Determination of Serum Antibodies to *Clostridium difficile* Toxin B in Patients with Inflammatory Bowel Disease

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Abstract: *Clostridium difficile* infection has increased in prevalence among patients with inflammatory bowel disease (IBD). Serum antibodies against *C. difficile* toxins have been detected in susceptible populations and may be protective; however, such antibodies have not been previously characterized in IBD patients. This study measured immunoglobulin G antibody levels to *C. difficile* toxin B in serum from IBD patients in remission and IBD patients in relapse. IBD patients demonstrated significantly higher antibody levels than non-IBD patients. In addition, a higher proportion of IBD patients in remission had positive antibody levels compared to IBD patients in relapse. Further characterization of antibody responses may elucidate understanding of susceptibility to *C. difficile* infection among IBD patients.

Clostridium difficile, an anaerobic, gram-positive, spore-forming bacilli, is the most common cause of nosocomial infectious diarrhea in developed countries.[1] Historically, the development of *C. difficile* infection (CDI) was characterized as colitis due to overgrowth of the pathogen among commensal bacteria with expression of toxin B.[2] Factors that increase patients' susceptibility to CDI include antibiotic exposure, advanced age, hospitalization, and immunosuppression, although the epidemiology of this disease is changing. Recently, the emergence of a hypervirulent strain of *C. difficile* (BI/NAP1/027) has been linked to an increase in the frequency and severity of cases of CDI.

Epidemiologic studies have also shown an increase in the prevalence and severity of CDI among inflammatory bowel disease (IBD) patients. Between 1998 and 2004, admissions related to CDI among patients with ulcerative colitis (UC) or Crohn's disease increased approximately 3-fold and 2-fold, respectively.[3] In addition, significantly higher mortality and surgery rates were observed with CDI in patients with UC compared to patients with Crohn's disease.[4] The management of CDI in patients with IBD remains challenging, as CDI can mimic a relapse of IBD,

Table 1. Baseline Characteristics of Non–Inflammatory Bowel Disease (IBD) Controls and IBD Patients

	Non-IBD controls	IBD patients in remission		IBD patients in relapse	
	(n=29)	Ulcerative colitis (n=13)	Crohn's disease (n=17)	Ulcerative colitis (n=12)	Crohn's disease (n=15)
Age (years)	29.7±9.68	38.6±11.9	48.4±15.9	40.4±15.9	34.1±12.1
Male (%)	20 (69.0)	5 (38.5)	9 (53)	5 (41.6)	4 (26.7)
White (%)	27 (93.1)	7 (53.8)	16 (94.1)	8 (66.7)	12 (80)
Recent (<3 months) use of corticosteroids (%)	–	0 (0)	0 (0)	6 (50)	9 (60)
Tumor necrosis factor inhibitor use (%)	–	7 (53.8)	5 (29.4)	2 (16.7)	5 (33.3)
Immunomodulator use (%)	–	4 (30.7)	6 (35.3)	5 (41.6)	3 (20)
5-aminosalicylic acid use (%)	–	7 (53.8)	11 (64.7)	6 (50)	5 (33.3)
Recent (<3 months) use of antibiotics (%)	0 (0)	2 (15.4)	4 (23.5)	6 (50)	7 (46.7)
Recent (<3 months) hospitalization (%)	–	0 (0)	0 (0)	0 (0)	3 (20)

exacerbate the severity of colitis, or exist as asymptomatic carriage.[5] Moreover, there is an ongoing debate about the risk of developing CDI in the IBD population, given these patients' use of antibiotics, steroids, and/or immunomodulator therapy. Schneeweiss and colleagues demonstrated a 3-fold increase in the risk of developing CDI with the use of corticosteroids but no additional risk with infliximab (Remicade, Janssen Biotech).[6] Data on the use of immunomodulators such as azathioprine, 6-mercaptopurine, and methotrexate are conflicting, and more studies will be needed to clarify the risk associated with these therapies.[7]

The role of the host immune response appears to be important in the outcomes of patients with CDI. Serum antibodies to toxins A and B have been suggested to be protective against colonization by *C. difficile* and recurrent disease.[1,8] Kyne and coauthors demonstrated elevated serum levels of immunoglobulin (Ig) G antibodies against both toxins in asymptomatic carriers of *C. difficile* compared to low levels of these antibodies in patients with diarrhea due to CDI.[1] In addition, low serum levels of anti–toxin B antibodies were associated with a significantly higher likelihood of recurrent CDI.[9]

Presently, the adaptive immune response to CDI in IBD patients has not been characterized. This observational study assessed IBD patients in remission and IBD patients in relapse, with the goal of detecting serum antibodies against toxin B from both the reference toxigenic strain VPI10463 ($TcdB_{HIST}$) and the hypervirulent strain BI/NAP1/027 ($TcdB_{HV}$).

Materials and Methods

Patients

This study was conducted primarily at an outpatient practice specializing in IBD. The study protocol was approved by the institutional review board of the University of Oklahoma Health Sciences Center, the academic institution with which the practice is affiliated, and all subjects gave written informed consent. IBD patients, both those with relapsing disease (n=27) and those in remission (n=30), were enrolled at the time of their scheduled visits. Patients were considered to be in relapse if they had 3 or more bowel movements per day, presence of bloody stools, abdominal pain, need for steroids or hospitalization, or dose escalation within the previous 3 months. Clinical remission was defined as the absence of these criteria. In addition, volunteers were enrolled from a preexisting registry of identified healthy patients (n=29); these individuals were screened for the absence of a history of IBD and

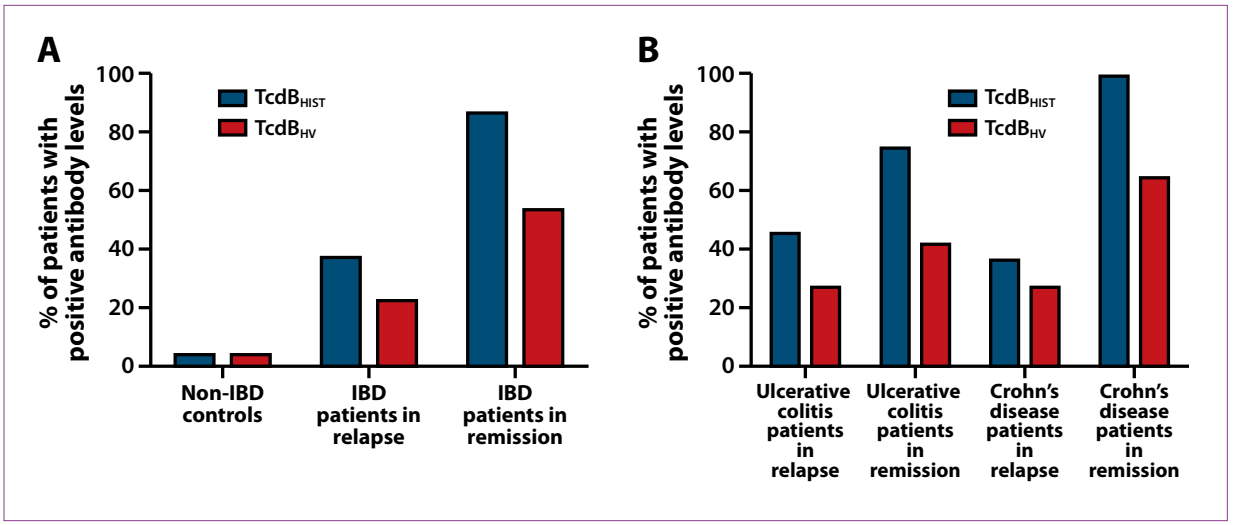

Figure 1. The proportions of patients with positive antibody levels.

IBD=inflammatory bowel disease; TcdB$_{HIST}$=toxin B from the reference toxigenic strain VPI10463; TcdB$_{HV}$=toxin B from the hypervirulent BI/NAP1/027 strain.

CDI. Two investigators reviewed de-identified medical records for data extraction. In particular, comorbidities, history of *C. difficile* stool toxin B testing, medications for IBD, and recent and concurrent antibiotic use were reviewed. Serum was obtained, coded, and stored at –20°C until analysis.

Enzyme-Linked Immunosorbent Assay

96-well polystyrene enzyme-linked immunosorbent assay (ELISA) plates were coated with 1 µg per well of either purified TcdB$_{HIST}$ or TcdB$_{HV}$. Assays were performed in duplicate as previously described.[10] Briefly, the plates were coated with antigen and kept overnight at 4°C. After appropriate washes and blocking with 0.1% bovine serum albumin (BSA), sera diluted at 1:100 in 0.1% BSA-Tween solution were added to the wells in duplicate and incubated for 2 hours. Plates were washed and incubated with anti–human IgG whole molecule secondary antibody conjugated to alkaline phosphatase. ρ-nitrophenyl phosphate disodium solution was used as substrate. A monoclonal mouse *C. difficile* toxin B antibody, diluted 1:100, was used as positive control. Plates were read at 410 nm on a microELISA plate reader when the positive control reached an optical density (OD) of 1.0. The relative OD of a given sample was defined as the average OD of the duplicate wells of the sample divided by the average OD of the duplicate positive control wells. A positive value was noted if a sample's average OD was equal to or greater than the average OD plus 2 times the standard deviation of the non-IBD control group at 1:100 dilution.[11]

Statistical Analysis

All analyses were performed with the GraphPad Prism 5 package. Data were checked for skewness, and an unpaired *t*-test was performed if the distribution of the values was Gaussian. If the distribution was not normal, a Mann-Whitney test was used. *P*-values less than .05 were considered to be statistically significant.

Results

Baseline characteristics of both IBD patients (n=57) and non-IBD controls (n=29) are shown in Table 1. Similar numbers of patients with Crohn's disease and UC were seen in both the remission and relapse groups. Positive antibody levels to TcdB$_{HIST}$ and TcdB$_{HV}$ were observed in 86.7% and 53.3% of IBD patients in remission, respectively, compared to 37.0% and 22.2% of IBD patients in relapse (Figure 1A). This trend was observed among both UC and Crohn's disease patients, although a much higher proportion of Crohn's disease patients in remission had positive antibody levels compared to Crohn's disease patients in relapse (Figure 1B). Stool TcdB data were sparse, as data were available for only 15 of the 27 IBD patients in relapse and only 4 of the 30 IBD patients in remission; as these data were not concurrent to this study, they did not provide any additional information for analysis.

Compared to non-IBD controls, both IBD patients in relapse and IBD patients in remission had significantly elevated levels of antibody to TcdB$_{HIST}$; the average antibody level for IBD patients in remis-

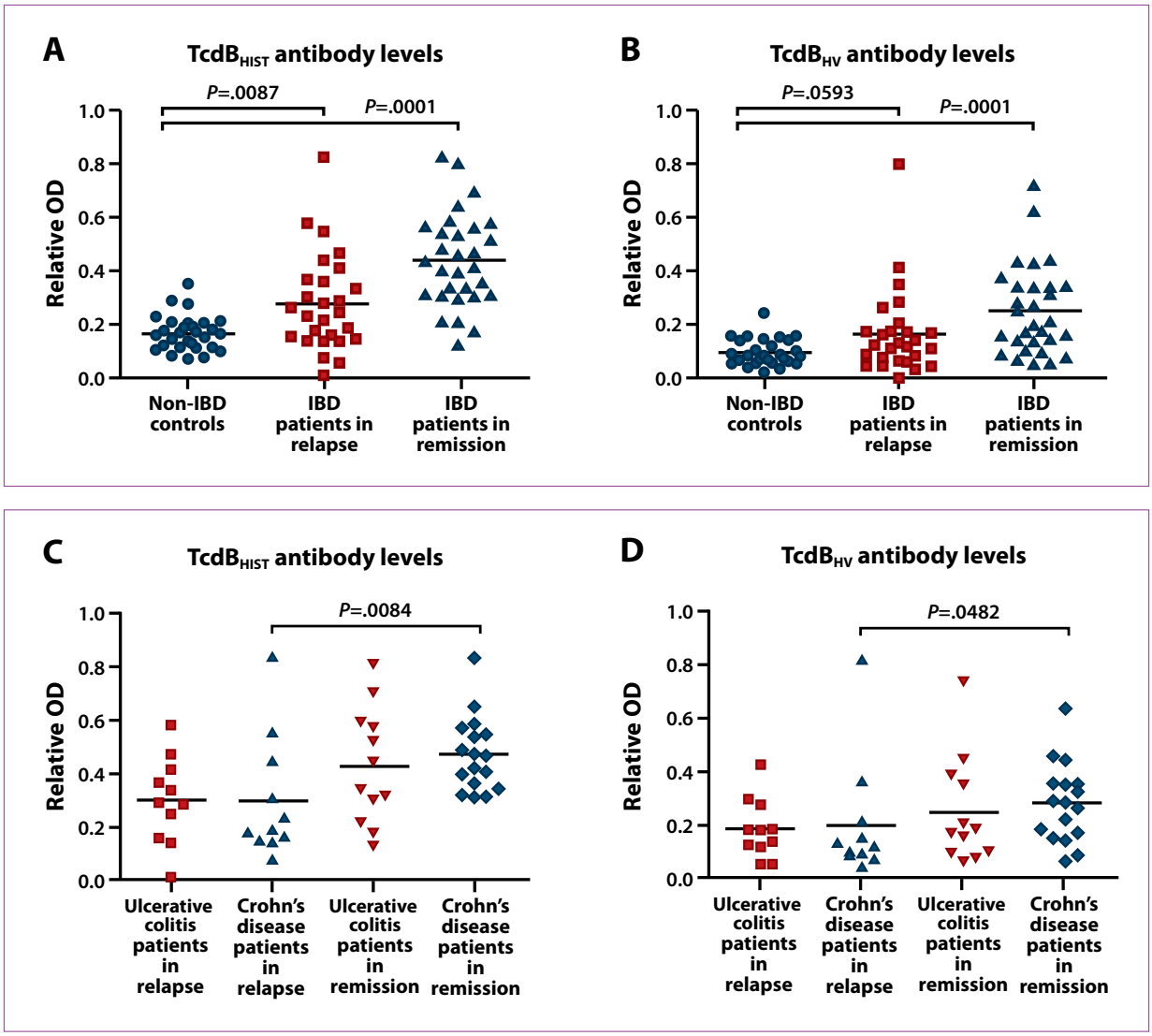

Figure 2. Scatter plots of antibody levels to toxin B from the reference toxigenic strain VPI10463 (TcdB$_{HIST}$) and the hypervirulent BI/NAP1/027 strain (TcdB$_{HV}$). Average optical density (OD) of serum samples is represented relative to the average OD of positive TcdB antibody. Horizontal bars represent mean relative OD.

IBD=inflammatory bowel disease.

sion was also higher than the average antibody level for IBD patients in relapse, but this latter difference did not reach statistical significance (Figure 2A). A similar observation was noted with TcdB$_{HV}$, although the difference between IBD patients in relapse and non-IBD controls did not reach statistical significance (Figure 2B). A correlation was observed between the proportion of patients with positive antibody levels and the average antibody levels among the individual groups (Figures 2C and 2D). Both UC and Crohn's disease patients in remission had higher antibody levels to TcdB$_{HV}$ and TcdB$_{HIST}$ than their counterparts who were in relapse; however, this difference was statistically significant only in the Crohn's disease group. Statistically significant differences were not observed with TcdB antibody levels when accounting for antibiotic, tumor necrosis factor inhibitor, immunomodulator, and/or corticosteroid use (data not shown).

Discussion

The increasing burden of CDI in IBD patients necessitates further understanding of the pathogenesis and impact of this disease on IBD outcomes. A protective role of serum

antibodies to both *C. difficile* toxins has been proposed in prior studies, but understanding of the serum immune response in CDI remains incomplete. The current study is the first to evaluate the presence of serum antibodies to TcdB from both a historical strain and the more recent hypervirulent strain in the IBD population.

We observed significantly elevated antibody levels to TcdB of both strains in IBD patients compared to non-IBD controls. Certainly, this observation deserves speculation about antigen exposure and adaptive responses among IBD patients. Clayton and coworkers observed that IBD patients in clinical remission had an increased frequency of detectable fecal *C. difficile* toxins compared to healthy adults.[5] Hence, whether the elevated serum antibody levels represent a higher rate of exposure to only TcdB or to antigens sharing similar epitopes with TcdB is not known. In addition, the current study observed higher antibody levels to TcdB of both strains among IBD patients in remission, particularly those with Crohn's disease, compared to their counterparts in relapse. The development of serum antibodies to a variety of commensal microbial antigens (ie, anti–*Saccharomyces cerevisiae* antibodies in Crohn's disease patients) has been characterized previously, although their role in IBD pathogenesis and their relationship with disease activity remain incompletely understood.[12] We speculate that the higher TcdB antibody levels among Crohn's disease patients may reflect a higher rate of colonization by *C. difficile* strains, which could be driven by an aberrant immune response. Whether the increased antibody levels observed in IBD patients in remission confer a protective status against CDI is unclear.

This observational study consisted of a relatively small cohort of IBD patients and, as such, is subject to limitation as to the conclusions that can be drawn from this study. Notably, the sample size lacked the power to detect significant differences in antibody levels between IBD patients in remission and IBD patients in relapse. Another limitation is the absence of concurrent stool TcdB data, which were largely unavailable, as the enrollment for this study was conducted predominantly in an outpatient setting. As such, asymptomatic carriage could not be determined. Finally, the majority of patients who were receiving antibiotics, primarily patients in the relapsing IBD cohort, were on metronidazole; in addition to its role for treating CDI, metronidazole also induces immunosuppression of peripheral blood lymphocytes.[13] Hence, the small sample size limits determination of whether the use of metronidazole or other antibiotics affected serum antibody levels to TcdB. Given the results of this study, future investigations could include a prospective study of a larger group, with an attempt to correlate serum antibody levels to *C. difficile* carriage in stool and/or CDI, as well as correlation between serum antibody levels and clinical status of patients' IBD. In addition, evaluation of toxin neutralization activity by serum toxin B antibodies may provide more insight into immune mechanisms in terms of both CDI and IBD pathogenesis.

In conclusion, patients with IBD are more likely to have serum antibodies to *C. difficile* toxin B. The observed variations in TcdB antibody levels may reflect increased exposure to antigens similar to TcdB and/or differences in host immune responses and underlying mucosal inflammation. Certainly, several variables—including the use of immunosuppressants, the extent of chronic inflammation, and other defects in host immune function—likely play a role in mucosal susceptibility to CDI and production of such antibodies. Further characterization of TcdB antibodies and their neutralizing activity will be needed to understand the clinical implication of this immune response.

References

1. Kyne L, Warny M, Qamar A, Kelly CP. Asymptomatic carriage of *Clostridium difficile* and serum levels of IgG antibody against toxin A. *N Engl J Med*. 2000;342:390-397.
2. McFarland LV, Surawicz CM, Stamm WE. Risk factors for *Clostridium difficile* carriage and *C. difficile*-associated diarrhea in a cohort of hospitalized patients. *J Infect Dis*. 1990;162:678-684.
3. Rodemann JF, Dubberke ER, Reske KA, Seo DH, Stone CD. Incidence of *Clostridium difficile* infection in inflammatory bowel disease. *Clin Gastroenterol Hepatol*. 2007;5:339-344.
4. Ananthakrishnan AN, McGinley EL, Binion DG. Excess hospitalisation burden associated with *Clostridium difficile* in patients with inflammatory bowel disease. *Gut*. 2008;57:205-210.
5. Clayton EM, Rea MC, Shanahan F, et al. The vexed relationship between *Clostridium difficile* and inflammatory bowel disease: an assessment of carriage in an outpatient setting among patients in remission. *Am J Gastroenterol*. 2009;104:1162-1169.
6. Schneeweiss S, Korzenik J, Solomon DH, Canning C, Lee J, Bressler B. Infliximab and other immunomodulating drugs in patients with inflammatory bowel disease and the risk of serious bacterial infections. *Aliment Pharmacol Ther*. 2009;30:253-264.
7. Sinh P, Barrett TA, Yun L. *Clostridium difficile* infection and inflammatory bowel disease: a review. *Gastroenterol Res Pract*. 2011;2011:136064.
8. Sánchez-Hurtado K, Corretge M, Mutlu E, McIlhagger R, Starr JM, Poxton IR. Systemic antibody response to *Clostridium difficile* in colonized patients with and without symptoms and matched controls. *J Med Microbiol*. 2008;57(pt 6):717-724.
9. Leav BA, Blair B, Leney M, et al. Serum anti-toxin B antibody correlates with protection from recurrent *Clostridium difficile* infection (CDI). *Vaccine*. 2010;28:965-969.
10. James JA, Gross T, Scofield RH, Harley JB. Immunoglobulin epitope spreading and autoimmune disease after peptide immunization: Sm B/B'-derived PPPGMRPP and PPPGIRGP induce spliceosome autoimmunity. *J Exp Med*. 1995;181:453-461.
11. Nguyen ML, Terzyan S, Ballard JD, James JA, Farris AD. The major neutralizing antibody responses to recombinant anthrax lethal and edema factors are directed to non-cross-reactive epitopes. *Infect Immun*. 2009;77:4714-4723.
12. Prideaux L, De Cruz P, Ng SC, Kamm MA. Serological antibodies in inflammatory bowel disease: a systematic review. *Inflamm Bowel Dis*. 2011 Nov 8. Epub ahead of print.
13. Fararjeh M, Mohammad MK, Bustanji Y, Alkhatib H, Abdalla S. Evaluation of immunosuppression induced by metronidazole in Balb/c mice and human peripheral blood lymphocytes. *Int Immunopharmacol*. 2008;8:341-350.