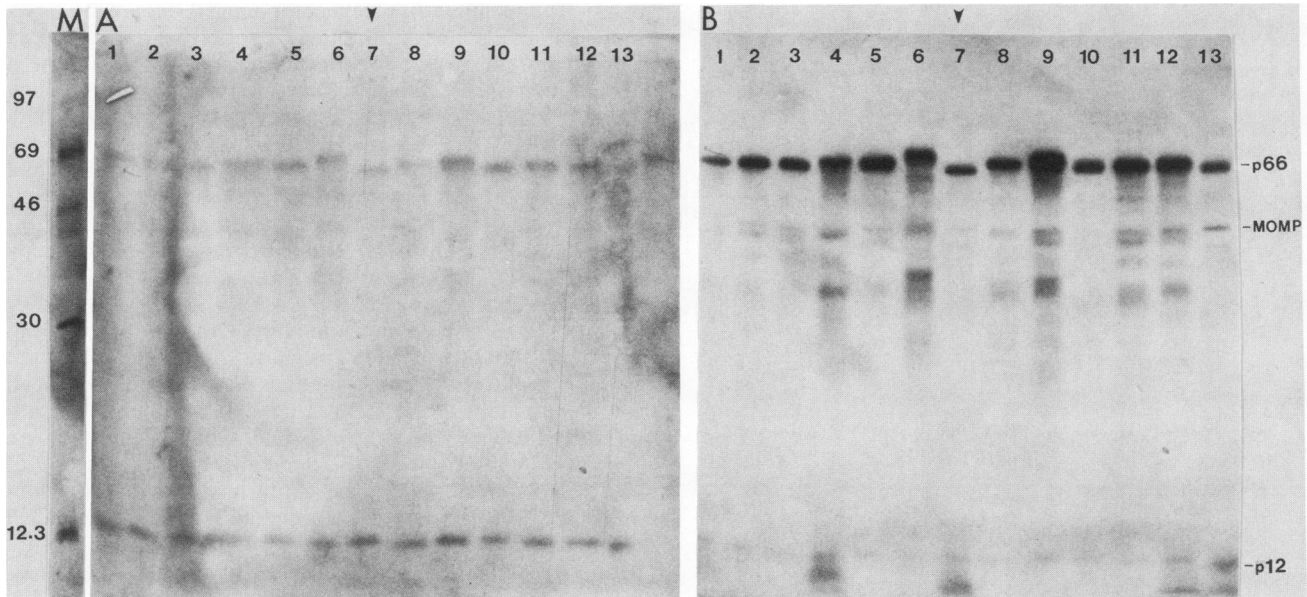
FIG. 4. Western blot analysis of sera from patient 7 against *C. jejuni* whole cells. Lane 7 contains whole-cell components from the homologous infecting strain. *C. coli* is in lane 13. Isolates in lanes 2 and 3 shared the same serotype antigen (Penner 56,37, Lior 28); isolates in lanes 8 and 11 also shared the same serotype antigen (Penner 1, Lior 2). The other strains had unique serotype antigens: lane 1 (Penner 17,10, Lior 16); lane 4 (Penner 16); lane 5 (Penner 18); lane 6 (Penner 5−,5+); lane 7 (Penner 2, Lior 4); lane 9 (Penner 16,4,13,3, Lior 7); lane 10 (Penner 16,4,13w, Lior 17); and lane 12 (Penner 4, Lior 1,17,24). (A) Acute-phase sera; (B) convalescent-phase sera. Molecular weight markers ($\times 10^3$) are shown in lane M.

antibody-binding pattern of sera from patient 4 was similar to that of patient 3 in that major antibody binding occurred with p66 and MOMP (data not shown).

Patient 7 had a pattern of antibody binding different from that of patient 3 (Fig. 4). Whole-cell binding only is shown. Acute-phase sera (Fig. 4A) reacted with two components, p66 and a 12,000-dalton component designated p12. Antibodies in these sera cross-reacted with the 13 strains shown on the blot, including a *C. coli* strain (lane 13). Strains in lanes 2 and 3 were of the same serotype (Penner 56,37, Lior 28) and strains in lanes 8 and 11 were of the same serotype (Penner 1, Lior 2). Convalescent-phase sera (Fig. 4B) reacted very strongly with p66, and other components were also detected. These sera reacted with a low-molecular-weight component that corresponded to LPS and was limited to the homologous strain (lane 7), the heterologous strains in lanes 4 and 12, and *C. coli* (lane 13). Sera from patient 8 showed a similar pattern; these sera reacted with p66 and p12 from all heterologous strains. In addition, reactivity was seen with the component corresponding to LPS, but cross-reactivity occurred with more strains. This suggested that the two sera (from patients 7 and 8) reacted with the component corresponding to LPS but were directed against different determinants.

Patient 2 had an antibody-binding pattern different from the other two patterns (Fig. 5). Both acute- and convalescent-phase sera showed essentially the same pattern, reacting with numerous components in whole cells and outer membranes. Many components detected in whole cells, such as p12, were not present in outer membranes. Reactivity with MOMP was detected, but the band did not appear to be a major one. p66 was the major, common component detected in all strains tested, including the homologous strain (lanes 3 and 4) and heterologous strains. A major reaction was also detected for components corresponding to p12 and LPS; these components reacted with the homologous strain and one heterologous strain (lanes 9 and 10). This restricted

cross-reactivity correlated with the serotype (Penner 1, Lior 2). Sera from patient 1 showed a similar pattern; p66 was the major band, and there were equivalent reactions with other components. Specific reactivity was observed for p12 and LPS from the homologous strain. Except for the p12 and LPS bands, patients 5 and 6 had similar antibody-binding patterns with the higher-molecular-weight components (data not shown).

Reaction with flagellin. Purified flagellin (Fig. 6) was used in the western blot analysis and reacted strongly with patient sera. This component corresponded to p66 in whole cells and outer membranes.

DISCUSSION

Few studies of the immunogenicity of *C. jejuni* cellular components during human infections have been done. Mills and Bradbury (14) used several extraction techniques to analyze antibody binding to campylobacter outer-membrane proteins. A clinical isolate from one patient and two reference strains were used. Several components in the outer membrane were immunogenic. Acute- and convalescent-phase sera used in their study appeared to react with a component corresponding to our p66 (extrapolated from photographs) that was present in membrane vesicles and in EDTA-lysozyme-treated and Sarkosyl-insoluble outer-membrane protein preparations. Whether this component is identical to our p66 is unknown. Other proteins, including MOMP and low-molecular-weight components, were immunogenic. Preinfection sera from this patient reacted with a number of outer-membrane components and differed from our normal, healthy sera. Sera from noninfected individuals reacted with MOMP only.

Blaser et al. (2) used both human and animal sera to detect immunogenic proteins of *C. jejuni*. Sera from rabbits immunized intravenously with *C. jejuni* reacted with a number of components in outer-membrane protein preparations and
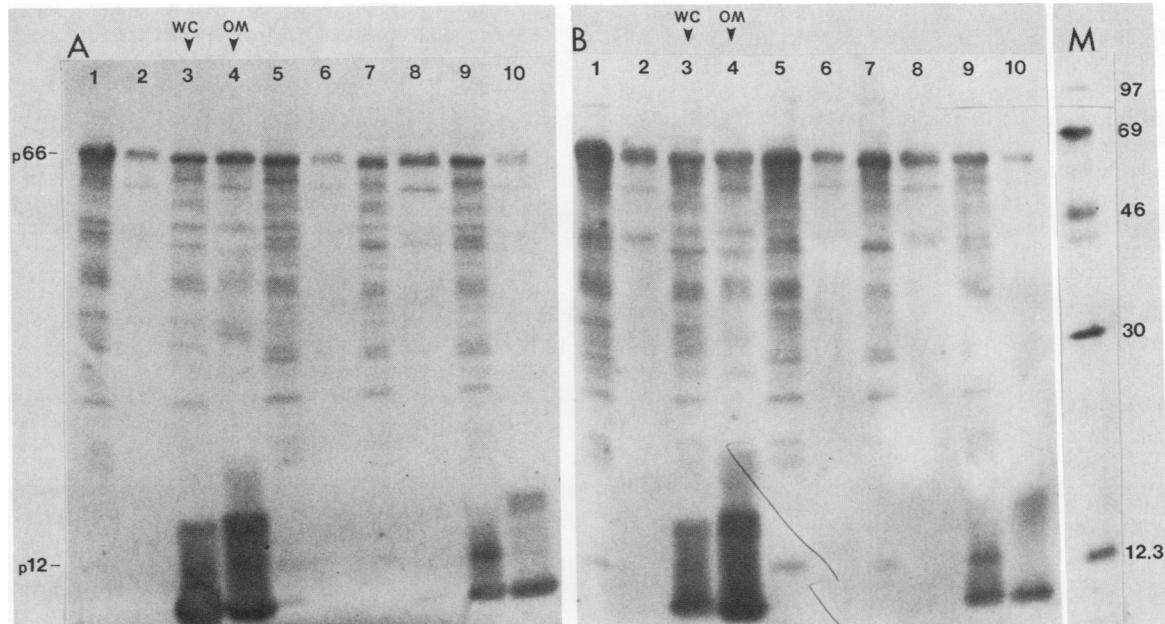
FIG. 5. Western blot analysis of sera from patient 2 against whole cells (WC) and outer membranes (OM). The homologous infecting strain is shown in lanes 3 and 4. Lanes 1, 5, 7, and 9 contain WC from heterologous clinical isolates; lanes 2, 6, 8, and 10 contain OM from corresponding heterologous clinical isolates. The same serotype antigens were shared by the strains as follows: lanes 3, 4, 9, and 10 (Penner 1, Lior 2); lanes 1 and 2 (Penner 16,4,13,3, Lior 4); lanes 5 and 6 (Penner 4, Lior 1,1,17,24); and lanes 7 and 8 (Penner 16,4,13w, Lior 17). (A) Acute-phase sera; (B) convalescent-phase sera. Molecular weight markers ($\times 10^3$) are shown in lane M.

differed from human sera. The significance of the rabbit immune response, however, cannot be assessed. Western blot analysis of human sera with a reference strain (PEN1) revealed somewhat more restricted binding patterns. MOMP appeared to be the most immunodominant protein, and major differences in antibody binding were not readily apparent when immunoglobin class-specific probes were used. A component near the 66,000-molecular-weight marker was detected by convalescent-phase sera in some western blots as well. This component was detected in both *C. jejuni* and *C. fetus* outer membranes. Blaser et al. (2) did note that a component with an approximate molecular weight of 62,000 was not recognized by human sera in radioimmuno-precipitation tests but was detected in western blot analyses.

Our study differs from previous studies because we showed that the immune response of patients with campylobacter enteritis varies when the western blot technique is used. It is clear from our results that several components present in whole cells and outer membranes, most notably p66 and MOMP, are immunogenic. However, the response to MOMP was not consistent, whereas p66 reacted strongly with almost all sera tested. LPS did not appear to produce consistent antibody responses. Some patients exhibited antibody activity against homologous LPS and heterologous LPS from a limited number of clinical isolates. Other patients showed no detectable antibodies against LPS. Most of the antibodies in infected sera cross-reacted with heterologous strains. These cross-reactions did not appear to be serotype related in most cases. These cross-reactions may have been due to denaturing procedures used to prepare samples or to the exposure of antigenic sites on nitrocellulose paper or both. We have not performed studies with radiolabeled *C. jejuni* from homologous and heterologous strains and immunoprecipitation to see whether the same cross-reactions occur, as suggested by Blaser et al. (2). Further studies are needed to confirm this with our sera.

A ca. 66,000-molecular-weight component, p66, was found to be the major immunodominant protein detected in both whole cells and outer membranes by most sera tested in this study. Further testing showed that p66 corresponds to
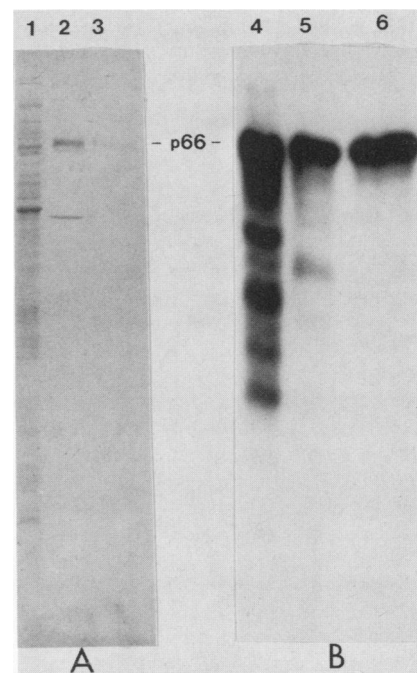

FIG. 6. Western blot analysis of purified *C. jejuni* flagellin protein. (A) Coomassie blue stain of whole cells (lane 1), partially purified flagellin (lane 2), and purified flagellin (lane 3). (B) Western blot analysis of whole cells and flagellin with patient sera against whole cells (lane 4), partially purified flagellin (lane 5), and purified flagellin (lane 6).

purified flagellin protein. We do not know, however, whether this component is a surface-exposed protein. Logan and Trust (13) and Blaser et al. (1) suggested that this may be so. The role of flagellin in relation to the pathogenicity of campylobacter enteritis is not known. However, the major antibody response to this component suggests that it elicits an antibody response early in the infection, as seen in its reactivity to acute-phase sera. Further studies are needed to assess the role of this protein in the infectious process.

ACKNOWLEDGMENTS

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