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MOLECULAR DIAGNOSTICS IN A HELMINTH-ENDEMIC AREA IN INDONESIA PROVIDES LEADS FOR THE LACK OF CLINICAL ALLERGY

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ABSTRACT

Background Helminth infections induce Th2 immunity with strong IgE responses, against parasite antigens and common allergens. Yet chronic helminth infections have been reported to be inversely associated with skin reactivity and clinical allergy.

Aim Molecular characterization of IgE responses against common allergen sources in a helminth-endemic area.

Methods A selection of 150 sera of school children from Flores Island, Indonesia, previously tested by ImmunoCAP and skin prick test (SPT) for IgE against house dust mite (HDM), cockroach, grass pollen (no SPT), peanut and shrimp were tested for IgE against 112 purified natural allergens by ImmunoCAP-ISAC microarray. ImmunoCAP inhibition was used to assess the role of cross-reactive carbohydrate determinants (CCD).

Results Of the 150 sera, 65% to 90% were positive by ImmunoCAP for the common inhalant and food allergen sources. At the molecular level, IgE against established major allergens was <10% for HDM and cockroach and absent for peanut and shrimp. Only for grass pollen (and tree pollen and walnut), IgE was detected in approximately half of the children, but selectively against natural glycoproteins. ImmunoCAP inhibition showed that CCD is at the basis of these IgE responses, most likely induced by helminths. Only venom allergens were frequently recognized without carrying CCD.

Conclusions ImmunoCAP-ISAC has revealed that in helminth-endemic areas, CCD contributes significantly to high sensitization levels to common allergen sources. Purified natural plant glycoproteins consequently have a negative impact on the specificity of such microarrays. These cross-reactive IgE responses are most likely part of the explanation of the inverse association between helminth infections and clinical allergy.

INTRODUCTION

The prevalence of asthma and allergic diseases has increased in affluent countries over recent decades, and the same development has now begun in many non-affluent countries[1]. At the same time however, helminth infections remain one of the major health problems in these countries, especially in tropical regions, where such infections are linked to poverty and rural living[2]. Both helminths and allergens are potent inducers of T helper 2 (Th2) responses that lead to high levels of immunoglobulin (Ig) E, mast cells, tissue eosinophilia and the secretion of Th2 cytokines such as interleukin (IL)-4, IL-5, IL-9 and IL-13[3].

Despite the similar immunological profiles of helminth infections and allergy, several cross-sectional studies have suggested a protective effect of helminth infection on atopic disorders[4,5], with a number of intervention studies supporting this notion[6-9]. It has been reported that high intensity helminth infection protects subjects from skin test reactivity to allergens[10,11]. Although not all studies confirm this, it has been proposed that the negative association between helminth infections and allergies might be the result of the strong immune-regulatory cell networks induced by chronic helminth infections that prevent pro-inflammatory Th2 responses from triggering disease in target organs[12,13]. However, there is also evidence for a parallel mechanism: the possibility that helminth infections are accompanied by IgE responses that are cross-reactive between helminth antigens and common environmental and/or food allergens, but of low biological activity and therefore associated with low or negligible skin prick test (SPT) reactivity to such cross-reactive allergens[14-16].

Laboratory diagnostics and skin test reagents to support the diagnosis of allergy have until recently always been based on allergen extracts. The increasing knowledge on the serological and clinical role of individual allergenic molecules in these extracts and their availability as purified natural or recombinant reagents has opened new diagnostic avenues. The introduction of so-called component-resolved diagnosis (CRD) based on individual allergen molecules has been instrumental in improving the clinical prognostic relevance of diagnostic tests and in identifying the origin of sensitization. Some individual allergen molecules have proven to be better predictors of clinical allergy[17] than whole extracts and have been shown to be risk factors for severe clinical phenotypes[18]. In addition, we now know that some allergens are biomarkers for primary sensitization to particular types of allergen sources, like group 5 allergen (e.g. Phl p 5) for grass pollen, group 1 and 2 allergens (e.g. Der p 1 and Der p 2) for house dust mite, and Ara h 2 for peanut sensitization[19]. On the other hand, cross-reactive structures have been identified that are present in many inhalant and food allergens sources, leading to positive IgE outcomes for many extract-based tests, without really revealing whether recognition is based on primary sensitization and/or cross-reactivity. The most relevant broadly cross-reactive structures are the pathogenesis-related (PR)-10 proteins (i.e. Bet v 1 and homologues in fruits and tree nuts)[20], profilins[21], tropomyosins[22] and lipid transfer proteins[23]. A special cross-reactive structure is not of protein but of carbohydrate nature, i.e. cross-reactive carbohydrate determinants (CCD)[14,24]. IgE against CCD has a very broad spectrum of cross-reactivity across the plant kingdom but also to glyco-proteins of invertebrate animals such as insects (venoms)

and parasites. Sensitization to CCD has first been shown to find its origin in pollen, bee and wasp venom allergies. In hay fever patients with strong pollen sensitization, IgE against CCD is most common and often accompanied by sensitization to profilin. It has been demonstrated convincingly that CCD-specific IgE is of poor biological activity and consequently of no clinical relevance[25]. Later it was suggested that helminth infections in humans and in cattle can play an important role in sensitization to CCD[16,26].

Many biomarkers for primary sensitization as well as cross-reactive allergens are now available as single diagnostic reagents on e.g. ImmunoCAP, but more recently, protein microarray technology has entered the field of molecular allergology, i.e. the ImmunoCAP-ISAC microarray with 112 individual purified natural and recombinant inhalant and food allergens[27,28]. This technology allows testing for individual IgE recognition profiles with a limited volume of serum, providing comprehensive information about likely sources of primary sensitization and cross-reactivity. For epidemiological research such technology has proven to provide an enormous resource of data[29] that can help elucidate the process of sensitizations and identify risk and protective factors for clinical phenotypes[30,31].

To the best of our knowledge, the present study is the first to characterize IgE profiles to allergen components using the ImmunoCAP-ISAC technology in a developing country in an area where helminths are endemic. Our aim was to elucidate the molecular basis of the extremely high prevalence of sensitization to common allergen sources (65% - 85%) but low SPT reactivity and virtually absent clinical allergy, observed amongst Indonesian school children.

METHODS

Study population

This study was part of a longitudinal study investigating the effect of anthelminthic treatment on malarial parasitemia and allergy, described in detail elsewhere[32,33]. In short, the study was conducted between 2008 and 2010 in a semi-urban (Nangapanda) and a rural (Anaranda) site, both located on Flores Island, where the infrastructure, access to electricity, shops and health care were low in the latter. A total of 1674 schoolchildren aged 5-15 years old were included in the study of allergic outcomes. Children were asked to undergo skin prick testing, provide blood for serum IgE testing, and a stool sample for diagnosis of soil-transmitted helminth infections. In addition, parents or guardians were asked to fill out a questionnaire to record self-reported allergic symptoms. Written informed consent was obtained from a parent or guardian of each child. This investigation was carried out within the ImmunoSPIN programme (www.immunospin.org) and approved by the Ethical Committee of the Medical Faculty, University of Indonesia, Jakarta and has been filed by the ethics committee of the Leiden University Medical Center, the Netherlands. The study was registered as clinical trial (ISRCTN83830814).

Due to budgetary restrictions, 150 serum samples could be analyzed in the current study. These were selected, based on plasma availability and complete information regarding skin prick test (SPT) reactivity, clinical symptoms of allergy as well as ImmunoCAP IgE data.

Questionnaires

Symptoms of asthma and atopic dermatitis in the past 12 months were recorded using a modified version of the International Study of Asthma and Allergy in Childhood (ISAAC) questionnaire as reported before[33].

Skin prick test

House dust mite (*Dermatophagoides pteronyssinus* and *D. farinae*), peanut, shrimp (kindly provided by Paul van Rijn from HAL Allergy Laboratories, Leiden, The Netherlands) and cockroach (*Blattella germanica*; Lofarma, Milan, Italy) were used for SPT. The SPT was considered positive when a wheal size was more 3 mm as described[33].

IgE antibody measurement

ImmunoCAP (ThermoFisher Scientific, Uppsala, Sweden) was used to measure IgE against house dust mite (*D. pteronyssinus*), cockroach (*B. germanica*), grass pollen (*Phleum pretense*), bromelain (*Ananas comosus*), peanut and shrimp, following to the manufacturer's instructions. Levels of ≥ 0.35 kU_A/L were considered positive.

For multiplex analysis, the ImmunoCAP-ISAC 112 with 112 allergen molecules (ThermoFisher Scientific) was used following manufacturer's instructions. Briefly, arrays were incubated with 30 µL of plasma for 2 hours. After washing and drying, arrays were incubated for 30 minutes with fluorescently labeled anti-human IgE. After further washing, arrays were read with a laser scanner. Results were expressed as ISAC standardized units (ISU)/L. Levels of >0.3 ISU/L were considered as positive.

Total IgE was measured by ELISA as described previously[32,33]. The results were expressed in International Units (IU/ml).

IgE inhibition assay

For ImmunoCAP inhibition assays, 4 separate pools of plasma were prepared, a HDM-, a cockroach-, a grass pollen- and a peanut-positive pool which each contained equal volumes (n=16, n=11, n=3, and n=18, respectively) of plasma samples that were positive (IgE levels ≥5 kU_A/L) for the respective allergen sources. Inhibitors used were bromelain (marker for CCD), SEA (soluble egg antigen of *Schistosoma haematobium*, a source of helminth CCD)[16] and *Ascaris lumbricoides* antigen (locally endemic parasite). Each 75 µL of pooled plasma was mixed with an equal volume of inhibitor dilutions (titrated inhibition) and pre-incubated at room temperature for 1 hour. Subsequently, samples were analyzed for HDM-, cockroach-, grass pollen- and peanut-specific IgE, following the normal ImmunoCAP protocol. Results were expressed as percentages of an uninhibited control (PBS)[16].

Parasitological examinations

Trichuris trichiura was detected by microscopy and a multiplex real-time PCR was used for detection of hookworms (*Ancylostoma duodenale*, *Necator americanus*), *A. lumbricoides*, and *Strongyloides stercoralis* DNA as detailed previously[32,33].

Statistical analyses

Statistical analyses were performed using IBM Statistical Package for Social Sciences (IBM Corp., Armonk, New York, USA) version 20. Descriptive data were expressed as means (± standard deviations), frequency (percentage of measured data) and geometric means [95% confidence intervals (CI)]. The correlation of IgE levels between natural or recombinant allergens or whole allergen extracts and CCD was examined with Spearman rank correlation coefficients. Logistic regression was used to examine the association between helminth infection and IgE reactivity to allergen component measured on ImmunoCAP-ISAC as well as the association between helminth infection and IgE reactivity to whole allergen extract tested on ImmunoCAP with adjustment for age, sex, and area. A *P-value* < 0.05 was considered statistically significant.

RESULTS

Sensitization by extract ImmunoCAP and SPT

Of the 150 sera selected for ImmunoCAP-ISAC, sensitization to cockroach, as assessed by extract ImmunoCAP, was highest (86.7%), followed by shrimp (79.3), HDM (74%) and peanut (64.7%). However, skin prick reactivity was much lower and highest for HDM (26.7%), followed by cockroach (24%), peanut (5.5%) and shrimp (4.7%). Total IgE was high with a geometric mean of 2816.4 IU/ml (95% CI, 2256.5-3515.2) (Table 1).

In this group, wheeze or eczema in the past 12 months was reported for only 5 children. None of the study subjects reported any symptoms of food allergy. With such small numbers it was not possible to use these data in further statistical analyses.

In this group of 150 subjects, 74 had stool samples and the prevalence of helminth infections was 93.2% (69/74). Analyzing characteristics of subjects providing stool samples and those who did not, showed no differences in atopic sensitization (SPT and IgE), age and sex.

Sensitization by ImmunoCAP-ISAC microarray

A total of 112 (74.7%) children recognized at least one allergen component. Of 112 allergens tested by ISAC, 46 allergens were recognized (Figure 1 and Table S1). The prevalence of recognition of purified natural glycosylated allergens was highest for those from grass pollen (nPhl p 4; 53.3%; nCyn d 1: 50.7%), followed by those from walnut (nJug r 2: 32.0%), and tree pollen (plane nPla a 2: 30.7%; cypress nCup a 1: 21.3%; Japanese cedar nCry j 1: 18,7%). IgE to MUXF3 representing the CCD glycan structures commonly found on these glycoproteins was detected in 21.3%. Recombinant allergens and non-glycosylated

Table 1. Characteristic of the study population

	N	n (%)
Age (mean in years, SD[#])	150	10.6 (2.9)
Sex (female)	150	83 (55.3)
Skin prick test		
House dust mite	150	40 (26.7)
Blattella germanica (cockroach)	150	36 (24.0)
Peanut	150	8 (5.3)
Shrimp	150	7 (4.7)
Specific IgE (cut off ≥ 0.35 kU_A/L)		
Dermatophagoides pteronyssinus	150	111 (74.0)
B. germanica	150	130 (86.7)
Peanut	150	97 (64.7)
Shrimp	150	119 (79.3)
Phleum pretense (grass pollen)	30	27 (90)
Ananas comosus (bromelain)	30	26 (86.7)
Total IgE (geometric mean, 95% CI)	133	2816.4 (2256.5-3515.2)
Helminth infection (n, %)		
Any helminths	74	69 (93.2)
Hookworms[1]	76	58 (76.3)
Ascaris lumbricoides[1]	76	32 (42.1)
Trichuris trichiura[2]	104	30 (28.8)

[#] SD: standard deviation. CI: confidence intervals. Helminth infection was diagnosed by [1]PCR, [2]microscopy.

natural allergens (walnut nsLTP, cockroach and shrimp tropomyosin) not substituted with CCD-type glycans (n=28) was found in < 3.4% with the exception of one recombinant tree pollen allergen (rPla a 1: 12.7%) and three recombinant venom allergens (honey bee rApi g 1: 18.7%; paper wasp rPol d 5: 36.7%; common wasp: 34.0%).

Mono sensitization was found in 12 children (8%), with 3 subjects positive for recombinant plane tree (rPla a 1), 3 subjects positive for paper wasp (rPol d 5), 2 subjects positive for nCyn d 1 and the other 4 subjects recognized different type of allergens (nPhl p 4, saltwort weed pollen [nSal k 1], dog [rCan f 2] and latex [rHev b 5]).

Correlation between IgE to natural allergens and CCD

The correlation of IgE (ImmunoCAP-ISAC) to natural glycosylated allergens nJug r 2, nCyn d 1, nPhl p 4, nCup a 1, and nPla a 2 and to CCD (MUXF3) was high: r = 0.77, 0.66, 0.72, 0.60, and 0.74, respectively; $P < 0.001$ (Figure S1). IgE levels to both extracts of grass pollen and peanut detected by ImmunoCAP were highly correlated with those against bromelain IgE (r=0.87 and 0.84, respectively; $P < 0.001$, Figure S2a-b). We found a weak correlation

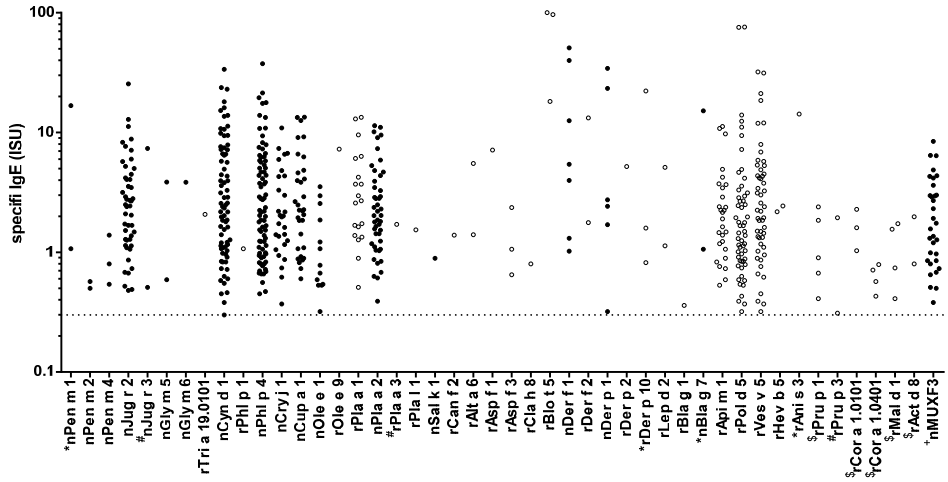

Figure 1. The levels of IgE to purified natural and recombinant allergens recognised on ImmunoCAP-ISAC (N=150). *Solid circles represent natural (n) component allergens, open circles represent recombinant (r) component allergens. Cross reacting allergen to: *tropomyosin, #non-specific lipid transfer protein (nsLTP), $the pathogenesis-related (PR)-10 protein, +Cross reactive carbohydrate determinants (CCDs). Each circle represents a single positive subject and the dotted line indicates the cut-off value for the ISAC assay (0.3 ISU/L). ISU, ISAC Standardized Unit. The allergen abbreviations are given in Table S1*

between bromelain IgE and IgE levels to cockroach (r= 0.53; P = 0.002) and a trend for HDM (r=0.32; P = 0.085) (Figure S2c-d).

IgE inhibition assay

Titrated CAP inhibition assays demonstrated that binding of IgE from peanut- and grass pollen-pooled samples was inhibited by bromelain (76.1% and 57.5% inhibition, respectively) and SEA (76.3% for peanut-pooled IgE and 45% for grass pollen-pooled IgE) whereas this was much less when the endemic helminth *A. lumbricoides* antigen was used for inhibition (25% for peanut-pooled IgE and 17% for grass pollen-pooled IgE) (Figure 2a-b). In HDM- and cockroach-pooled plasma; bromelain, SEA and *Ascaris* antigens could only inhibit up to 25% binding of IgE (Figure 2a-c).

Comparison of aero- and food-allergens measured on ImmunoCAP and ImmunoCAP-ISAC

Using ImmunoCAP, IgE sensitization to whole extract of HDM was detected in 74% of the study subjects. When the same samples were tested against HDM components on ImmunoCAP-ISAC, we found only up to 5% of the subjects showed IgE reactivity specific to natural or recombinant component of house dust mite (Figure 3a).

Of 113 subjects positive for IgE against cockroach extract on ImmunoCAP, only 2% of the children were reactive to cockroach allergen components; either nBla g 7 (1.3%) or rBla g 1 (0.7%) on ImmunoCAP-ISAC (Figure 3b). Peanut reactivity on CAP was observed

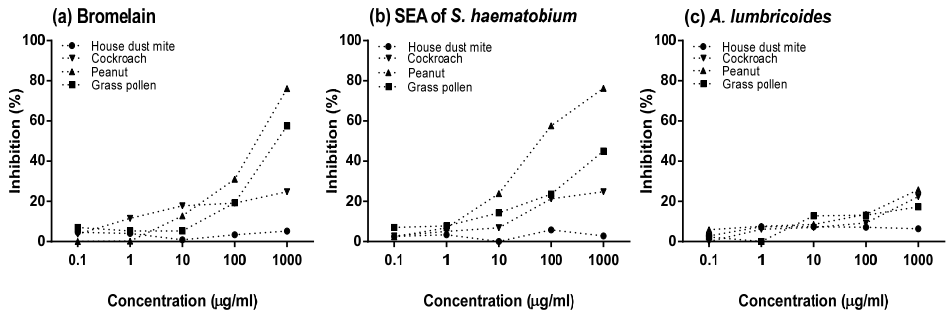

Figure 2. IgE inhibition assay*. IgE cross-reactivity of house dust mite, cockroach, peanut and grass pollen extract tested by using an IgE ImmunoCAP inhibition assay. Bromelain, soluble egg antigens (SEA) of Schistosoma haematobium and Ascaris lumbricoides were used as inhibitors and values were expressed as the percentage of IgE inhibition.*

in 97 subjects but none of them showed any reactivity to 6 peanut components present on ImmunoCAP-ISAC. Of 27 subjects positive to whole extract of timothy grass pollen in CAP assay, 21 (77.7%) recognized nPhl p 4 on ImmunoCAP-ISAC. However, no reactivity was observed to the other 7 recombinant allergens of grass pollen (Figure 3d).

Association between helminth infection and IgE to allergen components and whole allergen extracts

Given the high prevalence of any helminth infection (93.2%) in our study subjects, it is not possible to determine the association of any helminth with allergen reactivity. However, the analysis of the association with specific helminths such as hookworm (76.3%), *A. lumbricoides* (42.1%) and *T. trichiura* (28.8%) was possible. We found that having *A. lumbriodes* was positively associated with IgE reactivity to rVes v 5 on ImmunoCAP-ISAC (OR, 3.42; 95% CI 1.22-9.59; p=0.019). No other associations were observed. No association between specific helminths and IgE in ImmunoCAP was seen.

DISCUSSION

To our knowledge, this is the first study to investigate IgE reactivity using an IgE microarray assay involving school children in an area in a developing country where helminth infections are highly endemic. We found strong IgE reactivity to natural component allergens from the pollen group, walnut and CCDs. Similarly, we found high reactivity to recombinant venom allergens (paper wasp, common wasp and honey bee). The reason for high reactivity to the pollen group and peanut appeared to be due to cross reactivity with the well-known CCD structures the α(1,3)-linked fucose and β(1,2)-linked xylose[34,35] which are carbohydrate components of plant and insect glycoproteins but also in helminths[36]. Little IgE reactivity was found to recombinant allergens of pollens. Importantly, for HDM and cockroach, the high reactivity, again was not directed to recombinant versions of well-established major allergens

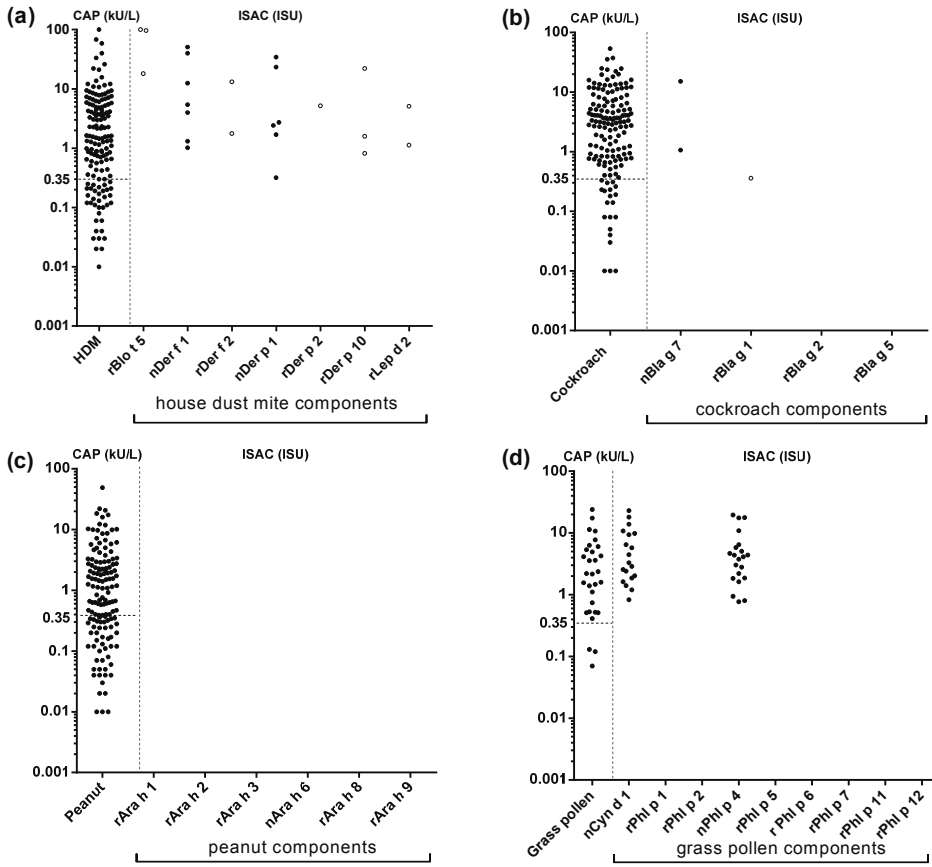

Figure 3. The levels of IgE to; (a) house dust mite, (b) cockroach, (c) peanut, and (d) grass pollen measured by ImmunoCAP and ImmunoCAP-ISAC. *The single allergens in ISAC are related to the whole allergen extracts tested in CAP. Solid circles represent natural (n) component allergens, open circles represent recombinant (r) component allergens.*

associated with primary sensitization. In this case IgE responses showed weaker associations to the typical CCD sources (MUXF3/bromelain), and more limited cross-reactivity in IgE inhibition. This leaves the question as to whether slightly different carbohydrate structures are responsible for sensitization and cross-reactivity or that other protein moieties thus far not present on the ISAC chip are involved. Since common allergens known to be associated with primary sensitization to house dust mite and cockroach were rarely recognized, sensitization to such "missing" allergens would most likely also be caused by other primary sources such as helminths. Some candidates have identified such as tropomyosin[22], but in our cohort this pan-allergen seems to be of limited importance (≤ 2% recognition). Other candidates not on the chip may be gluthation-S-transferases and paramyosins. As there is far less SPT or symptoms than IgE reactivity in this group studied, the data support the notion that in helminth endemic areas, IgE antibodies to a number of allergens arise form

cross reactivity between helminth derived or environmental carbohydrate structures and that these antibodies have poor functional properties.

Our results are in line with studies among subjects with and without allergic rhinitis in Philippines that showed an almost exclusive IgE reactivity to natural grass pollen allergens whereas non-glycosylated recombinant grass pollen allergens were hardly recognized[37]. Several studies in affluent countries have shown the existence of IgE antibodies directed to CCDs with negligible if any clinical relevance. Serum IgE from European pollen allergic patients cross-reacted with extracts from various allergenic foods which seemed to be tolerated[24]. Treating the extracts with periodate, which destroys the carbohydrate structures, abolished the reactions, indicating the involvement of carbohydrates in this cross reactivity[24]. Another study showed that elevated levels of IgE against peanut extract among grass pollen-sensitized European patients did not seem to translate to peanut SPT reactivity or clinical symptoms of peanut allergy[38]. In some of these patients, almost complete inhibition of IgE to peanut was possible with CCDs[38]. This was supported by another study, where about 42% of European pollen-allergic patients were found to have specific IgE to CCD marker bromelain without skin reactivity to this molecule[30]. The most convincing piece of evidence highlighting the lack of clinical relevance of CCD was provided by oral challenge with a purified glycoprotein polyvalently substituted with CCD[25].

In our study, we found that IgE against peanut as well as IgE to grass pollen were inhibited by bromelain (CCDs) which demonstrates that at least for pollen, anti-CCD IgE results in false-positive in vitro allergy test responses. Parasitic helminths carry carbohydrate structures which react with IgE. However, although bromelain could inhibit the ImmunoCAP of grass pollen and peanut, the extract of *A. lumbricoides,* which is endemic in our study area, was less potent. This indicates that either hookworm or *Trichuris* which are the other helminth species present in the study area might be the source of CCD. To support involvement of parasite-derived CCD, we used SEA from a non-endemic parasite that had been shown to be highly cross-reactive with peanut in a group of Ghanaian school children[16]. In the Ghanaian study, *A. lumbricoides* was also a poor inhibitor, as in our study. Using SEA, we found a strong inhibition of IgE to peanut and grass pollen. The characterization of the glycome of the endemic intestinal helminths should help us answer the question whether other cross reactive carbohydrate structures can explain the high IgE to peanut and grass pollen. The possibility that sources other than helminths in the rural area are responsible for the high cross reactive IgE seen in the area should also be studied further.

Although none of the study subjects showed IgE reactivity to natural bee venom allergen nApi m 4, we observed high IgE reactivity to recombinant venoms (rApi m 1, rPol d 5 or rVes v 5). However, why we find such high reactivity to venom allergens in children is not clear yet. Although it is possible that there is a true sensitization to venom allergens, it is also possible that the majority of positivity to honey bee/wasps represents sensitization to a peptide cross-reactive epitopes present on other insect proteins or helminths. We found that having *A. lumbricoides* increased the risk of IgE reactivity to rVes v 5, however, not to the other venom components. Therefore although the high venom IgE reactivity observed

in helminth endemic areas is not fully understood, it might be a cross-reactive response as a result of increased exposure to helminths which carry venom like proteins such as antigen 5. Although the clinical relevance of the high IgE to venoms needs to be formally investigated, there were no reports of clinical allergy to these allergens.

The strengths of this study include the microarray biochip which has allowed us to perform a comprehensive analysis of IgE reactivities to 112 component allergens from 51 source allergens, requiring only a small serum volume, which would have been impossible with traditional diagnostic tests. The ISAC chip is thus well suited where only small volumes of serum are available. However, a limitation of our study is that it was performed using a cross-sectional design in an area with no data on allergen exposure.

In conclusion, we provide evidence that there is cross-reactivity between IgE to allergens and CCD markers in a helminth-endemic area of Indonesia. Further epidemiological and clinical studies are needed to enable differentiation between IgE directed against protein structures that may be biologically active and IgE directed against carbohydrate moieties on glycosylated allergens that might be clinically irrelevant.

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SUPPLEMENTARY TABLES

Table S1. The prevalence of sensitization on ImmunoCAP-ISAC

Component allergens	N (%)	Component allergens	N (%)
Food components		*Cockroach*	
Shrimp (nPen m 2)	2 (1.3)	Cockroach (rBla g 1)	1 (0.7)
Shrimp (nPen m 4)	3 (2.0)	*Venom*	
Walnut (nJug r 2)	48 (32.0)	Honey bee (rApi m 1)	28 (18.7)
Soybean (nGly m 5)	2 (1.3)	Paper wasp (rPol d 5)	55 (36.7)
Soybean (nGly m 6)	1 (0.7)	Common wasp (rVes v 5)	51 (34.0)
Wheat (rTri a 19.0101)	1 (0.7)	*Latex*	
Grass pollen		Latex (rHev b 5)	2 (1.3)
Bermuda grass (nCyn d 1)	76 (50.7)	*Cross-reactive*	
Timothy grass (rPhl p 1)	1 (0.7)	Tropomyosin	
Timothy grass (nPhl p 4)	80 (53.3)	Anisakis (rAni s 3)	1 (0.7)
Tree pollen		Cockroach (nBla g 7)	2 (1.3)
Japanese cedar (nCry j 1)	28 (18.7)	*D. pteronyssinus* (rDer p 10)	3 (2.0)
Cypress (nCup a 1)	32 (21.3)	Shrimp (nPen m 1)	2 (1.3)
Olive pollen (nOle e 1)	13 (8.7)	nsLTP	
Olive pollen (rOle e 9)	1 (0.7)	Walnut (nJug r 3)	2 (1.3)
Plane tree (rPla a 1)	19 (12.7)	Peach (rPru p 3)	2 (1.3)
Plane tree (nPla a 2)	46 (30.7)	Plane tree (rPla a 3)	1 (0.7)
Weed pollen		PR-10 protein	
Plantain (rPla l 1)	1 (0.7)	Hazel pollen (rCor a 1.0101)	3 (2.0)
Saltwort (nSal k 1)	1 (0.7)	Hazelnut (rCor a 1.0401)	4 (2.7)
Animal		Apple (rMal d 1)	4 (2.7)
Dog (rCanf2)	1 (0.7)	Peach (rPru p 1)	5 (3.3)
Mold		Kiwi (rAct d 8)	2 (1.3)
Alternaria (rAlt a 6)	2 (1.3)	CCD	
Aspergillus (rAsp f 1)	1 (0.7)	nMUXF3	32 (21.3)
Aspergillus (rAsp f 3)	3 (2.0)		
Cladosporium (rCla h 8)	1 (0.7)		
Mite			
Blomia tropicalis (rBlo t 5)	3 (2.0)		
Dermatophagoides farinae (nDer f 1)	7 (4.7)		
D. farinae (rDer f 2)	2 (1.3)		
D. pteronyssinus (nDer p 1)	6 (4.0)		
D. pteronyssinus (rDer p 2)	1 (0.7)		
Lepidoglyphus destructor (rLep d 2)	2 (1.3)		

Natural (n) and recombinant (r) allergen components. The number positive (N) of 150 subjects examined.

SUPPLEMENTARY FIGURES

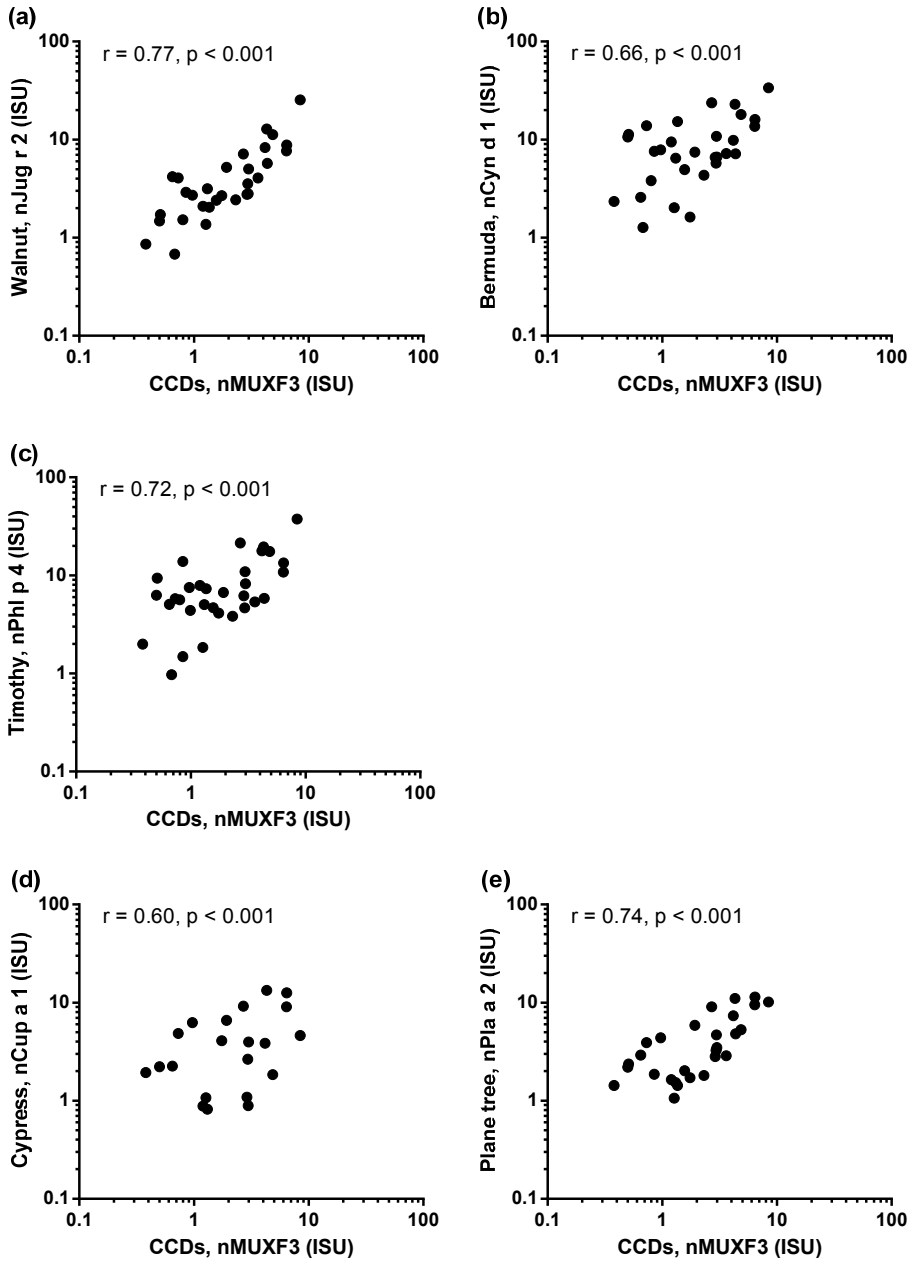

Figure S1. Correlation between IgE to CCD and natural allergens on ImmunoCAP-ISAC; *(a) walnut, (b) bermuda, (c) timothy, (d) cypress, and (e) plane tree. nMUXF3 is a marker glycoprotein for CCD reactivity on ISAC assay. r = correlation coefficient. ISU, ISAC Standardized Unit.*

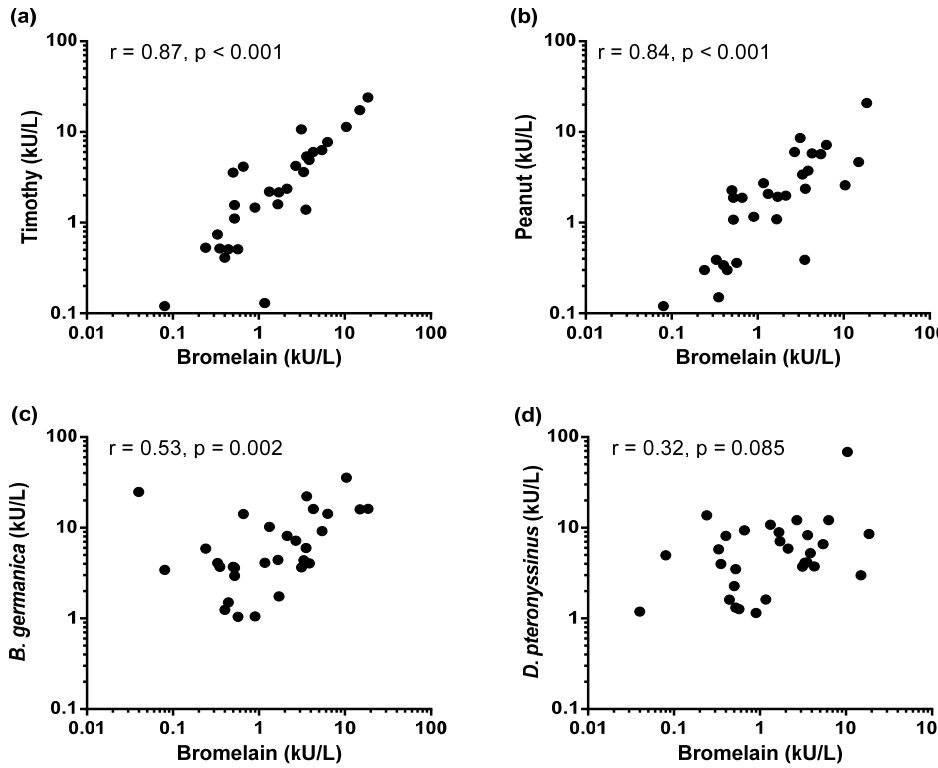

Figure S2. Correlation between IgE levels to bromelain and allergens on ImmunoCAP; *(a) timothy, (b) peanut, (c) cockroach, and (d) house dust mite. Bromelain is a marker glycoprotein for CCD reactivity. r = correlation coefficient.*