The effects of a synbiotic in infants with atopic dermatitis
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Chapter 4

Immunological differences between infants with IgE-associated and non-IgE-associated atopic dermatitis

Submitted
ABSTRACT

Background: Infants with atopic dermatitis (AD) that have elevated total and/or allergen-specific IgE levels (IgE-associated AD) seem to have more severe and persistent eczema and a higher chance to develop asthma or allergic rhinitis than infants with normal IgE levels (non-IgE-associated AD). We aimed to reveal immunological differences between infants with IgE-associated and non-IgE-associated AD.

Methods: Seventy-nine infants with AD, aged <7 months, were classified as having IgE-associated AD or non-IgE-associated AD. Plasma levels of CTACK, TARC, IL-5, IgG1 and IgG4, ex vivo cytokine responses by peripheral blood mononuclear cells, and percentage of regulatory T cells were determined.

Results: Infants with IgE-associated AD (n=50) were slightly older (5.2 vs. 4.4 months, \(P = 0.009\)) and had more severe eczema (SCORAD score 37 vs. 30, \(P = 0.005\)) than infants with non-IgE-associated AD (n=29). Infants with IgE-associated AD had higher eosinophil counts (\(P = 0.04\)), higher CTACK (\(P = 0.007\)) and IgG1 (\(P = 0.02\)) levels, higher ex vivo IL-4 (\(P = 0.002\)), IL-5 (\(P = 0.01\)) and IL-13 (\(P = 0.04\)) production and lower IL12p40p70 (\(P < 0.001\)) production than infants with non-IgE-associated AD. There were no significant differences in plasma IL-5, IgG4, TARC, ex vivo IL-10, IL-17, TGF-β and IFN-γ production, and numbers of regulatory T cells.

Conclusion: Infants with IgE-associated AD display a more pronounced Th2-biased immune profile than infants with non-IgE-associated AD. Whether this Th2 pattern indeed predisposes to a worse clinical prognosis will be elucidated in a long term follow-up study.

INTRODUCTION

Atopic dermatitis (AD) is a chronic, itching, inflammatory skin disease that often becomes manifest in infancy and perseveres in adults. The majority of AD patients have elevated total serum IgE levels and specific IgE antibodies or positive skin prick test reactions to common aero- or food allergens. Five to 45% of AD patients, however, do not have elevated total and/or specific IgE levels (1;2). It has been suggested that this non-IgE-associated form (also called intrinsic AD or atopiform dermatitis) should be considered as a separate disease entity (1;3;4).

Certain specific clinical characteristics have been reported for non-IgE-associated AD distinguishing it from IgE-associated AD, including milder disease with later onset, a female predominance and no relation to other allergic diseases (5;6). In adults, immunological differences between the IgE-associated and the non-IgE-associated form have been demonstrated. Peripheral blood lymphocytes of patients with IgE-associated AD express higher IL-4 and lower IFN-γ levels than those of patients with non-IgE-associated AD, whereas IL-5, IL-13 and absolute eosinophilic granulocyte numbers are similar (7;8).

In children, the distinction between IgE-associated and non-IgE-associated AD is less clear. Since serum IgE rises with age, children that are IgE-negative at one time point can convert to IgE-positive within a few months or years (9). For example, it has been demonstrated that more than half of skin prick-negative two year old children with AD, were skin prick-positive at age 11 (10). Therefore, in clinical practice and research, children with AD are often seen as one entity and the distinction between IgE-associated and non-IgE-associated AD is often not made. Also, immunological differences between children with IgE-associated and non-IgE-associated AD have not been extensively investigated.

Interestingly, significant differences in clinical outcome have been shown between children who develop IgE-associated AD at a young age (< 2 years) and children with AD that develop IgE-sensitization later in life or do not develop IgE-sensitization at all. Children with early IgE-sensitization seem to have more severe and more persistent eczema and a higher chance of developing asthma (45-60% vs. 14% in children with late or no sensitization) or allergic rhinitis (35% vs. 9%) later in life (10-12). It can be hypothesized that the early presence of IgE reflects an immunological predisposition to develop more severe allergic disease as exemplified by severe, persistent eczema and a high risk to develop allergic airway disease. Clarifying the immunological differences between infants with IgE-associated and non-IgE-associated AD may provide insight into the pathogenesis of developing more severe allergic disease, which could provide tools for diagnostic, therapeutic and preventive strategies.

There are several circulating markers for atopic disease, such as eosinophilic granulocyte count and IL-5, IgG1, which may be promoted by enhanced IL-5 levels (13), and IgG4, which is considered to dampen IgE responses (14), cutaneous T-cell attracting chemokine (CTACK) and thymus and activation-regulated chemokine (TARC), two chemokines that attract T cells to the skin and are related to AD severity (15-17). We aimed to assess differences in these markers between infants with IgE-associated AD and infants with non-IgE-associated AD. Additionally,
differences in ex vivo cytokine responses by peripheral blood mononuclear cells and numbers of regulatory T cells were assessed.

MATERIALS AND METHODS

Patients and study design
Ninety full-term infants, aged 0 to 7 months, fulfilling Hanifin and Rajka criteria for AD (18), were recruited for a prospective randomized controlled multi-centre trial investigating the effects of synbiotics on the severity of AD. Details of this study have been described elsewhere (19). Infants were ineligible if the SCORing Aopic Dermatitis (SCORAD) (20) score was < 15, if they had major medical problems or if they had used topical calcineurin inhibitors or systemic immunosuppressive drugs during the 4 weeks prior to collection of the blood sample. Written informed consent was obtained from both parents of all participating children. The protocol was approved by the Medical Ethics Committees of all participating centres. The trial is registered in the ISRCTN register: ISRCTN69085979. A 2-3 ml peripheral blood sample was drawn at baseline, i.e. before the intervention period, for analyses as described below.

IgE-associated and non-IgE-associated AD
Infants were classified as having IgE-associated AD if they had elevated total serum IgE levels (≥ 5 kU/L in infants < 3 months and ≥ 15 in infants > 3 months, reference values of the laboratory of the Academic Medical Center, Amsterdam) and/or elevated specific IgE antibodies (≥ 0.35 kU/L) against one or more common food- or aeroallergens. Total serum IgE and allergen-specific IgE against milk (f2), peanut (f13), egg (f245), fish (fx74), cat (e1) and house dust mite (d1) were determined using the CAP FEIA system (Phadia, Uppsala, Sweden) as indicated by the supplier.

Analysis of blood samples
Processing of blood
Total and differential leukocyte counts and total eosinophil counts were performed in EDTA-blood. EDTA-plasma samples were aliquoted and stored at -80˚C till analyses of immunoglobulins (IgE, specific IgE, IgG1 and IgG4), CTACK and TARC. Peripheral blood mononuclear cells (PBMCs) were isolated within 2 hours from heparin blood samples by standard density gradient techniques and approximately 7.5 x 10^6 cells per ampoule were cryopreserved in liquid-N2. IL-5 was determined as described before (21). For IgG1 and IgG4 reagents from Sanquin (Amsterdam, the Netherlands; M1325, M1802, anti-human IgG-biotin) were used. Duosets for CTACK and TARC were obtained from and applied to the recommendations by the supplier (R&D Systems, Abindon, UK).

Ex vivo cytokine production
For analysis, cryopreserved PBMCs were thawed, washed and incubated at 1.5x10^6 per ml culture medium (RPMI 1640 (Gibco, Invitrogen Ltd, Paisley, UK), containing 10% heat-inactivated FCS (Gibco), antibiotics and L-glutamine (Gibco) in the absence or presence of anti-CD3/antiCD28 (1XE 1 in 1000 dilution/5 µg/ml 1SE8) for 5 days at 37˚C and 5% CO2 Supernatants were harvested and stored at -20˚C until analysis. To be able to assess TGF-β, cells were incubated in serum-free medium (R&D; CCM010) instead of the RPMI medium, which was used in parallel to assess the other cytokines. The amount of several cytokines (IL-4, IL-5, IL-6, IL-10, IL-12p40p70, IL-13, IL-17 and IFN-γ) produced by stimulated PBMCs, was determined in a Luminex assay, according to the manufacturer’s protocol (Biosource, Camarillo, CA, USA). Briefly, standard solutions and samples were added to a mixture of antibody-coated beads, whereupon cytokines, bound to their specific beads, were detected using biotinylated secondary antibodies and streptavidin-RPE. The cytokine levels were determined by measuring the Fluorescence Intensity per bead per sample with the BioPlex System (Biorad Laboratories, Hercules, CA, USA). TGF-β was determined by ELISA (R&D; MAB240 and BAF240; according to the manufacturer’s protocol) The amount of each cytokine in the supernatant of stimulated cultures was adjusted for background by subtracting the amount in the medium-only supernatant.

Determination of regulatory T cells
Thawed PBMCs were washed in PBS containing 0.01% (w/v) NaN3 and 0.5% (w/v) BSA. A total of 500,000 PBMCs were incubated for 30 min at 4˚C with fluorescent-labelled conjugated monoclonal antibodies to the following surface markers (concentrations according to manufacturer’s protocols): anti-CD3-PECy7, anti-CD4-FITC, anti-CD25-PE and anti-CD8-PerCP-Cy5.5, CD127-APC AF750 (BD Biosciences, San Jose, CA, USA). For intracellular FoxP3 staining, cells were fixed after staining of surface markers and subsequently permeabilized in freshly prepared buffers from the FoxP3-staining kit (eBioscience, San Diego, CA, USA), and then incubated with anti-FoxP3-APC or rat isotype IgG2a-APC, according to the manufacturer’s instructions. After staining, cells were washed and analyzed using a FACS Canto flow cytometer and FlowJo software.

Statistical analysis
Parametric data were analyzed using unpaired t-tests. Non-parametric data were analyzed with the Mann-Whitney U test. To assess non-parametric associations, Spearman’s rank correlation coefficients were calculated. A P value < 0.05 was considered statistically significant. SPSS software (15.0) was used for all analyses.
RESULTS

Clinical characteristics

Blood samples were obtained from 79 infants, of whom 50 had IgE-associated AD. Infants with IgE-associated AD were slightly but significantly older and had significantly more severe eczema than infants with non-IgE-associated AD (Table 1).

Table 1. Clinical characteristics and IgE

<table>
<thead>
<tr>
<th></th>
<th>IgE-associated AD (n=50)</th>
<th>Non-IgE-associated AD (n=29)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male (%)</td>
<td>35 (68.6)</td>
<td>16 (53.3)</td>
<td>0.17</td>
</tr>
<tr>
<td>Age (months), mean (SD)</td>
<td>5.2 (1.4)</td>
<td>4.4 (1.4)</td>
<td>0.009</td>
</tr>
<tr>
<td>SCORAD index, median (range)</td>
<td>37 (16-70)</td>
<td>30 (17-45)</td>
<td>0.005</td>
</tr>
<tr>
<td>Total IgE (kU/L), median (range)</td>
<td>28.8 (5.2-631.0)</td>
<td>8.0 (2.3-14.3)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Specific IgE ≥ 0.35 kU/L, n (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Milk</td>
<td>12 (24%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Egg</td>
<td>30 (60%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peanut</td>
<td>27 (54%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fish</td>
<td>0 (0%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cat</td>
<td>9 (18%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HDM¹</td>
<td>1 (2%)</td>
<td></td>
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</table>

¹ House dust mite

Eosinophilic granulocytes and plasma IL-5

The peripheral eosinophil count was significantly higher in infants with IgE-associated AD (median 670x10^6/L, range 200-5570) than in infants with non-IgE-associated AD (490x10^6/L, range 50-2760, P = 0.04). Although the mean plasma IL-5 concentration was higher in infants with IgE-associated AD than in those with non-IgE-associated AD, this was not statistically significant (2.40 pg/ml, range 0.18-19.8, vs. 1.55 pg/ml, range 0.18-10.3, respectively, P = 0.29). IL-5 is a major maturation and differentiation factor of eosinophils in humans. In line herewith, eosinophil counts correlated significantly with plasma IL-5 concentration (Spearman’s rho = 0.46, P < 0.001).

IgG1 and IgG4

Total plasma IgG1 was significantly higher in infants with IgE-associated AD (5.8 mg/ml, range 0.9-66.0) than in infants with non-IgE-associated AD (4.4 mg/ml, range 0.5-13.5, P = 0.02). Total plasma IgG4 did not differ significantly between IgE-associated and non-IgE-associated AD (30.3 μg/ml, range 1.5-1290, vs. 15.4 μg/ml, range 1.2-406, respectively, P = 0.15).

CTACK and TARC

Circulating levels of these mediators in infants with IgE-associated and non-IgE-associated AD are shown in figure 1. CTACK was significantly higher in infants with IgE-associated AD than in infants with non-IgE-associated AD. TARC levels did not statistically differ between the two groups. Both CTACK and TARC levels correlated weakly with SCORAD score (rho = 0.25, P = 0.03 and rho 0.25, P = 0.04, respectively). After correction for SCORAD score, CTACK levels were still significantly higher in infants with IgE-associated AD than in infants with non-IgE-associated AD (ANCOVA with ln(CTACK) as dependent variable, P = 0.04).

Ex vivo cytokine production by PBMCs

Spontaneous cytokine production by PBMCs did not differ between infants with IgE-associated and non-IgE-associated AD (data not shown). Cytokine levels after stimulation with antiCD3/antiCD28 are shown in figure 2. Infants with IgE-associated AD had significantly higher levels of IL-4, IL-5, IL-13 and a significantly lower level of IL12p40p70 than infants with non-IgE-associated AD. Levels of IL-6 tended to be higher in infants with non-IgE-associated AD. IL-10, IL-17, TGF-β and IFN-γ did not significantly differ between the two groups. As some of the cytokines that differed between the IgE-associated and the non-IgE-associated group weakly correlated with age, we also performed the cytokine analyses after post hoc matching for age, in order to rule out a possible confounding effect. This did not significantly change the results.

There was no correlation between SCORAD score and IL-4, IL-5, IL-13 and IL12p40p70 (data not shown). The level of IL-4 after antiCD3/antiCD28 stimulation correlated significantly with IgE concentration (rho = 0.41, P < 0.001).

Regulatory T Cells

There were no differences between the IgE-associated and the non-IgE-associated group in the percentage of PBMCs that were CD3+ (63.1% vs. 62.9%, respectively, P = 0.96), CD4+CD8- (45.5% vs. 46.0%, respectively, P = 0.83), FoxP3+CD25+ (2.7% vs. 2.6%, respectively, P = 0.84) and FoxP3+CD25+CD127- (= regulatory T cells (22), 2.5% vs. 2.4%, respectively, P = 0.50).
Chapter 4

Immunological differences between infants with IgE-associated and non-IgE-associated atopic dermatitis

DISCUSSION

We demonstrated that infants with IgE-associated AD and non-IgE-associated AD have different immunological profiles. Infants with IgE-associated AD showed a more pronounced systemic Th2-dominated profile, with high circulating numbers of eosinophilic granulocytes and enhanced IL-4, IL-5, and IL-13 production by stimulated PBMCs, compared to infants with non-IgE-associated AD who showed higher IL-12p40p70 and IL-6 production.

So far, this is the first study that extensively investigated the immunological differences between IgE-associated and non-IgE-associated AD in infants. With this study we extended the findings reported by Park et al., who observed a higher eosinophil count in infants with IgE-associated AD than in infants with non-IgE-associated AD (23). Their data on IL-4 and IL-5 were based on analyses in only a minority of the participants, making it hard to draw any conclusions. Song et al. (15) studied CTACK and TARC levels in older children and found no differences between IgE-associated and non-IgE-associated AD. Their definition of IgE-associated AD (a total serum IgE > 100 kU/L and/or sensitization to more than one allergen), however, differed from our definition, which could explain the different findings since many infants that we included in the IgE-associated group would have been considered as non-IgE-associated in their study.

With a preponderant Th2 dominance at birth (24), the increased Th2-response in infants with IgE-associated AD, compared to non-IgE-associated AD, could be due to decreased regulatory T cell numbers and/or function, resulting in consolidation of the neonatal Th2 response. In our study, the percentages of systemic regulatory T cells nor the production of...
regulatory cytokines, IL-10 and TGF-β, differed between infants with IgE-associated and non-IgE-associated AD. We cannot exclude, however, that there are differences in regulatory T cell numbers in the skin. Interestingly, CTACK levels were increased in infants with IgE-associated AD, suggestive of an increased T cell recruitment to the skin. In addition, as CTACK is produced by keratinocytes, this suggests that local activation in the skin is enhanced in infants with IgE-associated AD as opposed to that in infants with non-IgE-associated AD. However, skin biopsy studies are needed to elucidate these hypotheses.

A strong point of our study is that we exercised great care to isolate and store PBMC within a fixed period as previous studies have indicated that variation in processing affects PBMC properties (25). A possible limitation of our study is that the group with IgE-associated AD was slightly older than the non-IgE-associated group, in line with a positive correlation between IgE levels and age. We consider it unlikely, however, that an age difference of less than one month influences the results that much. This was substantiated by repeating the analyses after post-hoc matching for age, which did not affect the results. Also, since the children participating in our study were very young, we can not exclude that some infants with non-IgE-associated AD will convert to IgE-associated AD in the (near) future. Even though the non-IgE-associated group might have been biased by inclusion of these children, we still found distinct differences between the two groups. In order to monitor changes over time, we will follow these infants up to age 5 years and measure total and specific IgE at several time points.

Our findings support the view that, also in infants, IgE-associated and non-IgE-associated AD are two different forms of the disease and maybe even different disease entities, as was suggested by Brenninkmeijer et al (5). It has been suggested that eczema that display IgE-sensitization before the age of 2 years have a poorer prognosis regarding their eczema and a higher chance of developing asthma or allergic rhinitis later in life than infants who do not display early IgE-sensitization (10-12). Our results are in line with these findings. Apparently, infants with early IgE-sensitization do not only have elevated IgE levels, but also show a full blown Th2 profile, which will result in events associated with chronic allergic inflammation, such as recruitment and activation of mast cells, basophils and eosinophils (26). Therefore, it seems conceivable that these children have more severe and persistent eczema and are more likely to develop other Th2-related diseases than infants with non-IgE-associated AD. If this is indeed true, it might be sensible to (repeatedly) test for allergic sensitization in all infants, as was previously suggested by Lowe et al (11). This will lead to better, tailor-made advice for parents of infants with AD regarding treatment and prognosis.

In conclusion, we demonstrated that infants with IgE-associated AD display a more pronounced Th2-biased immune profile than infants with non-IgE-associated AD. Whether this evident Th2 pattern predisposes to more persistent eczema and a higher risk of developing other allergic diseases has to be elucidated and will be investigated in a long term follow-up study.

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