Immunoblot Analysis of Serum Immunoglobulin G Response to Surface Proteins of Clostridium difficile in Patients with Antibiotic-Associated Diarrhea

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We examined by immunoblot analysis the serum immunoglobulin G antibody response to EDTA-extracted surface proteins of Clostridium difficile in 16 patients with antibiotic-associated diarrhea. For each patient, paired serum samples were tested against proteins of the infecting strain and of a collection strain (C253) known to belong to the electrophoretic group 2 pattern. Eight patients, all harboring group 2 C. difficile strains, exhibited responses to the proteins of the infecting strain; six patients showed increases in the level of antibodies between acute-phase and convalescent-phase sera. A great variability in the antigens recognized was found; however, seven patients possessed antibodies directed against an antigen of about 35 kilodaltons, corresponding to the major protein of group 2 strains. The sera of these seven patients cross-reacted also with the 35-kilodalton and other proteins of strain C253. Our data show that C. difficile proteins other than toxins can elicit an immune response in patients with C. difficile-associated disease; in this group of patients, the major surface protein of the group 2 strains was the antigen most often recognized.

A great number of studies in recent years have partially clarified the pathogenesis of Clostridium difficile-induced diseases, such as pseudomembranous colitis (PMC) and antibiotic-associated diarrhea and colitis (2, 5). It has been demonstrated that antibodies make the intestinal tract permissive to colonization or overgrowth by C. difficile (15), which produces at least two potent toxins (an enterotoxin and a cytotoxin) involved in the pathogenesis of disease (11, 14).

Immunity to C. difficile has been studied by various investigators but only in relation to antitoxin response (1, 10, 19). It has been found that specific antibodies to C. difficile toxins are evoked in many individuals. A rise in the level of these antibodies is often detectable in patients after PMC or antibiotic-associated colitis episodes.

The possibility that patients develop an immune response to clostridial antigens other than toxins has never been explored. Serologic and immunochemical studies (7, 17) have shown that C. difficile possesses a variety of surface antigens, both species and strain specific, and that some of them might be of importance in virulence, being more commonly associated with strains isolated from cases of disease.

In previous investigations (13, 16), we have used electrophoretic profiles of EDTA-extracted surface proteins of C. difficile to characterize different strains of the microorganism. Thus, we were able to demonstrate that most strains from cases of PMC or antibiotic-associated diarrhea, especially when isolated from nosocomial outbreaks, had similar electrophoretic patterns (group 2 pattern), characterized by a major polypeptide band of about 35 kilodaltons (kDa).

In the present study, the EDTA-extracted surface proteins of C. difficile have been used as antigens to examine by Western (immuno-) blot analysis the serum immunoglobulin G (IgG) response of patients with C. difficile-associated diarrhea.

MATERIALS AND METHODS

Clinical samples. Serum samples were obtained from 16 patients, 9 males and 7 females, 17 to 86 years old (median, 66 years), from three different hospitals in Italy.

In all patients, C. difficile-associated diarrhea was nosocomially acquired. Diagnosis was made on the basis of prior exposure to antibiotics, presence of diarrhea not attributable to any other obvious cause, and detection of C. difficile and its toxin in the feces (12). In all patients, routine stool cultures for common enteric pathogens were negative. Cultures for C. difficile on selective medium (CCFA) and cytotoxin assays were performed by standard procedures (6, 13). C. difficile isolates were identified by gas-liquid chromatography and biochemical tests (8), and all were able to produce cytotoxin in vitro.

In all patients diarrhea was severe enough to warrant therapy. Two patients were treated with oral teicoplanin (two doses of 400 mg), and the others were treated with oral vancomycin (four doses of 500 mg). Two patients had a relapse of C. difficile-associated diarrhea and responded to a second course of vancomycin.

Acute-phase sera were obtained from all patients at diagnosis (within 5 days from the onset of diarrhea), and convalescent-phase sera were obtained after a mean interval of 26 days (range, 12 to 60 days).

Sera from two control groups were also evaluated. The first group consisted of 10 hospitalized patients treated with antibiotics for pneumonia, without symptoms of intestinal disturbances, and the second group consisted of 8 patients with non-antibiotic-associated diarrheal disorders, with stool cultures negative for C. difficile (4 patients with Salmonella infection, 1 with Shigella infection, 1 with Crohn's disease, and 2 with ulcerative colitis).

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Antigen preparation and sodium dodecyl sulfate (SDS) gel electrophoresis. The antigens used in immunoblot assays were surface proteins of C. difficile extracted by EDTA (13).

Briefly, overnight cultures in 2% (wt/vol) proteose peptone-1% (wt/vol) yeast extract (Oxoid Ltd.) broth (100 ml) were harvested by centrifugation and washed twice in phosphate-buffered saline at pH 7.4. Bacterial cells were extracted with 10 mM EDTA in phosphate-buffered saline at 45°C for 30 min. The protein content was measured by protein assay (Bio-Rad Laboratories, Richmond, Calif.). Proteins from C. difficile strains isolated from the feces of patients and from a collection strain (C253) representative of the electrophoretic group 2 pattern were separated by SDS-polyacrylamide gel electrophoresis as previously described (13). Gels were stained with Coomassie blue or periodic acid-Schiff stain for carbohydrate detection (3). To further characterize the antigens relevant to the immunologic response, the EDTA extract from C. difficile C253 was incubated with 4 mg of protease K per ml (E. Merck AG, Darmstadt, Federal Republic of Germany) for 24 h at 37°C in phosphate-buffered saline (pH 7.4) prior to SDS gel electrophoresis.

For immunoblotting, approximately 5 µg of protein from each sample was applied to wells of a 9% acrylamide, 0.75-mm-thick slab gel. The discontinuous buffer system of Laemmli was used (9). In each run, prestained molecular weight standards (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) were included. Electrophoresis was performed at constant voltage (45 V) overnight.

Immunoblotting. After separation by SDS electrophoresis, the proteins were transferred onto nitrocellulose (NC) in a transblotting cell (Bio-Rad), using Tris-glycine buffer with 20% methanol (18) at 60 V (approximately 220 mA) and 4°C for 150 min.

After protein transfer, the NC was cut into strips. Guide strips were stained with amido black.

The NC strips were soaked overnight in Tris hydrochloride-buffered saline (0.9% NaCl, 10 mM Tris, pH 7.4) containing 3% bovine serum albumin and then incubated for 2 h with acute- or convalescent-phase serum diluted 1:25 in bovine serum albumin with 0.05% Tween 20. The NC strips were washed five times in 0.05% Tween 20-saline and then incubated for 1 h with anti-human IgG (γ-chain specific)-alkaline phosphatase conjugate (Sigma Chemical Co., St. Louis, Mo.) diluted 1:1,000 with bovine serum albumin. After this incubation, the NC strips were rinsed as described above. All incubations and washings were performed at room temperature on a rotary platform.

Binding of the enzyme-labeled antibodies was detected as described by Heard et al. (7), using a freshly prepared mixture in equal volumes of AP color development reagent naphthol phosphate (0.4 mg/ml) and fast red (6 mg/ml; Bio-Rad).

RESULTS

Analysis of EDTA-extracted surface proteins of C. difficile. When the C. difficile strains isolated from the 16 patients with C. difficile-associated diarrhea were compared for the EDTA-extracted proteins by SDS gel electrophoresis, it was apparent that the vast majority of strains (13 of 16) exhibited a common protein profile, characterized by a major band of approximately 35 kDa (Fig. 1). This profile was similar to that of strain C253 and was consistent with the previously described group 2 pattern (13). Only three of the isolates examined showed different protein profiles. No electrophoretic band reacted positively to periodic acid-Schiff stain, indicating the absence of carbohydrate that could be stained with periodic acid-Schiff stain.

Serum IgG response to EDTA-extracted proteins. Acute- and convalescent-phase serum specimens from each of the 16 patients with C. difficile-associated diarrhea were immunoblotted with the protein extracts of the infecting C. difficile strain (homologous strain) and of strain C253.

Serum specimens from eight patients did not contain any detectable IgG antibody to the antigens tested, as no bands could be identified in immunoblots with either sera. The serum specimens of the other eight patients were reactive with the proteins of the homologous strain. A large variability of response was evident regarding both the intensity of staining and the number of bands (Fig. 2). All of these patients harbored group 2 C. difficile strains and, with the exception of patient 12, all appeared to respond to the 35-kDa protein. For two patients (patients 9 and 14) no...
differences between blots with acute- and convalescent-phase sera were visible, while for six patients the appearance of one or more new bands or an increase of staining was seen in blots with the convalescent-phase sera. In patients 12 and 15, antibodies directed against various C. difficile proteins were present already in the acute-phase sera, but in these patients stronger staining of additional minor bands (of about 39 and 60 kDa, respectively) could be seen in the convalescent-phase serum blot. In four patients (patients 2, 6, 7, and 13) there was a clear increase in the level of antibodies binding to the 35-kDa antigen in the convalescent-phase sera, although only patient 6 clearly had more antibodies against the 35-kDa protein than against other C. difficile proteins. From this patient, three serum specimens were obtained and an increase in reactivity with time (until 6 weeks) was seen (data not shown). The second serum specimen of patient 7 reacted strongly, in addition to reacting with the 35-kDa antigen, with a number of protein bands ranging from approximately 20 to 45 kDa.

Figure 3 shows the cross-reaction of sera from patients with C. difficile-associated diarrhea to the protein antigens of strain C253. The sera of seven patients responded to proteins of strain C253 (the same patients who responded to proteins of the homologous strain, with the exclusion of patient 12), and all appeared to recognize the 35-kDa antigen. For most patients, other bands also appeared to be cross-reactive.

Sera from both control groups were immunoblotted with the protein extract of C. difficile C253. None contained any detectable IgG antibody directed against the antigens tested.

The absence of the antigen bands in immunoblots after treatment with proteinase K of the EDTA extract from strain C253 confirmed that the antigens were protein.

**DISCUSSION**

In the present study, we used immunoblot analysis to investigate the serologic response of patients with C. difficile-associated diarrhea to surface proteins of the clostridial cell and were able to demonstrate that 50% of the patients examined had antibodies to these antigens.

The observed rate of reactivity is not low compared with the reactivity reported by other investigators to C. difficile toxins, the principal virulence factors of this microorganism (4). In a study by Aronsson et al. (1), IgG antibodies to toxin B were found in 58% of patients with PMC and antibiotic-associated diarrhea, and antibodies to toxin A were found in none. Viscidi et al. (19) reported positive antibody titers to toxins A and B in 38 and 75% of patients with PMC, respectively. In both studies, it was possible to demonstrate an increase in antitoxin titers with paired serum samples from some patients.

In our study, we found antibodies to C. difficile surface proteins in eight patients and an apparent increase in the response between acute- and convalescent-phase sera in six patients. This is the first demonstration, to our knowledge, that C. difficile antigens other than toxins can elicit an immunologic response in patients. The observed variability in the response, however, is intriguing and may be due to different characteristics of the patients studied or may reflect the wide spectrum of diseases associated with the presence of C. difficile in the gut. We were not able to find any obvious correlation between the development of IgG response to the antigens examined and the clinical parameters of patients, such as age, underlying disease, or severity of diarrhea. No endoscopic investigation was carried out, so we could not correlate the development of antibody response with the presence of true colitis or the severity of colonic lesions. It is worth noting, however, that the two patients who had a relapse of C. difficile-associated diarrhea (patients 6 and 7) had very prominent responses to C. difficile proteins at the time of the second sample. This observation suggests that a prolonged presence of C. difficile in the gut is required to elicit a strong antibody response to clostridial cell surface antigens and that these antibodies are probably nonprotective. However, since the pathological process in this disease consists of bacterial colonization of the intestinal mucosa, other classes of immunoglobulins and especially secretory (fecal) IgA might be more relevant to protection. The involvement of this class of immunoglobulin in the patient response to C. difficile surface proteins deserves further evaluation.

In our study, the majority of the patients (13 of 16) and all of the 8 responders were infected with group 2 strains. Seven of these patients possessed antibodies which recognized the 35-kDa antigen both of the infecting strain and of strain C253. These data indicate that the major protein of group 2 strains is expressed in vivo and represents one of the principal antigens of these pathogenic strains.

We were not able to demonstrate antibodies reactive against C. difficile proteins in control groups of patients without diarrhea or with diarrhea of different etiology. We did not evaluate, however, patients asymptotically colonized by C. difficile, who, as recently reported (12), can actually outnumber the patients with C. difficile-associated diarrhea in some hospital settings. These patients will be evaluated to assess whether antibodies to C. difficile proteins can be considered a marker of disease or are elicited also in the carrier state.

In conclusion, we have demonstrated that surface proteins of C. difficile can elicit an immune response in patients, although the variability of the response and of the antigens recognized needs to be clarified by future investigations. Work is in progress to characterize the surface proteins of C. difficile and to investigate their possible role in the pathological process.

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LITERATURE CITED


