

Lower anti-echovirus antibody responses in children presenting to hospital with asthma exacerbations

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Summary

Background Rhinoviruses from the Enterovirus genus cause frequent infections and induce remarkably high titres of anticapsid antigen antibodies in asthmatics, while the prevalence of neutralising antibodies to the gut-trophic echoviruses from the same genus is diminished.

Objective To assess the absolute and specific antibody titres to VP1 antigens of the gut-trophic enteroviruses, echovirus 30 and Sabin 1 poliovirus, in asthmatic and non-asthmatic children.

Methods Recombinant polypeptides representing the VP1 capsid antigens of echovirus 30 and Sabin poliovirus 1 were produced. Their ability to bind IgG1 antibodies from the plasma of asthmatic ($n = 45$) and non-asthmatic ($n = 29$) children were quantitated by immunoassays that incorporated immunoabsorptions to remove cross-reactivity.

Results The IgG1 antibody titres and prevalence of antibody binding to echovirus 30 were significantly lower for asthmatic children compared to controls ($P < 0.05$) and inversely correlated with total IgE levels for the whole study population ($r = -0.262$; $P < 0.05$). There was no difference in the prevalence and titre between groups to the VP1 antigen of Sabin poliovirus. Anti-tetanus toxoid titres measured for comparison did not correlate with anti-echovirus or poliovirus, but correlated with anti-rhinovirus titres in controls but not asthmatics, where the titres were higher for the asthmatic group.

Conclusions and Clinical Relevance The associations of lower antibody titres of asthmatic children to echovirus reported here and those of our previous findings of a heightened response to rhinovirus suggest a dichotomy where respiratory enterovirus infection/immunity increases the probability of developing asthma and enteric infections lower the risk. This provides further support for the concept of intestinal infection playing a key role in the development of allergic respiratory disease.

Keywords asthma, children, echovirus, Enteroviruses, IgG1 antibody, poliovirus, rhinovirus, VP1

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Introduction

Children living in the immediate vicinity of farm animals and in unhygienic environments, and those born into large families exhibit a low prevalence of asthma and atopy [1]. This and the finding that children with a more diverse gut flora have a lower probability of becoming atopic [2, 3] have suggested that exposure to microbial products and infection might protect against the development of allergic sensitization and disease. However, the associations of a higher prevalence of

asthma with increased colonization of the respiratory mucosa with bacteria in infancy [4] and with a history of early respiratory virus infection [5, 6] are contrary to these observations. It therefore appears that the nature of the infection or microbial exposure is of critical importance, including the route of infection. With respect to gut infection, the incidence of microbially undefined childhood gastroenteritis has been shown to be associated with both an increased [7] and a decreased [8] risk of developing atopy. Sero-epidemiology found an association of enteric infections of

Hepatitis A and *Helicobacter pylori* and a reduced risk of young adults becoming atopic [9], but this was not found in a study by others [8].

Enteroviruses are a genus of RNA viruses that are phylogenetically grouped into 12 species (Enterovirus A-H, Enterovirus J, Human rhinovirus A-C), which were originally classically serotyped into polioviruses, coxsackie A, coxsackie B and echoviruses [10]. Evidence for an association of childhood infection with the gut-trophic enteroviruses and a reduced risk of childhood atopy has been obtained from a case-control study of echovirus infections. Atopic children had a significantly lower prevalence of neutralising antibodies against a panel of different serotypes of gut-trophic echovirus (EV 3, 5, 9, 11, 30) compared to non-atopic children [11]. These prevalence results (69% vs. 55%), however, give little measure of the size of the difference and the overall size of the response against echovirus antigens. As it has recently been shown that asthmatic children have increased titres of antibodies that bind the viral capsid protein 1 (VP1) capsid antigens of rhinovirus [12, 13], a lung-trophic enterovirus, the aim of this study was to assess the antigen binding titres to a gut-trophic echovirus VP1. Echoviruses are one of the most commonly isolated enteroviruses and the echovirus 30 used here has been noted for its high infection rates in children, frequently causing asymptomatic infection of the gut and minor illnesses in children, but can cause severe illness such as viral meningitis, particularly in parents following transmission from children [14–16]. It is also a common serotype found world-wide and in the study area [17]. We also report the IgG1 titre to the VP1 antigen of the oral vaccine Sabin poliovirus, an enterovirus almost eliminated from circulation that is not found in the study environment [18].

Materials and methods

Study population

Plasma was collected from 45 children aged 2–6 and taken within 24 h of them presenting with an asthma exacerbation to the Emergency Department (ED) of Princess Margaret Hospital for Children (PMH), the only paediatric tertiary referral hospital in Western Australia, as well as from 29 non-asthmatic control children of the same age range. All plasma was from a larger study collected over a period of 9 years (2002–2011) and was selected without reference to patient data except age. The controls had no history of doctor-diagnosed asthma and were either recruited from the community or were friends of ED asthmatic children. The demographics of the study population are given in Table 1. The ED subjects were given a doctor diagnosis of asthma on presentation to hospital with over 70% presenting with

Table 1. Characteristics of the study population

	ED asthmatics* (n = 45)	Non-asthmatic controls (n = 29)
Median age at recruitment in years	3.6	3.4
Age range in years	2.1–5.88	2.2–5.2
Male, %	64	48
Atopic†, %	78.6	20.0
HRV positive, n (%)	37 (82.2)	6 (20.7)
Daycare attendance, %	76.7	100

ED, Emergency Department; HRV, human rhinovirus.

*Doctor-diagnosed asthma exacerbation.

†Atopy was defined as at least one positive skin-prick test to 11 common allergens. A positive reaction was defined as a weal size that was larger than the negative control and greater than or equal to 3 mm in diameter.

symptoms classified as moderate or severe asthma according to the American Thoracic Society guidelines [19]. The molecular detection of respiratory viruses has been presented previously [20], and in this subset, 82% of the ED asthmatics were infected with human rhinovirus (HRV) and the majority (72.9%) with HRV-C. Of the asymptomatic controls, 21% also had HRV transcripts detectable by PCR with the detection rates of both groups being similar to others and the authors [13, 20]. For all but one of the ED asthmatic children, another plasma sample was obtained 6–26 weeks after the initial recruitment (median 12 weeks) when the children were clinically well. As reported by the parent/guardian, all subjects in the study had been vaccinated with cellular DTP vaccine at the time of sample collection. The study was approved by the PMH Human Ethics Committee, and parental/guardian written informed consent was obtained for each participant.

Antigens

Recombinant VP1 capsid proteins were produced in *Escherichia coli* as fusion proteins with glutathione-S-transferase at the N-terminus and a hexahistidine tag at the C-terminus using the vector pGEX-2T. Briefly, proteins from the soluble fraction of bacterial lysates were purified by affinity chromatography on glutathione agarose and then by size exclusion chromatography to obtain a discrete antigen of about 660 kDa corresponding to the size of a pentamer. The plasmid constructs used for the expression were made with synthetically produced VP1 coding region for echovirus 30 (E30) (accession AF128087.1) and as described [12] with synthetically produced VP1 coding regions for Sabin human poliovirus 1 (accession AY184219.1) and the VP1 antigens of two genotypes of each of the HRV species A, B and C.

Antibody assays

As described [12], IgG1 antibody binding titres were quantitated with a dissociation-enhanced immunofluorescence assay (DELFI[®]) using microtitre plate wells coated to achieve a standard coating of GST-fusion antigens as determined by titration with a monoclonal antigitathione-S-transferase antibody (Sigma-Aldrich, St Louis, MO, USA). Human myeloma IgG1 (Sigma-Aldrich) was used as a negative control as well as subsequently identified negative control sera. Absolute binding titres (ng/mL) of reference plasma for the VP1 antigens were calibrated by interpolating the results from side-by-side titrations of a humanized anti-Der p 2 IgG1 standard (Indoor Biotechnologies, Charlottesville, VA, USA) using GST-Der p 2 where the equivalence of the slopes was demonstrated. Antibody values below the limit of detection (500 ng/mL) were assigned 50% of the lower limit of detection (250 ng/mL). To remove any cross-reactive responses and thus obtain titres specific for Sabin and E30, an additional step was incorporated. This involved incubation of plasma in lysate mixtures of *E. coli* producing the VP1 antigens of HRV-A, HRV-B and HRV-C and depending on the target of the assay either E30 or Sabin using amounts demonstrated to be in excess [12]. The antitetanus toxoid assay was similarly conducted with the tetanus toxoid (TT) antigen (List Biological Laboratories Inc., Campbell, CA, USA). Sera were titrated against a titration curve of high-titre plasma selected for a reference and given a value of 1000 arbitrary units.

Statistical analyses

Differences in antibody binding between ED asthmatic children and non-asthmatic control children were

compared using the Mann-Whitney *U*-test. Differences in prevalence between these groups were compared using the Chi-squared test. Correlations were studied using Spearman's rho test. A $P < 0.05$ was considered significant. All analyses were performed using GRAPHPAD PRISM Software (La Jolla, CA, USA).

Results

Comparisons of the total antibody binding from the plasma of the ED asthmatics and non-asthmatic controls to the echovirus 30 and Sabin VP1 antigens showed that the mean titre for anti-echovirus response of the ED asthmatics was about fivefold less than that of the controls and significantly different ($P < 0.05$), while the mean titre for the total binding to Sabin was not different between the groups (Fig. 1a). When the cross-reactive antibodies were removed by absorption, the specific anti-echovirus titres for ED asthmatics were even lower and highly significant ($P < 0.005$), with a mean of 2406 ng/mL compared with 28 755 ng/mL for non-asthmatic controls (Fig. 1b). The mean specific anti-Sabin titres between the two groups were not significantly different. The prevalence of detectable anti-echovirus titres in the ED asthmatics (47%) was significantly less than the 76% prevalence in the healthy controls ($P < 0.05$). The prevalence of detectable specific antibodies to Sabin was 44% and 45% for the ED asthmatics and controls, respectively (not significantly different). When only the responders (children with measureable titres) were considered for the analyses (Fig. 2), the total and specific anti-echovirus titres remained significantly less ($P < 0.05$) for the ED asthmatic group. Comparison of the anti-echovirus titres with the age of children showed low titres of the ED asthmatic across the entire age range (Fig. 3), and there

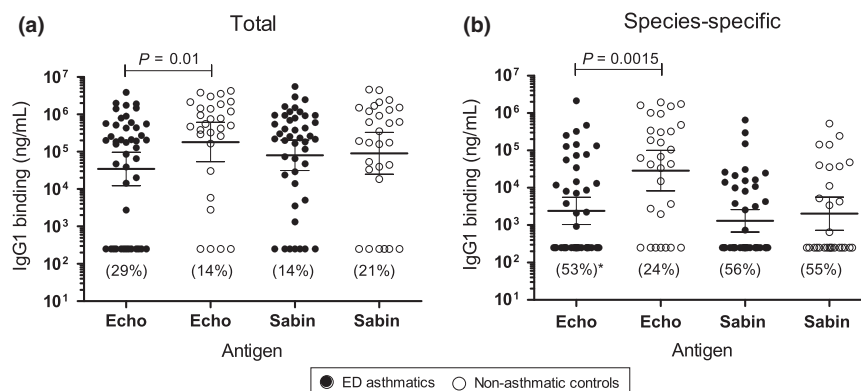


Fig. 1. Total (a) and species-specific (b) IgG1 antibody binding to echovirus 30 VP1 and human Sabin 1 poliovirus VP1 antigens in Emergency Department (ED) asthmatics and non-asthmatic children. The geometric mean and 95% confidence interval are indicated. Comparisons between the ED asthmatics ($n = 45$) and non-asthmatic controls ($n = 29$) were analysed using the Mann-Whitney test and the P values are shown where significant ($P < 0.05$). The prevalence (%) of subjects with titres below the assay limit of 500 ng/mL is indicated for each antigen. Comparison of prevalence between the two groups was analysed using the chi-squared test (*indicates $P < 0.05$).

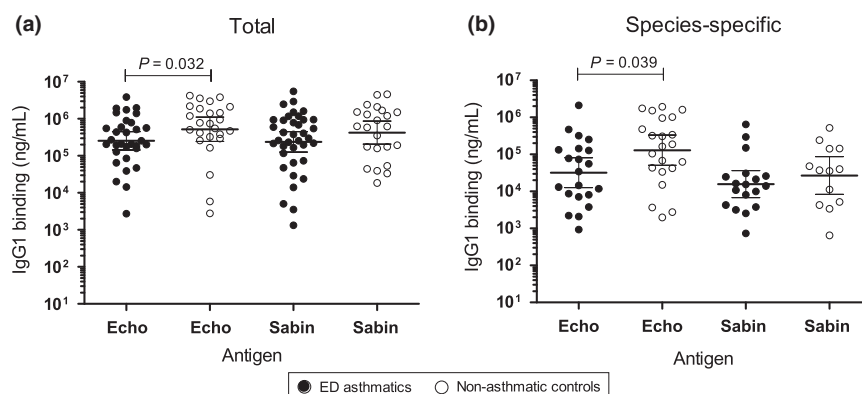


Fig. 2. Total (a) and Species-specific (b) IgG1 antibody binding to echovirus 30 VP1 and human Sabin 1 poliovirus VP1 antigens in Emergency Department (ED) asthmatics and non-asthmatic children with measurable titres. Graphs show geometric mean $\pm$ 95% confidence interval and significance (Mann-Whitney) when only those subjects with detectable titres in Fig. 1 were analysed.

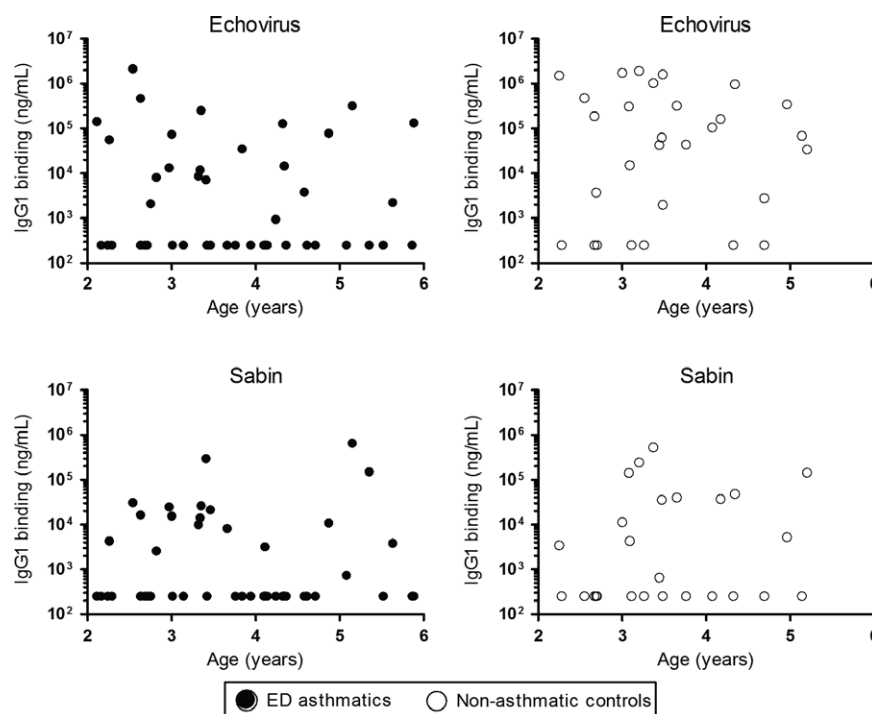


Fig. 3. Age distribution of specific IgG1 binding to echovirus 30 VP1 and Sabin 1 poliovirus VP1 in Emergency Department (ED) asthmatics and non-asthmatic children. As determined by the Spearman's rho test, there was no significant correlation between age and IgG1 titres for all groups.

was no obvious difference for anti-Sabin at any age. Follow-up convalescent samples collected for the ED asthmatics showed no change in the total and specific antibody titres to echovirus and Sabin VP1 compared to during an exacerbation (mean 1506 and 28 775 ng/mL specific IgG1 for echovirus in asthmatic and control and 1145 and 2033 ng/mL for Sabin).

To make a comparison with an injected vaccine antigen where non-specific effects on plasma titres might be revealed, the IgG1 antibody titres to TT were measured. There was a fourfold increase in the mean titre (Fig. 4a) of the ED asthmatics compared to controls

($P = 0.047$). The difference in number of responders was not significant ($P = 0.381$). Like the anti-echovirus titres, the difference appeared to be distributed across the whole age range for ED asthmatics (Fig. 4b); however, there was a positive correlation with age for non-asthmatic controls only (Fig. 4c; $P < 0.001$). The relationship between the anti-TT titres and those to other viruses was also assessed (Table 2). Correlations of antibody titres to HRV-A, already reported for the ED asthmatics [13], were included as like the anti-TT antibodies they were found to be increased in the ED asthmatics. For the asthmatics, all the correlations of

Table 2. Correlation of antitetanus toxoid antibodies with antiviral titres

	ED asthmatics	Non-asthmatic controls
Sabin poliovirus	-0.188 $P = 0.222$	0.194 $P = 0.314$
Echovirus	-0.168 $P = 0.277$	0.085 $P = 0.661$
Human Rhinovirus	-0.134 $P = 0.386$	0.670 $P < 0.0001$
A (HRV-A34)		

ED, Emergency Department.

anti-viral responses and IgG1 titres to TT were inverse but did not show statistical significance. For the healthy controls, all the correlations were positive and were statistically significant for HRV-A ($r = 0.670$, $P < 0.001$, Bonferroni $P < 0.006$). The ED asthmatics were further stratified based on childcare attendance, and childcare had no affect on either the prevalence of antibody binding or titre to echovirus, Sabin or TT (data not shown).

To study the effect of atopy, the relationship between total IgE and anti-IgG1 echovirus titres was examined. Interestingly, the IgG1 titres specific to echovirus were significantly and inversely correlated with total IgE levels in the whole study population ($r = -0.262$; $P < 0.05$). The atopic status also had an inverse correlation with titres specific to Sabin, but this was not significant ($r = -0.115$, $P = 0.341$). On the other hand, there was a trend towards a positive relationship with total IgE and TT antibody binding ($r = 0.237$; $P = 0.052$).

Discussion

On average the ED asthmatics had tenfold lower antibody titres specific to echovirus than the non-asthmatic controls. This was not found for titres to Sabin poliovirus, which showed no difference, nor to TT which, as will be discussed below, were higher in the asthmatic group. Importantly, the reduced anti-echovirus titres corroborate those found in a series of analyses made in Finland. The results from Korhonen et al. [11] showed the prevalences of neutralising antibodies to different echovirus serotypes were significantly lower in young children that became atopic by 5 years of age compared to those who did not. At 2 years, 69% of children who did not develop atopy had neutralising antibodies to at least one echovirus serotype compared to 55% of subjects that became atopic. This is mirrored by the 76% vs. 47% prevalence of detectable anti-echovirus antibody in the antibody binding results found in this study. Taken together, the results here and those of Korhonen et al. show that

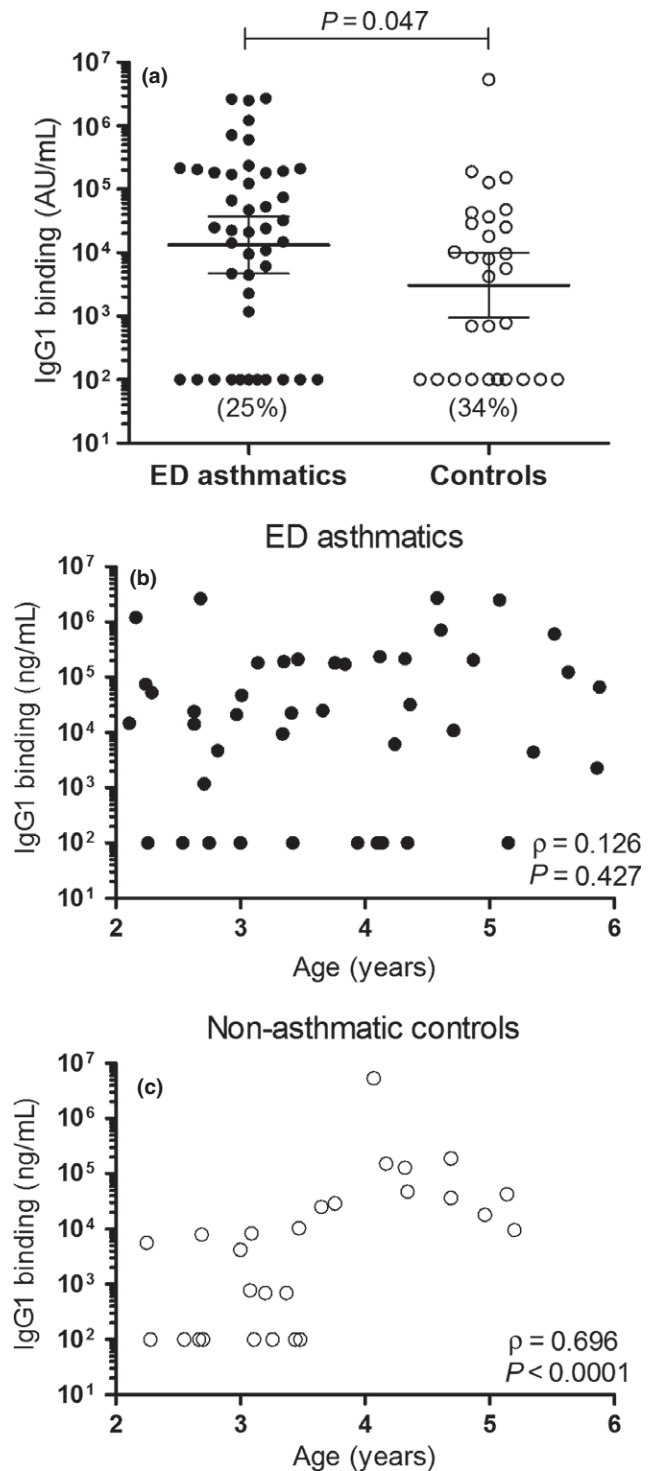


Fig. 4. IgG1 antibody binding responses to tetanus toxoid (TT). IgG1 binding (AU/mL) to TT in Emergency Department (ED) asthmatics and non-asthmatic controls (a). Graphs show geometric mean $\pm$ 95% confidence interval and significance (Mann-Whitney). The prevalence (%) of subjects with titres below the assay limit of 500 ng/mL is indicated for each antigen. Comparison of prevalence between the two groups was analysed using the chi-squared test. Correlation of age and IgG1 binding to TT in ED asthmatics (b) and non-asthmatic controls (c) are shown. Correlations were studied using Spearman's rho test.

the reduction in neutralising activity is accompanied by a large reduction in the size of the antigen binding titre. The significant difference in the amount of antibody binding in the plasma of the asthmatics shown here remained when only the responders were analysed indicating that it was not due to an absence of exposure. However, there was considerable overlap in the titres of the asthmatics and control subjects so the titres are likely to be either a marker of susceptibility, or one of a number of contributing factors that can lead to the development of asthma. The titres could be indicative of more frequent infections of entero- or other enteric viruses due to environmental circumstances and/or could be a marker of altered mucosal immunity. With respect to the current study, it should be noted that both the controls and asthmatics were from high socio-economic families that were recruited from geographically diverse areas with high rates of childcare so there is no obvious environmental reason for different incidences of infection.

The echovirus 30 was chosen because it is a common widely distributed serotype [17]. It is predominantly associated with gastroenteritis and is also one of the serotypes that can mediate the rarer occurrence of neurological disease. The amino acid sequence of its VP1 capsid protein shows 15–30% disparity from the sequences of the VP1 proteins from the other echovirus serotypes [21], which is typical for viruses within this group. This degree of sequence disparity is also found with the VP1 antigens of the coxsackie B viruses, which with echovirus, constitute the type B enteroviruses group, so they could have a high level of cross-reactivity with the specific antibodies reported here. Greater sequence disparity is found with the antigens of poliovirus and HRV-B (40%) [22] and with HRV-A and HRV-C (50%). As demonstrated here and more pointedly by Iwasaki et al. [12], there is substantial antibody binding cross-reactivity between all these viruses. Although absorbed out in the assay procedures, the cross-reactive responses might affect adaptive and innate immune responses during infection. Other picornaviruses such as Hepatitis A and parechovirus only have 10–20% amino acid sequence identity, levels far below that associated with cross-reactivity [23].

Measurements of enterovirus infection either from stool samples [24] or sero-conversion [25] have shown that about 50% of children have at least one enterovirus infection in the first year of life and T-cell responses have provided evidence to indicate this might be nearly 70% in the first 6 months [26]. In a very informative study that was able to serologically track a newly introduced echovirus serotype into a community, it was found that within a single year the echovirus had infected 75% of 2- to 4-year-olds and 90% of 4- to 6-year-olds [27]. A high prevalence of echovirus

infection was also found in the United Kingdom where they constituted 50% of isolates in a surveillance study of clinical fecal specimens [28]. Infections, as described for some viruses [29, 30], enteric commensal bacteria [31, 32] and bacterial pathogens [33], can accelerate the maturation of the immune system so that might hasten the production of the effector and regulatory responses that protect from the development of asthma and allergy. Feeding probiotics has been shown to protect infants from respiratory infection [34], as well as atopy and eczema [35, 36], and non-atopic and non-eczematic children have been found to have a greater diversity of enteric bacterial flora [2, 3]. Despite their effects on atopy and eczema probiotics have not been found to influence the incidence of asthma [37]. The viruses may have direct effects but could also act indirectly on the immune system by altering the bacterial microbiome [38, 39].

The lower anti-echovirus titres of the asthmatics contrast with the lack of a difference for the anti-Sabin titres and the higher anti-TT titres. The latter [40] vary with the vaccination regimen reaching a peak at age 5 but are fairly similar between 3 and 6 years. From the data (Fig. 4), the higher anti-TT titres of the asthmatics were found across the whole age range, as were the lower anti-echovirus titres. While the anti-TT responses showed that the reduced anti-echovirus titres in asthmatics were not due to a general immune hyporeactivity, they did question the interpretation of the previously described heightened antirhinovirus responses of asthmatics [13]. On further examination, the titres to TT in asthmatics proved to be inversely, but non-significantly, correlated with those to echovirus, rhinovirus and Sabin. The non-asthmatic controls were quite different and very interesting. The anti-TT titres were positively correlated with antiviral titres, and this was especially strong for HRV. The asthmatics thus appear to have a disconnection of a normal positive relationship found between the immune responsiveness to an injected antigen (tetanus) and rhinovirus infection. While these results with tetanus toxoid are very interesting, corroboration would be needed and would best be ascertained in concert with responses to other vaccine antigens. To date, two studies have reported no differences in the antitetanus toxoid antibody responses of atopic children [41, 42]. One that examined the minor Th2-type IgG4 subclass in 2-year-old atopic children [41] that might not be expected to be very informative, but the other study measured total IgG in 6-year-olds [42]. Contrary to the antibody measurements, studies have found increased Th1-type T-cell responses to TT in atopic children [42], and asthmatic adults [43].

Conflicting evidence has been found with respect to associations of gut infection and allergy. Cullinan

et al. [8] reported that the medical records of young adults with asthma showed a history biased to more gastrointestinal and less upper respiratory tract infection. Similarly a study of 6-year-old children found that children reported to have frequent recurrent gastrointestinal infection in the first 2 years of life had twice the risk of developing asthma [7]. Most of the infection at that age would be with rotavirus, an agent that can repeatedly infect children, being very prevalent in the study area. As only 3% of the subjects had frequent recurrent infections, they may constitute a special category and particular care must be made in interpreting the subjective and retrospective observations of parents that underpinned the study. In contrast to these studies, Matricardi and colleagues [9] provided serological evidence showing young adult asthmatics had less exposure to orofecal and food-borne bacteria and viruses.

The heightened antibody titres to rhinovirus A and B [13] and the lower titres of asthmatic children to echovirus reported here suggest a dichotomy where respiratory infection increases the probability of developing asthma and enteric infections reduce it. The different antibody responses could reflect either different prevalences of infection or different immune responsiveness to infection. As the subjects were recruited following an exacerbation requiring ED attention, the results warrant investigation of subjects with different degrees of allergic sensitization, different severities and manifestations of asthma. The tissue tropism of the enteroviruses is, however, complex,

and enteroviruses other than rhinovirus, including echovirus and related enterovirus type B, often cause clinically significant respiratory infections [44, 45] and have been proposed to exacerbate asthma attacks [46]. Several mechanisms could be proposed to produce the associations of asthma with lower anti-echovirus titres found both here and in the previous neutralising antibody studies. As the ED asthmatic subjects have contrastingly increased antibody titres to rhinovirus antigens, it is likely that the route of exposure and the effector and regulatory response to infection are important aspects that require further investigation.

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Conflict of interest

The authors declare no conflict of interest.

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