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***Clostridium difficile*-associated diarrhoea: bovine anti-*Clostridium difficile* whey protein to help aid the prevention of relapses**

Antibiotic treatment can usually help in symptomatic recovery in *Clostridium difficile*-associated diarrhoea (CDAD), but recurrent episodes of diarrhoea remain a major problem.^{1–4} Besides increasing the dose or extending the course of antibiotics, and using alternating or pulsed regimens, there are no effective alternatives to prevent relapses.⁵ Previously, we reported on anti-*Clostridium difficile* whey protein concentrate (anti-CD-WPC) made from the milk of cows immunised against *C difficile* and its toxins. Anti-CD-WPC is prepared using standard techniques used in the milk industry, and contains a high concentration of sIgA directed against *C difficile* and its toxins. It neutralises the cytotoxicity of *C difficile* toxins in vitro and protects hamsters against otherwise lethal *C difficile*-associated colitis.⁶

The aim of this study was to assess the preliminary efficacy of anti-CD-WPC in aiding the prevention of relapses in patients with CDAD confirmed by positive faecal *C difficile* toxin assay and culture before enrolment.^{7–8} After completion of at least 10 days of antibiotic treatment, patients received anti-CD-WPC for 2 weeks, with a follow-up period of 60 days. Patients provided written, informed consent.

Exclusion criteria were a history of milk intolerance, or inability to receive oral fluids. Patients received anti-CD-WPC 15 g/day, divided into 3 equal doses, for 14 days. Anti-CD-WPC was added to flat mineral water, stirred and taken in an empty stomach 1 h before each meal. Patients kept a diary to

Figure 1 Kaplan-Meier plot showing the fraction of *Clostridium difficile*-associated diarrhoea (CDAD) episodes resulting in a relapse of CDAD during and after receiving anti-*C difficile* whey protein concentrate (anti-CD-WPC). The immune whey preparation anti-CD-WPC was taken for 2 weeks (indicated in graph), after completion of at least 10 days of standard antibiotic therapy.

report stool frequency (stools/day) and consistency (scored as normal, semiformal or watery). A relapse of CDAD was declared if the patient reported looser stools compared with the day before (eg, change from normal to semiformal) and an increase in stool frequency for two consecutive days, or a single day with an increase of ≥ 3 stools, or any day with passage of >6 stools/day.

Tolerability and safety were monitored by self-report diaries, laboratory monitoring and active surveillance by interview, and has been reported elsewhere.⁹

We enrolled 101 patients (51 female; median age 74 years) with a total of 109 CDAD episodes (table 1). Most patients had underlying conditions that made them susceptible to acquiring CDAD (ie, older age cohort, antibiotic usage, surgery, stay in the intensive care unit, immunosuppressive medication).^{1–4} Eight patients did not complete anti-CD-WPC (1 died because of underlying disease, 4 stopped because of taste dislike, 1 relapsed and in 2 the attending doctor made a decision of no treatment). Of those who completed the course, five patients died within the 60-day follow-up because of underlying disease. Seven received anti-CD-WPC twice and one received it thrice.

In all, 11 (~10%) of 109 episodes were followed by a relapse of CDAD (table 1, fig 1). Faecal toxin assay and *C difficile* culture were positive in all cases. Patients with severe underlying disease were more likely (relative risk (RR) 11.7; 95% CI 1.5 to 96) to relapse than those without underlying condition, as were patients with *C difficile* PCR-ribotype 027 (RR 2.2, 95% CI 0.6 to 8.2).

The findings indicate that anti-CD-WPC, an immune whey protein concentrate directed against *C difficile* and its toxins, may aid the prevention of relapse in CDAD. This is based on the 10% relapse rate in those given anti-CD-WPC, compared with a 20–25% relapse rate in contemporary controls in the Dutch epidemic and published relapse rates from 20% to 47% in *C difficile* type 027 epidemics.^{3–5} The effect of anti-CD-WPC is probably mediated by sIgA antibodies, and this has important advantages over repeated use of antibiotics, because of its suppression of resident microbial bowel flora and potential for inducing antibiotic resistance. In some relapsing subjects, the amount of anti-CD-WPC appeared insufficient to neutralise the *C difficile* toxins in the faeces, and there may

Table 1 Characteristics of 101 patients with *Clostridium difficile*-associated diarrhoea (CDAD; 109 episodes), who had or did not have relapse of CDAD after 2 weeks of the immune whey anti-*C difficile* whey protein concentrate given after standard antibiotic treatment with oral metronidazole or vancomycin

Characteristic	Relapse	No relapse	p Value
Sex (n)			>0.30
Male	5	49	
Female	6	49	
Age (median and IQR; years)*	76 (9)	70 (27)	>0.20
Chronic Health Scoring System†			<0.01
Score 0	1	46	
Score 2	0	8	
Score 5	10	44	
Episode of CDAD			>0.20
First	6	59	
Second	4	23	
$\geq$ Third	1	16	
<i>C difficile</i> PCR-ribotype 027			>0.20
Yes	4	20	
No	7	75	
Not determined	0	3	
Antibiotic treatment of last episode			>0.30
Vancomycin	8	50	
Metronidazole	3	45	
In combination	0	3	

CDAD, *Clostridium difficile*-associated diarrhoea.

*IQR, interquartile range.

†Chronic Health Score in Acute Physiology, Age and Chronic Health Evaluation (APACHE) prognostic scoring system: angina pectoris New York Heart Association (NYHA) Class III–IV (n=21); biopsy-proven cirrhosis and documented portal hypertension (n=9); chronic obstructive pulmonary disease resulting in exercise restriction (n=28); renal insufficiency necessitating (chronic) dialysis (n=6); and immunocompromised status—for example, high-dose steroids, chemotherapy or an underlying haematological disease like chronic lymphatic leukaemia (n=31), according to Knaus.¹⁰

be room for improvement by raising the dose. The findings merit clinical development of anti-CD-WPC, and should be confirmed in a prospective placebo-controlled randomised trial.

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Is gliadin really safe for non-coeliac individuals? Production of interleukin 15 in biopsy culture from non-coeliac individuals challenged with gliadin peptides

Nowadays it is assumed that an innate immunity to gluten plays a key role in the

Table 1 Gliadin-challenged patients without coeliac disease

Patient no	Duodenal mucosa	Age (years)	Symptoms	Diagnosis
1	Normal	51	Unfiliated ferropenia	Undiagnosed (non-coeliac)
2	Normal	63	Pyrosis	Hiatus hernia, GORD
3	Chronic inflammation	46	Colic abdominal pain	Chronic gastritis
4	Chronic inflammation	54	Epigastric pain	Chronic gastritis
5	Normal	16	Diarrhoea	Protracted diarrhoea
6	Normal	71	Dyspepsia	Polyps in stomach, Helicobacter

GORD, gastroesophageal reflux disease.

The final diagnosis of the patients was non-coeliac in all the cases. Those with chronic inflammation had normal villi as well as no intraepithelial lymphocytosis. All the patients were biopsy cultured in basal medium and challenged with both the 19- and the 33-mer gliadin peptides. Patients 1–3 were also challenged with gliadin.

development of coeliac disease (CD).¹ This innate response, mediated by interleukin (IL) 15 and elicited by “toxic peptides”, like the 19-mer, through a DQ2-independent mechanism, induces epithelial stress and reprogrammes intraepithelial lymphocytes into natural killer (NK)-like cells² leading to enterocyte apoptosis and an increase in epithelium permeability. Thus, immunodominant peptides, like the 33-mer, can reach the lamina propria to trigger adaptive immunity. However, although an innate specific response in CD has been reported,³ no differential factors between patients with and without CD have been described controlling the innate immune response. Thus, since the toxic 19-mer elicits its harmful effect through a DQ2-independent mechanism, we hypothesise that the innate response is common in patients with and without CD, whereas the adaptive response is exclusive of susceptible patients with CD.

To test the hypothesis, biopsy cultures were taken from at least three patients with CD who are on a gluten-free diet (GFD) and three patients without CD (table 1). Biopsy specimens were challenged with crude gliadin and the gliadin synthetic 19-mer and deaminated 33-mer peptides after discarding the presence of lipopolysaccharide in all the cases. This was carried out at 100 µg/ml for only 3 h to imitate what are considered the normal timing and concentration in the gut after a normal meal. All biopsy specimens were then washed and cultured for another 21 h in new clean culture medium to determine whether an innate stimulus is reflected by an adaptive response. Each sample cultured in basal medium constituted an internal control. Innate immune mediators IL15 and nitrites were determined by western blot in the biopsy protein extract and by a Griess reagent system in the 3 h supernatants respectively. mRNA levels of adaptive immunity mediators like signal transducers and activators of transcription (STAT) 1, STAT3, tumour necrosis factor α , interferon (IFN) γ , IL23 (p19), IL27 (p28) and IL12 (p35) were determined by real-time polymerase chain reaction using β actine levels as house-keeping.

All patients with and without CD on GFD who were challenged with the gliadin solution produced IL15 when compared with the basal culture (fig 1A). Moreover, the IL15-mediated response in patients without CD was also triggered by the toxic 19-mer gliadin peptide (three of six) and, especially, by the 33-mer gliadin peptide (five of six). Importantly, none of the basal cultures produced this cytokine and, although not expected, the “non-toxic”

immunodominant 33-mer was also able to induce an innate response. Interestingly, this IL15 response was also confirmed by western blot in the supernatant of one GFD patient with CD and three patients without CD, who were on GFD (fig 1B), therefore, discarding an intracellular and non-biologically active IL15 response in patients without CD. We also found an increase in nitrite in the gliadin-challenged patients with CD who were on a GFD, although not in patients without CD. In a similar way, as expected, after the biopsy mRNA isolation, adaptive mediators (STAT1, STAT3, IFN γ) were only modified in GFD patients with CD. Finally, basal GFD-CD samples showed an 80-fold increase in IFN γ mRNA levels compared with non-CD basal samples (p value 0.002) and a slightly higher production of nitrites (p value 0.052).

We consider that, to our knowledge, this is the first time that an IL15-mediated innate response to gliadin and gliadin peptides is described in individuals without CD, as well as an IL15-mediated innate response to the “non-toxic” deaminated immunodominant 33-mer peptide.

The data obtained in this pilot study support the hypothesis that gluten elicits its harmful effect, throughout an IL15 innate immune response, on all the individuals. This innate response is found in both patients with and without CD, although the triggering of an adaptive response is CD specific. We propose that somehow patients with CD need to be DQ2 and also have a lower threshold for triggering an adaptive TH1 response. This lower threshold could be mediated by the higher basal levels of immune mediators, like IFN γ mRNA, found in patients with CD, a defect in the CD permeability or even a higher IL15-sensitive response under the same stimulus, which might be mediated by a higher density of IL15 receptor in patients with CD.⁴

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