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Protective antibodies against *Clostridium difficile* are present in intravenous immunoglobulin and are retained in humans following its administration

O. H. Negm,*† B. MacKenzie,‡ M. R. Hamed,*† O. A. J. Ahmad,* C. C. Shone,§ D. P. Humphreys,‡ K. Ravi Acharya,¶ C. E. Loscher,** I. Marszalowska,** M. Lynch,** M. H. Wilcox†† and T. M. Monaghan ‡‡

*School of Medicine, Queen's Medical Centre, University of Nottingham, Nottingham, NG7 2UH, UK, †Medical Microbiology and Immunology Department, Faculty of Medicine, Mansoura University, Mansoura, 35516, Egypt, ‡UCB Celltech, Slough, SL1 3WE, UK, §Public Health England, Salisbury, SP4, OJG, UK, ¶Department of Biology and Biochemistry, University of Bath, Bath, BA2 7AY, UK, **Immunomodulation Research Group, Dublin City University, Dublin 9, Ireland, ††Leeds Institute of Biomedical and Clinical Sciences, University of Leeds, Leeds, LS1 3EX, UK, and ‡‡NIHR Biomedical Research Unit in Gastrointestinal and Liver Diseases, Nottingham University and the University of Nottingham, Nottingham, NG7 2UH, UK

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Correspondence: Tanya M. Monaghan, NIHR Biomedical Research Unit in Gastrointestinal and Liver Diseases, Nottingham University and the University of Nottingham, Nottingham NG7 2UH, UK.
E-mail: tanya.monaghan@nottingham.ac.uk

Summary

The prevalence of serum antibodies against *Clostridium difficile* (CD) toxins A and B in healthy populations have prompted interest in evaluating the therapeutic activity of intravenous immunoglobulin (IVIg) in individuals experiencing severe or recurrent *C. difficile* infection (CDI). Despite some promising case reports, a definitive clinical role for IVIg in CDI remains unclear. Contradictory results may be attributed to a lack of consensus regarding optimal dose, timing of administration and patient selection as well as variability in specific antibody content between commercial preparations. The purpose of this study was to investigate retrospectively the efficacy of three commercial preparations of IVIg for treating severe or recurrent CDI. In subsequent mechanistic studies using protein microarray and toxin neutralization assays, all IVIg preparations were analysed for specific binding and neutralizing antibodies (NAb) to CD antigens *in vitro* and the presence of anti-toxin NAbs *in vivo* following IVIg infusion. A therapeutic response to IVIg was observed in 41% (10 of 17) of the CDI patients. Significant variability in multi-isotype specific antibodies to a 7-plex panel of CD antigens and toxin neutralization efficacies were observed between IVIg preparations and also in patient sera before and after IVIg administration. These results extend our current understanding of population immunity to CD and support the inclusion of surface layer proteins and binary toxin antigens in CD vaccines. Future strategies could enhance IVIg treatment response rates by using protein microarray to preselect donor plasma/serum with the highest levels of anti-CD antibodies and/or anti-toxin neutralizing capacities prior to fractionation.

Keywords: antibodies, *Clostridium difficile*, intravenous immunoglobulin

Introduction

Clostridium difficile (CD) is the leading cause of hospital-acquired infective diarrhoea, and is a global health problem [1]. The most prominent risk factor for disease development includes antibiotic use, which disrupts the gut microbiota, leading to loss of colonization resistance and subsequent *C. difficile* infection (CDI). Other major risk factors are prolonged hospital stay, increasing age and underlying co-morbidities [1]. CD exerts its major pathological effects through two proinflammatory and cytotoxic protein exotoxins, A and B. Some strains also produce a third protein toxin, known as binary toxin or CDT. Non-toxin virulence factors such as surface layer proteins (SLPs) also appear to be involved in pathogenesis [2–5]. Several clinical studies have shown previously that antibody-mediated immune responses to CD toxins A and B have an important role in asymptomatic carriage and predisposition to recurrent infection. Specifically, symptomless carriers of toxigenic CD and those who have had a single episode of CDI show more robust anti-toxin immune

responses than those with symptomatic and recurrent disease [6–10]. Circulating toxin A- and B-specific memory B cells have been detected after the development of CDI, strengthening the evidence for the importance of humoral immune responses against both toxins [11].

Early population prevalence studies also indicate that the majority of healthy adults have detectable antibodies to CD toxins A and B in their sera that are thought to arise from colonization in infancy or from repeated environmental exposure to CD in adulthood [12,13]. For this reason, polyclonal IVIg has been used off-label to treat both recurrent and fulminant CDI. Human intravenous immunoglobulin (IVIg) consists of purified plasma immunoglobulins from hundreds to thousands of healthy blood donors. Although several encouraging case reports highlight the potential benefits of IVIg, its definitive clinical role is still unclear, due mainly to the lack of robust evidence from randomized controlled trials [14–17]. Contradictory results obtained in respect to its clinical efficacy may be ascribed, in part, to the poor characterization of commercial IVIg preparations in terms of their specific antibody content.

The mode of action of IVIg remains poorly understood. While some attention has focused on the varying capacity of IVIg to treat recurrent CDI, presumably by neutralizing CD toxins A and B [14], the full repertoire of CD-associated protein targets of these complex preparations remains ill-defined, as do the subclass distribution of these specific antibodies. Furthermore, the exact prevalence, kinetics and individual variation of binding and neutralizing antibodies (NAb) against CD proteins in serum samples, including those exposed to IVIg, are poorly described. Microarray assays are a promising new tool for compositional bioanalysis of specific antibody content in patient sera and IVIg due to their high sensitivity, reproducibility and ease of use.

The aims of this study were to investigate retrospectively the efficacy of three different commercial preparations of IVIg used in our institution for treating severe or recurrent CDI and to determine if these preparations possess specific binding and neutralizing antibodies to CD antigens *in vitro*. In a second cohort of patients receiving IVIg for multiple indications, we also aimed to demonstrate the presence of protective serum anti-toxin NAbs *in vivo* following IVIg infusion.

Materials and methods

Patients and samples

We investigated retrospectively the efficacy of three commercial preparations of IVIg (Vigam® BPL, Privigen® CSL Behring and Intratect®; Biotest, Ringwood, UK) in the treatment of adult patients with protracted, recurrent or severe CDI at Nottingham University Hospitals NHS Trust between 2012 and 2015. CDI cases (cohort 1) were defined as patients with diarrhoea (at least three loose stools per day for at least 2 consecutive days) and cytotoxin-positive faeces. Medical records were reviewed for the following data: patient demographics, disease severity (ZAR score) [17,18], previous CDI treatment, IVIg type, timing (days from diarrhoea to infusion), dosage and response to treatment, complications of IVIg therapy, need for colectomy, in-hospital mortality and C. difficile-associated risk of death score (CARDS) [19]. In the Zar scoring system, a score of ≥ 2 denotes severe disease. The Zar criteria assign 1 point for each of the following: age > 60 years, albumin $< 2{\cdot}5$ mg/dl, white blood cell count $> 15 \times 10^9$/l, temperature $< 38{\cdot}3$°C, and 2 points each for endoscopic evidence of pseudomembranous colitis and admission to the intensive care unit [17]. For cohort 1, stored serum samples were not available for serological analysis. We therefore profiled sera from patients (cohort 2) before and immediately after administration of IVIg treatment for combined immunodeficiency disorder (CVID; $n = 5$, aged 47, range = 41–68 years), chronic inflammatory demyelinating polyneuropathy (CIDP; $n = 1$; aged 65 years) and CDI ($n = 1$, aged 71 years). All subjects provided written informed consent under approvals granted by the Nottingham Research Ethics Committee.

Antigen microarray

Binding of antibodies within IVIg preparations and patient sera to specific CD antigens were determined by using a previously validated CD protein microarray [20]. In brief, seven CD antigens, two positive controls: tetanus toxoid and lysates from *Candida albicans*, a negative control (printing buffer) and 10-point twofold serial dilutions of human Ig (matching the tested antibody isotype) were spotted onto aminosilane slides (Schott, Mainz, Germany) in quadruplicate using a MicroGridII arrayer (Digilab, Marlborough, MA, USA) and a silicon contact pin (Parallel Synthesis Technologies, Santa Clara, CA, USA). The seven CD antigens used in this study were: highly purified CD whole toxins A (200 μg/ml) and B (100 μg/ml; toxinotype 0, strain VPI 10463, ribotype 087), toxin B from a CD toxin B-only expressing strain (CCUG 20309; 90 μg/ml), precursor form of B fragment of binary toxin, pCDTb (200 μg/ml; produced from a wholly synthetic recombinant gene construct; amino acid sequence based on published sequence from 027 ribotype http://www.uniprot.org/uniprot/A8DS70) and purified native whole ribotype-specific (001, 002, 027) surface layer proteins (SLPs; all 200 μg/ml). Multi-isotype (IgG, IgG1, IgG2, IgG3, IgG4, IgA, IgA1, IgA2 and IgM) antibody levels in serum samples and in IVIg preparations were tested against the CD panel of antigens. Slides were scanned at 635 nm and the resultant images were processed with Genepix Pro-6 Microarray Image Analysis software (Molecular Devices Inc., Sunnyvale, CA, USA). Protein signals were determined after background subtraction using customized modules in the R statistical language to generate general mean of signal

levels. Specific isotype responses were interpolated against the internal isotype standard curve for each sample.

Antibody neutralization assay

A Caco-2 cell-based assay for anti-toxin A and anti-toxin B NAb was used as published previously [21]. Briefly, Caco-2 cells (HTB-37; American Type Culture Collection) were maintained in minimal essential medium (MEM) plus 20% fetal calf serum, 2 mM glutamine and non-essential amino acids at 37°C. Serum samples were diluted in the assay medium at three dilutions (1 : 10, 1 : 100 and 1 : 1000), then premixed with toxin A or toxin B [at 50% lethal dose (LD_{50})] for 1 h at 37°C before 50 µl of this mixture was transferred to the cells and incubated for 96 h. Following aspiration of the medium, 50 µl methylene blue [0·5% (wt/vol) dissolved in 50% (vol/vol) ethanol] was added to the cell culture and incubated for 1 h at room temperature. Then, the cells were washed gently with tap water (to remove excess stain) and air-dried. The cells were then lysed by adding 100 µl 1% (vol/vol) N-lauryl-sarcosine and incubated on a shaker for 15 min at room temperature. The cell biomass was determined by measuring the absorbance of each well on a BioTek Synergy2 (BioTeK, Winooski, VT, USA) plate reader at 405 nm. Toxin activity and working LD_{50} concentrations were defined empirically in preliminary experiments and for each individual batch/lot of toxin used.

Statistical analysis

All statistical analyses were performed on natural log-transformed data using GraphPad Prism version 6 (GraphPad software, San Diego, CA, USA). For non-paired data, the Mann–Whitney *U*-test and one-way analysis of variance (ANOVA) tests were applied as appropriate. For paired data, the Wilcoxon signed-rank test was used. Demographic data were presented as median and ranges. A *P*-value $\leq 0{\cdot}05$ was deemed statistically significant.

Results

Before IVIg treatment, all patients in cohort 1 had received high-dose oral vancomycin (500 mg four times daily) and intravenous metronidazole (500 mg four times daily). Responders to IVIg received a longer duration of antibiotics compared to non-responders (8 days, range = 1–11 days *versus* 2·5 days, range = 1–7 days), but this did not reach statistical significance ($P = 0{\cdot}1$). All patients received 0·4 g/kg of IVIg. Compared with non-responders ($n = 10$ of 17; aged 75, range = 58–85 years), responders to IVIg ($n = 7$ of 17; aged 82, range = 50–90 years) had lower ZAR disease severity (3, range = 1–6 *versus* 5, range = 2–8, $P = 0{\cdot}14$), CARDS risk of death scores (6, 3–15 *versus* 10·5, range = 2–14, $P = 3{\cdot}1$) and in-patient mortality (3 of 7 *versus* 7 of 10), although these findings did not reach statistical significance. There were no statistically significant differences between the treatment response subgroups in relation to co-morbidities using the Charlson co-morbidity index (CCI) (2, range = 0–4 *versus* 2, range = 0–4, $P = 0{\cdot}8$) or the duration of diarrhoeal symptoms prior to IVIg (16 days, range = 1–38 *versus* 13 days, range = 1–67, $P = 0{\cdot}9$). Furthermore, no differences were observed between the type of preparation, timing of administration or number of IVIg infusions received. No complications were reported for IVIg. Two patients underwent urgent colectomy in the non-responder group.

Specific antibody reactivities against CD proteins varied between the different commercial IVIg preparations, as shown in the heat-map in Fig. 1a. Briefly, all IVIg preparations showed IgG reactivity to all tested CD antigens, although a weaker response was observed to the SLPs. Vigam contained significantly higher levels of IgG1 antibodies against all toxins compared with Privigen and Intratect. Moreover, the antibody neutralization assay showed variability in percentage protection against CD toxins A and B between the different IVIg preparations. Here, Intratect at a 1 : 100 titration demonstrated a significantly lower protective capacity to neutralize CD toxin A compared with Vigam and Privigen (Fig. 1b).

For cohort 2, the microarray data showed post-IVIg infusion enhancement in the levels of total IgG, IgG1, IgG2 and IgG3 to CD antigens (native toxins A and B, both VPI 10463), binary toxin (pCDTb) and toxin B (CCUG 20309), in all patients' sera (Fig. 2a). A statistically significant increase ($P < 0{\cdot}05$) was observed in the levels of total IgG against all toxins tested following IVIg administration (Fig. 2b). Notably, the highest IgG binding response was against toxin B ($P = 0{\cdot}0006$, data not shown). However, there was no difference in post-IVIg NAb responses between toxins A and B ($P = 0{\cdot}0728$, data not shown). For IgG1, this increase was significant against toxin B, binary toxin (pCDTb) and toxin B (CCUG 20309) only. Moreover, IgG2 antibody levels were increased significantly ($P < 0{\cdot}05$) against toxin B and toxin B (CCUG 20309). Interestingly, following IVIg infusion, the level of IgG3 was increased against toxin A, toxin B, toxin B (CCUG 20309) and SLP027, but the magnitude was not statistically significant. Serum samples from all cohort 2 patients after IVIg infusion demonstrated significantly enhanced anti-toxin A and anti-toxin B antibody neutralization activities (Fig. 2c) at 1 : 10 dilution. However, the anti-toxin NAb effect was reduced at higher serum dilutions (data not shown).

Discussion

Although no patients in either cohort experienced complications attributable to IVIg therapy, only 41% of the CDI patients in cohort 1 showed a therapeutic response to IVIg,

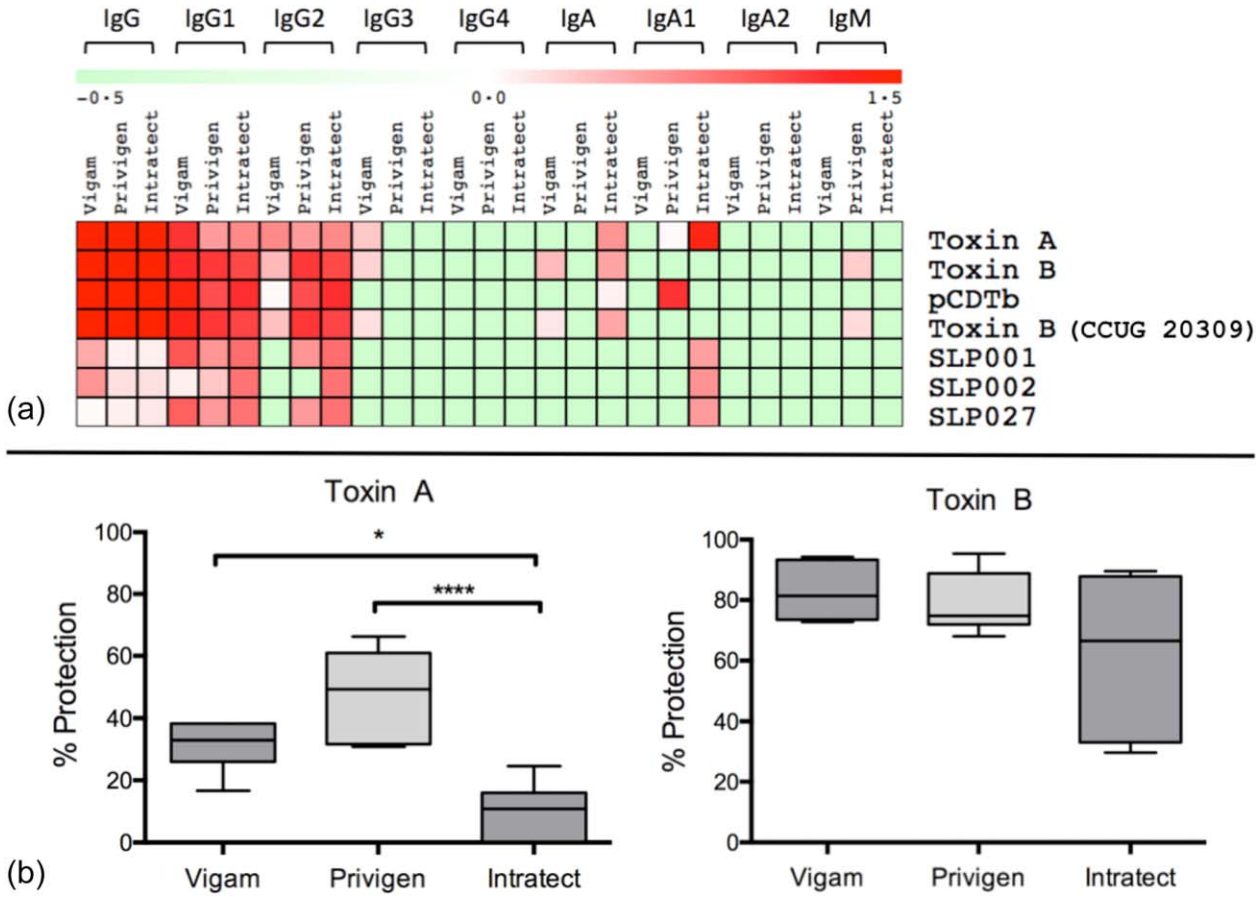

Fig. 1. Immune reactivity and neutralizing effect of intravenous immunoglobulin (IVIg) to *Clostridium difficile* antigens. (a) Reactivity of multi-isotype specific antibodies to *C. difficile* antigens in commercial IVIg preparations: heat-map produced by Multiple Experiment Viewer (MeV 4.9) illustrates the levels of specific antibody isotypes (IgG, IgG1, IgG2, IgG3, IgG4, IgA, IgA1, IgA2 and IgM) in three commercially available IVIg preparations; Vigam, Privigen and Intratect, against seven *C. difficile* antigens [toxin A (200 μg/ml, toxin B (100 μg/ml), pCDTb (200 μg/ml), toxin B (CCUG 20309; 90 μg/ml) and surface layer proteins (SLPs) 001, 002 and 027; all 200 μg/ml] using protein microarray technology. Colour code of the heat-map: green (low) to red (high) signal intensity. Signal values represented on the colour scale for the heat-map are $\log_2$-transformed from the arbitrary fluorescence units (AFU). Total IgG, IgG1 and IgG2 isotypes gave the highest binding reactivities against toxin A, toxin B, binary toxin (pCDTb) and toxin B (CCUG 20309). (b) IVIg neutralization efficacy against *C. difficile* native whole toxins A and B: percentage of protective neutralization effect of commercial IVIg products; Vigam, Privigen and Intratect against *C. difficile* toxins A and B. Each plot represents the median of triplicate experiments at 1 : 100 dilution. Intratect exhibits the lowest protective effect compared to Vigam and Privigen, particularly against toxin A. *P*-values of ****$\leq$ 0·0001; *$\leq$0·05 (one-way analysis of variance). [Colour figure can be viewed at wileyonlinelibrary.com.]

with two patients requiring emergency colectomy for fulminant CDI in the non-responder group. These findings are in keeping with an earlier observational study by Abougergi *et al.* [16], which revealed that 43% of patients survived their hospitalization with CDI colitis resolution following IVIg. These observations may reflect inadequate dosaging, delayed treatment, insufficient binding and/or neutralizing titres and more severe disease. Importantly, our findings show the limited efficacy of Intratect in neutralizing toxin A and suggest that Vigam or Privigen may be the preferred IVIg preparation of choice for use in the CDI population.

We believe that this is the first report that demonstrates the prevalence of CD anti-binary toxin and anti-SLP antibodies in all tested human IVIg preparations and in patient sera pre- and post-IVIg treatment. Our data also confirm the detection of protective anti-toxin A and anti-toxin B NAbs in patient sera following treatment. Variability in specific antibody content between the different IVIg preparations examined in this study and that reported in an earlier study by Salcedo *et al.* [14] may be due to the different geographical regions from which the plasma samples were collected and/or differences in CD exposure. Our binding data for IgG revealed significantly higher levels of anti-toxin B IgG in post IVIg sera. This finding seems to confirm the recent Merck monoclonal antibody Phase III trial, which showed that an anti-toxin B response was the prime determinant for preventing CDI relapse [22].

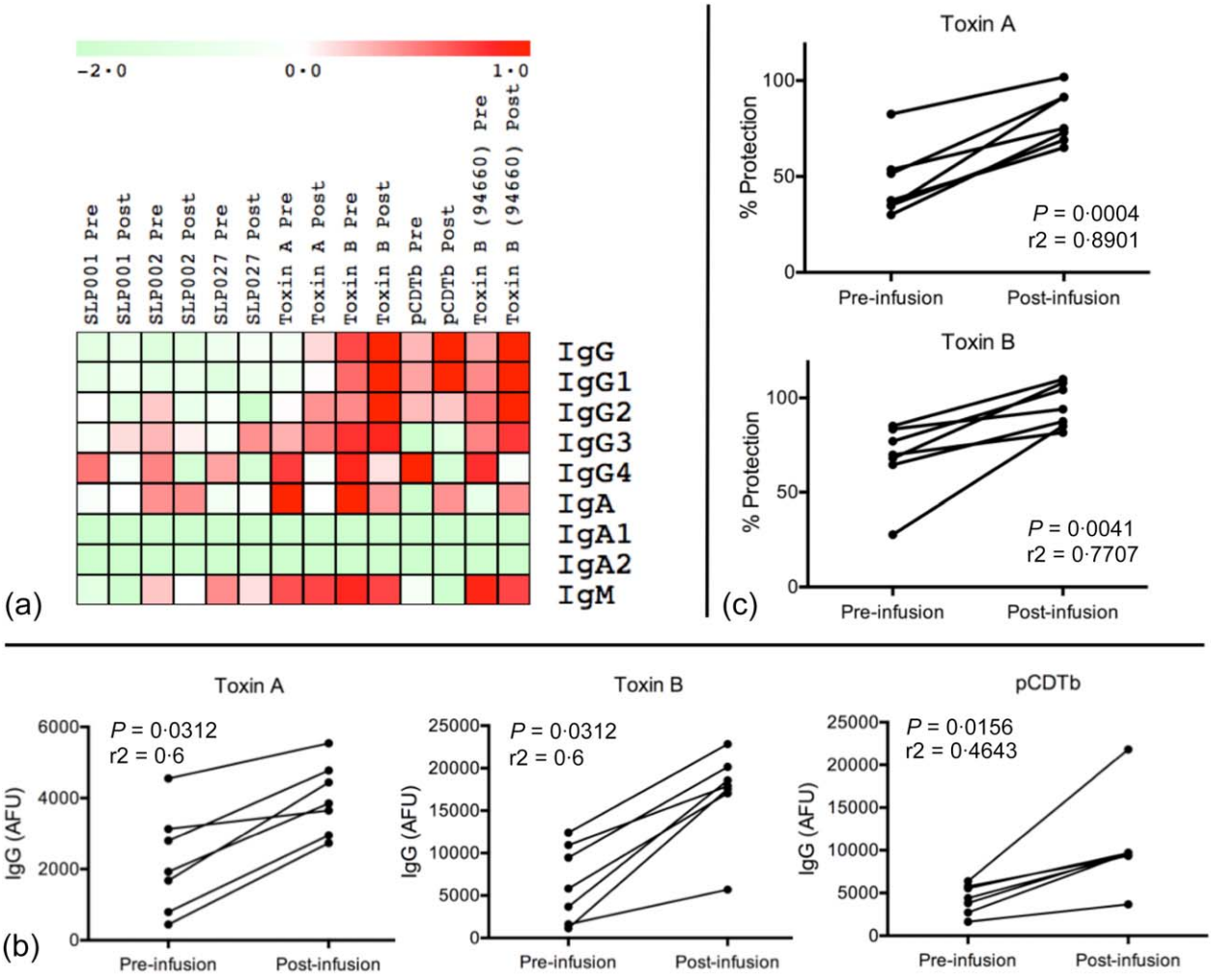

Fig. 2. Immune reactivity and neutralizing effect of patients' sera to *Clostridium difficile* antigens. (a) Comparison of antibody reactivities against *C. difficile* proteins in patients' sera before and after intravenous immunoglobulin (IVIg) infusion: heat-map produced by Multiple Experiment Viewer (MeV version 4.9) illustrates the expression level of the isotypes (IgG, IgG1, IgG2, IgG3IgG4, IgA, IgA1, IgA2 and IgM) in serum samples in seven patients before and after IVIg infusion against seven *C. difficile* antigens [toxin A (200 μg/ml), toxin B (100 μg/ml), pCDTb (200 μg/ml), toxin B (CCUG 20309; 90 μg/ml) and surface layer proteins (SLPs) 001, 002 and 027; all 200 μg/ml] using protein microarray technology. Colour code of the heat-map: green (low) to red (high) signal intensity. Signal values represented on the colour scale for the heat-maps are $\log_2$-transformed from the arbitrary fluorescence units (AFU). There was post-infusion enhancement of the total IgG, IgG1, IgG2 and IgG3 reactivities to toxin A, toxin B and pCDTb. (b) IgG responses to toxins A, B and binary toxin (pCDTb) pre- and post-IVIg administration. Pre- and post-IVIg IgG anti-toxin levels showing significant increase of total IgG against all toxins tested following IVIg infusion (Wilcoxon's signed-rank test). Each plot represents the median of triplicate experiments at 1 : 10 dilution. (c) Neutralization effect against *C. difficile* native toxins A and B following IVIg administration: comparison of pre- and post-infusion neutralizing antibody activities showed enhanced protective effect after IVIg infusions against *C. difficile* native toxins (toxins A and B). Each plot represents the median of triplicate experiments at 1 : 10 dilution. A significant increase in the protective effect against toxins A and B was noted in patient sera tested post-IVIg infusion (Wilcoxon's signed-rank test). [Colour figure can be viewed at wileyonlinelibrary.com.]

Enhanced IgG2 and IgG3 immunoreactivites seen following IVIg infusion may prove highly beneficial, given their more desirable molecular and functional attributes. Indeed, Katchar *et al.* [23] detected humoral immune deficiencies in the IgG2 and IgG3 subclasses directed towards toxin A in patients with recurrent CDI. The lack of a post-IVIg IgG4 response is perhaps indicative that the immune response has not been pushed through to repeat antigen challenge. Differences in observed toxin neutralizing efficiencies might be caused by a combination of anti-toxin antibody titres, as well as by individual differences in toxin potencies. Interestingly, none of the CVID and CIDP patients receiving three weekly IVIg infusions in cohort 2 had developed CDI previously. This may be because of the

presence of anti-toxin NAb in the IVIg, which may be contributing to protection against developing CDI. Although most CD protein toxins should be neutralized by IVIg treatment, we were unable to study anti-binary neutralizing capacities within the IVIg or patient sera. Moreover, we did not examine antibody affinities for the CD antigens described in this report. Although there were no stored sera available for cohort 1, we compared binding and NAbs pre- and post-infusion in a second small and mainly non-CDI cohort. It is noteworthy that the diarrhoeal symptoms of the CDI patient that received IVIg in cohort 2 resolved within 4 days of IVIg (Privigen) infusion.

These results, if confirmed in larger studies which will help with statistical significance, might be helpful for optimizing the type and dosage of IVIg used in adjunctive therapy for CDI, and further support a possible rationale for inclusion of SLPs and binary toxin antigens in future candidate CD vaccines. Further studies are required to measure antibody affinities and to clarify the precise contribution of different IgG subclasses to clinical protection or to disease pathogenesis. These studies could be achieved by purifying out the IgG subclass-specific antibodies and assessing their significance (including their potential to interfere with or block the action of other IgG subclasses) in well-validated functional assays [24]. For example, an early study compared purified subclass antibodies in Herpesvirus neutralization assays, determining that IgG3 and IgG4 had the greatest viral neutralizing ability despite not being the predominant subclass [25]. A further study of responses against human enterovirus 71 found that IgG1 and IgG2 fractions were the most effective at neutralization, and that IgG3 led to enhanced infection [26]. The knowledge obtained from IgG subclass studies, combined with a greater molecular understanding of IgG subclass properties, will facilitate the engineering and development of highly effective CD-specific monoclonal therapeutic antibodies. Despite ongoing debate as to the utility of IVIg for CDI, future strategies could attempt to enhance the opportunities of this drug to show therapeutic efficacy and survival through application of disease severity risk scores, which should prompt earlier identification of those patients who are likely to require and receive most benefit from IVIg [18,19]. A review of the severe cases of CDI published in the medical literature suggests that the earlier administration of IVIg may increase the likelihood of attaining therapeutic efficacy and survival [17]. Moreover, given that the concentration and anti-microbial specificities of the antibodies are not normally evaluated routinely in batches of commercial polyclonal IVIg, donor units delivered to the fractionation sites that have high antibody levels against CD antigens could be identified using microarray technology and stored in biobanks. Donor plasma/serum could even be prescreened before donation to identify optimal batches with the highest levels of CD-reactive IgG. Alternatively, the anti-CD activity of IVIg could be enhanced further by acquiring blood samples from patients convalescing from CDI or from vaccinated individuals. Such an enrichment strategy has been used successfully to treat viral diseases [27–29], and is regarded by the World Health Organization (WHO) as a potential treatment for Ebola virus disease [30]. While the breadth of protection may still be limited by ribotype or strain-specific differences in protein expression, hyperimmune IVIg (H-IVIg) may represent a more effective adjunct for CDI than the polyspecific IVIg that is currently employed clinically. In the absence of any randomized control trial data in the area of IVIg and CD (or registered active trials on ClinicalTrials.gov), this therapy should be studied in a head-to-head comparison with polyclonal IVIg and anti-toxin levels within patient sera should be correlated with clinical outcomes. Further studies may also be useful in determining if treating with IVIg for any indication is likely to reduce the risk of developing CDI in future.

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Author contributions

O. H. N., B. M. and O. A. J. A. performed the experiments. O. H. N. and M. R. H. transformed the data sets. C. C. S., D. P. H. and K. R. A. provided purified toxins and C. E. L., I. M. and M. L. provided the surface layer proteins for the study. M. H. W. proofread and edited the manuscript. T. M. M. designed the study, recruited all subjects, analysed the data sets and wrote the manuscript.

Disclosure

D. P. H. owns UCB stock options; M. H. W. has received honoraria for consultancy work, financial support to attend meetings and research funding from Astellas, AstraZeneca, Abbott, Actelion, Alere, AstraZeneca, Bayer, bioMérieux, Cerexa, Cubist, Da Volterra, Durata, Merck, Nabriva, Pfizer, Qiagen, Roche, Seres and Synthetic Biologics. All other authors have no conflicts of interest to declare.

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