

Clostridium difficile, atopy and wheeze during the first year of life

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Differences have been suggested to occur in the composition of intestinal microflora from allergic and non-allergic children. In this study we used a semi-quantitative enzyme-linked immunosorbent assay (ELISA) for the measurement of *Clostridium difficile*-specific immunoglobulin G (IgG) (CDIgG). CDiG was excellent in differentiating between adults with or without *Cl. difficile* colitis (absorbance levels, positive vs. negative controls: geometric mean (GM) 0.301, 95% CI: 0.289–0.314 vs. GM 0.167, 95% CI: 0.155–0.181; mean difference 1.8-fold, 95% CI: 1.65–1.95; $p < 0.0001$). We used this technique to investigate whether there are any differences between atopic wheezy infants and non-atopic non-wheezy controls. In a prospective cohort study ($n = 390$) 10 patients were identified at 1 year of age (atopic, history of recurrent wheeze) and matched (gender, month of birth, exposure to Der p 1, Fel d 1 and Can f 1) with a control group of infants (non-atopic, no history of wheeze). The patients had significantly higher *Cl. difficile*-specific IgG absorbance levels (GM 0.298, 95% CI: 0.249–0.358) compared with controls (GM 0.235, 95% CI: 0.201–0.274; mean difference 1.27-fold, 95% CI: 1.07–1.50; $p = 0.01$). These results suggest that there may be differences in the composition of intestinal microflora between allergic and non-allergic infants at 1 year of age, with allergic children having higher *Cl. difficile* IgG antibody levels.

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The intestinal microflora provides a continuous stimulation of the developing immune system and may contribute to the development of allergy (1,2). Differences have been observed in the composition of intestinal microflora between Estonian and Swedish infants (1); and allergic children in both countries were less often colonized with lactobacilli and bifidobacteria, but had higher counts of *Clostridium*, coliforms and *Staphylococcus aureus* compared to non-allergic children (2). We recently developed a semi-quantitative enzyme-linked immunosorbent assay (ELISA) for the measurement of *Cl. difficile*-specific immunoglobulin G (IgG) (CDiG) and used this assay to investigate whether there are any differences in CDiG between atopic wheezy infants and non-atopic non-wheezy controls at 1 year of age in a nested

case-control study within the National Asthma Campaign Manchester Asthma and Allergy Study (^{NAC}MAAS).

Methods

The ^{NAC}MAAS is a prospective, prenatally randomized, cohort study (a detailed description of which is given in refs 3 and 4). Briefly, all pregnant women and their partners were screened antenatally and babies at high-risk (both parents atopic) were followed-up prospectively. Environmental exposure to mite, cat, and dog allergens in dust samples from homes was determined using ELISAs (3).

Parents and children were invited into the clinic at the child's first birthday for completion of a validated questionnaire, skin-prick testing, and

collection of a blood sample. Within the cohort we carried out a case-control study to investigate whether there was any difference in CDIgG levels between children developing atopy and recurrent wheeze during the first year of life, and those who did not.

Cases and controls were categorized as follows:

- Cases: infants who at the 1-year follow-up visit were both atopic (skin-prick test positive to at least one allergen) and had a history of recurrent wheeze (three or more episodes).
- Controls: infants who at the 1-year follow-up visit were both non-atopic (skin-prick test negative) and had no history of wheeze.

Cases and controls were matched for gender, parental atopic status and history of allergic disease, month of birth, pet ownership, and the level of exposure to dust mite, cat and dog allergens.

Semiquantitative ELISA for *Cl. difficile*-specific IgG

Preparation of the antigen. *Cl. difficile* standard strain [American Type Culture Collection (ATCC) no. 11204] was cultured in 400 ml of culture medium (2% wt/vol protease peptone, 1% wt/vol yeast extract) in anaerobic conditions at 37°C for 36 h. The culture was centrifuged and the supernatant discarded. The sediment was washed three times with phosphate-buffered saline (PBS), and resuspended in 5 ml of PBS. The cells were broken using X-press (LKB, Uppsala, Sweden), the suspension was centrifuged at 5500 g for 20 min, and the resultant supernatant used as antigen at 10 mg/ml.

ELISA protocol. Immulon II flat-bottom ELISA plates (Dynatech, Chantilly, VA, USA) were coated with *Cl. difficile* antigen overnight at 4°C. Plates were washed three times with PBS containing 0.01% Tween-20 (PBS-T), pH 7.4, and incubated for 3 h at 37°C with PBS containing 3% skimmed milk (3% PBSM). After three further washes, 0.1 ml of undiluted serum was added. The plates were incubated for 2 h at 37°C, then washed three times and incubated for 1 h at room temperature with 0.1 ml/well of rabbit anti-human IgG (Sigma Chemicals, Poole, UK) diluted in PBSM. After a further three washes the plates were incubated for 1 h at room temperature with peroxidase-conjugated goat anti-rabbit IgG diluted in PBSM. Plates were washed a further three times and the assay was developed by adding 0.1 ml of 1 mM 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS; Sigma) in 0.07 M citrate phosphate buffer, pH 4.2. The plates

were read (at 405 nm) at 20-, 30-, 40- and 50-min time-points of assay development. Absorbance readings are directly proportional to the quantity of *Cl. difficile*-specific IgG antibody bound.

The ELISA data were validated using sera from 25 adults. Of those, 16 were negative controls (healthy volunteers with no evidence of *Cl. difficile* colitis) and nine were positive controls (patients with *Cl. difficile* colitis, i.e. appropriate clinical history, *Cl. difficile* isolated from the stool culture, toxin-positive in feces by ELISA; Techlab, Blacksburg, VA, USA).

The optical density (OD) data followed a log-normal distribution; the comparison between cases and controls was made using the paired-samples *t*-test.

Results

Cl. difficile-specific IgG levels (expressed as absorbance level) in two groups of adult controls are presented in Fig. 1(a). Semiquantitative ELISA was excellent at differentiating between patients with *Cl. difficile* colitis and healthy controls, and no overlap was seen between the groups. Absorbance levels were significantly higher in the positive controls [geometric mean (GM) 0.301, 95% CI: 0.289–0.314] compared to negative controls (GM 0.167, 95% CI: 0.155–0.181; mean difference 1.8-fold, 95% CI: 1.65–1.95; *p* < 0.0001).

Based on the outcome data from 390 high-risk infants reviewed at 1 year of age, we identified 10 pairs of cases (skin-prick test positive to at least one allergen and a history of recurrent wheeze) and controls (skin-prick test negative, no history of wheeze), who could be matched for all the criteria and whose parents gave consent for blood sampling. *Cl. difficile*-specific IgG levels (expressed as absorbance level) in these two groups of infants are presented in Fig. 1(b). Infants with a history of recurrent wheeze and a positive skin-prick test had significantly higher *Cl. difficile*-specific IgG absorbance levels (GM 0.298, 95% CI: 0.249–0.358) compared to skin-prick test-negative infants who had never wheezed (GM 0.235, 95% CI: 0.201–0.274; mean difference 1.27, 95% CI: 1.07–1.50; *p* = 0.01).

Discussion

The results of the current study support the concept of a difference in the composition of intestinal microflora between allergic and non-

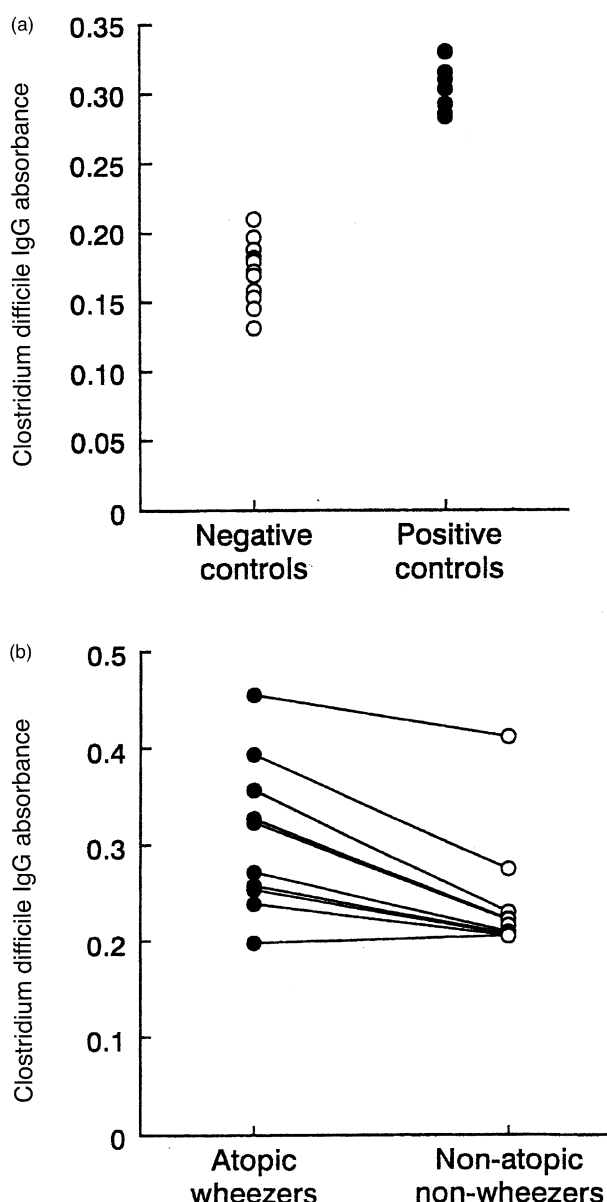


Fig. 1. (a) *Clostridium difficile*-specific immunoglobulin G (IgG) levels (expressed as absorbance) in nine positive controls (patients with *Cl. difficile* colitis, with *Cl. difficile* isolated from stool culture) and 16 healthy adult volunteers with no evidence of *Cl. difficile* colitis. (b) *Cl. difficile*-specific IgG levels (expressed as absorbance level) in a group of 20 infants at 1 year of age. Presented are 10 pairs of cases (skin-prick test positive to at least one allergen and a history of recurrent wheeze) and controls (skin-prick test negative and no history of wheeze).

allergic infants at 1 year of age. We used an indirect indicator of *Cl. difficile* in the intestinal microflora (i.e. CDIGG), which has the disadvantage of concentrating on a single species (albeit one that has been identified as potentially important, see ref. 2). Stool culture is less specific in that it is at best semi-quantitative, reveals numerous different bacterial species dependent on culture media, and the results may be difficult

to interpret as in previous studies which have used frozen stool samples. Qualitative seropositivity has been used in several previous studies on the prevalence of atopy in adults (5).

Differences in the composition of intestinal microflora between allergic and non-allergic infants have recently been confirmed by other methods, e.g. measurement of isocaproic acid (6), gas-liquid chromatography of bacterial cellular fatty acids, and quantitative fluorescence *in situ* hybridization of bacterial cells (7). Interestingly, isocaproic acid (which might be an indicator of *Cl. difficile*), was found in a greater proportion of allergic than non-allergic 1-year-old-infants (6). Furthermore, two prospective studies have demonstrated that differences in the intestinal microflora are present during the first few weeks of life, i.e. they preceded the development of atopy (7,8). Both of these studies have reported higher counts of clostridia in allergic infants at 3 weeks (7) and 3 months (8) of age, but not later. In addition, a prospective intervention study has recently suggested that probiotics (lactobacilli) administered during pregnancy and in early life might protect against the development of atopic dermatitis (9).

It is worth emphasizing that in the current study the cases and controls were closely matched according to a number of factors that may be important in the development of allergic disease (parental atopic status and history of allergic disease, gender, age, month of birth, environmental exposure to dust mite, cat and dog allergen in the home). Furthermore, they represented the extreme ends of a spectrum, i.e. those who were skin-prick test positive at 1 year of age and had three or more wheezy episodes during the first year of life were compared with non-atopic infants who had never wheezed. Several possible mechanisms could explain the differences observed. A high number of clostridia (assessed indirectly by measuring antibody concentration in the sera) could reflect the unbalanced gut microecology and be a primary cause of allergy (possibly by delaying immune deviation, see ref. 10). Alternatively, *Cl. difficile* could have a direct effect on the gut mucosa, or atopy itself may be associated with a mucosal condition promoting certain types of microflora (8).

In conclusion, our results suggest that there may be differences in the composition of intestinal microflora between allergic and non-allergic infants at 1 year of age, reflected in allergic children having higher *Cl. difficile* IgG levels. It is uncertain whether this is a primary or secondary phenomenon, and it is possible that the

observed difference is a consequence of allergic disease.

References

1. SEPP E, JULGE V, VASAR M, NAABER P, BJORKSTEN B, MIKELSAAR M. Intestinal microflora of Estonian and Swedish infants. *Acta Paediatr* 1997; 86: 956–61.
2. BJORKSTEN B, NAABER P, SEPP E, MIKELSAAR M. The intestinal microflora in allergic Estonian and Swedish 2-year-old children. *Clin Exp Allergy* 1999; 29: 342–6.
3. CUSTOVIC A, SIMPSON BM, SIMPSON A, HALLAM C, CRAVEN M, BRUTSCHE M, WOODCOCK A. Manchester Asthma and Allergy Study: Low allergen environment can be achieved and maintained during pregnancy and in early life. *J Allergy Clin Immunol* 2000; 105: 252–8.
4. CUSTOVIC A, SIMPSON BM, SIMPSON A, KISSEN P, WOODCOCK A. Effect of environmental manipulation in pregnancy and early life on respiratory symptoms and atopy during the first year of life: A randomised trial. *Lancet* 2001; 358: 188–93.
5. MATRICARDI PM, ROSMINI F, FERIGNO L, et al. Cross-sectional retrospective study of prevalence of atopy among Italian military students with antibodies against hepatitis A virus. *BMJ* 1997; 314: 999–1003.
6. BOTTCHE M, SANDIN A, NORIN E, MIDTVEDT T, BJORKSTEN B. Microflora associated characteristics in faeces from allergic and non-allergic children. *Clin Exp Allergy* 2000; 30: 590–6.
7. KALLIOMAKI M, KIRJAVAINEN P, EEROLA E, KERO P, SALMINEN S, ISOLAURI E. Distinct patterns of neonatal gut microflora in infants developing or not developing atopy. *J Allergy Clin Immunol* 2001; 107: 129–34.
8. BJORKSTEN B, SEPP E, JULGE K, VOOR T, MIKELSAAR M. Allergy development and the intestinal microflora during the first year of life. *J Allergy Clin Immunol* 2001; 108: 516–20.
9. KALLIOMAKI M, SALMINEN S, ARVILOMMI H, KERO P, KOSKINEN P, ISOLAURI E. Probiotics in primary prevention of atopic disease: A randomised placebo-controlled trial. *Lancet* 2001; 357: 1076–9.
10. OYAMA N, SUDO N, SOGAWA H, KUBO C. Antibiotic use during infancy promotes a shift in the T_H1/T_H2 balance toward T_H2 dominant immunity in mice. *J Allergy Clin Immunol* 2001; 107: 153–9.