Immunological properties of surface proteins of *Clostridium difficile*

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Sera from patients with *Clostridium difficile*-associated disease (CDAD) and sera from a control group were analysed by an ELISA to detect antibodies directed against four surface proteins and toxins A and B of *C. difficile*. The surface proteins were the flagellar cap protein FliD, the flagellin FliC, the adhesin Cwp66 divided into two domains, Cwp66-Nterminal and Cwp66-Cterminal, and the fibronectin-binding protein Fbp68. For each antigen, antibody levels in the CDAD patient group and in the control group were compared. In the CDAD patient group, the mean of the antibody levels decreased from Cwp66-Cterminal to Fbp68, FliD, toxins B and A, Cwp66-Nterminal and finally FliC, suggesting different immunogenic properties among these adhesins. For Cwp66-Nterminal, FliC, FliD and Fbp68, the antibody level observed in the control group was higher than in the CDAD group with a statistically significant difference whereas the antibody level for toxins A and B was not statistically different. In conclusion, this study suggests that during the clinical course of disease, *C. difficile* adhesins are able to induce an immune response which could play a role in the defence mechanism of the host.

Introduction

*Clostridium difficile*, a Gram-positive spore-forming intestinal pathogen, is frequently recovered from the hospital environment. Patients and hospital staff have been implicated as a major source of nosocomial acquisition (McFarland *et al.*, 1989). After disruption of the intestinal barrier by antibiotics, spores of *C. difficile*, acquired exogenously or endogenously, germinate and bacteria multiply in the intestine. *C. difficile* synthesizes two major toxins, toxins A and B, responsible for the clinical manifestations of the disease, diarrhoea, or in the worst case, pseudomembranous colitis (Lyerly *et al.*, 1988). However, different adhesins implicated in the colonization process have been described: (i) flagella, composed of the flagellin FliC and the flagellar cap protein FliD, involved in cell and mucus attachment (Tasteyre *et al.*, 2001a); (ii) a cell-surface protein with adhesive properties, Cwp66, with two domains, the N-terminal anchoring domain (Cwp66-Nterminal) and the C-terminal variable, surface-exposed domain (Cwp66-Cterminal) (Waligora *et al.*, 2001); (iii) a fibronectin-binding protein, Fbp68 (Hennequin *et al.*, 2003); (iv) S-layer proteins (Calabi *et al.*, 2002).

Immunoblot analysis of serum IgG response to EDTA-extracted surface proteins of *C. difficile* strains in patients with antibiotic-associated diarrhoea suggests that *C. difficile* proteins other than toxins can induce an immune response (Pantosti *et al.*, 1989). Mulligan *et al.* (1993) observed a higher level of serum immunoglobulins directed to *C. difficile* somatic cell antigens in asymptomatic carriers than in symptomatic patients, suggesting the importance of the immune response in the development of the disease.

The aim of this work was to assess the immune response directed to FliC, FliD, Cwp66 and Fbp68 in sera of 33 patients with *C. difficile*-associated disease (CDAD) compared to sera of control subjects in order to evaluate the immunogenicity of these adhesins and their role in the pathogenic process.

Methods

The fliC, fliD, cwp66-Nterminal, cwp66-Cterminal and fbp68 genes of the *C. difficile* strain 79-685 were cloned into the Escherichia coli expression vector pGEX-6P-1 and expression was induced with isopropyl β-D-thiogalactopyranoside. The recombinant proteins were purified by affinity chromatography on glutathione–Sepharose as previously described and quantified by the Bio-Rad protein assay.
(Tasteyre et al., 2000a, 2001b; Waligora et al., 2001; Hennequin et al., 2003). Toxins A and B were a kind gift from Dr M. Popoff, Institut Pasteur, Paris, France.

Samples were obtained from 33 patients (19 males and 14 females) with CDAD aged 6–95 years from various hospitals in Brussels, Belgium. The diagnosis of C. difficile disease was confirmed by culture and detection of toxin B in faecal samples by classical cytotoxic assay on Chinese hamster ovary cells (Mahe et al., 1987). The sera were obtained 1–3 weeks after the diagnosis. Control subject sera were composed of 35 sera from healthy women attending a maternity ward and 5 sera from children aged 1.5 months to 4.5 years with C. difficile negative stool culture (culture- and toxin-negative) from Jean Verdier hospital, AP-HP group, France.

Anti-FliC, FliD, Cwp66-Nterminal, Cwp66-Cterminal and Fbp68 and anti-toxin A and B antibodies were detected by an ELISA in sera. Wells of a 96-well microtitre plate (Immunomaxi; TPP) were coated with anti-toxin A and B antibodies were detected by an ELISA in sera. Wells were subsequently washed five times with PBS (pH 7.4) containing 0.1 % (v/v) Tween 20 (PBS-T). Blocking of remaining sites on the plastic was achieved by an overnight incubation with 1 % (w/v) BSA in PBS-T. After three washings, 100 μl of 1/100 diluted serum samples were added to the wells and incubated at room temperature for 90 min. After washings, positive reactions were detected by successive incubations with a goat anti-human polyvalent immunoglobulin conjugated to biotin (1/5000 dilution; Sigma) for 30 min at 37 °C and with a streptavidin–horseradish peroxidase conjugate (1/1000 dilution; Sigma) for 30 min at 37 °C. The final reaction was visualized by addition of 3,3',5,5'-tetramethylbenzidine (Sigma). After 10 min, the reaction was stopped with 100 μl of 0.9 M H2SO4. A450 values were measured using a microplate ELISA (Anthos II; Labtech Instruments). All samples in this study were treated simultaneously to avoid interassay variation.

The background level was defined as the mean absorbance measured with PBS-T-BSA as control for each protein tested. Samples yielding an absorbance five times greater than the background absorbance were reported as positive (Warny et al., 1994). The mean absorbance was calculated for each antigen for the CDAD patient group in order to evaluate the immunogenicity of each protein.

The specificity of the ELISA was further confirmed by an immune absorption test. Positive samples were preincubated with each protein at varying concentrations (3–50 μg ml−1).

Statistical analyses were done to compare the antibody level in the CDAD patient group with that in the control group and showed that antibody measurements were not normally distributed. Therefore, we used Wilcoxon’s rank score test to test the null hypothesis that there was no difference between patients with CDAD and patients from the control group. Similar analysis was done to compare in the CDAD patient group (i) the immunological response from males versus females and (ii) the antibody level between the different proteins tested. Analyses were done with the SAS software system, version 8.2. Statistical significance was set at P = 0.05. All P values were two-sided.

### Results and Discussion

For all the antigens tested, a dose-dependent reduction observed in the ELISA blocking test confirmed the specificity of the immunoenzymic reaction (data not shown). Statistical analysis revealed no significant difference in immune response to each C. difficile protein tested with respect to gender in the CDAD patient group.

In the CDAD patient group, according to the cut-off value defined above, the percentage of positive sera was 60 % for toxin A and only 25 % for toxin B. In the control group composed of 35 healthy women and 5 children aged 1.5 months to 4.5 years, 75 % of the sera were toxin A-positive and 24 % were toxin B-positive. In other survey studies, systemic antibody responses to toxin A or B were detected in 50–75 % of patients who developed C. difficile diarrhoea (Viscidi et al., 1983; Warny et al., 1994; Aronson et al., 1985). For each surface protein, the percentage of positive sera in the CDAD patient group and the control group was, respectively: Cwp66-Cterminal, 78 and 85 %; Cwp66-Nterminal, 6 and 10 %; Fbp68, 91 and 100 %; FliD, 87 and 98 %; and FliC, 21 and 53 %.

In the CDAD patient group, among the seven antigens tested the mean of the antibody levels decreased from Cwp66-Cterminal (A450 1.31 ± 0.14) to Fbp68 (A450 0.91 ± 0.09), FliD (A450 0.50 ± 0.06), toxin B (A450 0.49 ± 0.12), toxin A (A450 0.47 ± 0.1), Cwp66-Nterminal (A450 0.27 ± 0.04) and finally FliC (A450 0.20 ± 0.04) (Fig. 1). The Cwp66-Cterminal domain seems to be highly immunogenic. This domain is surface-exposed (Waligora et al., 2001) and can induce a strong immune response. In contrast, the mean of the absorbance for the N-terminal domain is significantly lower than for the C-terminal domain (P < 0.0001). This is probably because this domain is embedded in the cell wall (Waligora et al., 2001). The low immune response directed to FliC may result from the high variability of the surface-exposed antigenic part of the flagella. In fact, sequencing of the flagellin gene showed high conservation in the N-terminal and C-terminal regions but pronounced variability in the central domain (Tasteyre et al., 2000b). The N- and C-
terminal parts are responsible for secretion and polymerization of flagella, whereas the central region constitutes the surface-exposed antigenic part of the flagellar filament as described by Winstanley & Morgan (1997). This central region might be too variable to elicit antibodies specifically detectable with the FliC antigen originating from the C. difficile strain 79-685. Compared to FliC, the high mean level of antibodies directed to FliD (significant difference, $P < 0.0001$) could be explained by the presence of specific conserved domains, which could have a function in attachment to specific cell or mucus receptors (Tasteyre et al., 2001b). The localization of Fbp68 on the bacterial surface could be responsible for the high immune response against this protein. Furthermore, Fbp68 displays significant homology with the adherence and virulence protein A (PavA) of Streptococcus pneumoniae and FBP54 of Streptococcus pyogenes (Hennequin et al., 2003), so a cross-reaction might explain the high level of antibodies observed. Toxins seemed to be less immunogenic than adhesins. In fact, the mean absorbance values obtained for toxins A and B were significantly lower than for Cwp66-Cterminal, FliD and Fbp68 ($P < 0.0001$). In fact, it has been shown that toxins are highly variable (Rupnik et al., 1998).

For each antigen, we compared specific antibody levels in the CDAD patient group and in the control group. The difference in antibody level observed between these two groups for Cwp66-Nterminal, FliC, FliD and Fbp68 was statistically significant (Fig. 2), with a highest level in the control group (Cwp66-Nterminal, $P = 0.0043$; FliC, $P = 0.0001$; FliD, $P = 0.0034$; and Fbp68, $P = 0.0015$). This suggests that a defence mechanism of the host against these proteins could be important during the course of infection.

With regard to toxins A and B, the two groups were not statistically significantly different: for toxin A, $P = 0.55$, and for toxin B, $P = 0.79$ (Fig. 2). Interestingly, if we compare toxins A and B to the adhesins, the difference between the patient group and the control group was not significant as far as the toxins were concerned ($P = 0.5$), but was significant for the adhesins taken as a whole ($P = 0.004$). It should be noted that the antibody level was always higher for the

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Fig. 2. Antibody levels ($A_{450}$) against toxins A and B, Cwp66-Nterminal and -Cterminal, FliC, FliD and Fbp68 in the CDAD patient group and in the control group. Boxes, median, 25th and 75th percentiles; bars, 10th and 90th percentiles; square, outlying values. $P$ value, comparison of antibody levels between the two groups by Wilcoxon’s rank score test.
control group, except for Cwp66-Cterminal. This suggests that a low antibody level against adhesins can allow the colonization process.

Other authors have studied the immune response directed to non-toxic antigens. Mulligan et al. (1993) measured serum IgA and IgM antibodies against C. difficile somatic cell antigens and found higher antibody levels in asymptomatic carriers than in patients with C. difficile diarrhea. Similarly, serum levels of IgG antibody against non-toxic antigens (a sionicate of two non-toxigenic strains) were higher in the asymptomatic carriers than in the patients with diarrhea, but these differences were not statistically significant (Kyne et al., 2000). These results are concordant with our results on specific surface proteins comparing antibody levels in a CDAD patient group with those in a control group composed of asymptomatic subjects unknown to be healthy carriers or non-carriers. In our study, only one serum sample was analysed for each CDAD patient. It might be interesting to test several samples during the course of infection to follow the immune response during the pathogenic process. These investigations are under way.

In conclusion, this study suggests that during the clinical course of disease C. difficile adhesins are able to induce an immune response which could play a role in the defence mechanism of the host.

References


