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Human Antibody Response to *Clostridium difficile* Toxin A in Relation to Clinical Course of Infection

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This study investigated whether differences in fecal and serum antitoxin A antibody levels may account for the duration of *Clostridium difficile*-associated diarrhea (CDAD) and the occurrence of relapses. By an enzyme linked-immunosorbent assay, we tested 40 patients with CDAD including 25 patients without immunodeficiency and 15 patients receiving antineoplastic drugs. Two hundred eighty serum samples and 80 normal stool samples were investigated as controls. In nonimmunocompromised patients, serum immunoglobulin (IgG) and fecal IgA antitoxin A antibody titers were significantly higher in patients who suffered a single episode (n = 21) than in those with relapsing CDAD (n = 4) whose titers were at control levels. Of these 25 patients, eight suffered from diarrhea which lasted for more than 2 weeks. These patients had significantly lower serum- and feces-specific antibody levels than the others who presented symptoms of shorter duration. In cytostatic-treated patients, antitoxin A antibody levels were similar to controls, but relapses occurred in a single case. These data suggest an association between a defective humoral response to toxin A and a more severe form of *C. difficile* infection. They also indicate that other host-related factors control the severity of CDAD and remain to be elucidated.

Clostridium difficile is a common cause of diarrhea after antibiotic therapy and induces a spectrum of diseases ranging from mild diarrhea to pseudomembranous colitis (2, 22). Infants frequently harbor *C. difficile* and its toxins, but most remain asymptomatic (20, 33). Pathogenic strains produce two protein exotoxins, toxin A and toxin B (2, 10, 22, 23). In animal models, toxin A but not toxin B induces diarrhea, infiltration of the intestinal mucosa with neutrophils, mucosal inflammation, and epithelial cell necrosis (21, 26, 30, 31). Therefore, toxin A is considered to be the main causative agent of *C. difficile*-associated diarrhea (CDAD).

Two-thirds of healthy adults have serum immunoglobulin G (IgG) and intestinal secretory IgA (sIgA) antibodies to toxin A (17, 32). These antibodies may inhibit in vitro toxin A binding to its specific intestinal receptor (17). Whether they are involved in the relative resistance of infants to toxin A is unknown. In CDAD, rising titers of serum antibodies to toxin A were found in up to two-thirds of patients (32), and low levels of IgG to toxin A were reported in children with chronic relapsing CDAD (19). These data suggest that the humoral response to toxin A may be an effector of host defenses.

The aim of this study was to determine whether differences in fecal and serum antitoxin A antibody levels could account for the variation in the time duration of CDAD and the susceptibility to relapses in adults with CDAD.

MATERIALS AND METHODS

Patients. Forty patients were selected on the basis of their having diarrhea, the presence of a toxin A-producing *C. difficile* strain in their stools, and a positive fecal cytotoxin test. Diarrhea was judged from the macroscopic aspect of the stools and by questioning the patient and/or his physician. The following exclusion criteria were used: additional enteropathogen in stool culture, celiac disease, Crohn's disease, ulcerative colitis, recent intestinal surgery, intestinal neoplasm, and acquired immunodepression of any origin except that caused by antineoplastic therapy. The group of patients included 29 patients hospitalized in our institution, 10 hospitalized in three other hospitals, and a single outpatient who was seen at our institution. All patients had a history of antibiotic intake. Each CDAD episode was treated with vancomycin, and the usual dose was 250 mg four times daily for at least 1 week. A relapse was defined as the recurrence of CDAD within the first 2 months after the therapy had been discontinued.

Patients were divided into two groups. The first group included 25 patients, i.e., 7 males and 18 females (median age, 70 years; range, 4 to 83 years), without any immunosuppressive therapy. Of these, four females of 40, 53, 66, and 67 years of age suffered two, five, two, and two relapses, respectively, and colitis. None of these patients had a clinical history of immunodeficiency or an abnormal lymphocyte count. The severity of relapses tended to be less than the acute episode. However, in the patient with five relapses, the last episode was the most severe and was life threatening. Two of these four patients (53 and 66 years of age) were hospitalized for diarrhea and dehydration, and a case of severe pseudomembranous colitis was found at admission. The two patients developed hypoproteinemia (lowest values observed, 48 and 37 g/liter) and hypogammaglobulinemia (5.8 and 6 g/liter), but these levels returned to a normal range at the resolution of symptoms. The 40-year-old patient acquired CDAD after surgery and suffered diarrhea for 3 weeks despite vancomycin therapy. The next two episodes were shorter, and the patient did not require hospitalization. The 67-year-old patient responded well to vancomycin and was the only outpatient in this study. Of the patients without relapses, five underwent a colonoscopy, and colitis was found in four.

The second group included 15 patients, i.e., 9 males and 6 females (median age, 32 years; range, 4 to 69 years), with

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leukopenia caused by antineoplastic drugs (median leukocyte count, 900/μl). Of these, eight had leukemia, three had lymphoma, and one each had seminoma, myeloma, vesical urothelioma, and vesical rhabdomyosarcoma. This last one suffered four relapses and had unspecific colitis.

Serum and fecal samples. One hundred fifty-three serum samples and 141 stool samples were obtained (median number per patient, three serum and three stool specimens). The fresh feces from the patients of our institution were processed, aliquoted, and frozen at −80°C. The samples collected in the other hospitals were stored at −20°C before being processed in our laboratory. All samples were stored at −80°C until use. The mean duration of serological follow-up was 24 days for nonimmunocompromised patients (range, 13 to 234 days) and 70 days in immunocompromised patients (range, 25 to 120 days). The time from onset of diarrhea to the first serum sample was 4.9 ± 2.7 days, and the time from the onset of diarrhea to culture was 3.1 ± 3.2 days.

All stool specimens were obtained during the diarrhea. Two group comparisons of serum-specific antibody levels were calculated by using the highest level observed in each patient. Stool antitoxin A IgA levels were compared by using the median value, since these may vary in consecutive samples obtained at intervals of a few days.

Control subjects. Serum samples from 280 control subjects (136 males and 144 females) were tested for IgG and IgA antitoxin A levels. One hundred eighty-five were healthy blood donors, and 95 either younger than 20 years (n = 50) or older than 65 (n = 45) were seen at the emergency care unit of our institution and did not suffer diarrhea. Stool specimens were obtained from 80 additional healthy volunteers, including 30 children of up to 2 years of age (median age, 1 year).

***C. difficile* identification.** Strains were isolated from stools as described previously (11) and identified by gas-liquid chromatography (13). Fecal cytotoxin was detected by using HeLa cells, and the specificity of the cytopathic activity was confirmed by neutralization with *Clostridium sordellii* antitoxin (Wellcome). In vitro toxin A production was detected by an enzyme-linked immunosorbent assay (ELISA) using a commercially available kit (Premier *C. difficile* Toxin A; Meridian Diagnostics, Cincinnati, Ohio). Serogroups were identified by slide agglutination and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (8, 9).

Toxin A purification. Toxin A was purified from *C. difficile* VPI 10463 culture supernatant by ammonium sulfate precipitation, anion-exchange chromatography on DEAE-Sephacel (Pharmacia, Uppsala, Sweden), and acetate buffer precipitation (29). On a gradient polyacrylamide gel under native conditions and with high-molecular-weight markers (Pharmacia), toxin A migrated as a single band of 540,000 Da, in agreement with a previous report (25). Toxin A was cytotoxic on HeLa cells and caused a strongly positive result with the Premier *C. difficile* Toxin A kit.

Fecal IgA concentration. One volume of stool sample was diluted with 2 volumes of phosphate-buffered saline (PBS) at 4°C. The suspension was vigorously vortexed, and the volume of the stool homogenate was measured to calculate the original stool volume. After centrifugation at 1,000 × g for 20 min at 4°C, the supernatant was supplemented with 2 mM phenylmethylsulfonyl fluoride (12), a protease inhibitor, and centrifuged at 12,000 × g for 15 min. The IgA concentration was determined in the supernatant by ELISA. Microtiter plates (Maxisorb; Nunc, Roskilde, Denmark) were coated overnight at 4°C with rabbit immunoglobulins to human alpha-chains (Dako, Glostrup, Denmark) at 1/1,000 in PBS. The plates were saturated with PBS with 0.05% (vol/vol) Tween 20 (PBST) and 1% bovine serum albumin (PBST-BSA). Twofold dilutions of stool homogenate in 1% PBST-BSA were incubated for 1 h at room temperature. To detect bound IgA, horseradish peroxidase-labeled rabbit anti-human IgA (Dako) was diluted 1/1,000 in PBST-BSA, and this mixture was incubated for 1 h at room temperature. The plates were washed three times with PBST between incubation steps. The diammonium salt of 2,2′-azino-bis(3-ethyl-benzthiazoline sulfonate-6) (Sigma) in citrate-phosphate buffer was used as the substrate. After incubation for 30 min at room temperature, the optical density at 415 nm (OD_{415}) was measured with a microplate reader (Bio-Rad). Serial twofold dilutions of purified colostral sIgA (7) were used as the standard, and the results were expressed in weight per original stool volume (in micrograms per milliliter).

Fecal IgG concentration. Fecal IgG concentrations were estimated by ELISA by following the same steps as those used for fecal IgA. Microtiter plates (Polysorb; Nunc) were coated with rabbit immunoglobulins to human gamma-chains (Dako) at 1/1,000 in PBS. Fourfold dilutions of fecal supernatant in 1% PBST-BSA were incubated for 1 h at room temperature. To detect bound IgG, horseradish peroxidase-labeled rabbit anti-human IgG (Dako) was diluted 1/1,000 in PBST-BSA. Serial twofold dilutions of a serum control (Ortho Diagnostic System, Beerse, Belgium) was diluted in a pool of 10 normal fecal supernatants and used as the standard. The ODs were similar to those obtained with PBST-BSA as the diluent. Results were expressed in weight per original stool volume (in micrograms per milliliter).

Antitoxin A ELISA. Microtiter plates (Polysorb; Nunc) were coated with toxin A as described previously (17). Serum samples were diluted 1/100 in PBST-BSA and incubated for 1 h at room temperature. Fecal supernatants were normalized at 10 μg of IgA per ml in PBST-BSA before testing and incubated overnight at 4°C. Serum and intestinal antibodies were detected with horseradish peroxidase-labeled rabbit anti-human IgA or IgG (Dako) diluted 1/1,000 in PBST-BSA. Horseradish peroxidase-labeled rabbit anti-human secretory component (Dako) was used for assaying for fecal secretory antitoxin at a 1/200 dilution in PBST-BSA. Control sera positive for specific IgG and IgA and stool samples positive for IgA, sIgA, and IgG were obtained from patients with CDAD. Overnight incubation of all controls with increasing concentrations of toxin A induced a dose-dependent reduction of OD values. The mean OD observed when the sample was substituted by PBST-BSA was defined as the background level. In all assays, samples yielding an OD five times greater than the background OD were reported as positive. Specific serum antibody titers were expressed in arbitrary units (AU) by using OD values of serial dilutions of a highly positive serum control. The value of 5 AU was placed arbitrarily as the cutoff level. Fecal antibody titers of 10 μg of IgA per ml were expressed as the test OD_{415} divided by the background OD_{415} (OD_t/OD_b) and this value was multiplied by (fecal total IgA concentration × 10^{-1}) to express specific activity per milliliter of stool. All samples were tested in triplicate and in two assays. The intra-assay coefficient of variation was less than 5% in all assays. A rise was determined as a difference greater than twice the coefficient of variation of the controls.

Statistical methods. The unpaired two-tailed Student's t test was used to compare sex and age-related IgG and sIgA antitoxin A levels in controls. The differences in antibody levels between patients were assessed by using the Mann-Whitney U test because the distribution of these variables had a positive skewness. Antibody levels were expressed as the mean ±

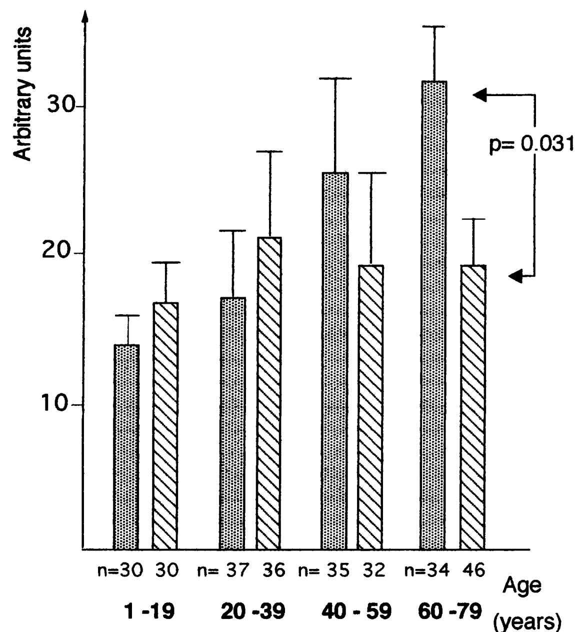

FIG. 1. Age- and sex-related levels of serum antitoxin A IgG in control subjects. Specific IgG levels increased with age and were significantly higher in men than in women above 60 years of age (P = 0.031). Symbols: ▩, males; ▧, females.

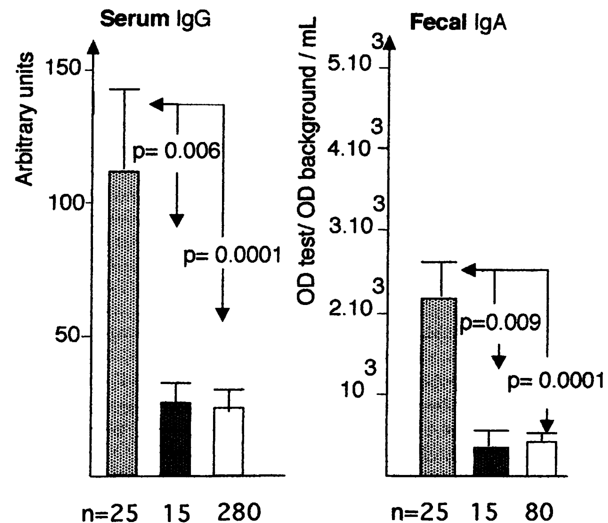

FIG. 2. Antitoxin A serum IgG and fecal IgA levels in nonimmunocompromised ▩ and cytostatic-treated ■ patients and in control subjects (□). Nonimmunocompromised patients had significantly higher serum and fecal antibody levels than cytostatic-treated patients and controls.

standard error. Calculations were performed with the Statview II Tm (Abacus Concepts, Berkeley, Calif.).

RESULTS

Serum antibody levels to toxin A. In control subjects, 72% (n = 165) of adults and 40% (n = 12) of children up to 2 years of age were positive for IgG to toxin A, while 51% (n = 117) and 20% (n = 33), respectively, were positive for IgA. In subjects older than 60 years, males had significantly higher IgG antitoxin A antibody levels than females (P = 0.031; Fig. 1). Patients without immunosuppressive therapy had higher IgG and IgA antitoxin A levels than control subjects (P = 0.0001 for each isotype) and cytostatic-treated patients (P = 0.006 and 0.0012, respectively; Fig. 2). Increases in IgG and IgA titers were observed in 68 and 52% of nonimmunocompromised patients, respectively (mean rises for IgG and IgA, 95 and 20 AU, respectively), and in 20% and none of cytostatic-treated patients, respectively (mean rise for IgG, 8 AU).

Fecal antibody levels to toxin A. In control subjects, higher nonspecific fecal IgA levels were found in children up to 2 years of age (n = 30) than in adults older than 22 years (n = 37; mean ± standard error, 830 ± 142 versus 386 ± 98 μg/ml; P = 0.011). Forty percent (n = 12) of these children and 70% (n = 26) of the adults were positive for sIgA to toxin A (P = 0.017). No significant difference in mean antitoxin A sIgA levels was observed between these two groups (478 OD_t/OD_b ml^{-1} in children versus 371 in adults; P = 0.6). A good correlation was found between specific IgA and sIgA levels (r^2 = 0.81).

Nonimmunocompromised patients had total fecal IgA levels significantly higher than those of cytostatic-treated patients (2,085 ± 443 versus 935 ± 230 μg/ml; P = 0.039) and controls. The coefficients of variation of fecal IgA levels were 145% in controls, 111% in nonimmunocompromised patients, and 80% in cytostatic-treated patients. Specific fecal IgA and sIgA levels were significantly higher in nonimmunocompromised than in cytostatic-treated patients (P = 0.0068 and 0.0015, respectively; Fig. 2) and in controls. The correlation between specific IgA and sIgA levels was not as good as that in controls (r^2 = 0.5), and the OD values were about 50% higher when anti-alpha-chain conjugate was used than when anti-secretory component conjugate was (the results of sIgA were not taken into account for reasons explained in Discussion).

Twelve stool samples from six nonimmunocompromised patients with high levels of serum-specific IgG were positive for IgG to toxin A (median OD_t/OD_b, 19; median sample dilution factor, 310), while all controls were negative. In 20 normal stool samples, IgG levels were below 0.2 μg/ml in 17 subjects and were 3, 4, and 9 μg/ml in three. In positive stool samples for IgG to toxin A, the mean IgG concentration was 740 μg/ml (range, 126 to 1,920 μg/ml; standard deviation, 635 μg/ml).

Relation of Antitoxin A levels to the duration of diarrhea. In nonimmunocompromised patients, serum IgG and fecal IgA antitoxin A titers were lower in patients in whom the duration of diarrhea was longer than 2 weeks, including the four patients with relapses (P = 0.001 and 0.0055, respectively; Fig. 3A). All cytostatic-treated patients, except one, responded to vancomycin therapy within 1 week, despite low specific antibody levels. The duration of CDAD within the two groups cannot be compared since a prolonged or relapsing disease was a positive selection bias.

Relation of Antitoxin A levels to the occurrence of relapses. The four patients with relapsing CDAD had specific IgG and fecal IgA levels significantly lower than those without a relapse (P = 0.033 and 0.025, respectively; Fig. 3B). The 67-year-old patient who suffered a mild form of relapsing CDAD was the only patient of this study with higher IgA levels (i.e., 60 AU, the third highest value of all patients) than IgG levels (i.e., 12 AU). The 53-year-old patient received intravenous gamma globulin (400 mg kg^{-1}, Sandoglobulin; Sandoz, Brussels, Belgium) during the fifth relapse, causing a rise in IgG to toxin A from 10 to 30 AU (19). She recovered within the first week

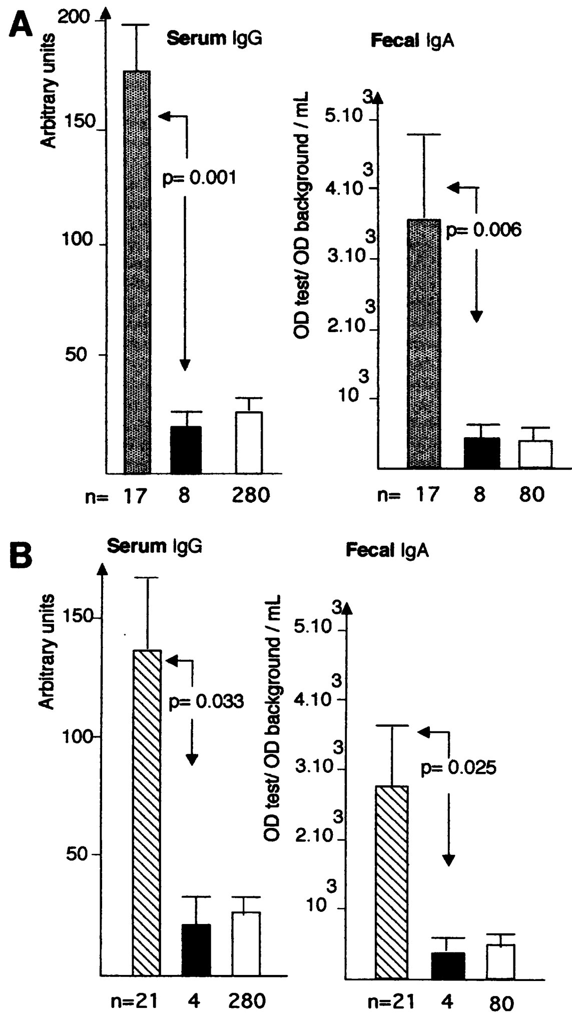

FIG. 3. Antitoxin A serum IgG and fecal IgA levels in nonimmunocompromised patients and controls. (A) Relation of antibody levels to the time duration of CDAD. Eight patients who suffered prolonged CDAD (>2 weeks), including four with relapses, had serum and fecal titers significantly lower than those with symptoms of shorter duration. Symbols: ▧, <2 weeks; ■, >2 weeks; □, controls. (B) Relation of antibody levels to the presence of relapses. Serum IgG and fecal IgA antitoxin A levels were significantly higher in patients who presented a single episode ($n = 21$) than in those with relapsing CDAD ($n = 4$) who had titers at control levels. Symbols: ▧, no relapse; ■, relapses; □, control.

after the first dose and remained asymptomatic during the 7 months of the follow-up. Contrary to medical advice, the patient who was afraid of a new relapse prolonged the course of vancomycin for 4 months after receiving intravenous gammaglobulin, thus making its benefit impossible to assess.

Of the 15 cytostatic-treated patients, only one suffered three relapses and remained negative for serum IgG to toxin A (i.e., 1 to 4 AU) and for specific fecal IgA levels (1 OD_t/OD_b ml^{-1}) despite a high fecal IgA concentration (2,300 μg/ml). He had the lowest antibody levels of this group. The other patients did not relapse and had antibody levels similar to those of nonimmunocompromised patients with relapses.

***C. difficile* typing.** In each of the four patients with relapsing CDAD, all consecutive positive cultures yielded strains with identical serogroups (six *C. difficile* strains of serogroup A1, six of serogroup G, three of serogroup H, and four of serogroup A1).

DISCUSSION

In normal adults, we found serum IgG and sIgA to toxin A in two-thirds of the subjects, in agreement with previous studies (17, 32). The finding of an age-related prevalence of fecal sIgA to toxin A is consistent with the previous observation that the prevalence of serum antitoxin A antibodies rises in the first years of life (32). Although unexplained, the sex-related difference in serum-specific IgG levels may be significant because CDAD was found 1.3 to 1.8 times more often in women than in men in a large epidemiologic survey (1).

Several animal studies suggested that antitoxin A antibodies may play a protective role in CDAD. In hamsters, active and passive immunization against *C. difficile* toxin A and B or toxin A alone was protective against subsequent clindamycin-induced ileocecitis (18, 21). In gnotobiotic mice, the intravenous administration of monoclonal antibodies to toxin A was protective against induced fatal cecitis (5). In humans, low serum antitoxin A IgG titers comparable to those of an age-matched control group were reported in six children with relapsing CDAD, in agreement with our results in nonimmunocompromised patients (19). Moreover, the observation of high levels of IgA to toxin A in the patient with mild relapsing CDAD seems interesting because a recent report by others suggests that, in convalescent-phase sera from patients with CDAD, serum IgA, but not IgG, neutralizes toxin A (15).

However, the hypothesis of a protective role of antitoxin A antibodies is called into question by two observations, namely, the mild form of CDAD in cytostatic-treated patients despite low antitoxin A levels and the detection by others of high specific IgG and IgA antibody levels in two of three patients with relapsing CDAD (14). These divergences suggest two comments. First, our data suggest an association between antitoxin A antibody levels and the course of CDAD not a causal relation. The possibility that the antibody levels are unimportant may not be excluded. Second, those data suggest that additional host-related factors control the severity of the CDAD. Therefore, studies on the role of immunity to toxin A based on a few patients selected on the basis of different criteria, regardless of the underlying diseases, may lead to conflicting conclusions. Among immunological factors that could affect the response to *C. difficile* infection, the relationships between the *C. difficile* toxins and the cells of the immune system may have an important pathogenic role. On rabbit ileum, the toxin A-induced intestinal damage was suggested to be caused by the release of inflammatory mediators following the mucosal infiltration by neutrophils and mononuclear cells (26, 30, 31). In agreement with this hypothesis, in vitro toxin A stimulated chemotaxis and chemokinesis in human granulocytes (27). Finally, toxin B enhanced the ability of gamma interferon-treated monocytes to secrete tumor necrosis factor as a probable consequence of the disorganization of the cytoskeleton (28).

The cause of the relative resistance of infants to *C. difficile* toxins remains unknown. In our control group, we did not observe a significant difference in levels of sIgA to toxin A between children under 2 years of age and adults, suggesting that the relative resistance of infants to toxin A is not mediated through these antibodies. These data must be interpreted carefully since the functional activity of these antibodies was not investigated.

The observation of serum IgG to toxin A in some stool

specimens was confirmed by the presence of large amounts of fecal IgG. These data agree with the finding by others of a 100-fold increase in IgG in the cecal contents of gnotobiotic mice with induced *C. difficile* colitis (4). Although partially investigated, the potential degradation of IgG by the fecal proteases may be minimal because the inflammation process also transfers serum proteins that inhibit trypsin or trypsin-like enzymes (4).

Although significant differences in feces-specific antibody levels were consistent with the hypothesis we tested, these data must be interpreted carefully. In normal jejunal secretions, the mean IgA monomer/total IgA ratio is 0.07 (16). In feces, this ratio is about 0.27 in normal specimens and rises to about 0.42 during intestinal inflammation, mainly because various amounts of serum IgA monomers are transferred by exudation (24). Since this ratio was not investigated, the fecal IgA concentrations of patient samples are consistently overestimated, leading to an underestimation of specific sIgA levels (6). Moreover, an unknown proportion of intestinal dimeric IgA may not have the secretory component because its availability may be reduced by the ulceration of the intestinal epithelium or may be insufficient to face the rise in polymeric IgA supply (3). These considerations probably explain the difference in correlation between specific fecal IgA and sIgA levels in patients ($r^2 = 0.5$) versus controls ($r^2 = 0.81$) and the weak sensitivity of sIgA testing in patients. For these reasons, we took into account only the feces-specific IgA levels obtained with the anti-alpha-chain conjugate.

In summary, a defective antibody response to toxin A was found in patients with relapsing and prolonged CDAD. However, a mild form of CDAD was observed in immunocompromised patients despite low antitoxin A antibody levels. The antibody response to toxin A may play a protective role in CDAD, but other host-related factors that control the severity of the disease remain to be elucidated.

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