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ACTIVE AND PASSIVE IMMUNISATION AGAINST *CLOSTRIDIUM DIFFICILE* DIARRHOEA AND COLITIS*

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SUMMARY

Clostridium difficile, a Gram-positive bacterium, is the major cause of hospital-acquired infectious diarrhoea and colitis in industrialised nations. *C. difficile* colonisation results from antibiotic administration and subsequent loss of protection provided by intestinal flora. *C. difficile*-induced colitis is caused by the release of two exotoxins, toxin A and B. Host factors including advanced age, pre-existing severe illness and weakened immune defences predispose individuals to symptomatic infection. The generation of antibody responses to toxin A through natural exposure is associated with protection from disease. In addition, an inability to acquire immunity to toxin A puts individuals at risk for recurrent and/or severe disease. Immunological approaches for the management of this disease are being developed which could reduce the reliance on antibiotics for treatment and allow for re-establishment of the natural barrier provided by an intact commensal flora. An active vaccine and various immunotherapeutic strategies under evaluation may prove to be effective against severe or relapsing *C. difficile* infection.

INTRODUCTION

Clostridium difficile is a Gram-positive, anaerobic, spore-forming bacterium that is commonly found in the environment. The organism is transmitted by the faecal-oral route through the ingestion of resistant spores that survive passage through the stomach, ultimately residing in the colon. Antimicrobial therapy creates an ecological niche, which allows *C. difficile* spores to germinate in the colon. The bacterium colonises the luminal surfaces of colonic epithelial cells and produces two large exotoxins (toxins A and B), which are principally responsible for the disease manifestations associated with this infection. *C. difficile* is currently the most frequent cause of nosocomial infectious diarrhoea (*McFarland* et al., 1989; *Kelly* et al., 1994) and is responsible for an estimated $1 billion in health care costs annually in the US alone (*Kyne* et al., 2002).

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INCIDENCE, RISK FACTORS AND MODES OF TRANSMISSION

The incidence of *C. difficile* carriage in the healthy adult population is ~1-3%. The rate of colonisation increases substantially to ~20% with antibiotic usage (*McFarland* et al., 1989), due to the alteration of the protective commensal flora. Up to 60% of healthy neonates and infants are colonised with *C. difficile* without clinical symptoms (*Larson* et al., 1982; *Viscidi* et al., 1981). The total number of *C. difficile*-associated diarrhoea (CDAD) cases is estimated to be at least 300,000 annually in the US. The incidence can be higher in hospitals, nursing homes and other long-term care facilities where CDAD outbreaks occur. In such settings, diarrhoea helps disseminate the spores that can be found on environmental surfaces, equipment and staff clothing and can be difficult to eradicate. Viable spores have been cultured from various surfaces years after being deposited (*Fekety* et al., 1981; *Kim* et al., 1981). Such contamination constitutes a continuous source of infection for those at risk for CDAD. A variety of factors increase the risk of acquiring CDAD. The most important factor is antibiotic therapy with certain agents, but advanced age (>65), serious underlying illness, an institutional setting and immunodeficiency due to AIDS or chemotherapy, also increase the risk of developing disease. Despite the fact that up to 31% of high-risk hospitalised patients are colonised with *C. difficile*, only a subset develops disease symptoms (*McFarland* et al., 1989; *Samore* et al., 1994).

CLINICAL SYMPTOMS, DIAGNOSIS AND TREATMENT

C. difficile colonisation of adults produces a spectrum of clinical symptoms ranging from asymptomatic carriage to life threatening pseudomembranous colitis. Diarrhoea often appears 1-2 weeks after initiation of antibiotic therapy, which can be accompanied by modest fever and abdominal cramping. Moderate or severe colitis develops in a subset of patients and the most serious form of the infection, pseudomembranous colitis, carries the risk of intestinal perforation and death.

The diagnosis of *C. difficile* infection most commonly relies on the detection of toxin in stool filtrates. A toxin-specific enzyme-linked immunosorbent assay (ELISA) is often employed due to the quick turn-around time and ease of use. The most sensitive and specific test measures the cytotoxic activity in stool supernatants but this method takes up to 3 days for results and requires cell culture capabilities. The anaerobic culture of *C. difficile* from stool does not necessarily aid in diagnosis since non-toxigenic strains exist and these are not associated with disease. However, this method is useful for identifying isolates that are toxin assay negative but are toxigenic under appropriate growth conditions and also for defining the asymptomatic carrier state.

The treatment of CDAD typically involves the cessation of the offending antibiotic, initiation of oral metronidazole or vancomycin therapy and fluid replacement. Metronidazole is generally considered a first-line therapeutic for CDAD due to cost and the concern that oral vancomycin might induce the emergence of vancomycin-resistant enteropathogens. The response rate to initial therapy is ~95% but up to 20% of

patients relapse within 1-2 weeks of completing a course of antibiotics (*Teasley* et al., 1983). The risk of relapsing disease increases markedly with each additional relapse such that individuals who have experienced two or more relapses have a 65% risk of further recurrence (*McFarland* et al., 1994). In one study, it was found that over 50% of the relapse incidents are due to reinfection with a different *C. difficile* strain rather than recrudescence of the primary infection (*Wilcox* et al., 1998). *C. difficile* organisms isolated following a recurrent episode are sensitive to antimicrobial therapy, indicating that relapsing disease is not due to the acquisition of antibiotic resistance. Further exposure to *C. difficile* and/or the imbalance in the normal intestinal flora perpetuated by metronidazole or vancomycin therapy likely contribute to the development of relapsing disease. Host factors associated with protection from primary and recurrent CDAD will be discussed below.

TOXIN STRUCTURE AND MECHANISMS OF ACTIONS

C. difficile is a non-invasive organism which possesses multiple virulence factors that aid in colonisation and may promote disease. These include various adherence factors such as flagellar proteins (*Tasteyre* et al., 2001), surface layer proteins (*Calabi* et al., 2002), and a surface-exposed adhesin (*Waligora* et al., 2001). In addition, all pathogenic strains of *C. difficile* express one or two large exotoxins (A and B) and the majority expresses both toxins. Toxin A (308 kDa) and toxin B (270 kDa) exhibit 49% amino acid identity. These toxins belong to the large clostridial cytotoxin (LCT) family (*von Eichel-Streiber* et al., 1996; *Warny* and *Kelly*, 2003). This family includes *Clostridium sordellii* haemorrhagic and lethal toxins and *Clostridium novyi* alpha toxin. LCT family members are structurally and functionally related proteins with the following properties: 1) high molecular weight; 2) an amino-terminal enzymatic domain; 3) a central hydrophobic region; and 4) a carboxy-terminal domain carrying carbohydrate recognition sequence repeats. The C-terminal region functions as a multivalent lectin which recognises host cell surface carbohydrate receptors. This high avidity binding allows for internalisation by target cells via receptor-mediated endocytosis in clathrin-coated pits (*von Eichel-Streiber* et al., 1996). Certain cultured cell lines do not possess toxin receptors yet these cells effectively internalise toxin A and B (presumably by fluid-phase endocytosis) and become intoxicated, but this requires higher toxin concentrations than for receptor-bearing cells (*Tucker* et al., 1990). Oligosaccharide receptors for toxin A are expressed on the apical membranes on intestinal epithelia of small animals (hamsters, rabbits and certain mouse strains) and in humans. A toxin B receptor has not been identified, which is consistent with the insensitivity of animals to toxin B incubated administered orally (see below). The central hydrophobic region is believed to be necessary for the translocation of the toxins from endocytic vesicles into the cytoplasm, where the toxins interact with their GTPase substrates. Following endosomal acidification, the toxins undergo structural changes that expose the hydrophobic region (*Qa'Dan* et al., 2000), forming potassium permeable channels which facilitate translocation to the cytosol (*Barth* et al., 2001). The enzymatic domain catalyses the transfer of glucose from UDP-glucose as donor molecule to threonine 35/37 of members

of the Rho family of small GTP-binding proteins (including Rho, Rac, cdc42) (*Just* et al., 1995a,b). This covalent addition irreversibly inactivates these proteins, which regulate the actin cytoskeleton, among other functions. The loss of actin cytoskeletal network is lethal to cells and causes a distinctive rounded cell phenotype, which is exploited for the diagnosis of toxigenic *C. difficile* in stool filtrates.

ROLE OF TOXINS IN DISEASE

Studies in animals have contributed greatly to our understanding of the pathogenicity of toxins A and B. When administered orally, the purified toxins are capable of inducing the full spectrum of disease manifestations typical of *C. difficile* infection. Purified toxin A possesses potent enterotoxic and pro-inflammatory activity, as determined in ligated loop studies in mice, rats, hamsters and rabbits (*Kurtz* et al., 2001; *Lyerly* et al, 1982) Toxin A is also cytotoxic to cultured cells in low nanogram quantities. By contrast, toxin B does not exhibit enterotoxic activity in animals but is a more potent cytotoxin than toxin A (*von Eichel-Streiber* et al., 1996). When administered intragastrically, toxin A is lethal to mice and hamsters but toxin B is not (*Lyerly* et al., 1985). The toxins appear to act synergistically when co-administered by the intragastric route, suggesting that toxin A may initially affect epithelial integrity allowing entry of the more potent cytotoxin, toxin B. Indeed, after mechanically compromising the epithelial barrier, toxin B can cause systemic toxicity and death (*Lyerly* et al., 1985). The hamster is a natural model of *C. difficile* diarrhoea and colitis and some laboratory colonies experience outbreaks of *C. difficile* infection (*Chang* and *Rohwer*, 1991). A single dose of oral clindamycin to hamsters followed by intragastric inoculation with toxigenic *C. difficile* organisms produces fulminant disease symptoms (diarrhoea, ruffled fur, lethargy, etc) leading to death within 2-3 days. Necropsy reveals severe haemorrhagic caecitis. This model of *C. difficile* diarrhoea and colitis is a stringent test for vaccines and immunotherapies.

By contrast to the observations with toxin-producing strains, intragastric administration of culture filtrates from non-toxigenic strains does not result in disease (*Lyerly* et al., 1985), confirming the principal role of the toxins in the pathogenicity of *C. difficile*. Recently, certain related strains (serogroup F) have been shown to possess a toxin A-B+ phenotype. Examination at the genetic level revealed that these strains do not have an intact toxin A gene but do express an unusual variant toxin B (*Chaves-Olarte* et al., 1999). Strains of this phenotype have been associated with clinical disease, suggesting that toxin B alone can cause intestinal symptoms in humans (*Alfa* et al., 2000; *Johnson* et al., 2001; *Limaye* et al., 2000). *Ex vivo* studies using human colonic explants indicated that toxin B can induce a loss in transepithelial resistance and pro-inflammatory cytokine signalling consistent with enterotoxic activity (*Riegler* et al., 1995). In addition, toxin B was recently found to possess enterotoxic and pro-inflammatory activity in human intestinal xenografts in immunodeficient (*scid*) mice (*Savidge* et al., 2003). The enterotoxic potential of toxin B in humans is an important consideration for the design of vaccines and immunotherapies, as discussed below.

VACCINATION WITH *C. DIFFICILE* TOXOIDS IN ANIMAL MODELS

One approach to defining the roles of toxins A and B in the pathogenesis of *C. difficile* infection has been to examine the protective capacity of toxin-specific immunity in animals.

Active vaccination

Animals have been vaccinated with various forms of *C. difficile* toxoids ranging from crude culture filtrates to partially purified preparations. Hamsters vaccinated parenterally with formalin-inactivated toxins (toxoids) A and B in culture filtrate, but not individual toxoids in culture filtrate, were protected from lethal ileocaecitis induced with clindamycin and toxigenic *C. difficile* (*Fernie* et al., 1983; *Kim* et al., 1987; *Libby* et al., 1982). *Kim* et al. (1987) found that toxoid A plus B and toxoid A alone (but not toxoid B) similarly protected hamsters from fatal *C. difficile* challenge, while culture filtrate from non-toxigenic *C. difficile* strains did not confer protection. Although the different immunisation schemes, antigen dose levels and adjuvant formulations employed in these studies make direct comparisons of these findings difficult, the protection afforded by toxin-specific immunity was clear.

The rapid onset of fulminant, lethal disease in hamsters represents a rigorous test of vaccine-induced immunity because of the requirement to neutralise the enterotoxicity, mucosal damage and inflammation mediated by the toxins as well as the systemic toxicity due to toxins entering the circulation. While initial vaccine studies used protection from lethal disease as the primary efficacy measure, more detailed assessments of protection from enterotoxicity and diarrhoea were carried out which provided proof-of-principle in support of the development of an effective vaccine against CDAD. An evaluation of the routes of delivery of an inactivated culture filtrate vaccine in hamsters assessed protection from both lethal disease and diarrhoea and found that a sequential combination of intranasal and intraperitoneal immunisation with vaccine plus cholera toxin and Ribi adjuvants, respectively, provided complete protection from death and diarrhoea, suggesting that induction of both systemic and mucosal immunity was necessary for optimal protection (*Torres* et al., 1995).

Using a more purified toxoid preparation, various clinically compatible vaccination regimens were tested in hamsters to determine the routes of administration capable of eliciting protection from death and diarrhoea (*Giannasca* et al., 1999). The combination of rectal immunisation with *E. coli* heat-labile toxin adjuvant and intramuscular (i.m.) administration with alum provided full protection from *C. difficile* challenge, irrespective of the sequence employed. Intranasal or intragastric vaccination in combination with i.m. administration was partially protective against diarrhoea, as was i.m. vaccination with alum. While assessing the requirement of alum adjuvant during i.m. administration, it was unexpectedly found that the toxoid preparation without adjuvant was best able to consistently protect hamsters from diarrhoea and death of all the regimens tested, and it elicited high levels of serum toxin A and B neutralising activity, as determined in the cell culture assay. No detectable anti-toxin antibodies were found in saliva or faeces, suggesting that serum antibodies were the principal effector molecules.

In order to define the domains of the large toxins that contain protective epitopes, recombinant peptides have been generated and evaluated in small animal models. A large portion of the cell-binding domain of toxin A was cloned

and expressed in *E. coli* (*Price* et al., 1987). This 104 kDa polypeptide retained its ability to agglutinate rabbit erythrocytes. Antibodies raised against this peptide neutralised the enterotoxic activity of native toxin A in the rabbit intestinal loop assay (*Lyerly* et al., 1990) and a mAb (PCG-4) generated with this antigen had similar neutralising activity (*Lyerly* et al., 1986). When hamsters were vaccinated with this peptide, partial protection from death and diarrhoea due to *C. difficile* challenge was observed, thereby establishing a role for this toxin A domain in protective immunity. Another polypeptide from this cell-binding domain of toxin A was generated and used to explore the intranasal route of immunisation in mice (*Ward* et al., 1999a). This antigen induced specific antibodies in both serum and lung lavage fluid but not in small intestinal secretions.

Because *C. difficile* disease manifestations in humans are largely confined to the intestinal mucosa, delivery systems capable of presenting non-toxic domains of toxin A to intestinal immune induction sites have been explored. Various length polypeptides spanning the cell-binding domain of toxin A were expressed as fusions with tetanus toxin C fragment in an attenuated *Salmonella typhimurium* vaccine strain known to be effective against murine typhoid disease (*Ward* et al., 1997). Following intragastric (i.g.) immunisation of mice, it was found that one construct containing 14 toxin A carbohydrate recognition domain (CRD) repeats generated serum anti-toxin A responses. Using a less attenuated *aro A, aroD S. typhimurium* host strain given to mice by the i.n. or i.g. routes, these 14 CRD repeats generated toxin A-binding and -neutralising antibodies in serum (*Ward* et al., 1999b). Analysis of pulmonary and intestinal lavage samples subsequent to i.n. or i.g. vaccination, respectively, revealed that toxin A-specific IgA was induced. Using another live vector delivery system, 720 amino acids comprising most of the toxin A cell-binding domain was expressed as a fusion protein with the signal sequence of *E. coli* haemolysin A in an attenuated *Vibrio cholerae* vaccine strain and used to orally immunise rabbits (*Ryan* et al., 1997). It was found that anti-toxin A serum IgG antibodies were induced and protection from enterotoxicity was demonstrated using the ligated ileal loop assay. The host range specificity of these live vectors limits their evaluation to susceptible species, making it difficult to compare with findings generated in the hamster model.

Passive immunisation

Because active vaccination elicits both cellular and humoral immune responses, passive vaccination with immune sera has been employed to define the relative roles of the two branches of the immune system in protection from *C. difficile* diarrhoea and colitis.

Oral administration

Toxin-mediated diseases typically require the production of toxin-specific antibodies for protection. Because *C. difficile* intoxication begins with release of toxin molecules at the luminal surfaces of the caecum and colon, investigators have examined whether anti-toxin preparations administered orally could neutralise enterotoxicity. Bovine antibodies have been tested as a means to provide protection against various enteric pathogens following oral delivery (*Korhonen* et al., 2000). A *C. difficile* bovine IgG concentrate was prepared by immunising gestating cows with culture filtrate toxoid and processing the resulting colostrum. This antibody formulation contained toxin binding and neutralising activity and was able to prevent diarrhoea and death in hamsters

when administered before and during clindamycin/*C. difficile* challenge (*Kelly* et al., 1996; *Lyerly* et al., 1991).

In order to define the toxin polypeptide domains which could elicit antibodies with protective activity when delivered orally, *Kink* and *Williams* (*Kink* and *Williams*, 1998) created multiple recombinant peptides that together spanned the entire toxin proteins. These peptides were used to immunise hens for the production of egg IgY antibodies, which were orally administered to hamsters to assess passive protection from CDAD in a prophylactic and therapeutic setting. They observed that antibodies to the cell-binding domains of both toxins were most effective in eliciting toxin-neutralising antibodies. Administration of anti-toxin A neutralising antibodies alone prior to challenge was sufficient to prevent disease, while neutralising antibodies to both toxins was required for complete therapeutic protection from death and diarrhoea. Furthermore, hamsters effectively treated with antibodies did not develop relapsing disease months after treatment was halted.

Parenteral administration

Early *C. difficile* studies established a principal, if not exclusive, role for humoral immunity in protection from disease. Prior to the availability of *C. difficile* toxin-specific antisera, *C. sordellii* anti-toxin was tested for cross-reactivity and passive immune protection in hamsters (*Allo* et al., 1979). This anti-toxin preparation neutralised the cytotoxicity of *C. difficile* toxins and was able to fully protect animals from death while significantly preventing diarrhoea when administered by the i.m. route on three consecutive days surrounding clindamycin challenge. These observations suggested that circulating antitoxin could indeed confer protection from enterotoxicity. However, the very high antitoxin doses administered perhaps clouded the physiological relevance of these results.

The ability of circulating anti-toxin IgG to mediate intestinal protection was further established by subsequent studies in mice and hamsters. The intravenous administration of IgG monoclonal antibodies directed against the cell-binding domain of toxin A to gnotobiotic mice and subsequent oral challenge with *C. difficile* resulted in complete protection from death and diarrhoea (*Corthier* et al., 1991). Polyclonal antibodies with toxin neutralising activity induced with toxoid vaccine were administered to hamsters by the i.p. route and were able to protect animals from oral challenge in a dose-dependent manner (*Giannasca* et al., 1999).

MECHANISMS OF PROTECTION IN ANIMAL MODELS

The ability of an antitoxin antibody preparation to convey full protection from oral *C. difficile* challenge in mice and hamsters indicates that antibodies are the essential effector molecules in these animal models. The oral administration of toxin-specific antibodies is capable of neutralising the enterotoxicity and mucosal inflammation caused by the toxins presumably by intercepting the toxins in the intestinal lumen rendering them inactive. This "immune exclusion" likely models the action of secretory antibodies elicited via mucosal vaccination with toxoid or natural exposure to *C. difficile* toxins. Indeed, colonic aspirates from *C. difficile* patients were shown to possess toxin A-specific secretory IgA capable of inhibiting toxin A binding to receptors (*Kelly* et al., 1992).

The ability of serum antibodies to

prevent enterotoxicity and mucosal damage is mechanistically less obvious. The protective role for circulating antibodies in *C. difficile* animal models suggested that toxin-specific IgG was the critical effector molecule because of the elevated IgG titres relative to IgA and IgM levels, but the contribution of IgA or IgM antibodies could not be dismissed. Furthermore, rodents including mice and hamsters possess an efficient hepatobiliary transport system for serum IgA and IgM, which directs substantial amounts of polymeric immunoglobulins into the intestinal tract (*Delacroix* et al., 1985; *Vaerman* and *Langendries*, 1997). Accordingly, the most conclusive evidence for the role of anti-toxin IgG in protection from enterotoxicity was provided by the i.v. administration of monoclonal IgG to gnotobiotic mice (*Corthier* et al., 1991). While the precise mechanism by which anti-toxin IgG neutralises enterotoxicity has not been established, the direct effects of the toxins on epithelial cells probably play a role. Toxins A and B have been shown to increase the permeability of polarised intestinal epithelial cells (*Hecht* et al., 1988,1992) through the specific inactivation of Rho proteins which regulate tight junctions and their interaction with the actin cytoskeleton. Thus, the barrier function of intestinal epithelium appears to be highly sensitive to the action of the toxins and the increase in epithelial permeability may lead to enhanced paracellular transport of soluble molecules including antibodies. In support of this hypothesis, intravenously-administered anti-toxin A monoclonal was detected in the caecal contents of gnotobiotic mice following oral challenge with toxigenic *C. difficile* but not in unchallenged mice (*Corthier* et al., 1989). If this model is correct, the "leakage" of serum proteins into the intestinal lumen can occur in the absence of fluid loss (diarrhoea) or gross changes in the epithelium, as described for hamsters protected by parenteral immunisation with different toxoid vaccine preparations (*Giannasca* et al., 1999; *Kim* and *Rolfe*, 1989).

ANTIBODY RESPONSES TO TOXINS IN HUMANS

Many healthy adults (~60%) have detectable serum IgG and IgA to toxins A and B (*Viscidi* et al., 1983) despite only a small population (2-3%) being colonised (*Kelly* and *Lamont*, 1998), as determined by culturing stool on selective media. It is not known if the prevailing responses in adults are a reflection of childhood exposure or sub-clinical infection(s) as adults. The ability to mount an effective immune response following exposure to *C. difficile* appears to impact the course of disease expression. Indeed, only a small proportion of high-risk hospitalised patients develop symptomatic infection while up to 31% are colonised with *C. difficile* (*McFarland* et al., 1994; *Samore* et al., 1994). Following symptomatic infection, many individuals develop anti-toxin A and B antibodies in serum (*Viscidi* et al., 1983; *Aronsson* et al., 1985), including toxin neutralising IgA (*Johnson* et al., 1995), as well as in stool and this response appears to be associated with protection from subsequent infection. The important role of acquired immunity to this disease is supported by the observations that individuals with recurrent *C. difficile* diarrhoea were found to mount poor anti-toxin responses despite repeated exposure to these antigens (*Aronsson* et al., 1985; *Leung* et al., 1991; *Warny* et al., 1994). A comprehensive prospective analysis of hospitalised patients receiving antibiotics

revealed that the development of anti-toxin A IgG in the serum of colonised patients was associated with asymptomatic carriage of *C. difficile* (*Kyne* et al., 2001). Patients who developed elevated serum anti-toxin A IgG titres in response to colonisation were 48 times less likely to suffer from diarrhoea than those who did not. Furthermore, patients who developed circulating antitoxin A IgG antibodies soon after a primary episode of *C. difficile* diarrhoea were much less likely to experience recurrent diarrhoea (*Kyne* et al., 2000). Thus, two recent prospective studies strongly suggest that the magnitude and kinetics of the IgG response to toxin A play an important role in determining the clinical outcome of *C. difficile* infection.

These data also raise the intriguing possibility that circulating anti-toxin A IgG antibodies may act as effector molecules in immune protection from *C. difficile* diarrhoea in humans. The observation that total stool IgG levels are elevated in patients with *C. difficile* diarrhoea (*Warny* et al., 1994), consistent with the results previously described in experimentally-infected mice (*Corthier* et al., 1989), allows one to speculate that serum exudation may facilitate access of circulating antibodies to the intestinal lumen where antibody neutralisation of enterotoxicity and protection of the intestinal mucosa may occur.

IMMUNOLOGICAL APPROACHES TO CLINICAL MANAGEMENT

Because antimicrobial therapy is the principal inciting agent for CDAD, the need for non-antibiotic approaches for the clinical management of this disease is apparent. Interventions that allow for the restoration of the commensal flora and exploit its protective effect hold the greatest promise for primary prevention and secondary prophylaxis. Active and passive immunisation strategies are being developed which may yield effective alternatives to anti-microbial therapy for use in certain clinical settings.

Active vaccination

The development of an investigational vaccine comprised of a partially purified preparation containing inactivated toxins A and B is in progress. This parenteral toxoid vaccine was recently tested in young, healthy volunteers for safety and immunogenicity (*Kotloff* et al., 2001). The *C. difficile* vaccine was administered by intra-muscular injection to volunteers at one of three dose levels (6.25 mg, 25 mg, and 100 mg) with or without aluminum hydroxide adjuvant on days 1, 8, 30 and 60. The vaccine was generally well tolerated with some local injection site soreness, which was mainly associated with the aluminum hydroxide adjuvant. Analysis of toxin A-specific IgG responses in serum by ELISA showed that all subjects seroconverted, and exhibited a range of 42- to 92-fold increases over baseline across all doses and formulations. Toxin A-neutralising titres, as determined in the cell culture cytotoxicity assay, were elevated 32- to 43-fold over baseline. Positive anti-toxin B IgG responses were seen in 90% of volunteers. Anti-toxin faecal IgA was stimulated less frequently than in serum, as might have been expected following parenteral vaccination. The potent immune responses elicited by the vaccine suggested that a vaccine might prove useful as an immunological alternative to anti microbial therapy.

Although the antibody titres stimulated through vaccination were substantial, the magnitude of these responses relative to those associated with protection from symptomatic infection was unknown. In order to bridge these data, the sera from clinical study described

above were tested in the standardised ELISA use to demonstrate the relationship between anti-toxin IgG levels and resistance to hospital-acquired *C. difficile* diarrhoea and recurrent illness. The kinetics of toxin A-specific IgG induction during the course of vaccination was assessed relative to the "threshold" level associated with protection (*Aboudola* et al., 2003). It was found that, across all dose levels and formulations, 57% of subjects reached or surpassed the threshold by day 15 of the vaccine course. Furthermore, by day 90, all subjects exceeded this level and were found to have a median titre 50-fold higher than the threshold. Because the toxoid vaccine elicited substantial toxin A-neutralising titres, this activity was also measured in sera from asymptomatic *C. difficile* carriers as well as those with *C. difficile*-associated diarrhoea. It was found that none of the patients with diarrhoea developed neutralising antibodies while only 1 of 18 carriers demonstrated a detectable titre, suggesting that this functional activity does not correlate with protection from symptomatic infection. In summary, parenteral immunisation with *C. difficile* toxoid vaccine elicits toxin A-binding antibody titres which greatly exceed the levels associated with protection from disease symptoms.

The inability to mount substantial *C. difficile* toxin-binding antibody responses despite repeated exposure is a hallmark of recurrent *C. difficile* diarrhoea. Because this syndrome is particularly difficult to treat, new approaches which may prove beneficial to these sufferers are needed. The positive findings with the toxoid vaccine in healthy volunteers prompted an initial pilot test of the vaccine in three patients with chronic, relapsing *C. difficile* diarrhoea to assess safety and immunogenicity (*Sougioultzis* et al., 2004). In this open label study, volunteers undergoing vancomycin therapy were administered 50 mg doses of the vaccine without adjuvant on days 0, 7, 28 and 56. The patients continued vancomycin therapy until the fourth vaccine dose on day 56.

All three subjects remained free of recurrent CDAD for the two month follow-up period in the absence of vancomycin. These preliminary observations suggest that active vaccination may be an effective strategy for treatment of recurrent CDAD. Larger controlled clinical studies will be needed to establish this approach as an immunological alternative to long-term antimicrobial therapy.

Passive vaccination

The prevalence of serum antibodies against *C. difficile* toxins A and B in healthy populations has prompted investigators to test the therapeutic activity of intravenous immune globulin (IVIG) preparations derived from plasma donors in individuals experiencing severe or recurrent *C. difficile* infection. In the first application of IVIG treatment for *C. difficile* infection, five children with relapsing *C. difficile* colitis who were found to have lower anti-toxin A IgG titres than healthy children were administered 400 mg/kg IVIG containing toxin A- and B-specific IgG antibodies (*Leung* et al., 1991). All treated children responded favourably to therapy, with resolution of colitis symptoms and diarrhoea and clearance of toxin B from stool samples. IVIG therapy has also shown promise in the treatment of adults with severe and/or recurrent *C. difficile* diarrhoea and colitis (*Beales*, 2002; *Salcedo* et al., 1997; *Warny* et al., 1995).

These case reports provide proof-of-principle that intravenously administered antibodies can confer rapid protection from the enterotoxic and inflammatory actions of *C. difficile* toxins. The limited availability of IVIG precludes its general

use as a therapeutic for severe or recurrent *C. difficile* diarrhoea. An intriguing alternative to standard IVIG preparations, which rely on anti-toxin antibodies raised in response to natural exposure to the organism, is the production of hyper-immune globulin derived from volunteers immunised with *C. difficile* toxoid vaccine. This strategy is being employed to produce an immune globulin preparation which a higher specific activity than IVIG developed from source plasma. The dose requirements for therapeutic activity would need to be determined empirically in clinical trials.

The oral administration of anti-*C. difficile* antibodies has also been explored for the treatment of severe or recurrent CDAD. The bovine IgG preparation found to be effective in animal models, as described above, was evaluated in a clinical study aimed at determining the survival of bovine IgG following passage through the GI tract (*Kelly* et al., 1997). The preparation was administered in liquid form or within enteric capsules and the effect of antacid treatment or therapy with a proton pump inhibitor was also assessed. The degradation of bovine IgG during transit by intestinal proteases was found to substantially reduce the activity of anti-*C. difficile* antibodies recovered in stool. Further development of this approach has not been reported.

The passive therapies described above rely on the activity of polyclonal anti-toxin antibodies. Although monoclonal antibodies (mAbs) against toxins A or B have been produced for many years, only recently have they entered development as therapeutics intended for clinical evaluation. However, the selection of antibody clones with the best chance of demonstrating clinical activity is complicated by several factors. Firstly, both toxins A and B will likely need to be neutralised for optimal efficacy and because no cross-neutralising mAbs have been reported, it is likely that at least two antibodies will be required. Secondly, because the toxins possess distinct functional domains for which their respective roles in human disease have not been precisely defined, choosing the critical epitopes within the large toxin molecules may be difficult. Thirdly, since the cell-binding domains of toxins A and B are comprised of 30 and 19 carbohydrate binding sites, respectively, the adherence to target cells may be difficult to block with a single mAb. In addition, sequence variation amongst toxin types may reduce the activity of mAbs against certain *C. difficile* strains. In total, the use of monoclonal antibodies as therapeutics represents a novel strategy against CDAD that may require more than one mAb component for optimal clinical efficacy.

Nevertheless, monoclonal therapeutics are being developed as alternative strategies. Mice expressing human immunoglobulin gene repertoires are being employed to generate human IgG antibodies against *C. difficile* toxin A and possibly toxin B. In addition, recombinant human antibodies specific for toxins A and B have been engineered into corn as a cost-effective production system. These interesting approaches may be evaluated in the clinic in the near future. The mounting interest in developing immune-based strategies for combating *C. difficile* disease validates the belief that symptomatic infections which arise due to insufficient host responses can be managed through active or passive immunisation. Furthermore, these immunological interventions also allow for the restoration of the natural protective barrier of an intact commensal flora, which together should reduce the reliance on antibiotics for treatment of this iatrogenic infection.

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LITERATURE

Aboudola, S., Kotloff, K.L., Kyne, L., Warny, M., Kelly, E.C., Sougioultzis, S., Giannasca, P.J., Monath, T.P., and Kelly, C.P.: *Clostridium difficile* vaccine and serum immunoglobulin G antibody response to toxin A. Infect. Immun. 71, 1608-1610 (2003).

Alfa, M.J., Kabani, A., Lyerly, D., Moncrief, S., Neville, L.M., Al-Barrak, A., Harding, G.K., Dyck, B., Olekson, K., and Embil, J.M.: Characterization of a toxin A-negative, toxin B-positive strain of *Clostridium difficile* responsible for a nosocomial outbreak of *Clostridium difficile*-associated diarrhea. J. Clin. Microbiol. 38, 2706-14 (2000).

Allo, M., Silva, J. Jr., Fekety, R., Rifkin, G.D., and Waskin, H.: Prevention of clindamycin-induced colitis in hamsters by *Clostridium sordellii* antitoxin. Gastroenterology 76, 351-355 (1979).

Aronsson, B., Grantsrom, M., Mollby, R., and Nord, C.E.: Serum antibody response to *Clostridium difficile* toxins in patients with *Clostridium difficile* diarrhoea. Infection 13, 97-101 (1985).

Barth, H., Pfeifer, G., Hofmann, F., Maier, E., Benz, R., and Aktories, K.: Low pH-induced formation of ion channels by *Clostridium difficile* toxin B in target cells. J. Biol. Chem. 276, 10670-10676 (2001).

Beales, I.L.: Intravenous immunoglobulin for recurrent *Clostridium difficile* diarrhoea. Gut 51, 456 (2002).

Calabi, E., Calabi, F., Phillips, A.D., and Fairweather, N.F.: Binding of *Clostridium difficile* surface layer proteins to gastrointestinal tissues. Infect. Immun. 70, 5770-5778 (2002).

Chang, J. and Rohwer, R.G.: *Clostridium difficile* infection in adult hamsters. Lab. Anim. Sci. 41, 548-552 (1991).

Chaves-Olarte, E., Low, P., Freer, E., Norlin, T., Weidmann, M., von Eichel-Streiber, C. and Thelestam, M.: A novel cytotoxin from *Clostridium difficile* serogroup F is a functional hybrid between two other large clostridial cytotoxins. J. Biol. Chem. 274, 11046-11052 (1999).

Corthier, G., Muller, M.C., Elmer, G.W., Lucas, F., and Dubos-Ramare, F.: Interrelationships between digestive proteolytic activities and production and quantitation of toxins in pseudomembranous colitis induced by *Clostridium difficile* in gnotobiotic mice. Infect. Immun. 57, 3922-3927 (1989).

Corthier, G., Muller, M.C., Wilkins, T.D., Lyerly, D., and L'Haridon, R.: Protection against experimental pseudomembranous colitis in gnotobiotic mice by use of monoclonal antibodies against *Clostridium difficile* toxin A. Infect. Immun. 59, 1192-1195 (1991).

Delacroix, D.L., Malburny, G.N., and Vaerman, J.P.: Hepatobiliary transport of plasma IgA in the mouse: contribution to clearance of intravascular IgA. Eur. J. Immunol. 15, 893-899 (1985).

Fekety, R., Kim, K.H., Brown, D., Batts, D.H., Cudmore, M., and Silva, J. Jr.: Epidemiology of antibiotic associated colitis; isolation of *Clostridium difficile* from the hospital environment. Am. J. Med. 70, 906-908 (1981).

Fernie, D.S., Thompson, R.O., Batty, I., and Walker, P.D.: Active and passive immunization to protect against antibiotic associated caecitis in hamsters. Dev. Biol. Stand. 53, 325-323 (1983).

Giannasca, P.J., Zhang, Z.X., Lei, W., Boden, J.A., Giel, M.A., Monath, T.P., and Thomas, W.D. Jr.: Serum antitoxin antibodies mediate systemic and mucosal protection from *Clostridium difficile* disease in hamsters. Infect. Immun. 67, 527-538 (1999).

Hecht, G., Pothoulakis, C., LaMont, J.T., and Madara, J.L.: *Clostridium difficile* toxin A perturbs cytoskeletal structure and tight junction permeability of cultured human intestinal epithelial monolayers. J. Clin. Invest. 82, 1516-24 (1988).

Hecht, G., Koutsouris, A., Pothoulakis, C., LaMont, J.T., and Madara, J.L.: *Clostridium difficile* toxin B disrupts the barrier function of T84 monolayers. Gastroenterology 102, 416-423 (1992).

Johnson, S., Sypura, W.D., Gerding, D.N., Ewing, S.L., and Janoff, E.N.: Selective neutralization of a bacterial enterotoxin by serum immunoglobulin A in response to mucosal disease. Infect. Immun. 63, 3166-3173 (1995).

Johnson, S., Kent, S.A., O'Leary, K.J., Merrigan, M.M., Sambol, S.P., Peterson, L.R., and Gerding, D.N.: Fatal pseudomembranous colitis associated with a variant *Clostridium difficile* strain not detected by toxin A immunoassay. Ann. Intern. Med. 135, 434-438 (2001).

Just, I., Selzer, J., Wilm, M., von Eichel-Streiber, C., Mann, M., and Aktories, K.: Glucosylation of Rho proteins by *Clostridium difficile* toxin B. Nature 375, 500-503 (1995a).

Just, I., Wilm, M., Selzer, .J, Rex, G., von Eichel-Streiber, C., Mann, M., and Aktories, K.: The enterotoxin from *Clostridium difficile* (ToxA) monoglucosylates the Rho proteins. J. Biol. Chem. 270, 13932-13936 (1995b).

Kelly, C.P., Pothoulakis, C., Orellana, J., and LaMont, J.T.: Human colonic aspirates containing immunoglobulin A antibody to *Clostridium difficile* toxin A inhibit toxin A-receptor binding. Gastroenterology 102, 35-40 (1992).

Kelly, C.P., Pothoulakis, C., and LaMont, J.T.: *Clostridium difficile* colitis. N. Engl. J. Med. 330, 257-262 (1994).

Kelly, C.P., Pothoulakis, C., Vavva, F., Castagliuolo, I., Bostwick, E.F., O'Keane, J.C., Keates, S., and LaMont, J.T.: Anti-*Clostridium difficile* bovine immunoglobulin concentrate inhibits cytotoxicity and enterotoxicity of *C. difficile* toxins. Antimicrob. Agents Chemother. 40, 373-379 (1996).

Kelly, C.P., Chetham, S., Keates, S., Bostwick, E.F., Roush, A.M., Castagliuolo, I., LaMont, J.T., and Pothoulakis, C.: Survival of anti-*Clostridium difficile* bovine immunoglobulin concentrate in the human gastrointestinal tract. Antimicrob. Agents Chemother. 41, 236-241 (1997).

Kelly, C.P. and Lamont, J.T.: *Clostridium difficile* infection. Annu. Rev. Med. 49, 375-390 (1998).

Kim, K.H., Fekety, R., Batts, D.H., Brown, D., Cudmore, M., Silva, J. Jr., and Waters, D.: Isolation of *Clostridium difficile* from the environment and contacts of patients with antibiotic-associated colitis. J. Infect. Dis. 143, 42-50 (1981).

Kim, P.H., Iaconis, J.P., and Rolfe, R.D.: Immunization of adult hamsters against *Clostridium difficile*-associated ileocecitis and transfer of protection to infant hamsters. Infect. Immun. 55, 2984-2992 (1987).

Kim, P.H. and Rolfe, R.D.: Characterization of protective antibodies in hamsters immunized against *Clostridium difficile* toxins A and B. Microb. Ecol. Health Dis. 2, 47-59 (1989).

Kink, J.A. and Williams, J.A.: Antibodies to recombinant *Clostridium difficile* toxins A and B are an effective treatment and prevent relapse of *C. difficile*-associated disease in a hamster model of infection. Infect. Immun. 66, 2018-2025 (1998).

Korhonen, H., Marnila, P., and Gill, H.S.: Bovine milk antibodies for health. Br. J. Nutr. 84, S135-S146 (2000).

Kotloff, K.L., Wasserman, S.S., Losonsky, G.A., Thomas, W. Jr., Nichols, R., Edelman, R., Bridwell, M., and Monath, T.P.: Safety and immunogenicity of increasing doses of a *Clostridium difficile* toxoid vaccine administered to healthy adults. Infect. Immun. 69, 988-995 (2001).

Kurtz, C.B., Cannon, E.P., Brezzani, A., Pitruzzello, M., Dinardo, C., Rinard, E., Acheson, D.W., Fitzpatrick, R., Kelly, P., Shackett, K., Papoulis, A.T., Goddard, P.J., Barker, R.H. Jr., Palace, G.P., and Klinger, J.D.: GT160-246, a toxin binding polymer for treatment of *Clostridium difficile* colitis. J. Antimicrob. Chemother. 45, 2340-2347 (2001).

Kyne, L., Warny, M., Qamar, A., and Kelly, C.P.: Asymptomatic carriage of *Clostridium difficile* and serum levels of IgG antibody against toxin A. N. Engl. J. Med. 342, 390-397 (2000).

Kyne, L., Warny, M., Qamar, A., and Kelly, C.P.: Association between antibody response to toxin A and protection against recurrent *Clostridium difficile* diarrhoea. Lancet 357, 189-193 (2001).

Kyne, L., Hamel, M.B., Polavaram, R., and

Kelly, C.P.: Health Care Costs and mortality associated with nosocomial diarrhea due to *Clostridium difficile*. Clin. Infect. Dis. 34, 346-353 (2002).

Larson, H.E., Barclay, F.E., Honour, P., and Hill, I.D.: Epidemiology of *Clostridium difficile* in infants. J. Infect. Dis. 146, 727-733 (1982).

Leung, D.Y., Kelly, C.P., Boguniewicz, M., Pothoulakis, C., LaMont, J.T., and Flores, A.: Treatment with intravenously administered gamma globulin of chronic relapsing colitis induced by *Clostridium difficile* toxin. J. Pediatr. 118, 633-637 (1991).

Libby, J.M., Jortner, B.S., and Wilkins, T.D.: Effects of the two toxins of *Clostridium difficile* in antibiotic-associated cecitis in hamsters. Infect. Immun. 36, 822-829 (1982).

Limaye, A.P., Turgeon, D.K., Cookson, B.T., and Fritsche, T.R.: Pseudomembranous colitis caused by a toxin A (-) B(+) strain of *Clostridium difficile*. J. Clin. Microbiol. 38, 1696-1697 (2000).

Lyerly, D.M., Lockwood, D.E., Richardson, S.H., and Wilkins, T.D.: Biological effects of toxins A and B of *Clostridium difficile*. Infect. Immun. 35, 1147-1150 (1982).

Lyerly, D.M., Saum, K.E., MacDonald, D.K., and Wilkins, T.D.: Effects of *Clostridium difficile* toxins given intragastrically to animals. Infect. Immun. 47, 349-352 (1985).

Lyerly, D.M., Phelps, C.J., Toth, J., and Wilkins, T.D.: Characterization of toxins A and B of *Clostridium difficile* with monoclonal antibodies. Infect. Immun. 54, 70-76 (1986).

Lyerly, D.M., Johnson, J.L., Frey, S.M., and Wilkins, T.D.: Vaccination against lethal *Clostridium difficile* enterocolitis with a nontoxic recombinant peptide of toxin A. Curr. Microbiol. 21, 29-32 (1990).

Lyerly, D.M., Bostwick, E.F., Binion, S.B., and Wilkins, T.D.: Passive immunization of hamsters against disease caused by *Clostridium difficile* by use of bovine immunoglobulin G concentrate. Infect. Immun. 59, 2215-2218 (1991).

McFarland, L.V., Mulligan, M.E., Kwok, R.Y., and Stamm, W.E.: Nocosomial acquisition of *Clostridium difficile* infection. N. Engl. J. Med. 320, 204-210 (1989).

McFarland, L.V., Surawicz, C.M., Greenberg, R.N., Fekety, R., Elmer, G.W., Moyer, K.A., Melcher, S.A., Bowen, K.E., Cox, J.L., Noorani, Z., et al.: A randomized placebo-controlled trial of *Saccharomyces boulardii* in combination with standard antibiotics for *Clostridium difficile* disease. JAMA 271, 1913-1918 (1994).

Price, S.B., Phelps, C.J., Wilkins, T.D., and Johnson, J.L.: Cloning of the carbohydrate-binding portion of the toxin A gene of *Clostridium difficile*. Curr. Microbiol. 16, 55-60 (1987).

Qa'Dan, M., Spyres, L.M., and Ballard, J.D.: pH-induced conformational changes in *Clostridium difficile* toxin B. Infect. Immun. 68, 2470-2474 (2000).

Riegler, M., Sedivy, R., Pothoulakis, C., Hamilton, G., Zacherl, J., Bischof, G., Cosentini, E., Feil, W., Schiessel, R., LaMont, J.T., et al.: *Clostridium difficile* toxin B is more potent than toxin A in damaging human colonic epithelium *in vitro*. J. Clin. Invest. 95, 2004-2011 (1995).

Ryan, E.T., Butterton, J.R., Smith, R.N., Carroll, P.A., Crean, T.I., and Calderwood, S.B.: Protective immunity against *Clostridium difficile* toxin A induced by oral immunization with a live, attenuated *Vibrio cholerae* vector strain. Infect. Immun. 65, 2941-2949 (1997).

Salcedo, J., Keates, S., Pothoulakis, C., Warny, M., Castagliuolo, I., LaMont, J.T., and Kelly, C.P.: Intravenous immunoglobulin therapy for severe *Clostridium difficile* colitis. Gut 41, 366-70 (1997).

Samore, M.H., Degirolami, P.C., Tlucko, A., Lichtenberg, D.A., Melvin, Z.A., and Karchmer, A.W.: *Clostridium difficile* colonization and diarrhea at a tertiary care hospital. Clin. Infect. Dis. 18, 181-187 (1994).

Savidge, T.C., Pan, W.H., Newman, P., O'Brien, M., Anton, P.M., and Pothoulakis, C.: *Clostridium difficile* toxin B is an inflammatory enterotoxin in human intestine. Gastroenterology 125, 413-420 (2003).

Sougioultzis, S., Kyne, L., Drudy, D., Keates, S., et al.: *Clostridium difficile* toxoid vaccine in recurrent *C. difficile* associated diarrhea. Submitted for publication (2004).

Tasteyre, A., Barc, M.-C., Collignon, A., Boureau, H., and Karjalainen, T.: Role of FliC and FliD flagellar proteins of *Clostrid-*

ium difficile in adherence and gut colonization. Infect. Immun. 69, 7937-7940 (2001).

Teasley, D.G., Gerding, D.N., Olson, M.M., Peterson, L.R., Gebhard, R.L., Schwartz, M.J., and Lee, J.T. Jr.: Prospective randomized trial of metronidazole versus vancomycin for *Clostridium difficile*-associated diarrhea and colitis. Lancet 2, 1043-1046 (1983).

Torres, J.F., Lyerly, D.M., Hill, J.E., and Monath, T.P.: Evaluation of formalin-inactivated *Clostridium difficile* vaccine administered by parenteral and mucosal routes of immunization in hamsters. Infect. Immun. 63, 4619-4627 (1995).

Tucker, K.D., Carring, P.F., and Wilkins, T.D.: Toxin A *of Clostridium difficile* is a potent cytotoxin. J. Clin. Microbiol. 28, 869-871 (1990).

Vaerman, J.P. and Langendries, A.: Hepatobiliary transport of IgA in the golden Syrian hamster (*Mesocricetus auratus*). Immunol. Lett. 55, 19-26 (1997).

Viscidi, R., Willey, S., and Bartlett, J.G.: Isolation rates and toxigenic potential of *Clostridium difficile* isolates from various patient populations. Gastroenterology 81, 5-9 (1981).

Viscidi, R., Laughon, B.E., Yolken, R., Bo-Linn, P., Moench, T., Ryder, R.W., and Bartlett, J.G.: Serum antibody response to toxins A and B of *Clostridium difficile*. J. Infect. Dis. 148, 93-100 (1983).

von Eichel-Streiber, C., Warfolomeow, I., Knautz, D., Sauerborn, M., and Hadding, U.: Morphological changes in adherent cells induced by *Clostridium difficile* toxins. Biochem. Soc. Trans. 19, 1154-1160 (1991).

von Eichel-Streiber, C., Boquet, P., Sauerborn, M., and Thelestam, M.: Large clostridial cytotoxins - a family of glycosyltransferases modifying small GTP-binding proteins. Trends Microbiol. 4, 375-382 (1996).

Waligora, A.-J., Hennequin, C., Mullany, P., Bourlioux, P., Collignon, A., and Karjalainen, T.: Characterization of a cell surface protein of *Clostridium difficile* with adhesive properties. Infect. Immun. 69, 2144-2153 (2001).

Ward, S.J., Douce, G., Dougan, G., Wren, B.W.: Delivery of non-toxic fragments of *Clostridium difficile* toxin A to the mucosal immune system. Rev. Med. Microbiol. 8, S34-S36 (1997).

Ward, S.J., Douce, G., Dougan, G., and Wren, B.W.: Local and systemic antibody responses induced by intranasal immunization with the nontoxic binding domain of toxin A from *Clostridium difficile*. Infect. Immun. 67, 5124-5132 (1999a).

Ward, S.J, Douce, G., Figueiredo, D., Dougan, G., and Wren, B.W.: Immunogenicity of a *Salmonella typhimurium* aroA aroD vaccine expressing a nontoxic domain of *Clostridium difficile* toxin A. Infect. Immun. 67, 2145-2152 (1999b).

Warny, M., Vaerman, J.P., Avesani, V., and Delmée, M.: Human antibody response to *Clostridium difficile* toxin A in relation to clinical course of infection. Infect. Immun. 62, 384-389 (1994).

Warny, M., Denie, C., Delmee, M., and Lefebvre, C.: Gamma globulin administration in relapsing *Clostridium difficile*-induced pseudomembranous colitis with a defective antibody response to toxin A. Acta Clin. Belg. 50, 36-39 (1995).

Warny, M. and Kelly, C.P.: Pathogenicity of *Clostridium difficile* toxins. In: Microbial pathogenesis and the intestinal epithelial cell (Ed. Hecht, G.). ASM Press, Washington, D.C., 503-524 (2003).

Wilcox, M.H., Fawley, W.N., Settle, C.D., and Davidson, A.: Recurrence of symptoms in *Clostridium difficile* infection - relapse or reinfection? J. Hosp. Infect. 38, 93-100 (1998).