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ORIGINAL ARTICLE

Humoral response to *Clostridium difficile* in inflammatory bowel disease, including correlation with immunomodulatory treatment

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Key words

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Abstract

Background and Aim: An abnormal immune response to intestinal bacteria has been observed in Crohn's disease (CD). *Clostridium difficile* infection incidence and severity are increased in CD, but reports on the humoral response have provided conflicting results. We aimed to shed light on the possible role of *C. difficile* in CD pathogenesis by paying attention to the influence of immunomodulatory treatment on the humoral response.

Methods: A total of 71 consecutive outpatients with CD, 67 with ulcerative colitis (UC), and 121 healthy controls were analyzed for serum IgA and IgG to *C. difficile* toxins A and B.

Results: IgA levels were similar in all study groups. IgG to toxin A was increased similarly in CD and UC ($P = 0.02$ for both). In contrast, IgG to toxin B was elevated only in CD patients not receiving disease-modifying anti-inflammatory bowel disease drugs (DMAID) ($n = 16$) ($P = 0.0001$), while the CD medication subgroup ($n = 47$) had a level similar to healthy controls. The UC results were not influenced by DMAID treatment.

Conclusion: Our findings add support to the idea of a disturbed interaction between intestinal cells and the microbiota being part of the CD disease mechanism. An abnormal immune response to *C. difficile* toxin B may be a critical component of this interaction.

Introduction

Clostridium difficile is an anaerobic bacterium that can be part of the normal intestinal flora, but with pathogenic strains producing the two major exotoxins, toxin A and toxin B, with each being capable of mediating infection.[1–4] Patients with inflammatory bowel disease (IBD), including Crohn's disease (CD) and ulcerative colitis (UC), have an increased risk of developing *C. difficile* infection[5] and asymptomatic colonization.[6] An excess of antibiotic use in the early childhood of IBD patients has been reported[7]; this may be causally related to the overrepresentation of *C. difficile*, perhaps indirectly via an altered gut microbiota, losing its protective role against *C. difficile* expansion.[8]

Serum IgG antibodies against these toxins are protective in humans.[2,9] The first report on the antibody response to *C. difficile* in IBD was limited to the analysis of IgG to toxin B and noted an increased level with a tendency to be most marked in CD.[10] A few IBD patients were included in a cystic fibrosis study, with a tendency for IgG to toxin A, but not to toxin B, to become generated during ongoing *C. difficile* infection.[11] Another study failed to demonstrate any increase in antitoxin IgG due to *C. difficile* infection in IBD patients.[12] Several factors may have contributed to these discrepant observations, such as patient characteristics involving remission, relapse, *C. difficile* infection (prior or ongoing), IBD treatment types, previous intestinal resection, and control subject characteristics as well as enzyme-linked immunosorbent assay (ELISA) methodology. However, anti-tumor necrosis factor (anti-TNF) treatment has been shown not to be associated with an excess of *C. difficile* infection[13], although it does mediate a lowered humoral response to hepatitis B vaccine expansion.[14] The present study is an attempt to shed more light on *C. difficile* seroreactivity in IBD by paying attention to the possible influence of immunomodulatory treatment and by applying the previously well-documented[9]

ELISA method to relatively large IBD outpatient material. The results may increase our understanding of IBD pathogenesis and aid in identifying effective monoclonal antibodies to *C. difficile* toxins for treatment.[15]

Methods

Patients and healthy controls. This is a prospective study on outpatients, primarily with scheduled visits, at three clinics in southern Sweden specialized in IBD. The study was approved by the local medical ethics committee of Lund University (permit number LU 552-03), and written informed consent was obtained from all patients.

Sera from 138 consecutive patients with established IBD (76 females and 62 males, aged 18–82 years; mean 45 years), including 71 with CD (45 females and 26 males, aged 18–78 years; mean 43 years) and 67 with UC (31 females and 36 males, aged 19–82 years; mean 47 years), were analyzed. Data on disease duration, previous intestinal resection, smoking habits, and frequency as reported by the patients during the recent year of all types of infections are listed in Table 1. The indicated type of treatment was ongoing at the time of inclusion in the study and sampling of serum (shown in Table 2). Disease-modifying anti-IBD drugs are referred to by us as DMAID. The panel of controls was composed of 121 randomly selected sera from healthy blood donors (51 females and 70 males, aged 20–66 years; mean 45 years) and was obtained from the Southern Sweden Microbiology Biobank.

Antibody analysis. Patient and healthy control sera were stored at −20°C until analysis. Levels of serum antibodies against toxins A and B were measured in doublets by ELISA as previously described[7] with minor modifications, using 96-well polystyrene microtiter plates coated with either toxin A or toxin B and mouse monoclonal antibodies to toxin A and toxin B, respectively, serving as positive controls (tgcBIOMICS, Mainz, Germany). Patient sera were diluted 1:800 for analysis of IgA and 1:1600 for IgG. Secondary antibodies were horseradish peroxidase-conjugated. One patient serum used in our previous studies was included on each plate as an additional and manufacturer-unrelated control.

Table 2 DMAID data

	CD	UC
Total patients	71 (100%)	67 (100%)
No data on DMAID	8 (11%)	16 (24%)
DMAID-treated	47 (66%)	26 (39%)
Azathioprine, total	42 (89%†)	12 (46%†)
Only azathioprine	31	9
+ TNF inhibitor	10	1
+ Mesalazine	1	2
TNF inhibitor, total	12 (26%†)	2 (8%†)
only TNF inhibitor	2	1
+ Azathioprine	10	1
Mesalazine, total	3 (6%†)	15 (58%†)
Only mesalazine	2	13
+ azathioprine	1	2
Methotrexate	1	0

†% of DMAID-treated patients.
The data shown are: *n* (%).
CD, Crohn's disease; TNF, tumor necrosis factor; UC, ulcerative colitis.

Statistics. Antibody measurement data were not normally distributed; the analyses were performed using the Mann–Whitney U test. As is common practice, we call the finding statistically significant if the relevant *P*-value is lower than 0.05. All statistical analysis is performed as described in the reference literature.[16]

Results

Serum IgG to toxin A was found to be increased in the IBD patients to a similar extent in CD and UC ($P = 0.02$, as compared to healthy controls), with ELISA median values 1.41 for CD, 1.51 for UC, and 1.24 for healthy controls (Fig. 1a). IgG to toxin B was elevated significantly among the CD patients ($P = 0.002$, as compared to healthy controls) and somewhat among UC ($P = 0.06$, as compared to healthy controls); the median for CD was 1.54, for UC 1.36, and for the healthy controls 1.21 (Fig. 1b). The IgA results showed very similar medians for all three subject groups (for toxin A 0.55–0.60 and for toxin B 0.26–0.31), with no significance (results not shown).

We sought to determine if the observed IgG elevation among IBD patients was influenced by DMAID treatment. A

Table 1 Patient characteristics

	CD no DMAID	CD DMAID	UC no DMAID	UC DMAID	Healthy controls
Female/male (*n*)	11/5	29/18	10/15	12/14	51/70
Age < 45 years (*n*, %)†	7, 44	29, 62	9, 36	11, 42	60, 50
Disease duration ≥10 years (*n*, %)†	6, 38	24, 51	10, 43‡	11, 42	NA
Intestinal resection (*n*, %)†	11, 69	24, 51	0	1, 4	NA
Smokers (*n*, %)†	2, 13§	15, 33§	7, 28	1, 4	ND
Infections >1 per year (*n*, %)†	10, 71‡	35, 74	12, 48	11, 42	ND

†There was no significant difference in frequency between the disease-modifying anti-inflammatory bowel disease drugs (DMAID) and the no DMAID groups.
‡Information is lacking in two patients.
§Information is lacking in one patient.
CD, Crohn's disease; UC, ulcerative colitis; NA, not applicable; ND, no data.

majority of the CD patients (47/71; 66%) and a smaller fraction among UC (26/67; 39%) were treated with at least one type of DMAID (Table 2); these figures may represent an underestimate because we lack information on treatment status for 8 patients with CD and 16 with UC. Azathioprine and TNF inhibitors dominated in CD and mesalazines and azathioprine in UC.

There was no correlation between treatment and IgG to toxin A in either CD or UC (results not shown). There was a trend, but with no significance, for a higher IgG to toxin B in UC patients treated with DMAID compared with the UC subgroup with no such drug (Fig. 2). Among UC patients with a mesalazine as the only DMAID drug (13 of all 26 DMAID-treated UC), there was also a nonsignificantly higher level of IgG to toxin B, and there was no significant difference between untreated UC and all DMAID UC or with DMAID except mesalazine. However, while CD patients not receiving medication had a most marked elevation of IgG to toxin B compared with healthy controls ($P = 0.0001$), the CD patients taking DMAID presented with a normal level; medians were 1.99, 1.21, and 1.35, respectively (Fig. 2). There were 2 CD patients taking mesalazine as the only DMAID type, precluding a meaningful evaluation of the effect of this drug among all 47 DMAID-treated CD patients. We analyzed if the different levels of IgG to toxin B between treated and untreated CD patients could be attributed to a single type of DMAID, but no statistical significance due to any of the DMAID types (listed in Table 2) was found (as all but five DMAID-treated patients had azathioprine and only three had mesalazine, it was statistically meaningful only to note a slight trend ($P = 0.22$) for higher IgG to toxin B in patients with TNF inhibitors compared with DMAID except TNF inhibition).

There was no significant difference among DMAID-treated CD patients regarding the presence of the potentially confounding factors listed in Table 1: patient age, disease duration, intestinal resection, smoking habits, or number of infections.

Discussion

Both the incidence and severity of *C. difficile* infection have increased dramatically in the general population during the past two decades[4], and there has been an even higher increase among patients with IBD.[5] The humoral immune response to the *C. difficile* toxins A and B is known to be protective in humans,[2,9] but previous reports on seroreactivity to *C. difficile* in IBD patients have provided conflicting results.[10–12] In summary, we found IgA levels in IBD to be no different from healthy controls but IgG to toxin A to be elevated similarly in both CD and UC and IgG to toxin B to be elevated only in CD. Our findings are in agreement with Shakir *et al.*,[10] who reported that IBD patients have a higher level of IgG to toxin B than non-IBD control subjects, probably including a higher level in CD than in UC (there were no statistical data). Findings differing from ours may be attributed to inclusion of subjects with ongoing or recent *C. difficile* infection. Hughes *et al.*[12] observed elevated IgA to both toxins in patients with infection and no IgG abnormalities in IBD; a strength of this study is more detailed clinical information than in our material, including ruling out a significant difference in antibiotic use during the prior 6 months between IBD patients and healthy controls. A study focusing on cystic fibrosis found a small number of IBD patients (3 CD and 7 UC) with ongoing *C. difficile* infection to have a trend suggesting high IgG to toxin A but low IgG to toxin B (IgA was not analyzed).[11]

We interpret our finding in CD and UC of a high level of IgG to toxin A to reflect a memory of a normal immune response to *C. difficile* infection, agreeing well with *C. difficile* infection

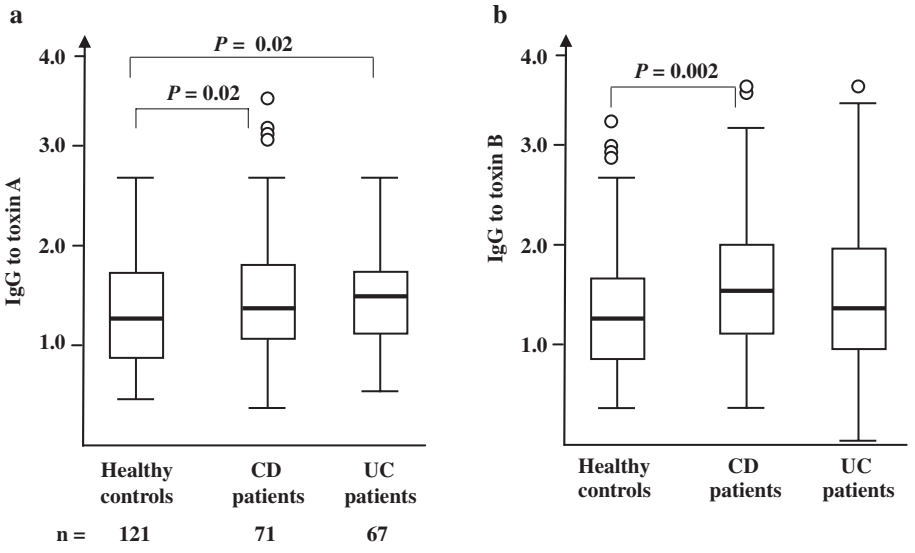

Figure 1 IgG to *Clostridium difficile* toxins A and B. Enzyme-linked immunosorbent assay arbitrary spectrophotometric absorbance unit median values are shown. Boxes represent values between quartiles 1 and 3, and a thick line indicates the median. Whiskers show the max and min values located above the top or below the bottom of the box, respectively, within a 1.5 interquartile distance. Circles denote outliers values located outside a 1.5 interquartile distance. CD, Crohn's disease; UC, ulcerative colitis.

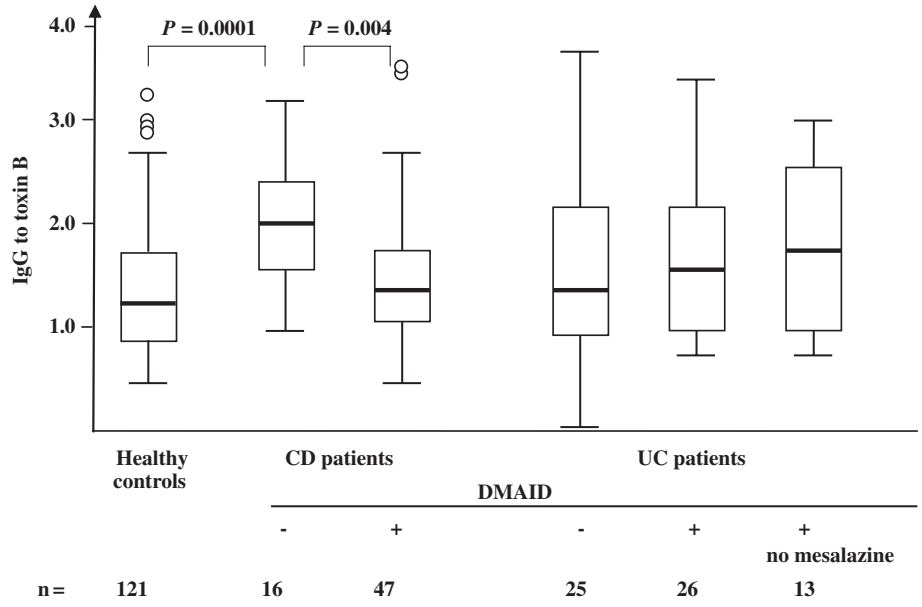

Figure 2 IgG to *Clostridium difficile* toxin B, with patient groups subdivided according to if they, at the time of blood sampling, were receiving disease-modifying anti-inflammatory bowel disease drugs. Enzyme-linked immunosorbent assay values are shown. Boxes represent values between quartiles 1 and 3, and a thick line indicates the median. Whiskers show the max and min values located above the top or below the bottom of the box, respectively, within a 1.5 interquartile distance. Circles denote outliers values located outside a 1.5 interquartile distance. CD, Crohn's disease; UC, ulcerative colitis.

being more common in IBD than in non-IBD controls. Next, we consider our most striking finding, namely, a highly significantly ($P = 0.0001$) elevated level of IgG to toxin B among CD patients without immunomodulatory DMAID treatment, coupled with a normal level among the DMAID-treated CD subgroup.

How should the different response to toxin B between treated and untreated CD patients be interpreted? Is it because of the medication per se or the therapeutic effects on disease activity? We cannot conclude an answer, but because there was no difference in IgG to toxin B between the types of DMAID, we speculate that an effect of a drug itself is unlikely. Although it seems plausible that the intestinal pathology typical for CD, with deeper intestinal wall layers rich in immune cells involved compared with UC, would lead to more heavy exposure to *C. difficile* antigen, the remarkably high level of IgG to toxin B in CD patients not receiving DMAID remains to be explained because we find it unlikely that only one of the two toxins, namely, toxin B, will reach into deeper layers. Instead of heavy exposure of intestinal immune cells to *C. difficile* antigens, we propose that an abnormal immune response to toxin B is involved.

Considering the seemingly endless complexity of the intestinal immune system, interacting dynamically with luminal antigens and recently shown to include mucosa-resident T cell-dependent memory B cells, it is possible that a heightened IgG reactivity with toxin B, persisting after infection, may be part of an abnormal immune response in CD.[8,17] Our proposition closely follows the suggestions made in a report showing enhanced development in CD of IgG against many intestinal commensal bacteria, leading the authors to conclude that there is a generalized increased IgG response to the intestinal microbiota, with no specific species being involved in CD pathogenesis.[18] However, our finding of markedly different reactivity to two separate toxins from the same species serves as an indicator that, amid a background of a generalized hyperreactivity, there may be bacterial factors playing a role in CD pathogenesis.

Assuming that an abnormal immune response in CD is mediating the strong production of IgG to toxin B, then can analysis of this IgG possibly be an early diagnostic marker? It might be a more efficient marker, one that is more readily analyzed on a routine basis, than the fecal microbiome pattern suggested to be a potential early disease marker in CD.[19] Such a diagnostic tool is needed because it is now acknowledged that DMAID treatment of IBD should ideally be initiated already before the bowel has become severely damaged.[20,21]

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