Bovine antibody-enriched whey to aid in the prevention of a relapse of *Clostridium difficile*-associated diarrhoea: preclinical and preliminary clinical data

Jaap T. van Dissel,[1] Nanda de Groot,[2] Charles M. H. Hensgens,[2] Sandra Numan,[1] Ed J. Kuijper,[3] Peter Veldkamp[1,4] and Jan van 't Wout[1,5]

[1,3]Department of Infectious Diseases[1] and Department of Medical Microbiology[3], Center for Infectious Diseases, Leiden University Medical Center, Leiden, The Netherlands

[2]MucoVax bv, Niels Bohrweg 11–13, Leiden, The Netherlands

[4]Department of Medicine, Division of Infectious Diseases, University of Pittsburgh, USA

[5]Department of Internal Medicine, Bronovo Hospital, Bronovolaan 5, The Hague, The Netherlands

Correspondence
Jaap T. van Dissel
j.t.van_dissel@lumc.nl

In a pilot study, the feasibility of immune whey protein concentrate (40 %; immune WPC-40) to aid the prevention of relapse of *Clostridium difficile* diarrhoea was evaluated. Immune WPC-40 was made from milk after immunization of Holstein–Frisian cows with *C. difficile*-inactivated toxins and killed whole-cell *C. difficile*. Immune WPC-40 contained a high concentration of specific sIgA antibodies, and was effective in neutralizing the cytotoxic effect of *C. difficile* toxins in cell assays *in vitro*. Immune WPC-40 conferred protection from otherwise lethal *C. difficile*-associated caecitis in hamsters. To obtain preliminary data in humans, 16 patients (10 male; median 57 years) with toxin- and culture-confirmed *C. difficile* diarrhoea were enrolled in an uncontrolled cohort study. Nine had a history of relapsing *C. difficile* diarrhoea. After completion of standard antibiotic treatment, the patients received immune WPC-40 TID for 2 weeks; it was well tolerated and no treatment-related adverse effects were observed. In all but one case, *C. difficile* toxins had disappeared from the faeces upon completion of treatment. During a follow-up period of median 333 days (range 35 days to 1 year), none of the patients had suffered another episode of *C. difficile* diarrhoea. These preliminary data suggest that immune whey protein concentrate-40 may be of help in the prevention of relapse of *C. difficile* diarrhoea.

INTRODUCTION

Clostridium difficile is the leading cause of nosocomial infectious diarrhoea, typically affecting the elderly patient with underlying disease who has received antimicrobial therapy during an extended hospital stay. Underlying disease, old age, a long hospital stay and prior antimicrobial therapy, notably with broad-spectrum antibiotics such as cephalosporins, are factors that disrupt the endogenous bowel flora and increase the rate of acquisition of *C. difficile.* As a result, individual cases of *C. difficile*-associated pseudomembranous colitis and hospital-associated outbreaks of diarrhoea occur (Kelly *et al.*, 1994; McFarland *et al.*, 1989; Stoddart & Wilcox, 2001; Barbut & Petit, 2001; Bignardi,

1998). With an estimated half a million cases occurring annually in the United States, the infection adds significant costs to patient care (Stoddart & Wilcox, 2001; Spencer, 1998; Wilcox *et al.*, 1996).

C. difficile-associated diarrhoea (CDAD) relapses in about 10–20 % of cases (Kelly *et al.*, 1994; Stoddart & Wilcox, 2001; Barbut & Petit, 2001). Such patients are not only prone to further relapse, but also create a reservoir from which the bacterium may spread to susceptible individuals (Stoddart & Wilcox, 2001; Barbut & Petit, 2001). Treatment of CDAD consists of the discontinuation of implicated antibiotics in an effort to restore normal bowel flora (McNulty *et al.*, 1997), and all but mild cases receive specific therapy with metronidazole and/or vancomycin. Patients who relapse with diarrhoea may receive an additional cycle of antimicrobial therapy, thus triggering new recurrences. In these cases, alternative treatments to eliminate *C. difficile* bowel colonization and toxin production have been tried, including

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Abbreviations: CDAD, *C. difficile*-associated diarrhoea; WPC, whey protein concentrate.

toxin-binding resins such as cholestyramine (Burbige & Milligan, 1975), antimicrobial combinations comprising vancomycin and rifampicin or bacitracin (Tedesco, 1982), probiotics like *Saccharomyces boulardii* (Surawicz *et al.*, 1989; McFarland *et al.*, 1994), *Lactobacillus* GG (Bennett *et al.*, 1996; Thomas *et al.*, 2001), and administration of stools from healthy human relatives (Aas *et al.*, 2003). Many of these treatments, however, are only modestly effective and may present problems of their own, such as the emergence of vancomycin-resistant enterococci, or that cholestyramine cannot be given in severe CDAD in combination with vancomycin because it binds this antibiotic (Taylor *et al.*, 1980; Kurtz *et al.*, 2001).

In the present study, we report preliminary data on the use of specific polyclonal antibody-enriched immune whey protein concentrate (immune WPC-40; MucoMilk). The pilot study in humans was preceded by a study in hamsters that provided evidence that immune WPC-40 effectively protects against lethal caecitis caused by *C. difficile*, without relapse after discontinuation of the treatment.

METHODS

Bacteria and culture filtrate. The *Clostridium difficile* strain VPI 10463 was grown overnight at 37 °C in medium composed of 2 % yeast extract (Oxoid), 2 % soya peptone (Oxoid), 1 % glucose, 1.04 g K_2HPO_4 l^{-1}/0.68 g KH_2PO_4 l^{-1} (Merck) and 1 g $NaHCO_3$ l^{-1} (Merck) for the production of whole cells. To harvest culture filtrate for toxins, *C. difficile* was cultured for 6 days. The cell component of the immune-stimulant was measured at OD_{600} and set to contain between 10^8 and 10^9 bacterial cells ml^{-1}; the toxoid component was based on a fixed toxin A concentration measured by densitometry, using toxin A purified to >95 % homogeneity as standard. Inactivation of whole bacterial cells and culture filtrate was done by adding formaldehyde to a final concentration 1 % (stock 37 %, Merck), followed by incubation overnight at 4 °C and subsequently for 1–2 h at 37 °C. Next, formaldehyde was removed by diafiltration, to ensure a final concentration of < 0.001 %.

Immune whey protein concentrate preparation. Polyclonal antibody-enriched immune whey protein concentrate 40 % (immune WPC-40) was prepared from cow's milk, after immunization of Holstein–Frisian cows with formaldehyde-inactivated whole *C. difficile* cells (strain VPI 10463) and toxoid prepared from the *C. difficile* culture filtrate. The composition of the immune-stimulant (both in terms of inactivated whole bacterial cells, as well as toxoid concentration) was kept constant throughout the study, and for the preparation of the WPC-40 used in this study, a single batch was prepared.

Immunization comprised repeated nasal (mucosal; every 2 weeks), subcutaneous (every 2 months) and supramammary lymph node administration (percutaneous; once every month), and was carried out during the lactation period after calving when cows give mature milk. This resulted in a concentration of *C. difficile*-specific sIgA just as high as that in the colostrum of cows immunized in an identical fashion (see Results). Normal farm breeding and selection techniques were applied to harvest mature milk.

Immune WPC-40 was prepared via standard milk industry techniques. Fat was removed by centrifugation, and caseins were removed by acid precipitation or during cheese production. The whey fraction was pasteurized, concentrated by ultrafiltration and spray-dried to the final whey powder by DMV International (Veghel, The Netherlands) and

MucoVax (Leiden, The Netherlands). This powdered milk preparation represented concentrated bovine milk, lacking only the casein fraction, and fulfilled the stringent Dutch agricultural and food criteria for milk for human consumption. Analysis of immune WPC-40 showed that it had the following major components: protein (38.3 %, of which approximately 10 % were immunoglobulins), fats (1.9 %), lactose (33.5 %), ash (residue) (18 %) and moisture (4.8 %). Appropriately labelled aluminium-foil sachets were filled with 5 g whey. When dissolved in 100 ml water, the lactose content of one sachet (1.7 %) did not exceed the lactose content of raw milk (~4.6 %). The lactose and mineral content of each sachet equalled that of approximately 50 ml of normal milk for human consumption. Sachets were stored at room temperature (15–20 °C) in the dark.

***C. difficile*-specific sIgA and IgG.** Toxin A- and *C. difficile*-specific sIgA and IgG antibody concentrations in immune WPC-40 were determined by ELISA. Microtitre plates (96 wells, Greiner) were coated with *C. difficile* whole bacterial cells or toxin A in coating buffer (74 mM $NaHCO_3$, 26 mM Na_2CO_3, pH 9·6) for 2 h at 70 °C or 37 °C, respectively. The plates were kept overnight at 4 °C, washed three times with PBS and blocked with 2 % gelatin (Difco) diluted in 0.05 % Tween 20 (Sigma) in PBS (PBST). Next, plates were incubated for 1 h at 37 °C and washed three times with PBS. Samples were diluted in 0.2 % gelatin/PBST and added to each well. Plates were incubated for 1·5 h at 37 °C and washed three times with PBS. Monoclonal anti-bovine IgA (α-chain) or IgG1/2 (γ-chain) (ECACC IL-A71/IL-A2) were labelled with digoxigenin-3-O-methylcarboxy-ε-aminocaproic acid-N-hydroxysuccinimide ester (Boehringer Mannheim) and diluted in 0.2 % gelatin/PBST. Next, the monoclonal antibodies were added to measure specific sIgA or IgG. Plates were incubated for 1·5 h at 37 °C, washed three times with PBS, and then a 1 : 3000 dilution of anti-digoxigenin-POD Fab fragments (Boehringer Mannheim) in 0.2 % gelatin/PBST was added. After another incubation for 1·5 h at 37 °C, plates were washed three times with PBS and developed by adding 2·5 mg ml^{-1} ABTS substrate (2,2-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt, Sigma) diluted in phosphate/citrate buffer (50 mM, pH 4·2) at 37 °C for 30 min. The optical densities of the plates were measured at 415 nm. ELISA units for sIgA and IgG were calculated by comparison of the measured optical densities to those of a pool of undiluted immune colostrums, which for both immunoglobulins were set arbitrarily at 1000 units ml^{-1}.

Inhibition of cytotoxicity. The neutralizing effect of immune WPC-40 was tested in two distinct tissue-culture assays (96 wells) using HeLa cells or IMR-90 human fibroblasts, and assessed by cell rounding (i.e. cytotoxicity) after exposure to *C. difficile* culture filtrate or toxins A and B purified from the *C. difficile* culture filtrate. The minimum concentration of purified toxin to give a 50–100 % cell rounding was 15 ng ml^{-1} for toxin A and 30 pg ml^{-1} for toxin B (Sullivan *et al.*, 1982), as determined on IMR-90 fibroblasts. For *C. difficile* culture filtrate (typically 1·2 mg protein ml^{-1}) assayed on HeLa cells, the minimum cytotoxic dose was 10×5^6, i.e. the sixth fivefold dilution of the culture filtrate. In the latter case, the neutralization assay was set up as follows: the culture filtrate was diluted in fivefold steps ending in a dilution of 10×5^7; this dilution was no longer toxic to HeLa cells. Immune and control whey was added to these dilutions. Whey made from the colostrum of cows immunized with the identical immune-stimulant, as well as commercially available goat antiserum (Techlab), were used as positive (i.e. neutralizing) control samples. Next, the culture-filtrate whey sample (1 : 1), containing the immune WPC-40 or control whey, or positive controls, was added to HeLa cells and incubated for 24 h at 37 °C under 7 % CO_2. The minimum cytotoxic dose was the lowest concentration of toxin that was toxic to fibroblast or HeLa cells.

Animals. Golden Syrian hamsters weighing $\sim$100 g were obtained from Charles River Laboratories. Animal studies were carried out by M.

Warny and colleagues, Department of Gastroenterology, at the Beth Israel Deaconess Medical Center, Harvard Medical School.

Hamster model of *C. difficile*-induced caecitis. Groups of ten hamsters were challenged with a toxigenic strain of *C. difficile* ($\sim10^4$ cells of VPI 10463) by gastric tube 24 h after intraperitoneal clindamycin administration (1 mg per 100 g body weight), as described by Chang *et al.* (1978). For the animal study, the only difference in the immune WPC-40 preparation procedure was that the caseins were precipitated by acid, then dialysed against 100 mM sodium bicarbonate and sterilized by filtration prior to gastric administration. Control WPC was prepared similarly from milk of non-immunized cows. Ten-times-concentrated immune WPC-40 and control WPC were prepared by ultrafiltration. The following five treatment regimens were used in this study (for every intervention a group of ten animals was used): (1) no treatment intervention; (2) control WPC prepared from milk of non-immunized cows; (3) immune WPC-40 prepared from milk of immunized cows; (4) control WPC prepared from milk of non-immunized cows concentrated ten times; (5) immune WPC-40 prepared from milk of immunized cows concentrated ten times. Treatments 1) to 5) were carried out in a blinded fashion. In the treatment groups, 1 ml of the different whey preparations was administered by feeding tube 3 h before bacterial challenge, 3 h after challenge and subsequently every 8 h for three successive days (i.e. resulting in 11 ml of whey for the complete treatment). One control group of ten hamsters did not receive any whey.

The hamsters were monitored for diarrhoea and survival for up to 4 weeks after bacterial challenge. In this model, unprotected animals develop an acute caecitis that typically follows a lethal course within 48–72 hours (Chang *et al.*, 1978). The histological features of this caecitis are similar to those of human *C. difficile*-associated colitis.

Design of the human pilot study. We performed a prospective uncontrolled cohort study of immune WPC-40 (MucoMilk) at the Leiden University Medical Center (LUMC), Bronovo Hospital in The Hague and several regional hospitals. Patients were enrolled after completing a 10–14 day course of either metronidazole and/or vancomycin per os for CDAD, as confirmed by toxin assay and positive faeces culture of *C. difficile*. The choice and duration of antimicrobial therapy was decided by the attending physicians. Patients with a milk allergy were not eligible for the study. The protocol used in this study was reviewed and approved by the Institutional Review Boards at the LUMC and Bronovo Hospital. Nine patients were offered MucoMilk off-protocol, as other measures had failed to prevent relapses of CDAD. All patients gave informed consent according to GCP standards prior to enrolment.

The evaluation (though not statistically validated) focused on the occurrence of a clinical relapse of CDAD as end-point. The secondary end-point was the disappearance from the faeces of *C. difficile* toxin. A relapse of diarrhoea was defined as either an increase of more than two bowel movements a day for two consecutive days, compared to the bowel movements in the days prior to that, or a change to a looser consistency of stool for two consecutive days, compared to previous bowel movements. In the laboratory, the demonstration of toxin production by *C. difficile* was required in faeces obtained from relapse diarrhoea.

Patients and intervention. A health screen was performed to identify underlying conditions, active diseases and prior episodes of CDAD. Current medications and previously taken antimicrobial medications were recorded. Subjects enrolled in the study received immune WPC-40 (MucoMilk) three times daily for 2 weeks. MucoMilk was administered within 1 day of the cessation of antibiotic therapy. For each dose, one sachet containing 5 g immune whey powder was added to 100 ml lukewarm uncarbonated mineral water. The dosage was not adjusted to take account of age, weight or other factors. After brief stirring, the mixture was ingested on an empty stomach 1 h before each meal. After 2 weeks, remaining sachets were counted to assess compliance.

Subjects were asked to report any perceived adverse effects, and were seen weekly by a study nurse. At the end of the 14-day study period, the performance status (Karnofsky score of min. 10 to max. 100), a medical check-up and general health questionnaire were repeated and any changes were recorded. Before and after completion of the study, venous blood was sampled for the determination of creatinine and the liver enzymes alanine aminotransferase (ALT) and aspartate aminotransferase (AST). In the later phase of the pilot study (seven subjects), a diary was kept by the patients to record daily the number and consistency (reported as normal = 1, intermediate = 2, watery = 3) of their bowel movements.

Toxin assay and culture of *C. difficile* in faeces. Faeces was collected before and after finishing MucoMilk therapy, and thereafter in case of diarrhoea to detect a possible relapse, and evaluated for *C. difficile* by culture and toxin assay. Samples were processed within 24 h of collection. Stool specimens treated with the ethanol-shock selection procedure were plated onto non-selective blood agar plates and CDMN plates, both of which contained 32 mg moxalactam l^{-1} and 12 mg norfloxacin l^{-1}, as previously described (Aspinall & Hutchinson, 1992). Plates were incubated in anaerobic conditions at 37 °C for 3 and 7 days, respectively. Colonies of Gram-positive rods with subterminal spores were tested for the production of L-proline-aminopeptidase and for hydrolysis of aesculin (Fedorko & Williams, 1997). The procedure allowed for a gross estimate of the number of viable micro-organisms in standardized samples. Also, samples were assayed for toxin A and toxin B production by use of commercial enzyme immunoassays and conventional tissue-culture cytotoxicity tests (ELFA, VIDAS CDA2, Biomerieux).

RESULTS

Immune WPC-40 (MucoMilk) preparation

Holstein–Frisian cows were immunized during the first and second lactation period after calving; the immunization schedule consisted of priming by several intranasal (mucosal) administrations and then by boosting through supra-mammary lymph-node stimulation. Whey prepared from the milk of cows thus immunized contains high concentrations of specific antibodies against toxin A and toxin B, as well as whole bacterial cells. The immunization protocol predominantly enhanced the specific sIgA response in the mature milk: the specific sIgA concentration of the immune milk reached at least the concentration in pooled colostrums of identically immunized cows, that of IgG about one-tenth of that concentration (Fig. 1). Thus, the data indicate that the relative concentration of specific sIgA versus IgG in whey prepared from the milk of immunized cows was about a 100 : 1. In contrast, in the mature milk of the cows before immunization, concentrations of sIgA and IgG antibodies against toxin A, toxin B and whole bacterial cells amounted to less than 10 U ml^{-1}. In immunized cows, high titres were maintained throughout the period of mature milk production (i.e. the lactation period, cf Fig. 1). This enabled us to collect large amounts of immune milk, and therefore the readily available mature (immune) milk rather than colostrum was used as starting material for production of immune whey.
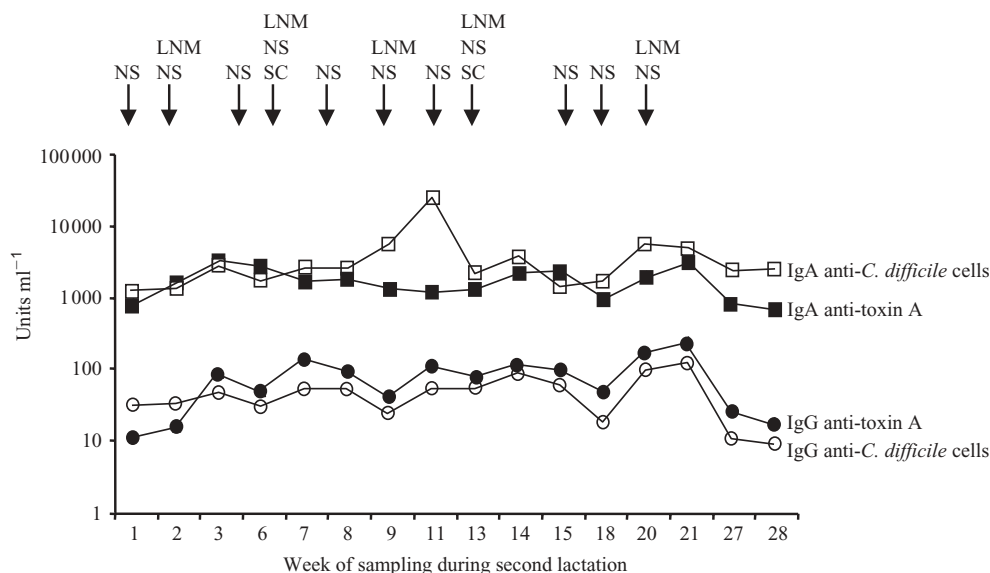
Fig. 1. The concentration of specific sIgA (upper lines) and IgG (lower lines) against *C. difficile* cells and toxin A was measured in the milk of a pool of 12 immunized Holstein−Frisian cows, sampled over a period of 28 weeks. The antibody units were standardized against the titres of a pool of undiluted colostrum of identically immunized cows; the levels of antibodies reached in the colostrums were arbitrarily set at 1000 U sIgA ml^{-1} and 1000 U IgG ml^{-1}. Thus, during the whole period of immune stimulation, the sIgA in samples of mature milk was at a concentration identical to that reached in colostrum, whereas the concentration of IgG remained at about one-tenth of that in colostrum. Immunizations were administered as follows: intranasal (NS); percutaneous, supramammary lymph node (LNM); subcutaneous (SC). In milk of cows before immune stimulation, the (cross-reacting) sIgA and IgG antibodies amounted to less than 10 U ml^{-1}.

Neutralization by MucoMilk of toxin-induced cytotoxicity

The neutralizing activity of immune WPC-40 was tested in tissue culture, with cytotoxicity assayed by cell rounding after exposure to *C. difficile* culture filtrate (assayed in HeLa cells) or purified toxins (assayed in IMR-90 human fibroblasts). Immune WPC-40 reduced the cytotoxicity of the *C. difficile* culture filtrate by 3125-fold (range 625−15 625), that of purified toxin A about 40-fold (600 ng ml^{-1}) and that of purified toxin B 332-fold (10 ng ml^{-1}). Control whey prepared from the milk of non-immunized cows did not reduce the cytotoxicity of *C. difficile* culture filtrate or purified toxins.

Animal study

Immune WPC-40 conferred protection in hamsters challenged with toxigenic *C. difficile*.

Hamsters were given either whey from immunized cows or whey from non-immunized cows at different concentrations, and monitored for survival over a 2-week period after intragastric administration of toxigenic *C. difficile*. All animals given *C. difficile* but no treatment with whey or control whey died of caecitis, whereas none of uninfected hamsters died (Fig. 2). Immune WPC-40 protected the animals effectively, as evidenced by their high survival rate: in hamsters given immune WPC-40 or concentrated immune WPC-40 the survival was very high, 90 % and 80 %,

respectively, compared to 0 % survival in the control group ($P < 0.005$). The ten-times concentration of the immunoglobulin fraction of immune whey did not increase the already high survival rate of treated hamsters. However, the control whey had some positive effect in the control group, since the survival rate increased from 0 to 20 %. This may be explained by the presence of other bactericidal proteins, such as lactoferrin and lysozyme, or by the cross-reaction of non-specific immunoglobulins.

Significantly, during a 4-week observation period, none of the hamsters developed *C. difficile* disease, despite stopping administration of the immune whey 3 days after bacterial challenge. Hamsters given control whey developed severe diarrhoea, and the moribund animals had, by pathology, acute mucosal inflammation of the caecum with oedema, neutrophil infiltration and ulceration. In contrast, the colon mucosa of hamsters that were given immune whey did not show signs of inflammation at 4 weeks after bacterial challenge (data not shown).

Human study

We included 16 patients (10 male, median age 57, range 6–78, years). In one instance, that of patient no. 6, the parents gave permission to enrol their son, aged 6 years. All patients had received antibiotics in the month prior to CDAD, either because of proven (recurrent) infections, fever during immunosuppression for an underlying malignant or auto-
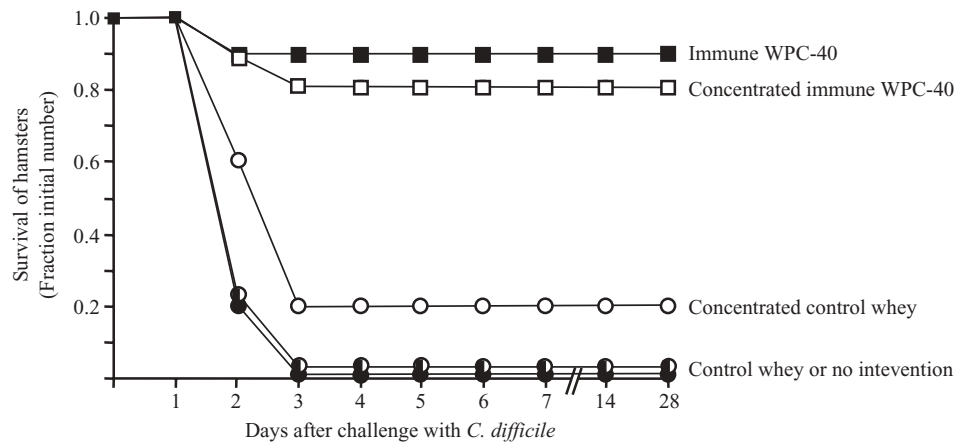
Fig. 2. Groups of ten hamsters received toxigenic *C. difficile* by gastric tube, 24 h after clindamycin. Whey was administered by feeding tube for three successive days. The following regimens were used: (1) no intervention (negative controls), (2) control whey prepared from milk of non-immunized cows, (3) immune WPC-40 prepared from milk of immunized cows, (4) control whey prepared from milk of non-immunized cows ten-times concentrated, and (5) immune WPC-40 prepared from milk of immunized cows ten-times concentrated. The hamsters were monitored for diarrhoeal symptoms and survival for up to 4 weeks after bacterial challenge.

immune disorder, or prophylaxis for an operative procedure (Table 1). Seven individuals were included after finishing therapy of their first episode of CDAD. The remaining nine patients had a history of one or more relapses of CDAD; the last relapse prior to enrolment occurred on average 3 weeks (range 2–4) earlier. One typical case of a patient with relapses of CDAD, and his course after MucoMilk, is illustrated in Fig. 3(a).

Adverse effects

All but one patient completed the 2-week course of Muco-Milk. This patient died from an acute neurological complication of a congenital Rathke's cyst in the mesencephalon on the tenth day of the 2-week course. None of the patients experienced an adverse effect related to the use of MucoMilk, and the preparation was well tolerated. Furthermore, compared with values at the start of treatment, the patients' Karnofsky score did slightly improve (mean 65 before treatment, 70 after treatment; Table 1), whereas determinations of serum liver enzymes (ALT before 8–43, after 7–41 U l^{-1}; AST before 17–52, after 17–41 U l^{-1}) and creatinine (before 49–165 μmol l^{-1}, after 57–129 μmol l^{-1}) did not change during the study.

Relapse of CDAD

With a median follow-up period of 333 days (range 35–365), none of the patients experienced a relapse of CDAD (Table 1). One patient experienced an episode of diarrhoea due to *Campylobacter* sp. 8 days after completing the 2-week course of MucoMilk. Faecal cultures were negative for *C. difficile* and its toxins. The patient responded favourably to macrolide treatment, and no other diarrhoeal episodes were reported in follow-up.

In the seven individuals who kept a diary, stool consistency and bowel movements improved during MucoMilk therapy and returned to normal towards the end of the first week (Fig. 3b).

Faecal toxin and enumeration of *C. difficile*

In 14 out of 15 patients, the faeces toxin assay had become negative in faeces samples taken in the week after stopping MucoMilk, and in the single patient that was at first positive the assay became negative within one more week (Table 1). Also, *C. difficile* could no longer be cultured from the faeces in 9 out of 15 subjects (Table 1). A rough estimate of the number of colonies in standardized samples of faeces indicated that, in those patients who remained culture-positive, the numbers of *C. difficile* had reduced. This reduction of faecal colonization of *C. difficile* was also observed in preliminary experiments using real-time PCR (directed to the toxin B gene as target) to quantitatively detect faecal colonization. For instance, four consecutive faecal samples of patient no. 1 tested for *C. difficile* by real-time PCR revealed decreased CT values, indicating a reduction of faecal colonization. The four *C. difficile* isolates from these samples were also investigated by PCR-ribotyping, and belonged to a single ribotype. This real-time technique is currently the subject of further investigation for the quantitative measurement of *C. difficile* in faecal samples (E. J. Kuijper, unpublished data).

DISCUSSION

The findings of the present pilot study suggest that immune whey protein concentrate, in other words a polyclonal sIgA-enriched whey directed against *C. difficile* toxins and whole bacterial cells, has the potential to reduce the rate of relapse of CDAD in humans, while being safe and well tolerated. These

Table 1. Demographic and clinical information for 16 patients with *Clostridium difficile*-associated diarrhoea (CDAD) given supplementary therapy with MucoMilk

Abbreviations: CLL, chronic lymphatic leukaemia; COPD, chronic obstructive pulmonary disease; GI, gastrointestinal tract; UT, urinary tract; V, vancomycin; M, metronidazole.

Patient no.	Sex, age (years)	Inciting condition of antibiotic usage/underlying condition	Episode of *C. difficile* infection*	*C. difficile* infection treated with:	*C. difficile* culture/toxin†	Karnofsky score start/finish‡	Follow-up (days)§
1	Male, 78	*Salmonella* bacteraemia/CLL, COPD	3	M/V	+/−	80/80	>1 year
2	Male, 75	Fracture – osteosynthesis	1	M	−/−	30/40	8‖
3	Female, 73	Neuroborreliosis	1	M	+/−	70/70	>1 year
4	Male, 70	Cardiac valve prosthesis operation	2	M/V	−/−	50/60	>1 year
5	Male, 67	Cardiac bypass operation	4	M/V	+/+¶	80/80	>1 year
6	Male, 6	GI-dysplasia	1	M	−/−	40/40	>1 year
7	Male, 75	UT infection/fracture – osteosynthesis	1	M	−/−	50/50	341
8	Female, 18	Immunosuppression for Crohn's colitis	1	M/V	+/−	90/100	333
9	Male, 78	UT infection	1	V	−/−	70/80	300
10	Male, 50	M. Kahler, ASC (autologous stemcell) transplant	0	M	+/−	80/80	287
11	Male, 19	Rickettsiosis	0	M	−/−	90/100	243
12	Female, 52	Cystitis/Rathke's cleft cyst operation	0	M	‖	40/−‖	−‖
13	Female, 24	Antibiotics for gastroenteritis in Peru	0	M	−/−	90/90	107
14	Male, 31	Multi-trauma, multiple antibiotics	0	M	−/−	30/40	68
15	Female, 24	Cauda syndrome – abscess, UT infection	0	M	−/−	70/70	63
16	Female, 57	Pancreatitis – multiple infections	0	M	+/−	70/70	35

*Immune whey started after primary episode (0) or first to fourth relapse (1–4) of CDAD.

†Outcome of faecal culture/toxin test in the week after completion of MucoMilk. +, positive; −, negative.

‡Karnofsky score (min. 10 to max. 100) before and 1 week after completion of MucoMilk.

§Days of follow-up at 22 March 2004.

‖Patient no. 2 died in another hospital from an acute cardiac cause 8 days after his last dose of MucoMilk. Patient no. 12 died on the tenth day of the 14-day course of MucoMilk from an acute neurological deterioration, likely bleeding from a cerebral cyst.

¶Outcome of faecal culture/toxin test 1 week later was +/−.
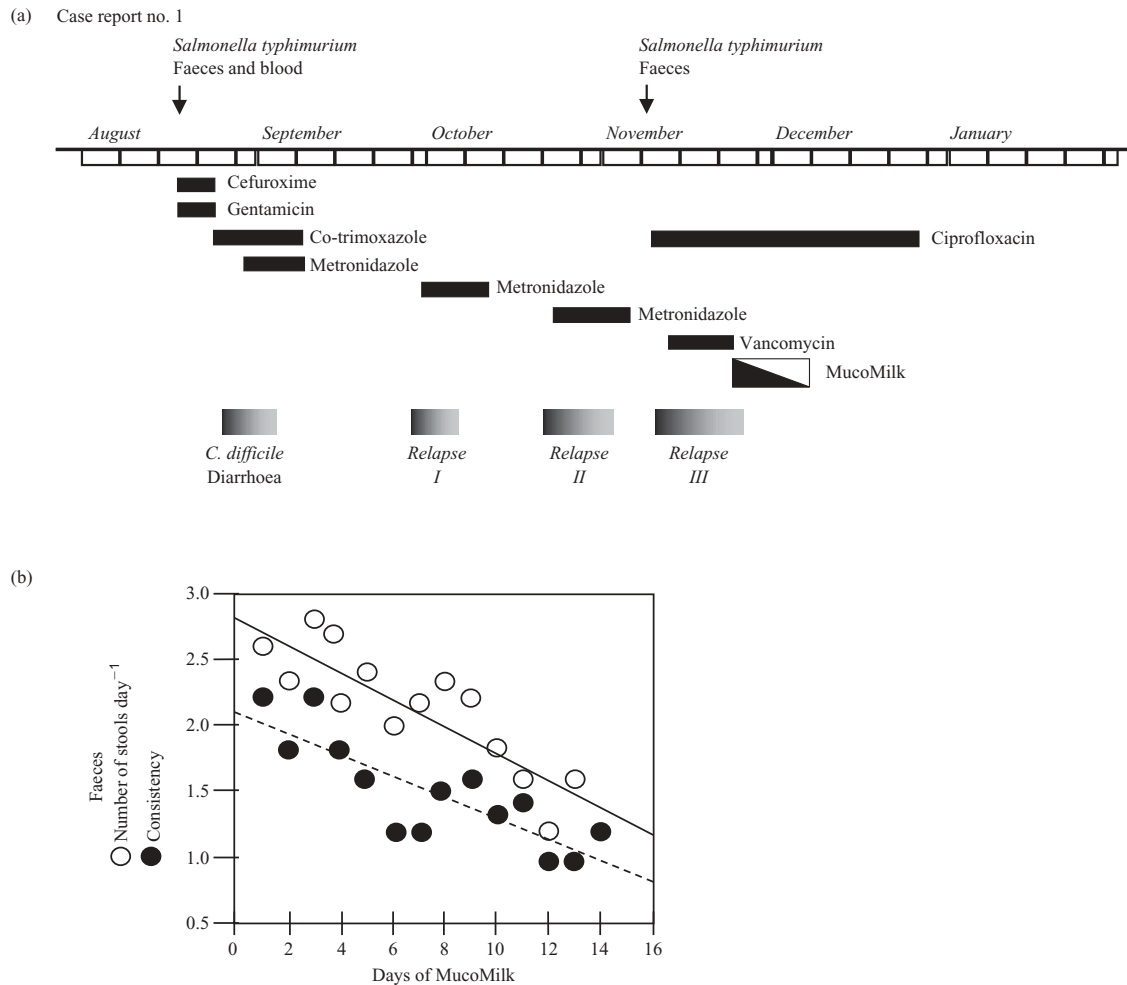
Fig. 3. Typical cases of relapsing *C. difficile*-associated diarrhoea (CDAD), treated with a 14-day course of MucoMilk. (a) Case no. 1 was a 78-year-old male with chronic lymphocytic leukaemia and secondary hypogammaglobulinaemia, who received antibiotics regularly for recurrent respiratory tract infections. He was hospitalized because of a traumatic haemorrhage in an enlarged spleen, and fever due to *Salmonella* bacteraemia. After empiric therapy with cefuroxime and gentamicin, he received co-trimoxazole. During his hospital stay, he developed CDAD. This episode and two subsequent relapses were treated with metronidazole. Subsequently, he had a fourth episode of CDAD that coincided with the use of ciprofloxacin for a relapse of *Salmonella* enteritis. Following treatment with vancomycin, MucoMilk was started. (b) Mean number of passage of stools, and consistency of stools per day, in seven patients given a 2-week course of MucoMilk, after standard antibiotic treatment of CDAD. Consistency was scored daily as normal (1), intermediate (2) or watery (3).

preliminary data in humans are supported by the finding that immune WPC-40, but not control whey, contains high concentrations of sIgA antibodies directed against whole bacterial cells and *C. difficile* toxins, and that this is effective in the neutralization *in vitro* of the cytotoxic effect of *C. difficile* culture filtrate and toxins. It is also protective in a hamster model of *C. difficile*-associated caecitis that otherwise follows a lethal course within days. Furthermore, after completion of immune WPC-40 treatment in our *C. difficile* culture- and toxin-positive patients with CDAD, in 14 out of 15 patients the faeces cytotoxicity assay had become negative, while culture of *C. difficile* was either negative or showed reduced numbers of bacteria. This coincided with the normalization of the frequency and consistency of the stools.

At present, the preliminary findings are promising for a role for immune WPC-40 in the prevention of relapse of CDAD. None of the enrolled patients suffered a further relapse after the MucoMilk, despite the fact that many in this group had had one or more episodes of diarrhoea prior to treatment and were at risk of relapse. For instance, in a randomized placebo-controlled trial of *Saccharomyces boulardii*, the relapse rates in patients given placebo were 24 % and 65 % in those with an initial episode and those with a recurrent episode of CDAD, respectively (McFarland *et al.*, 1994). Thus, one would have expected to see at least some new CDAD episodes in our patients. However, given the obvious methodological limitations of the present uncontrolled cohort study, the use of immune whey will now be subjected to a formal randomized placebo-controlled trial.

Consistent with an earlier report on anti-*C. difficile* bovine immunoglobulin IgG concentrate prepared from the colostral milk of Holstein cows (Kelly *et al.*, 1996), in this study the immune WPC-40 neutralized the cytotoxic effects of purified toxins A and B on cultured human cells, whereas control bovine whey concentrate had no toxin-neutralizing activity. In hamsters, the immune WPC-40 proved highly effective in protecting against lethal *C. difficile* caecitis. Significantly, despite withdrawal of the immune WPC-40 3 days after bacterial challenge with a highly toxigenic strain, in a 4-week observation period, none of the hamsters developed *C. difficile* disease. This finding is in contrast to a previous report, in which treatment of hamsters with colostral bovine IgG directed only against toxin A and B and not whole bacterial cells, though effective in protecting hamsters against *C. difficile*-induced caecitis as long as the colostral IgG preparation was administered, did not prevent disease when treatment was stopped (Lyerly *et al.*, 1991). Probably, immune WPC-40, which contains a high concentration of sIgA directed against whole bacterial cells as well as toxins, helped reduce bowel colonization and promoted the elimination of the micro-organism. Consistent with this notion, after the completion of immune whey therapy in our *C. difficile* culture- and toxin-positive patients, in 14 out of 15 patients the faeces cytotoxicity assay was negative, and in most colonization with *C. difficile* was eliminated or reduced.

The immune WPC-40 must pass through the stomach and intestinal tract, and retain sufficient activity in the stool. It has previously been demonstrated that orally taken bovine IgG directed against *C. difficile* toxins resists digestion in the human stomach and upper intestinal tract to the extent that it retains specific anti-*C. difficile* toxin A binding and neutralizing activity in the stools (Warny *et al.*, 1999; Kelly *et al.*, 1997). The administration of antacid or proton pump inhibitors did not add significantly to its activity in this respect (Warny *et al.*, 1999; Kelly *et al.*, 1997). In the present whey preparation, the neutralizing activity depends to a large extent on specific sIgA; this immunoglobulin is generally more resistant than IgG to degradation in the stomach and intestine (Fagarasan & Honjo, 2003). This was corroborated by an *in vitro* digestion system that mimics the dynamic conditions in the stomach and small intestine (Minekus & Havenaar, 1996) and showed that sIgA was less degraded than IgG: the immunoglobulins survived passage through this digestion system by 60 % and 40 %, respectively (Internal report on 'TIM1' by *TNO* Nutrition and Food Research, Zeist, The Netherlands). These findings suggested that, following the addition of immune whey powder to uncarbonated mineral water, the suspension could simply be taken on an empty stomach approximately 1 h before a meal.

Patients who suffer multiple relapses of CDAD present a major therapeutic problem, because repeated cycles of antibiotics may trigger more relapses. In such cases, various therapies, including cholestyramine (Burbige & Milligan, 1975; Kurtz *et al.*, 2001), *Lactobacillus* GG (Bennett *et al.*, 1996; Thomas *et al.*, 2001), *Saccharomyces boulardii* (Surawicz *et al.*, 1989; McFarland *et al.*, 1994), and even

replacement of the colonic flora from the stools of healthy relatives (Aas *et al.*, 2003), have been tried. In paediatric patients, intravenous immunoglobulin has been administered (Leung *et al.*, 1991). Many of these alternative non-antibiotic treatments, however, have been found to be only moderately effective (Stoddart & Wilcox, 2001; Surawicz *et al.*, 1989; McFarland *et al.*, 1994; Thomas *et al.*, 2001). Also, in a large placebo-controlled randomized trial, no benefit of prophylactic therapy with *Lactobacillus* was shown (Thomas *et al.*, 2001). The effectiveness in preventing mortality in the animal model of lethal *C. difficile* caecitis and our preliminary findings in humans suggest that immune WPC-40 consisting of polyclonal antibody-enriched immune whey is potentially of use to reduce the relapse rate of *C. difficile*-associated diarrhoea. This issue will now be addressed in a prospective, randomized controlled trial.

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