Journal of Medical Microbiology (2008), **57**, 717–724 DOI 10.1099/jmm.0.47713-0

Systemic antibody response to *Clostridium difficile* in colonized patients with and without symptoms and matched controls

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It has been proposed that patients who develop *Clostridium difficile*-associated disease (CDAD) do so because they are unable to mount an adequate immune response. Serum was collected from three groups of elderly in-patients: (i) cases (n=21) of CDAD, being toxin A/B-positive; (ii) carriers (n=21) asymptomatic for CDAD (no diarrhoea) but at least toxin or culture positive; and (iii) controls (n=26) asymptomatic for CDAD and negative for both *C. difficile* toxin and culture. The age and gender of each group were compared, and the colonizing strains were ribotyped and toxinotyped. Serum antibodies (IgG and IgM) were measured by ELISA using different antigen preparations: EDTA extract (containing cell-surface proteins and carbohydrates), guanidine hydrochloride extract (surface-layer proteins), aqueous phenol-extracted lipocarbohydrate (LC); crude toxin (dialysis culture supernatant) and purified toxin A. LPS from *Escherichia coli* was used as a control antigen. Antibodies were also tested for toxin neutralization on tissue monolayers and for binding to EDTA-extracted antigens by Western blotting. IgG antibody measurements to cytomegalovirus (CMV) were included as an indicator of potential immunosenescence. Results showed that the patient groups were well matched by age and gender, and the colonizing strains were similar in cases and carriers, being predominantly ribotype 001 and toxinotype 0. By ELISA, IgG levels to most of the antigens were highest in the cases and lowest in the controls, with the exception of antibodies to the LC, which were higher in the controls than the cases. Levels in the carriers tended to be of intermediate level or similar to the controls. For all antigens, the levels of IgM were not significantly different among cases, carriers and controls. Serum from all groups was able to neutralize the cytotoxic action of toxin on both Vero and Caco2 cells, and all to a similar extent. Western blots showed an overall higher level of IgG antibodies to the EDTA-extracted antigens in the cases. The results of the CMV ELISA showed that specific IgG was detected in more cases (78 %) than carriers and controls (both 65 %), but this difference in seropositivity was not significant. The conclusion is that, during symptomatic infection, patients respond to protein antigens of *C. difficile* in a manner typical of a secondary antibody response, with no evidence that an inability to respond predisposes to the appearance of symptoms.

Received 23 October 2007
Accepted 30 January 2008

INTRODUCTION

Cases of *Clostridium difficile*-associated disease (CDAD) continue to increase with a seemingly concomitant higher proportion of patients with more-severe disease requiring surgery or contributing to death. In many parts of Europe and North America, this has coincided with the emergence of a hypervirulent strain, known as ribotype BI, NAP1 or 027 (Bartlett, 2006). Such strains have the potential to cause more-severe disease by producing higher levels of toxin (Warny *et al.*, 2005).

However, there is the belief that the immune response of the host is crucial in determining the outcome of colonization with *C. difficile* and may be independent of the infecting strain. The ability of the host to produce specific antibodies against toxins and/or cellular antigens (Mulligan *et al.*, 1993; Kyne *et al.*, 2000) and to initiate the

Abbreviations: AP, alkaline phosphatase; CDAD, *Clostridium difficile*-associated disease; CMV, cytomegalovirus; LC, lipocarbohydrate; SLP, surface-layer protein.

most appropriate cellular response (Johal *et al.*, 2004) have been postulated as key factors to explain the variability in the response of the host to colonization with *C. difficile* by conferring protection against the negative effects of the bacterium. In particular, the inability of a patient to mount an adequate antibody response to the toxins (or other antigens) may be crucial for their predisposition to recurrent episodes of disease after an initial resolution of symptoms (Kyne *et al.*, 2001). It is considered by some that the host response to non-toxin antigens is as important as that to toxins, by preventing adhesion or other pathogenic mechanisms (Drudy *et al.*, 2004; Péchiné *et al.*, 2007).

Most of the world's population has been infected at some point in their lives with cytomegalovirus (CMV). Once infected, the virus usually lies dormant until the immune system is weakened, and only causes problems in vulnerable groups such as babies, pregnant women or the immunocompromised, for example transplant patients, those on chemotherapy for malignancies and human immunodeficiency virus patients. In these groups, infection can severely affect organs including the eye, liver, gastrointestinal system and nervous system (Ogilvie, 2002). Recently, immunosenescence, a condition recognized in the elderly and linked to oligoclonal expansion mainly of $CD8^+$ T cells, has been linked to seropositivity to CMV (Pawelec *et al.*, 2006). Infection with CMV at any age can bring about these changes in T-cell subsets that are similar to age-related changes, with possible consequences for susceptibility to other infections (Looney *et al.*, 1999). The CMV status of a patient might therefore be linked to susceptibility to *C. difficile*. CMV has also been reported as a cause of pseudomembranous colitis in cases of severely immunocompromised patients (such as AIDS, leukaemia and transplant patients), both on its own (Olofinlade & Chiang, 2001; Cadena *et al.*, 2007) and during co-infection with *C. difficile* (Ives & Smith, 1996; Riva *et al.*, 2005). Such a co-infection with CMV in patients carrying *C. difficile* might help to precipitate the symptoms associated with CDAD, and therefore patients carrying both CMV and *C. difficile* might be at a higher risk of developing symptoms.

The main aim of this study was to explore the different abilities of patients to mount an antibody response to toxins and cell-associated antigens by comparing the outcome of colonization with *C. difficile* in terms of antibody responses in three groups of hospitalized elderly patients: (i) confirmed cases of CDAD, (ii) asymptomatic carriers of *C. difficile*, and (iii) asymptomatic controls who were negative for *C. difficile*. Secondary aims were to identify any differences among the patient groups in terms of gender or age, the strains infecting them and their CMV status.

METHODS

Bacterial strains. An example of the local epidemic ribotype 1, toxinotype 0 strain, MPRL 338a (McCoubrey, 2002), was chosen as the strain from which antigens were extracted, as many of the patients were likely to be colonized with this strain. Throughout the study, strains were maintained as spore suspensions in cooked meat broth at room temperature.

Patients and collection of specimens. Patients were over 65 years of age, in long-stay hospitalization and under antibiotic treatment. Those with dementia were not considered, as they could not consent for themselves. Blood and stool samples were collected in accordance with the Local Research Ethics Committee consent from every major hospital in Edinburgh, with the majority – cases, carriers and controls – from the Royal Victoria Hospital, the main geriatric assessment and rehabilitation hospital for the north of Edinburgh. Blood was allowed to clot overnight at 4 °C, and serum was separated by centrifugation and stored at −20 °C.

Patients were arranged retrospectively into three groups: (i) cases, diagnosed CDAD cases (toxin A/B positive), symptomatically colonized with *C. difficile*; (ii) carriers, asymptomatic for CDAD (no diarrhoea), but at least toxin A/B positive or culture positive; and (iii) controls, asymptomatic for CDAD, and both *C. difficile* toxin (A/B) negative and culture negative. The cases were selected by the requirement that they were toxin positive in stools to avoid misdiagnosis of the symptoms. If the patient had diarrhoea or was suspected to have CDAD but was toxin negative in stool samples, they were not considered for the study, independent of their colonization status. It was not possible to interrogate patients or see their notes to see whether they had experienced CDAD or diarrhoea previously.

The timings of collection of blood and stool samples from the patients were as close together as possible, ideally within 24 h. However, it should be noted that for all patients, and in particular for the carriers and controls, it was impossible to know their carriage status at the precise time of sampling and, if they were carrying *C. difficile*, for how long they had been colonized.

Culture of stool samples and identification of *C. difficile*. Stools were cultured directly on Brazier's cefoxitin cycloserine egg yolk (CCEY) agar (Oxoid) and incubated at 37 °C under anaerobic conditions (10 % H_2, 10 % CO_2, 80 % N_2) for 24–48 h. Positive results were seen within that period. If negative, the plates were incubated for a further 48 h to confirm the negative results. Preliminary identification of *C. difficile* was carried out by colony morphology, a characteristic smell, visualization of motility by phase-contrast microscopy and chartreuse fluorescence under long-wave UV light (Wood's lamp). Further identification included a Gram film and toxin assay.

Toxin assays. Toxin was measured either in fresh stools (less than 24 h after collection) or following isolation of pure cultures of *C. difficile* and growth at 37 °C under anaerobic conditions (10 % H_2, 10 % CO_2, 80 % N_2) for 5–6 days in proteose peptone yeast extract medium (Deacon *et al.*, 1978) using a commercially available kit (*C. difficile* toxin A+B; Techlab) following the instructions of the manufacturer.

Ribotyping. PCR ribotyping was carried out following the method described by O'Neill *et al.* (1996). Specific oligonucleotide primers complementary to the 3′ end of the 16S rRNA gene (5′-CTGGGGTGAAGTCGTAACAAGG-3′, nt 1445–1466) and the 5′ end of the 23S rRNA gene (5′-GCGCCCTTTGTAGCTTGACC-3′, nt 1–20) were used to amplify the variable-length intergenic spacer region. Primers were obtained from MWG.

The total volume of the PCR was 100 µl containing PCR buffer [20 mM Tris/HCl (pH 9.0), 50 mM KCl], 2.25 mM $MgCl_2$, 2 U *Taq* polymerase (all from Invitrogen), 200 µM each dNTP (Amersham Pharmacia Biotech), 50 pmol each primer and 10 µl template DNA. Initial denaturation was carried out at 94 °C for 3 min. The mixtures

were then subjected to 35 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min and extension at 72 °C for 2 min. A final elongation step was included at 72 °C for 5 min.

The amplification products were concentrated to a final volume of 12–15 μl by heating at 75 °C for 100 min, and run on 3 % MetaPhor agarose gels (ABgene) at 80 V for 2.5 h. A 100 bp DNA ladder (ABgene) was used as a size marker.

Patterns were identified by comparison with those already included in the laboratory database. The concentrated PCR products of those that were different were sent to the Anaerobe Reference Unit in Cardiff (UK) to be identified.

Toxinotyping. Toxinotyping was carried out following the method described by Rupnik *et al.* (1997, 1998). The first 3 kb of the toxin B gene (*tcdB*, PCR fragment B1) and the 3 kb repetitive region of the toxin A gene (*tcdA*; PCR fragment A3) were detected and characterized by RFLP. Primers were obtained from MWG. The primers used were: A3C (5′-TATTGATAGCACCTGATTTATAT-ACAAG-3′) and A4N (5′-TTATCAAACATATATTTTAGCCATATA-TC-3′) for *tcdA* (3100 bp amplicon); and B1C (5′-AGAAAA-TTTTATGAGTTTAGTTAATAGAAA-3′) and B2N (5′-CAGATAA-TGTAGGAAGTAAGTCTATAG-3′) for *tcdB* (3100 bp amplicon).

PCRs were performed in a final volume of 50 μl containing PCR buffer [20 mM Tris/HCl (pH 9.0, 50 mM KCl], 3 mM $MgCl_2$, 1 U *Taq* polymerase (all from Invitrogen), 200 μM each dNTP (Amersham Pharmacia Biotech), 15 pmol each primer and 5 μl template DNA. For amplification of A3 fragments, 1 % W-1 detergent (Invitrogen), tetramethylammonium chloride (final concentration 10^{-4} M; Sigma) and BSA (final concentration 0.1 mg ml^{-1}; Promega) were also added. After initial denaturation at 93 °C for 3 min, B1 products were amplified for 30 cycles and A3 products for 35 cycles of annealing and extension at 47 °C for 8 min, and denaturation at 93° for 4 s. Final extension was performed at 47 °C for 10 min.

Amplified fragments were subjected to restriction enzyme digestions using *Acc*I and *Hinc*II for B1, and *Eco*RI for A3. Restriction enzymes were obtained from New England BioLabs. Incubation with 1 μl (in a final volume of 20 μl) of the enzymes was carried out at 37 °C for 2 h. In the restriction of B1 with *Hinc*II, 10 % BSA (Promega) was also added. After electrophoresis of the digestion products in 1 % agarose gels, toxinotypes of all tested isolates were identified as described by Rupnik *et al.* (1997, 1998).

Production of crude toxin by dialysis culture. Crude toxin was produced as described by Sánchez-Hurtado & Poxton (2008) (this issue). The supernatant, which contained toxins A and B together with other extracellular products, including surface-layer proteins (SLPs) (shown by SDS-PAGE), but none of the culture medium components, was filter-sterilized and stored at 4 °C.

Purification of toxin A. Toxin A was purified as described by Sánchez-Hurtado & Poxton (2008).

EDTA extraction of surface proteins and extraction of SLPs. EDTA extraction of surface proteins and extraction of SLPs were carried out as described by Sánchez-Hurtado & Poxton (2008).

Extraction of surface lipocarbohydrate (LC). This method was based on those described previously by Poxton & Cartmill (1982) and Sharp & Poxton (1986), and was carried out as described by Sánchez-Hurtado & Poxton (2008). LPS from an *Escherichia coli* R mutant was taken from our laboratory stocks. It had been prepared by petroleum/chloroform/phenol extraction and complexed with polymyxin B, and was prepared and used in ELISA as described by Gibbs *et al.* (2004).

ELISA. PolySorp plates (Nunc) were coated with 100 μl of the appropriate concentration of the chosen antigen in antigen buffer [0.05 M sodium carbonate buffer (pH 9.6), 0.02 % sodium azide] and incubated overnight in darkness at room temperature. Plates were washed in PBS (pH 7.4; Oxoid) four times and blocked with 100 μl TG buffer [0.05 M phosphate buffer (pH 7.4) containing 2 % teleostean gelatin and 0.1 % Tween 20] for 90 min. After four washes in PBS, the plates were incubated for 90 min with 100 μl of the chosen serum diluted 1 : 10 in antiserum buffer [0.05 M sodium phosphate buffer (pH 7.4) containing 0.085 % NaCl, 0.05 % Tween 20 and 0.02 % sodium azide] and then washed four times as above. The plates were then incubated with a 1 : 10 000 dilution of anti-human (IgG or IgM) alkaline phosphatase (AP) conjugate (Sigma) in antiserum buffer for 90 min and developed with AP substrate (Sigma tablets) in AP substrate buffer [0.05 M sodium carbonate (pH 9.8) containing 1 mM $MgCl_2$]. The A_{405} was measured at 15 min intervals for 1 h in an ELISA plate reader (Anthos Reader 2001) with the reference filter at 620 nm. All assays were carried out at least twice.

Three different negative controls (buffer only, antigen and no serum, serum and no antigen) were included. Background levels were in the range of 0.03–0.05 absorbance units (405/620 nm). A standard positive control [pooled serum from young (under 65 years old) healthy, non-hospitalized volunteers] was included on each plate to ensure reproducibility of the assay, and each set of assays was carried out on the same one or two plates from the same batch.

Western blotting. Surface proteins were extracted with EDTA as described above. Samples were mixed in equal volumes of double-strength sample buffer, heated at 100 °C for 3–5 min and then analysed by 10 % bisacrylamide/Tris PAGE (Invitrogen) following the manufacturer's instructions and using MOPS SDS running buffer supplied by the manufacturer. Gels were run at 150 V until the dye front reached the bottom of the gel (~30–45 min). The separated antigens were transferred to a nitrocellulose membrane at 150 V for 100 min at 4 °C. The membrane was then blocked with 8 % skimmed milk powder (Marvel) in PBS overnight at 4 °C. After three 10 min washes in PBS at room temperature, the membrane was incubated with rocking in a 1 : 1000 dilution of human serum in 1 % skimmed milk powder/PBS/0.05 % Tween 20 for 3 h at room temperature. The membrane was then washed three times (10 min each, room temperature) with 1 % skimmed milk powder/PBS/0.05 % Tween 20, followed by incubation with secondary antibody (anti-human IgG horseradish peroxidase-conjugate) at a 1 : 10 000 dilution in 1 % skimmed milk powder/PBS/0.05 % Tween 20 for 90 min. After three 10 min washes in 1 % skimmed milk powder/PBS/0.05 % Tween 20 at room temperature, the membrane was incubated with enhanced chemiluminescence solution (containing 250 mM luminol and 90 mM *p*-coumaric acid in DMSO) for 5–10 min and analysed by autoradiography using Hyperfilm ECL (Amersham Biosciences) and an X-Ray film developer (Protec).

Cell culture. The Vero cell line is a well established cell line originally obtained from kidney epithelium of the African green monkey, and has been used extensively for testing *C. difficile* cytotoxicity. The Caco2 lineage was obtained from human colonic tumour and is used as a model for the human intestine. This lineage spontaneously differentiates after reaching confluency. Vero cells and Caco2 cells were used from our frozen stocks and grown as described by Sánchez-Hurtado & Poxton (2008). A 10 % inoculum (10^6 cells ml^{-1}) was used to inoculate new flasks.

Although it might have been preferable to use the Caco 2 cells after they had differentiated, it was difficult to ensure that every layer of cells was exposed to the same concentration of toxin. Therefore for both lines the cells were used when they had just reached confluency.

Neutralization of cytotoxic effect with serum. The wells of microtitre plates were seeded with 100 µl cells (Vero or Caco2) and incubated for 24 h until they reached confluency. Non-purified toxin was added to the wells (10 µl 150 µg toxin ml^{-1} solution) and incubated for 3 days with and without the addition of equal volumes (10 µl) of pooled serum from each of the groups of patients or 10 µl medium in the case of blanks. At the end of the incubation period, an MTT assay was used to measure cell viability, as described by Sánchez-Hurtado & Poxton (2008).

CMV IgG ELISA. A commercially available ELISA diagnostic kit for CMV (IBL Hamburg) was used as specified by the manufacturer.

Statistics. All tests were performed with the GraphPad Prism 4 package. Data were checked for normality and then compared with each other. If the distribution of the values was Gaussian, an unpaired *t*-test was performed. If the distribution was not normal, a Mann–Whitney test was used.

RESULTS AND DISCUSSION

Patients

Initially, 69 patients consented to take part and had blood samples taken. Of these, 21 were diagnosed as cases of CDAD by testing positive for toxin A + B. One patient had CDAD-like symptoms but was toxin A + B negative and so was discarded from the study. The remaining 47 were asymptomatic for CDAD (no diarrhoea). However, following stool culture, 21 were found to carry the bacterium (carriers) and 26 were CDAD and *C. difficile* negative (controls). Stool samples were obtained from all asymptomatic patients within the 24 h prior to blood sampling. Of the 21 symptomatic patients, stools were not obtained for culture from 3 of them, but they had already tested positive for toxins A + B in the routine diagnostic laboratory tests. All of the stool samples were tested for toxins A + B as described above and cultured on CCEY agar. Thus, the final 68 patients investigated consisted of 21 cases, 21 asymptomatic carriers and 26 controls.

Of the 18 cases from whom a stool sample was available, 13 produced positive cultures of *C. difficile*. Of the five culture-negative stools, three had been frozen for several days before culturing and two were from patients being treated with metronidazole at the time of sampling.

All of the patients included in the study (n=68) were elderly with an age range from 61 to 95 years, with just one patient under 66. This patient was accepted for the study because she had a degenerative condition that made her biologically older than her chronological age and she was hospitalized in a geriatric unit. The mean age of the group was 81.9 years and the gender ratio was 2 : 1 female : male (44 females, 22 males, 2 not recorded). Among the cases, this ratio decreased to 1.7 : 1 female : male (12 females, 7 males, 2 not recorded), the carriers were 2 : 1 (14 females, 7 males) and controls had a slightly higher proportion of females, 2.3: 1 (18 females, 8 males). Overall, the gender ratio was not considered significantly different among the groups. The mean age of the subgroups was 82.9 years for cases, 82.8 for carriers and 80.5 for controls. Although the mean age of the control group was 2 years younger than the cases and controls, these differences were not significant.

Strains of *C. difficile* isolated

The isolates from cases and carriers were characterized to see whether disease could be explained by the strain with which the host was colonized. From the culture-positive stool samples, 32 isolates (13 cases and 19 carriers) were selected for genotyping by PCR ribotyping and toxinotyping, as well as being tested for the ability to produce toxin *in vitro*. The isolates were predominantly ribotype 001 (n=23, 72 %), followed by ribotype 106 (n=4, 12.5 %), with single examples of ribotypes 010, 023, 046, 070 and 139. Most were toxinotype 0 (n=29, 91 %), with one each of toxinotypes I and IV, and one non-toxigenic isolate. These reflect the types of strain isolated previously from patients in south-east Scotland, where Mutlu *et al.* (2007), upon investigating 149 isolates, found that 75.8 % were ribotype 001, 8.1 % were ribotype 106 and 96.6 % were toxinotype 0. The hypervirulent strain ribotype 027 was not found in this population.

Symptomatic patients all produced toxin-positive stools by definition, and the isolates subsequently obtained from them all produced toxin *in vitro*. Of the asymptomatic patients investigated by culture, 19 were culture positive. Of the subsequent cultures, 18 produced toxin when incubated *in vitro*. The one that did not produce toxin after 1 week of incubation was confirmed as a non-toxigenic strain belonging to ribotype 010 and was non-toxinotypable as described above.

Of the common ribotypes, 001 was found in 9 cases and 14 carriers, and 106 in 1 case and 3 carriers, both reflecting the proportion of cases and carriers. Similarly, the 29 toxinotype 0 strains were found in 11 cases and 18 carriers, with the 2 uncommon toxinotypes both being in cases and the non-toxin-producing ribotype 010 in a carrier.

Antibody response to toxin and cell-surface antigens

Serum levels of IgG and IgM antibodies against crude and pure toxin A, EDTA-extracted surface antigens, SLPs and the LC of *C. difficile*, as well as the core of *E. coli* LPS, were measured by ELISA. The results are summarized in Fig. 1(a) for IgG and Fig. 1(b) for IgM. Overall, for most of the antigens, both IgG and IgM levels were largely similar among the three groups of patients, with a tendency for highest levels in the cases and lowest in the controls.

In Fig. 1(a), the IgG antibody levels against non-purified toxin were highest in the cases. When the means were compared, there was a significant difference between the cases and controls (P=0.042), but not between cases and carriers (P=0.138). However, when antibodies against

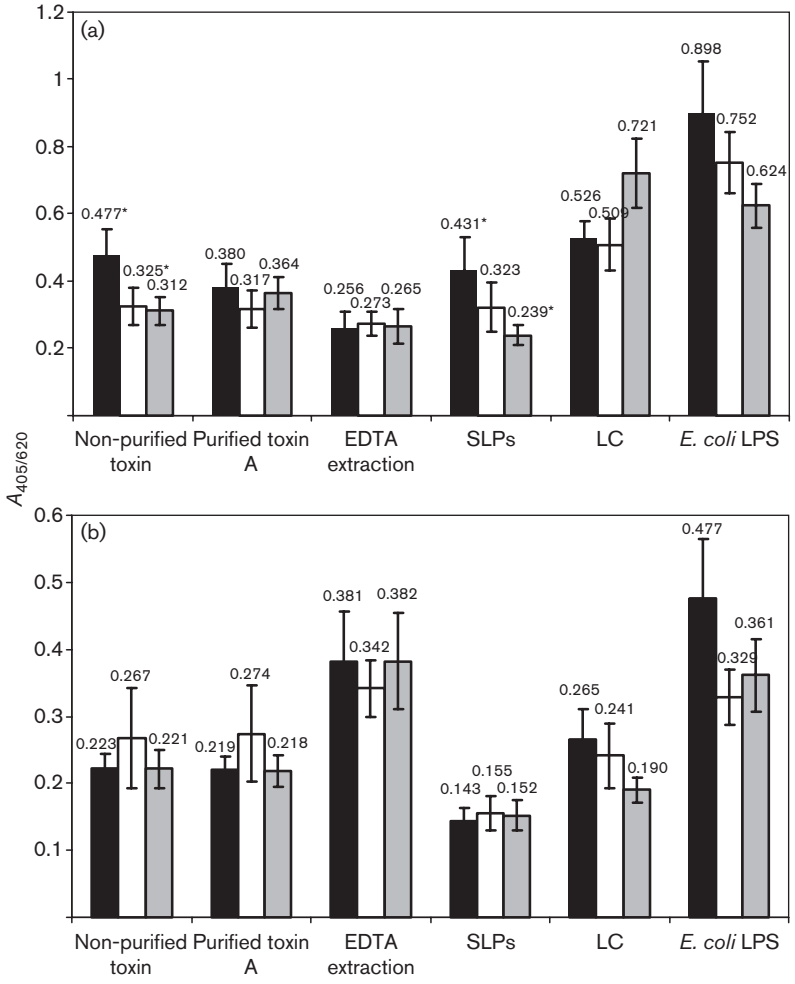


Fig. 1. Antibody responses to antigens of *C. difficile* and *E. coli* LPS analysed by ELISA for IgG (a) and IgM (b). Results are shown for cases (n=21, black bars), carriers (n=21; white bars) and controls (n=26; grey bars), and are given as mean absorbance values ± SEM. *, $P<0.05$.

purified toxin A were measured, no differences were found among the groups.

The IgG antibody levels against the EDTA extract, which contains surface proteins and carbohydrates, were the same for the three groups ($P>0.750$). However, when the antibody levels against the guanidine hydrochloride extraction (which contains the two SLPs) of the same standard strain were analysed, differences were found. These were similar to the non-purified toxin, where levels were highest in the cases. Means were analysed statistically and again differences were significant between cases and controls ($P=0.042$), but not between cases and carriers ($P=0.373$). The levels of antibodies against the surface LC of *C. difficile* were highest in the control group, but this difference was not statistically significant. No difference between the cases and controls was seen.

The results for IgM antibodies (Fig. 1b) were similar to the IgG levels, but no statistically different levels were measurable among any of the groups. The IgM levels against the surface proteins, both EDTA (surface proteins and carbohydrates) and guanidine hydrochloride (SLPs) extractions of a standard strain, were equal among all three groups. This was similar for the surface LC of *C. difficile*, where no significant differences were found.

The cases had the highest levels of IgG antibodies against the core of *E. coli* LPS, followed by the carriers and then the controls (Fig. 1a). For IgM (Fig. 1b), the highest levels were again in the cases, but the lowest levels were in the carriers. However, none of these differences were statistically significant.

Antibodies against the core of *E. coli* LPS were measured as controls with a view to investigating whether the differences among the groups were specific to *C. difficile* or were due to the general state of the immune system. The core oligosaccharide of *E. coli* LPS is an immunogenic antigen to which everyone is exposed, and antibodies – both IgG and IgM – can be detected in everyone (Poxton, 1995). The kinetics of circulating anti-LPS core antibodies have been proposed to be a good indicator of systemic exposure to LPS/endotoxin during sepsis (Bennett-Guerrero *et al.*, 1997). If there has been increased LPS exposure to the systemic immune system during damage to the affected gut in the cases, then it would be expected that the antibody levels might be boosted. In contrast, if the

patients were endotoxaemic/septic, which is not usual in CDAD patients, then the levels of anti-LPS antibodies would be expected to fall.

The functionality of the antibodies in the sera was measured by neutralization of toxin-induced cytotoxicity in Vero and Caco2 cells, and the results are shown in Fig. 2. It can be seen that the sera from each of the three groups (cases, carriers and controls) neutralized the toxin when compared with the toxin-only control ($P<0.0001$, asterisk not shown on figure). However, there were no significant differences among the sera from the three different groups.

The results of Western blotting on the EDTA-extracted antigens indicated that the serum pooled from cases gave a stronger signal and reacted with more bands than the sera from carriers and controls. There was a particularly strong reaction to two bands of approximately 50 and 35 kDa, which correspond to the SLPs, and a third, smaller band of just under 15 kDa (results not shown). Those patients symptomatically colonized by *C. difficile* had a higher level of IgG against several surface proteins compared with those not colonized or colonized but without symptoms.

In summary, and contrary to our initial hypothesis, cases (patients symptomatically colonized with *C. difficile*) did not have lower levels of antibodies to *C. difficile* compared with carriers when measured by three different methods: ELISA, neutralization of cytotoxicity and Western blotting against surface proteins, and their ability to respond to specific *C. difficile* antigens and to LPS did not appear to be compromised; perhaps, in retrospect, this is not surprising. The results of this study agree with other studies that did not find significant differences in the levels of systemic antibodies between CDAD cases and controls (Drudy *et al.*, 2004; Johal *et al.*, 2004). Péchiné *et al.* (2005) showed that CDAD patients had lower levels of specific antibodies to different cell-wall antigens than women in maternity wards and children.

In all of the patients tested, including the controls, and in large numbers of healthy individuals who have not been reported on in this study, there is a detectable level of pre-existing antibodies to *C. difficile*. These antibodies will be produced throughout life and perhaps initially stimulated in infancy by constant environmental exposure to *C. difficile* itself and perhaps to other clostridia, such as *Clostridium sordellii*, which possess cross-reacting antigens. It seems likely that antibody levels begin to be boosted following colonization and that this is further enhanced by the disease itself, as is typical of a secondary antibody response.

Kyne *et al.* (2000) found that the symptomatic patients had lower levels of IgG against toxin A and of IgM against non-toxin antigens, but they also found that the symptomatic patients had on average more-severe diseases and that a higher percentage was admitted to intensive care units. This information was not available for our study. However, it is possible that the cases had more-severe underlying diseases than the carriers, and that perhaps it is this that is compromising a protective immune response against *C. difficile* before CDAD develops. The healthier carriers may be able to mount a protective response prior to the development of CDAD.

Another possibility is that the key feature is the point at which antibodies are produced, rather than on the ability per se to produce the antibodies or the levels of antibodies produced. If individuals are exposed for long enough to *C. difficile* before their gut microbiota is severely affected, they might be able to produce specific antibodies that could be protective, even if the microbiota is affected afterwards. The level of antibodies before infection with *C. difficile* might also be crucially important in stopping symptoms developing. Another point to note is that, for ethical reasons, patients that were medically very unwell, or indeed terminally ill with CDAD, were not approached to join the

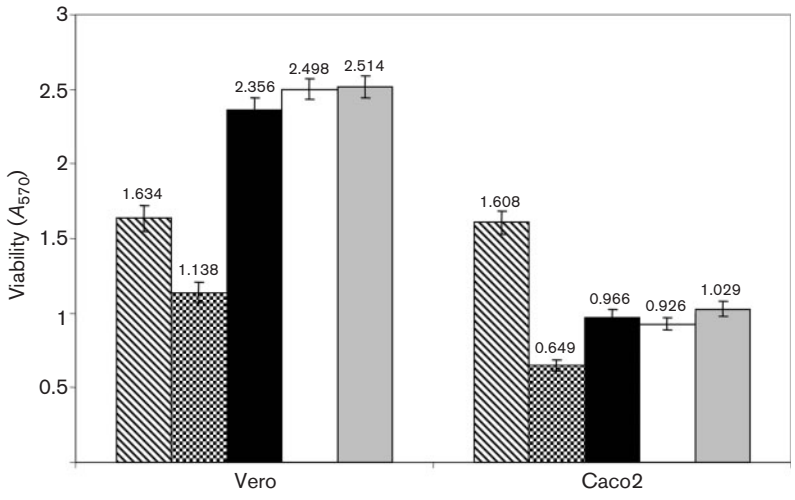


Fig. 2. Neutralization of toxin cytotoxicity by addition of pooled serum from each of the patient groups. Results are shown as mean A_{570} values ± SEM for the 21 cases, 21 carriers and 26 controls. Each test was carried out in duplicate. Hatched bars, unchallenged cells; spotted bars, cells + toxin; black bars, cells + toxin + sera from cases; white bars, cells + toxin + sera from carriers; grey bars, cells + toxin + sera from controls.

study. It is therefore possible that the immune response of these individuals would have differed from that found in our cases.

However, the fact that the antibody levels were only a little higher in the cases might indicate that, even if they are not significantly immunocompromised, their immune system could be inefficient due to age (immunosenescence) and/or other diseases and infections. The mean age of the patients of our study was 82 years and only patients aged 65 or over were recruited. In the study by Kyne *et al.* (2000), patients of all age groups were included, with 23 % being under 65 years, with a resultant mean age of 74.

The significantly higher levels of IgG against non-purified toxin and against SLPs is an indication that, at least at one point, cases were able to respond to *C. difficile*. Note that the non-purified toxin contained SLPs, and this may be the reason why there were higher levels of antibody. Measuring IgM levels is probably not very useful, as all of the subjects were probably already producing antibodies and infection would only produce a boosting of a secondary response, with a primary response being unlikely.

Antibodies to CMV

The levels of IgG against CMV were measured using a commercial ELISA kit. Tests were performed in duplicate on 64 of the patients (18 cases, 20 carriers and 26 controls). The results are summarized in Fig. 3. Of the 64 patients, 44 (69 %) tested positive for CMV antibodies: 14 were cases, 13 were carriers and 17 were controls. One, a carrier, was in the grey area between positive and negative, and the remaining 19 (4 cases, 6 carriers and 9 controls) were negative. These levels are above the normally expected levels of 40–60 % in 'mid-adult life', but this can increase to over 90 % in those with 'multiple intimate exposures' (Ogilvie, 2002). A higher proportion of the cases of CDAD tested positive, with a 13 % difference between cases (78 %) and both carriers and controls (both 65 %). If CMV had reactivated in the patients, it might suggest that cases are in fact more ill – with regard to other illnesses for which they are in hospital – than carriers or controls. More work is needed to support this hypothesis, but it is consistent with the findings of Kyne *et al.* (2000) who analysed the severity of the primary disease in the patients of their study. *C. difficile* is an opportunistic pathogen, and the inadequate immune response that other authors have found may be related to, but not necessarily causing, CDAD. Co-infection of *C. difficile* and CMV or other opportunistic pathogens might be an area worth exploring further.

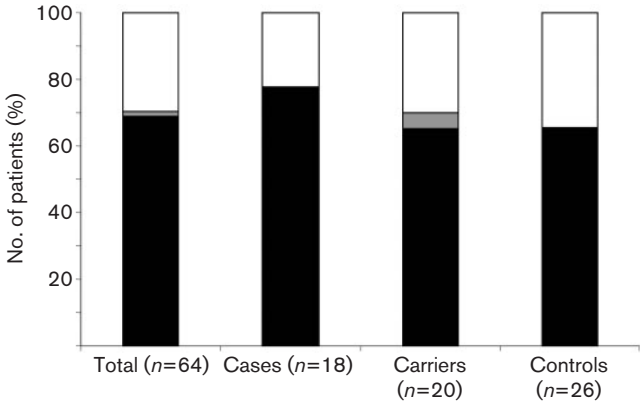


Fig. 3. Proportions of patients giving positive and negative results for anti-CMV IgG by ELISA. The 'grey zone' indicates results that were between positive and negative levels. Black, CMV infection; grey, 'grey zone'; white, no infection.

In conclusion, the patients with CDAD appeared to be able to mount an immune response to *C. difficile* antigens that was better or at least equivalent to the response in asymptomatic carriers of the bacterium. The basis of their predisposition to develop symptoms is not simply an inability to respond; it might well be due to the timing and specificity of the response – both in the gastrointestinal tract (mucosal response) and in the systemic circulation. Future areas of study should include longitudinal studies of both systemic and mucosal responses.

ACKNOWLEDGEMENTS

This study was supported by a grant (CZG/1/159) from the Chief Scientist Office, Scottish Government Health Department, and K. S.-H. was supported by a grant from Fundación Mutua Madrileña. We thank Jon Brazier and colleagues at the Anaerobe Reference Unit, Cardiff, UK, for help in identifying some of the ribotypes.

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