Chapter 6

Humoral immune response as predictor of recurrence in *Clostridium difficile* infection

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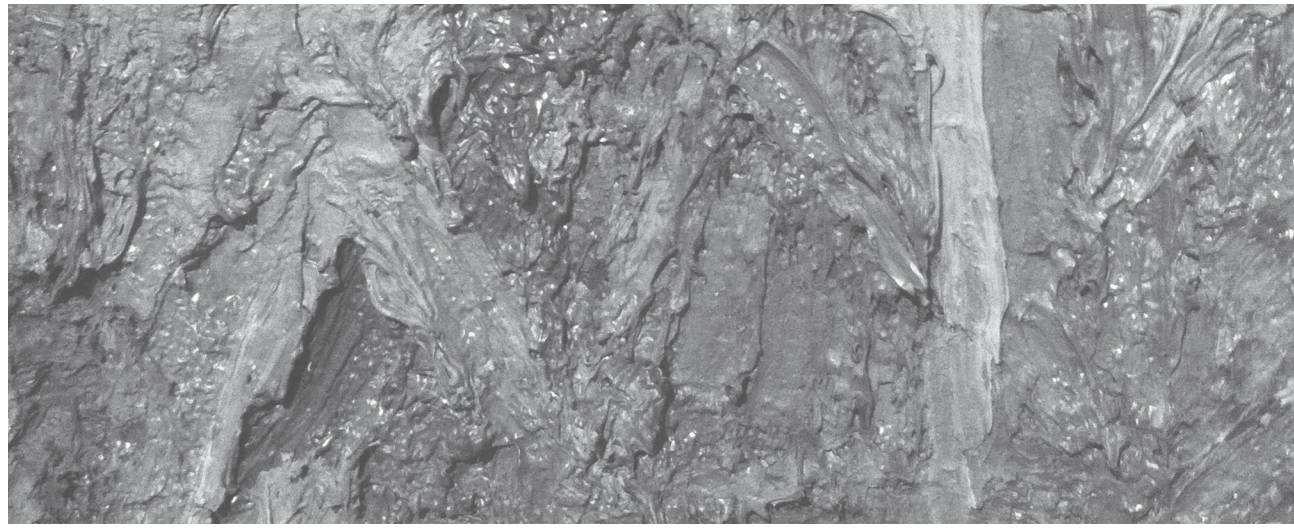

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Abstract

Low serum concentrations of antibodies directed against large clostridial toxins (TcdA and TcdB) have been associated with a higher risk of recurrence of *Clostridium difficile* infection (CDI) after successful antibiotic treatment. However, there are conflicting reports. Herein, we compared serum levels of antibodies of patients with a single episode of CDI with those of patients who subsequently suffered a recurrence. We used a serum bank from patients who received an experimental whey protein product following successful antibiotic treatment for CDI. We determined levels of IgA and IgG directed against TcdA, TcdB and non-toxin cell surface antigens in serum collected directly and three weeks after completing a 10 days course of antibiotic treatment for CDI. We also developed an objective flow cytometry-based assay to determine the proportion of cells exhibiting cytopathic effect after exposure to TcdB. Using this method, we measured sera's TcdB-neutralizing capacity. We compared the results for patients without a subsequent recurrence with those of patients who suffered a recurrence within 60 days after completing the antibiotic treatment. Advanced age, comorbidity other than immunocompromised state and low serum levels of anti-TcdA and TcdB antibodies were associated with recurrence, whereas serum levels of antibodies directed against cell surface antigens were not. Serum TcdB-neutralizing capacity, which correlated only weakly with serum IgG anti-TcdB, was not significantly associated with recurrence.

Key words: *Clostridium difficile*, toxins, antibodies, recurrence, IgA, IgG

Introduction

Clostridium difficile infection (CDI) is an important problem in healthcare facilities. Spores of this bacterium are ingested and bacteria may colonize the gut after germination. Colonization of the gut may lead to carriage or disease, which ranges from mild self-limiting diarrhea to fulminant life-threatening colitis. Mild disease may subside after withdrawal of antibiotics. Moderate and severe disease usually respond to oral metronidazole, glycopeptides, or fidaxomicin, but often recur. Factors associated with the outcomes of exposure to spores, colonization of the gut and disease include recent exposure to antibiotics, virulence of the *C. difficile* strain, advanced age, severe comorbidity, the use of proton pump inhibitors and the presence of antibodies directed against the large clostridial toxins TcdA and TcdB and/or other antigens [1].

However, the role of humoral immunity in CDI is unclear due to conflicting reports on the association between the humoral immune response and disease outcome [2-11]. Important issues include the type of antibodies (secretory IgA or IgG) involved, whether serum antibodies reflect mucosal immunity in the gut, whether TcdA or TcdB must be neutralized, which toxin epitope is the most important one, and whether antibodies directed against antigens other than toxin matter.

To investigate the possible relationship between the humoral immune response and the outcome of CDI, we compared serum levels of IgA and IgG directed against TcdA, TcdB and non-toxin cell surface antigens of patients with a single episode to those of patients with a subsequent CDI recurrence.

Methods

Serum samples

Serum samples were taken during a prospective cohort study [12] into the safety and preliminary efficacy of a whey protein concentrate made from milk of cows immunized against *C. difficile* to prevent recurrences after successful antibiotic treatment of CDI [13]. CDI was suspected clinically and confirmed by a positive fecal toxin assay and culture. Patient data registered on enrollment included age, sex, disease severity and chronic comorbidity according to the Acute Physiology, Age and Chronic Health Evaluation (APACHE) II prognostic system [14], previous episodes of CDI, and antibiotic treatment before starting the whey protein concentrate. After completion of 10 days of standard antibiotic therapy and reaching clinical remission of CDI, participating patients received this whey protein concentrate orally for two weeks with a follow-up period of 60 days. Outcome measures were CDI recurrences. Recurrence was declared if the patient reported looser stools according to a three-grade visual

scale, in comparison to the day before and an increase in stool frequency for two consecutive days, or a single day with an increase of ≥ 3 stools, or any day with passage of > 6 stools/day, and a positive *C. difficile* toxin stool test (Vidas, BioMérieux, Marcy l'Etoile, France) and culture. Among cultured strains, those strains belonging to PCR-ribotype 027 were identified as described earlier [15]. Serum samples were taken from these patients on the day they started on the whey protein concentrate (i.e., after 10 days of antibiotic therapy), and a second time 18 to 21 days later.

Enzyme-linked immunosorbent assay (ELISA) for the determination of serum anti-TcdA and anti-TcdB IgA and IgG

Wells of flat-bottom 96-well high-binding plates (Greiner Bio-One, Frickenhausen, Germany) were incubated with 100 μL per well of toxin in a 100 mM carbonate buffer (pH9.6) overnight at 4 °C. For the IgA antitoxin assays the wells were coated with 1.5 μg/mL of TdA [Mucovax, Leiden, Netherlands] or 1.0 μg/ml of TcdB [kindly provided by dr. H. Feng, Tufts University Cummings School of Veterinary Medicine, Grafton, MA] and for the IgG assays wells were coated with 1.0 μg/mL of TcdA or TcdB. The plates were then washed 3 times with phosphate-buffered saline (PBS; pH 7.4) as well as between every two incubation steps. Next, the plates were incubated with 200 μL of blocking buffer (2% wt/v gelatine in PBS containing 0.05% v/v Tween-20) for 1 hour at 37 °C. Thereafter, serial dilutions of serum samples diluted in 0.2% wt/v gelatine in PBS with 0.05% v/v Tween-20 were transferred to the plate (100 μL/well) and incubated for 90 minutes at 37 °C. All serum samples were tested in duplicate. The dilution buffer was used as a negative control. Subsequently, the plates were incubated with 100 μL of 4,000 x diluted polyclonal rabbit anti-human IgA antibodies conjugated with horseradish peroxidase (HRP) or 6,000 x diluted HRP-conjugated polyclonal rabbit anti-human IgG antibodies (DakoCytomation, Glostrup, Denmark) for 90 minutes at 37 °C. Lastly, the plates were incubated with 2.5 mg/mL of filtered 2,2'-azoni-bis(3-ethylbenzothiazoline-6-sulphonic acid) [ABTS] (Roche, Basel, Switzerland) in substrate buffer [47.2% of 100 mM citric acid and 52.8% of 100 mM Na_2HPO_4; pH 4.2] (100 μL/well) and 0.015% v/v H_2O_2 for 30 minutes at 37 °C. The absorbance was measured at 405 nm. For standardization, serum samples with high level of anti-TcdA immunoglobulins (IgA or IgG) were pooled, assigned an arbitrary value of 10,000 units (U) per mL, and used in all assays as standard.

ELISA for the determination of serum IgG directed against other antigens

The following non-toxin antigens were prepared from PCR ribotypes 001 and 027 as previously described: an EDTA extract representing the entire cell surface layer, a guanidine hydrochloride extracted S-layer proteins, an aqueous phenol extracted lipoteichoic acid (LTA) analogue (only from ribotype 001) [16] and whole flagella (mechanically sheared and purified on a cesium chloride gradient) as described by Hancock and Poxton [17]. ELISAs for IgG levels to these antigens were performed as described by Sanchez-Hurtado et al [16].

Flow cytometric assay to determine the capacity of serum to neutralize TcdB

We developed an objective method to determine the level of TcdB-neutralizing antibodies in sera. Briefly, 3T3 cells, a spontaneously immortalised cell line derived from embryonic mouse fibroblasts, were cultured in a 96-wells tissue culture plate until a semi-confluent monolayer was formed. Serial dilutions of TcdB in culture medium were preincubated 1:1 with fourfold diluted heat-inactivated patient serum for one hour at room temperature on a microplate shaker. Next, we incubated these mixtures of toxin and serum (100 μL per well) with the cells at 37 °C for one hour. Heat-inactivated pooled human serum (Lonza, Basel, Switzerland) was used as a standard. Toxin diluted in culture medium to a concentration of 50 ng/mL was used as a positive control and culture medium only as a negative control. After washings, the cells were cultured overnight and the degree of CPE was assessed microscopically. Next, we removed the cells from the wells by mechanically detaching them and then examined approximately 10,000 cells per sample on a FCSCalibur (Becton and Dickinson, La Jolla, CA). Results are expressed as the mean fluorescence intensity (MFI) in arbitrary units. The MFI of the cell population was used as a measure of the proportion of cells showing CPE. The MFI's for the positive control well and the negative control well were considered to characterize a cell population with maximum CPE and an unaffected cell population, respectively. For practical purposes, the mean of these MFI values was considered to represent 50% CPE. The MFI for the toxin dilution pre-incubated with patient serum that resulted in 50% CPE was divided by the value for the toxin dilution pre-incubated with standard serum resulting in 50% CPE to yield a standardized measure of the toxin-neutralizing capacity (Table 1).

Statistical analysis

The distributions of continuous variables were compared with a Mann-Whitney *U* test and proportions with the β test. The strength of the relationship between two continuous variables was estimated by Kendall's tau-b. Continuous variables were dichotomized using the median for the entire population as a cut-off. For the association between dichotomized variables and recurrence, odds ratios (ORs) with 95% confidence intervals were calculated. To examine the presence of confounding, categorical variables with a strong and clear association (OR > 2 and $P < 0.2$) with recurrence were introduced into a multivariate logistic regression model. IBM SPSS Statistics 20.0 software was used for the calculations.

Table 1 Comparison of the flow cytometric and light microscopical assessment of the toxic effect of TcdB on 3T3 cells.

In short, semi-confluent monolayers of 3T3 cells were exposed for 1 hour at 37 °C to various amounts of TcdB that had been pre-incubated with eight-fold diluted patient serum for 1 hour at ambient temperature (B-E), or as positive control to 50 ng/ml TcdB (A) and as negative control to medium alone (F). After washing and subsequent culturing overnight at 37 °C, the cytopathic effect (CPE) of the toxin was estimated by visual inspection or, after mechanical detachment of the cells from the wells, quantitated by flow cytometry, ie the median fluorescence intensity (MFI) of the forward scatter. We assumed that the mean of the MFI values for the positive control and the negative control (in this example: 409 arbitrary units [AU]) represents a cell population showing 50% CPE. The serum-preincubated TcdB concentration that resulted in a cell population with 50% CPE (in this example extrapolated to a TcdB concentration of 4.4 ng/L) is divided by the corresponding, pooled reference serum preincubated , TcdB concentration to yield a standardized measure of the TcdB-neutralizing capacity.

Treatment	MFI	Light microscopy
TcdB 50 ng/mL	310 AU	All cells showing CPE
TcdB 8.3 ng/mL with patient's serum	332 AU	More cells showing CPE than unaffected cells
TcdB 5.6 ng/mL with patient's serum	366 AU	Cells showing CPE mixed with unaffected cells
TcdB 3.7 ng/mL with patient's serum	436 AU	Cells showing CPE mixed with unaffected cells
TcdB 2.5 ng/mL with patient's serum	481 AU	More unaffected cells than cells showing CPE
Medium alone	508 AU	No cells showing CPE

[b]RR for the association of the variable with recurrence.

Results

Of 120 CDI patients whose data were present in the database, 16 (13.3%) suffered a toxin-confirmed recurrence. Table 2 shows patient characteristics. Advanced age and severe comorbidity were predictors of recurrence. However, immunocompromised state was not. Treatment with vancomycin was non-significantly associated with subsequent recurrence, probably reflecting more severe CDI for which treatment with vancomycin was preferred over metronidazole.

Table 2 Characteristics of CDI patients after completion of 10 days of antibiotic treatment

	Recurrence		No recurrence		
	N	Result	N	Result	*P*
Epidemiological characteristics:					
Age (median and IQR)	15	77 (73 – 84)	99	69 (52 – 79)	0.057
Age > 73 years (%)	15	80	99	46	**0.013**
Male sex (%)	16	38	103	50	0.371
Liver cirrhosis with portal hypertension* (%)	16	0	100	10	0.186
Heart failure NYHA class IV (%)	16	31	99	18	0.225
Severe pulmonary disease* (%)	16	44	98	28	0.189
Receiving chronic dialysis (%)	16	13	100	6	0.341
Immunocompromised* (%)	16	25	98	34	0.492
Any of the above comorbidities (%)	16	81	98	56	0.057
Any of the above comorbidities without immunocompromised status (%)	16	75	98	43	**0.017**
Recurrent CDI episode vs. first episode (%)	16	44	102	40	0.788
Number of previous CDI episodes (median andIQR)	16	0 (0 – 1)	102	0 (0 – 1)	0.839
Recent episode treated with metronidazole (%)	16	31	102	49	0.185
Recent episode treated with vancomycin (%)	16	69	102	48	0.123
Clinical, hematological, biochemical and microbiological characteristics:					
Leukocyte count [10^9/L] (median and IQR)	15	7.6 (6.1 – 15.3)	72	9.0 (6.6 – 13.3)	0.536
Creatinine [μmol/L] (median and IQR)	16	83 (61 – 124)	101	82 (62 – 127)	0.949
Serum albumin [g/L] (median and IQR)	13	38 (27 – 39)	66	31 (23 – 37)	0.092
APACHE II score (median and IQR)	12	2 (0 – 4)	57	1 (0 – 3)	0.380
Episode of CDI caused by PCR ribotype 027 (%)	12	50	60	28	0.142

* According to the Chronic Health Points score of APACHE II

CDI = *Clostridium difficile* infection. N = number of patients for whom information was available. IQR = interquartile range. Results printed in boldface have reached statistical significance ($P < 0.05$).

Table 3 shows IgA and IgG anti-TcdA and anti-TcdB antibody levels and TcdB-neutralizing levels in sera from patients with and those without recurrence. Low serum levels of IgA anti-TcdA and IgG anti-TcdB three weeks after completing 10 days of antibiotic treatment for CDI were most clearly associated with recurrence, as well as a decrease in serum IgG anti-TcdA during this time period. Some of these associations reached statistical significance. Interestingly, serum levels of anti-toxin A/B antibodies decreased in many patients during the three weeks after antibiotic treatment.

Low serum TcdB-neutralizing capacity was not a predictor of recurrence, although none of the patients with a higher serum neutralizing capacity than the reference serum suffered a recurrence. There was no correlation between anti-TcdB IgA level in serum and its capacity to neutralize TcdB (day 0: $r = 0.155$, $P = 0.185$; day 18: $r = 0.185$, $P = 0.129$) and only a weak correlation between anti-TcdB IgG level in serum and its capacity to neutralize TcdB (day 0: $r = 0.253$, $P = 0.036$; day 18: $r = 0.309$, $P = 0.011$).

Serum levels of IgG directed against any of the non-toxin cell-surface antigens after antibiotic treatment did not differ between patients with subsequent recurrence and those without subsequent recurrence (data not shown).

Antibody levels and neutralizing capacity were dichotomized using the median of the entire population of patients as a cut-off. Odds ratios for the association with recurrence were calculated (Table 4). To investigate possible confounding, the odds ratios for these dichotomized antibody levels were corrected for age over 73 and the presence of severe comorbidity. Serum levels of IgA anti-TcdB directly after antibiotic treatment and IgG directed against both toxins three weeks later and a decrease in serum IgG anti-TcdA were associated most strongly with recurrence.

Table 3 Antibodies against *C. difficile* antigens in serum of CDI patients with and those without recurrence

	Recurrence			No recurrence			
	N	Median	IQR	N	Median	IQR	*P*
Day 0:							
Serum IgA anti-TcdA [arbitrary units]	14	327	128 – 576	74	477	197 – 1027	0.133
Serum IgG anti-TcdA [arbitrary units]	14	580	162 – 1286	75	350	166 – 1430	0.696
Serum IgA anti-TcdB [arbitrary units]	15	986	659 – 1155	67	1603	665 – 2894	0.067
Serum IgG anti-TcdB [arbitrary units]	15	810	390 – 1423	65	974	537 – 3904	0.392
Serum TcdB-neutralizing capacity [proportion of that of reference pooled serum]	7	0.42	0.30 – 0.85	33	0.45	0.40 – 1.15	0.485
Day 18 – 21:							
Serum IgA anti-TcdA [arbitrary units]	14	183	0 – 389	76	423	198 –1255	**0.009**
Serum IgG anti-TcdA [arbitrary units]	14	237	0 – 815	75	486	186 – 1660	0.121
Serum IgA anti-TcdB [arbitrary units]	11	835	387 – 1001	68	1280	561 – 2551	0.133
Serum IgG anti-TcdB [arbitrary units]	11	446	224 – 892	68	954	527 – 2932	**0.030**
Serum TcdB-neutralizing capacity [proportion of that of reference pooled serum]	4	0.46	0.37 – 0.61	33	0.60	0.40 – 1.10	0.354
Increase between day 0 and day 18 – 21:							
Serum IgA anti-TcdA [arbitrary units]	14	-73	-372 – 2	74	-28	-297 – 66	0.620
Serum IgG anti-TcdA [arbitrary units]	14	-175	-795 – -20	74	0	-92 – 263	**0.007**
Serum IgA anti-TcdB [arbitrary units]	11	-36	-711 – 263	44	-451	-1485 – 83	0.323
Serum IgG anti-TcdB [arbitrary units]	11	-568	-2022 – 99	43	-358	-1007 – 263	0.499

CDI = *Clostridium difficile* infection. N = number of patients for whom information was available. IQR = interquartile range. Day 0 = the day after completing 10 days of antibiotic treatment. Results printed in boldface have reached statistical significance ($P < 0.05$).

Table 4 Dichotomized variables as predictors of CDI recurrence

	Recurrence		No recurrence					
	N	%	N	%	OR	95% CI	aOR	95% CI
Epidemiological characteristics:								
Age > 73 years	15	80	99	46	**4.80**	**1.28 – 18**		
Comorbidity other than immunocompromised status	16	75	98	43	**4.00**	**1.21 – 13**		
Antibody levels:								
Day 0:								
Serum IgA anti-TcdA ≤ 435 units	14	64	74	47	2.01	0.61 – 6.56	3.04	0.78 – 12
Serum IgG anti-TcdA ≤ 408 units	14	36	75	53	0.49	0.15 – 1.59	0.49	0.13 – 1.76
Serum IgA anti-TcdB ≤ 1271 units	15	80	67	43	**5.24**	**1.35 – 20**	**4.57**	**1.06 – 20**
Serum IgG anti-TcdB ≤ 949 units	15	53	65	49	1.18	0.38 – 3.63	1.39	0.39 – 4.98
Serum TcdB-neutralizing capacity ≤ 0.44	7	57	33	49	1.42	0.27 – 7.34	1.03	0.17 – 6.16
Day 18 – 21:								
Serum IgA anti-TcdA ≤ 366 units	14	71	76	46	2.93	0.84 – 10	**6.06**	**1.40 – 26**
Serum IgG anti-TcdA ≤ 460 units	14	64	75	48	1.95	0.60 – 6.37	3.31	0.85 – 13
Serum IgA anti-TcdB ≤ 1212 units	11	82	68	46	**5.37**	**1.08 – 26**	**6.53**	**1.05 – 40**
Serum IgG anti-TcdB < 854 units	11	73	68	47	3.00	0.73 – 12	3.25	0.65 – 16
Serum TcdB-neutralizing capacity < 0.60	4	75	33	52	2.82	0.27 – 30	4.50	0.25 – 81
Increase between day 0 and day 18 – 21:								
Serum IgA anti-TcdA ≤ -42 units	14	57	74	49	1.41	0.45 – 4.64	2.08	0.57 – 7.62
Serum IgG anti-TcdA ≤ -22 units	14	79	74	45	**4.56**	**1.17 – 18**	**5.35**	**1.22 – 23**
Serum IgA anti-TcdB ≤ -419 units	11	36	44	55	0.48	0.12 – 1.87	0.50	0.10 – 2.49
Serum IgG anti-TcdB ≤ -362 units	11	55	43	49	1.26	0.33 – 4.75	0.93	0.18 – 4.77

CDI = *Clostridium difficile* infection. N = number of patients for whom information was available. Day 0 = the day after completing 10 days of antibiotic treatment. OR = odds ratio. 95% CI = 95% confidence interval. aOR = odds ratio adjusted for age > 73 years and comorbidity other than immunocompromised status. Results printed in boldface have reached statistical significance (P < 0.05).

Discussion

The main findings of this study in patients with CDI are that advanced age, comorbidity and low serum levels of antibodies directed against TcdA and TcdB were associated with recurrence, whereas serum levels of antibodies directed against cell surface antigens were not.

The strong points of this study pertain to the study protocol and analytic methods. First, the data derive from a prospective study in which patient characteristics and sera were collected according to a standardized protocol. Second, blood was collected at two time points, which allowed for the analysis of the dynamics of antibody levels. Third, standardized ELISAs for antibodies against toxins A and B and an objective assay for toxin-neutralization capacity of sera were developed. Clostridial toxin neutralization assays are commonly based on microscopic assessment of cypathic effect (CPE) on cells. In these assays, a cell population showing 50% CPE is used to compare the rate of toxin neutralization. Because there are grades of CPE (e.g., loss of length and number of cell processes, gradual rounding of cells), the proportion of cells showing CPE may vary per visual field and certain unaffected cells that are not fully attached to the surface may appear to show CPE, we felt that assessing 50% CPE microscopically is subjective. Unfortunately, the relatively small number of patients with recurrence and the fact that all patients were treated with a whey protein concentrate containing antibodies against *C. difficile* and its toxins may have compromised statistical power to detect differences between CDI patients with recurrence and those without recurrence. Another limitation of the present study pertains to quantitation of the anti-toxin antibody levels in sera instead of fecal samples. Currently, it is not known to what extent serum antibody levels reflect mucosal immunity, which is probably the relevant part of the immune system given that CDI is not an invasive infection. Finally, the analysis of toxin-neutralizing capacity of the sera was limited to TcdB, as this toxin was considered the most important one in the pathogenesis of CDI according to scientific consensus at the time of experimentation.

Several studies have found associations between humoral immunity and the clinical course of CDI. For instance, it has been reported that serum IgG directed against TcdA, and less convincingly, IgG directed against TcdB and other antigens [5] as well as all classes of antibodies directed against whole cell *C. difficile* [8] are associated with disease instead of carriage. Warny [11] found low serum IgG and fecal sIgA directed against TcdA to be associated with a longer duration of illness and a higher risk of recurrence. Others reported that low levels of IgM directed against various antigens and low levels of IgG directed against TcdA, but not TcdB or other antigens predict recurrence [6]. Aronsson [2] identified low serum IgG directed against TcdB to be a better predictor of recurrence than low serum IgG directed

against TcdA. Leav [7] found IgG directed against the receptor-binding domain of TcdB and to a lesser extent that of TcdA, but not against whole toxins, and Drudy [3] reported IgM directed against surface-layer proteins to predict recurrence. By contrast, Johnson [4] and Sánchez-Hurtado [9] found that humoral immune responses did not influence the clinical course of CDI. Recently, Solomon [10] reported no correlation between serum IgG anti-TcdA and IgG anti-TcdB and recurrence, but did find a relationship between low levels of these antibodies and 30-day all-cause mortality, most of which was at least partly attributed to CDI.

Several findings raise the possibility that serum anti-toxin antibody levels are not causally related with recurrences. In this study, for example, the serum antitoxin antibody levels in most of the patients with and without recurrence decreased during the first three weeks after completion of the antibiotic course. In addition, immuno-compromised state was not associated with recurrence. Furthermore, in contrast to serum antibodies directed against toxins, the TcdB-neutralizing capacity of sera did not predict recurrence. It could be that low serum antibody levels are caused by fecal protein loss from a severely inflamed colonic mucosa. The severity of colonic inflammation may itself be associated with the risk of recurrence. The fact that several studies have found hypo-albuminemia to predict recurrence [18] and our study found patients with subsequent recurrence to recover less from hypo-albuminemia than patients without recurrence (data not shown) support this hypothesis. On the other hand, there are arguments against this hypothesis. Low levels of antibody against non-toxin, cell-surface antigens were not associated with recurrence. Moreover, the administration of parenteral monoclonal antibodies against the C-terminus of TcdA and TcdB has been found to prevent recurrences [19].

In conclusion, low serum anti-TcdA and anti-TcdB antibodies are associated with a higher risk of recurrence. However, further studies into the humoral immune responses in CDI, simultaneously measuring serum and fecal antibody levels at several time points, as well as measuring fecal protein loss (e.g., by fecal alpha1-antitrypsin clearance), may help to distinguish between a causal relationship and confounding.

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