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Fecal Lactoferrin, Interleukin-1β, and Interleukin-8 Are Elevated in Patients with Severe *Clostridium difficile* Colitis

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Twenty-two patients with *Clostridium difficile* colitis as determined by positive enzyme immunoassay for toxin A were evaluated for fecal inflammatory markers and their relationship to the severity of illness. Fourteen of 22 specimens were positive for fecal lactoferrin (FLF), with titers from 1:50 to 1:800. Nine of 10 stools tested had ratios of interleukin-1β (IL-1β) to IL-1 receptor antagonist (IL-1ra) of >0.01. Seventeen of 22 specimens also had elevated IL-8 concentrations, and 12 of 14 had elevated IL-1β concentrations. A review of the 18 available patient records revealed that fecal IL-8 concentrations, IL-1β/IL-1ra ratios, and FLF titers were significantly higher in patients with moderate to severe disease than in patients with mild disease. These findings suggest that the proinflammatory effects of *C. difficile* may directly influence clinical characteristics of human disease.

Clostridium difficile is the leading cause of antibiotic-associated colitis and a major source of nosocomial morbidity and mortality worldwide (2). While there has been considerable progress in the understanding of the pathogenesis of this disease, a number of important questions relevant to its prevention and treatment remain.

Disease due to *C. difficile* develops when the organism is allowed to proliferate in the colon, most commonly after antibiotic use has eliminated competing flora. *C. difficile* then releases two high-molecular-weight toxins, toxin A and toxin B, which are responsible for the clinical manifestations, which range from mild, self-limited watery diarrhea to fulminant pseudomembranous colitis, toxic megacolon, and death (2).

Although toxin A and toxin B are known to glucosylate small GTP-binding proteins of the Rho superfamily, producing cellular intoxication (7, 13, 14), the ways in which this enzymatic activity leads to the clinical manifestations of *C. difficile* disease are not known.

We designed this study to test the hypothesis that the clinical manifestations of *C. difficile* colitis are associated with the severity of intestinal inflammation, as measurable by stool assays. We examined stool specimens and clinical features of 22 patients with *C. difficile* colitis in order to determine whether the fecal inflammatory markers lactoferrin, interleukin-8 (IL-8), IL-1β, and IL-1 receptor antagonist (IL-1ra) correlate with the severity of the disease or with the degree of inflammation as determined by fecal lactoferrin (FLF) titer.

MATERIALS AND METHODS

Stool sample preparation. The patient group consisted of 22 people whose physicians requested a *C. difficile* stool examination and whose stools were positive for toxin A by enzyme immunoassay (Tox-A-Test; TechLab, Blacksburg, Va.). Stools were tested for lactoferrin titer by latex agglutination (Leuko-Test; TechLab); those with no agglutination at a dilution of 1:50 were considered negative, while positives were diluted further to obtain lactoferrin titers.

For cytokine testing, stools were thawed and diluted 1:2 (wt/vol) in phosphate-buffered saline (Gibco BRL, Gaithersburg, Md.) containing leupeptin (2.5 μg/ml; Sigma, St. Louis, Mo.), aprotinin (0.1 mg/ml; Sigma), and 0.5 mM 4-(2-aminoethyl)benzenesulfonyl fluoride or phenylmethylsulfonyl fluoride (Sigma). Samples were centrifuged for 10 min at 10,000 × *g*, and the supernatants were passed through 0.44-μm-pore-size filters to remove debris if present. The supernatants were kept at −70°C prior to testing.

Cytokine assays. IL-8 and IL-1β were measured by enzyme immunoassay (Quantikine; R&D Systems, Minneapolis, Minn.). IL-1ra was measured by radioimmunoassay as described previously (6). The lower limits of detection were 10 pg/ml for IL-8, 2.5 pg/ml for IL-1β, and 1,000 pg/ml for IL-1ra. The two samples beyond these detection limits for IL-1ra were retested by enzyme immunoassay (R&D Systems).

Data analysis. Charts were reviewed in a blinded fashion to assess the clinical severity of illness. Eighteen of 22 charts were available for review. Particular emphasis was placed on the severity of the diarrhea, underlying or comorbid conditions, peripheral leukocyte (WBC) count, and patient temperature at the time the stool sample was sent. All patients were treated successfully for their attacks; no data on subsequent relapses were available.

Disease was considered severe if any of the following was present: diarrhea severe enough to produce clinical signs of volume depletion and to require hospitalization, WBC count of >10,000/μl, or temperature of >38.3°C. The latter two criteria were disregarded if other illnesses were clearly responsible (such as in one patient with fever due to streptococcal sepsis and another with a WBC count of 13,200/μl shortly after a generalized tonic-clonic seizure but that promptly returned to normal). Twelve of the 18 patients met these criteria for severe disease.

Fecal inflammatory markers were compared between the mild and severe groups by the Mann-Whitney U test. Correlations between fecal inflammatory values from individual patients were compared by the Spearman rank order correlation. All statistics were calculated by using Minitab 10.5 Power Mac software.

RESULTS

The clinical characteristics and results of stool assays are shown in Table 1. Fourteen of 22 samples were positive for lactoferrin, occasionally with high titers (median, 1:133; range, 1:50 to 1:800). Seventeen samples also had detectable IL-8 (mean, 3,950.3 pg/ml; range, 10.9 to 20,896). All samples tested had detectable IL-1ra (mean, 31.8 ng/ml; range, 0.213 to 106), and all but two had elevated IL-1β (mean, 7,758 pg/ml; range, 11 to 30,444).

There were strong correlations among the fecal inflammatory markers measured from individual patients. The fecal IL-8 concentration correlated with the IL-1β concentration, the IL-1–IL-1ra ratio, and the FLF titer ($R = 0.903$, 0.913, and 0.684, respectively; all $P < 0.001$), and the FLF titer correlated with the fecal IL-1β concentration and the IL-1β/IL-1ra ratio ($R = 0.654$ and 0.564; $P < 0.01$ and <0.025, respectively). Moreover,

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TABLE 1. Patient characteristics[a]

Patient age (yr)	No. of WBCs/μl[b]	T_{max} (°C)	Symptom score[c]	Comorbid condition(s)	Overall score[d]	FLF titer	Fecal IL-8 (pg/ml)	Fecal IL-1β (pg/ml)	Fecal IL-1ra (ng/ml)	Fecal IL-1/IL-1ra ratio
36	41,900	39.4	3+	Major trauma, UTI	Severe	1:400	2,531	25,586	56.9	0.45
61	6,200	37.0	1+	Short bowel	Mild	1:50	<10	73.8	93.6	0.001
52	4,900	36.8	3+	Chemotherapy for head-neck cancer	Severe	1:50	19,590	30,444	>100	≤0.30
7	13,300	37.3	2+	Recent hemorrhagic colitis (EHEC)	Severe	1:400	28	94.7	3.5	0.027
81	13,700	NA	NA	Ureteral obstruction, UTI	Severe	1:400	626	418	3.4	0.12
44	NA	NA	1+	Appendectomy	Mild	<1:50	<10	<2.5	0.0213	ND
73	10,600	37.2	2+	COPD, CAD	Severe	1:400	8,828	22,838	35	0.65
55	6,800	37.6	3+	Infected peritoneal dialysate	Severe	<1:50	17	57.7	20.7	0.003
33	800	38.7	1+	Chemotherapy, sinusitis, streptococcal bacteremia	Mild	<1:50	<10	<2.5	2.1	≤0.001
86	13,200	37.8	1+	Seizure, UTI	Mild	<1:50	<10	11.1	7.5	0.001
86	6,800	37.2	3+	Dementia	Severe	1:50	24.5	ND	ND	ND
23	10,000	39.7	2+	Acute leukemia	Severe	<1:50	<10	ND	ND	ND
78	21,100	37.5	2+	Aortic aneurysm repair	Severe	1:50	141.1	ND	ND	ND
69	400	38.1	1+	Acute leukemia	Mild	<1:50	10.9	ND	ND	ND
69	19,600	38.5	2+	Myocardial infarction	Severe	<1:50	699	ND	ND	ND
44	NA	36.7	2+	UTI, relapsing *C. difficile* diarrhea	Mild	<1:50	24.5	ND	ND	ND
55	12,800	39.0	2+	Intracranial hemorrhage	Severe	1:200	6,431	ND	ND	ND
39	14,200	37.9	3+	Anal sphincterotomy	Severe	1:800	20,896	ND	ND	ND

[a] Abbreviations used: NA, not available; T_{max}, maximum temperature in illness; COPD, chronic obstructive pulmonary disease; CAD, coronary artery disease; UTI, urinary tract infection; EHEC, enterohemorrhagic *Escherichia coli*; ND, not determined.
[b] Peripheral WBC count at time stool sample sent.
[c] Scores: 3+, diarrhea severe enough to require hospitalization; 2+, clinical evidence of volume depletion or weight loss but no need for hospitalization; 1+, no diarrhea or no volume depletion or weight loss.
[d] Severe disease, temperature of >38.3°C, WBC count of >10,000/μl without other reasons, or symptom score of 3+; mild disease, all other patients.

there was a strong relationship between the lactoferrin titer and the IL-1β/IL-1ra ratio in that 9 of 10 stools with positive lactoferrin titers and 0 of 4 without had an IL-1β/IL-1ra ratio of >0.01 ($P = 0.005$ [Fisher's exact test]), which is the average minimum value at which IL-1 receptor activation has been reported in vitro (8).

Eighteen of the 22 stool specimens came from patients whose charts were available for review. The data obtained from these charts and the stool analyses are shown in Table 1. Six patients qualified as having mild disease, while the other 12 were considered to have severe disease. As shown in Fig. 1, the patients with severe disease had significantly higher fecal IL-8 concentrations ($P = 0.0045$), FLF titers ($P = 0.021$), and fecal IL-1β/IL-1ra ratios ($P = 0.025$) than patients with mild disease.

DISCUSSION

Considerable progress has been made recently toward understanding the basic pathogenesis of *C. difficile* colitis. However, it is not yet clear how the consistent and severe inflammatory changes caused by toxins A and B in vitro translate into the wide range of colonic pathology seen in patients.

There is accumulating evidence that the host inflammatory response to *C. difficile* is responsible for many of the pathophysiologic changes. In ligated rabbit ileal loops, for example, hemorrhagic fluid accumulation induced by toxin A can be prevented by inhibitors of cyclooxygenase, phospholipase A_2 (10), platelet-activating factor (11), and substance P (22). In addition, several lines of evidence suggest that neutrophil recruitment is an early and consistent feature of *C. difficile* colitis. Neutrophil infiltrates are seen in the rabbit colon within 1 h of toxin A instillation and precede the development of histologic colitis (5). The characteristic pseudomembranes seen in *C. difficile* colitis in humans consist of masses of neutrophils along with necrotic cells and debris (16). Moreover, the histologic and secretory effects of toxin A in rabbit ileal loops can be blocked by pretreatment with antibody to the neutrophil adhesion molecule CD11/CD18 (15).

While mucosal infiltration of neutrophils is clearly prominent in *C. difficile* colitis, only 28 to 63% of methylene blue-stained stool samples from patients with *C. difficile* disease reveal WBCs (18, 19), limiting the diagnostic value of this test. For this reason several groups have measured FLF, which is a stable product of neutrophil secondary granules. FLF is a reliable marker of intestinal inflammation, since titers of >1:50 are not seen in healthy controls but are almost always present in highly inflammatory diseases like shigellosis. Recent studies have shown that FLF titers detect bacterial colitis with greater sensitivity than microscopic examination for fecal WBCs (18, 25, 26, 29) and even stool culture (28). In fact, in a multivariate regression model, an elevated FLF titer predicts a positive *C. difficile* toxin assay in the setting of nosocomial diarrhea better than any other clinical or laboratory parameter (18). Despite this, the sensitivity of elevated FLF titer in *C. difficile* disease is only 60.5 to 75% (12, 18, 29).

In this study we examined the degree of intestinal inflammation in patients with diarrhea due to *C. difficile* by measuring several inflammatory markers in stool samples. There is precedent for this approach to studying intestinal diseases. Several groups have measured fecal cytokine concentrations in shigellosis and inflammatory bowel disease (4, 17, 21, 23, 24) and correlated these with tissue cytokine expression; the latter was shown to correlate with severity of illness in shigellosis (24).

Measurements of the clinical severity of *C. difficile* colitis have varied among published studies, and there is no consensus formula for determining severity in a particular patient. However, several studies have used criteria similar to those we used in this study, namely, fever, WBC count, and severity of diarrhea (1, 3, 27). Using these criteria, we determined that *C. difficile* colitis is associated with elevations in fecal proinflammatory cytokine concentrations in severe but not mild cases. Second, we extended the findings of Manabe et al. (18), who

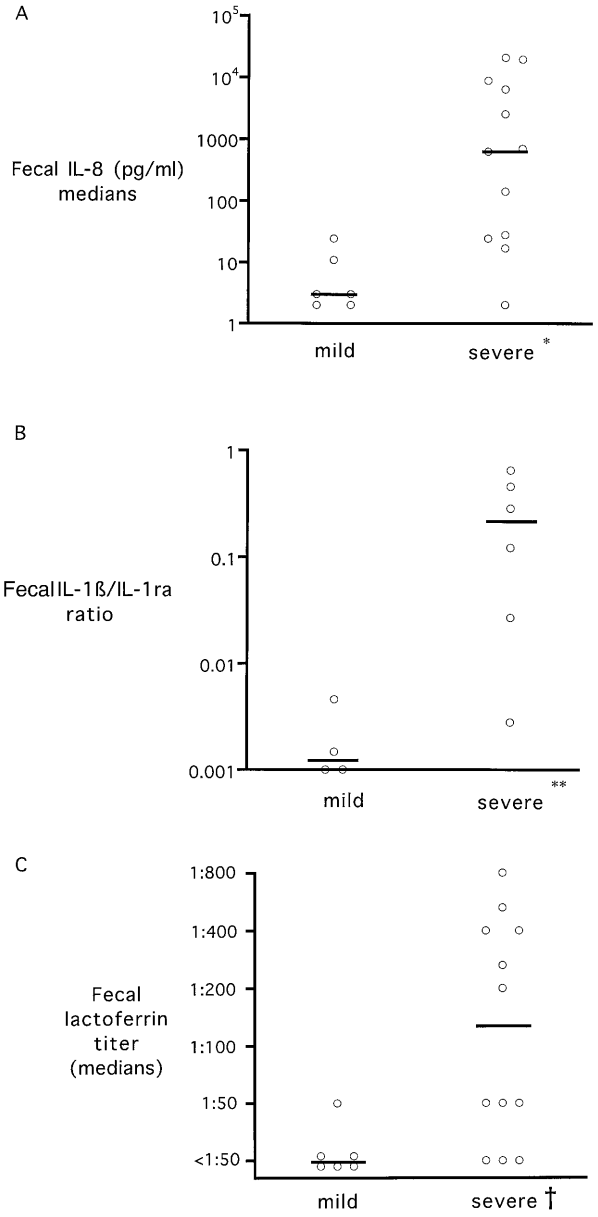

FIG. 1. Fecal inflammatory markers from patients with either mild *C. difficile* colitis (enzyme immunoassay-positive for toxin A but WBC count of <10,000/μl, peak temperature of <38.3°C, and hospitalization or intravenous hydration not required) or severe colitis (enzyme immunoassay-positive for toxin A and not meeting above-mentioned criteria). (A) Fecal IL-8 concentration was measured by enzyme immunoassay. *, $P = 0.005$, severe versus mild (Mann-Whitney). (B) Fecal IL-1β concentration was measured by enzyme immunoassay, and IL-1ra was measured by radioimmunoassay. **, $P = 0.025$, severe versus mild. (C) FLF titers were measured by latex agglutination and serial dilution. †, $P = 0.021$, severe versus mild.

found that elevated FLF titers are common in *C. difficile* colitis, to show that these titers tend to be higher in patients with more severe disease. Of 12 patients with moderate to severe disease, 9 had elevated FLF titers, compared to only 1 of 6 with mild disease.

Taken together, these findings lend support to the hypothesis that *C. difficile* colitis is an inflammatory condition which results from a cascade of effects of toxins A and B on cells present in the intestinal epithelium. Moreover, this study raises the possibility that the FLF latex agglutination test may be a useful step in the diagnostic algorithm for *C. difficile* colitis. While an undetectable FLF titer clearly cannot be used to rule out *C. difficile* disease, it may help in the diagnosis of patients with a severe clinical presentation. Our findings suggest that patients with FLF titers of ≤1:50 are unlikely to have severe systemic signs and symptoms of *C. difficile* colitis and that alternative diagnoses (e.g., other nosocomial infections) should be sought for patients with fever and leukocytosis whose FLF titers are <1:50. Moreover, there should be a very high suspicion for *C. difficile* in patients hospitalized for more than 3 days who have elevated FLF titers, since previous studies have shown that other enteritides associated with elevated FLF titers (e.g., shigellosis, salmonellosis, campylobacteriosis) are extremely uncommon in this setting.

Further prospective studies are indicated to clarify the role of the FLF latex agglutination test in the diagnostic evaluation of patients with diarrhea. In particular, it would be useful to know if the FLF titer, in combination with objective clinical parameters like fever, WBC count, and severity and duration of diarrhea, can form a model to predict the likelihood of *C. difficile* colitis. The application of such an algorithm could help both to improve the cost-effectiveness of testing for *C. difficile* toxins and to identify patients most likely to have *C. difficile* colitis and who might benefit from empiric therapy while toxin assay results are pending.

Finally, the results of this study may help to identify a subset of patients with *C. difficile* colitis who, for whatever reason, have only mild disease. These patients, with FLF titers of ≤1:50, undetectable fecal IL-8, and minimal signs of systemic disease, might be likely to resolve their disease with conservative therapy alone (withdrawal of antibiotics, oral rehydration) rather than requiring treatment with metronidazole or vancomycin, both of which are associated with significant relapse rates (16) and contribute to the emergence of resistant pathogens (9, 20). Organizing a prospective, randomized trial of patients with positive *C. difficile* toxin assays and negative FLF tests would be one way to approach this question.

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