The Role of Microbiota in Allergy

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Key Words
Allergy · Asthma · Eczema, atopic · Gut microbiota · Oral tolerance

Abstract
The hygiene hypothesis links the growing epidemic of clinical manifestations of allergy, atopic eczema, allergic rhinoconjunctivitis and asthma to reduced exposure to microbes at an early age as a result of environmental changes in the industrialized world. These include improved sanitation and living conditions, vaccinations and antimicrobial therapy, together with declining family size and changes in dietary intake. The discovery of three subgroups of regulatory T cells has revolutionized the original immunological basis of the hygiene hypothesis, the so-called T helper 1/T helper 2 paradigm. In a case of defective oral tolerance allergy ensues. Recent experimental and clinical findings clearly indicate that both the development and maintenance of oral tolerance is dependent on these immunosuppressive regulatory T cells. Moreover, gut microbiota has been demonstrated to be crucial for the appropriate expression and function of the regulatory T cells, thus connecting microbiota closely to allergy. Indeed, many of the cross-sectional studies have shown a different composition of gut microbiota in children with atopic eczema and healthy controls. Most of the published prospective follow-up studies so far have also found that alterations in gut microbiota precede development of allergy. Changes in the amount of bifidobacteria, clostridia and Escherichia coli have been the most common findings in these studies. There has been, however, considerable variation in the settings of different prospective studies, making it difficult to interpret probable causes for variable results. The future studies addressing the issue should not only use novel molecular techniques of gut microbiota assessment, but also take into consideration several other aspects discussed in this paper.

Definitions of Allergy and Allergic Diseases

Definitions related to allergy and allergic diseases have varied considerably in different clinical studies making comparisons between the studies difficult. To help these comparisons a nomenclature for allergy for global use has been proposed [1]. Allergy is a hypersensitivity reaction initiated by specific antibody-mediated or cell-mediated immunologic mechanisms. The term ‘hypersensitivity’ describes objectively reproducible symptoms or signs initiated by exposure to a defined stimulus at a dose tolerated by normal persons [1].

Allergic diseases, manifested in atopic eczema, rhinoconjunctivitis and asthma, are the most common chronic diseases in the Western world [2]. Eczema is a chronic or relapsing itchy skin inflammation with typical lesions and locations. Eczema is called atopic if it is associated with immunoglobulin E (IgE) production demonstrated either by positive skin prick tests or elevated antigen-specific IgE antibodies [1]. However, the association between atopy and eczema in general has recently been shown to be quite weak [3]. It should also be pointed out that the
association does not necessarily mean causality. For example, the majority of children with eczema and a low increase in antigen-specific IgE to food (i.e. children with atopic eczema) actually tolerate the food without skin or other symptoms [4]. Moreover, due to the immunological balance dominating in utero, the type 2 T helper (Th2) phenotype, the one facilitating IgE production, is universal at an early age. Consequently, a significant overlap in the concentrations of interleukin (IL)-4, the key Th2 cytokine, and IgE antibodies prevails between atopics and non-atopics at an early age [5]. These findings are not surprising in view of the mechanisms of oral tolerance described below.

Allergic rhinoconjunctivitis causes nasal and ocular immunologically mediated hypersensitivity symptoms, such as itching, sneezing, increased secretion and blockage [1]. Asthma is a chronic inflammatory disorder of the airways, which is associated with airway hyper-responsiveness that leads to recurrent episodes of wheezing, breathlessness, cough and chest tightness [6]. Asthma resulting from immunological reactions is called allergic asthma [1]. However, although wheezy atopic infants have reversible airflow obstruction typical for asthma, they do not have similar epithelial reticular basement membrane thickening and eosinophilic inflammation in endobronchial biopsies as older school-age children and adults with asthma have [7]. Strictly speaking, these infants should not be regarded asthmatic at all, despite the asthma-like symptoms and abnormal IgE production, since findings in their endobronchial biopsies did not differ from those of non-atopic infants without asthma-like symptoms [7]. The examples above demonstrate that patients with an identical clinical diagnosis (e.g. asthma) may greatly differ in their immunological phenotypes.

**Inflammatory Cascade in Allergy – a Duel between Inflammatory and Suppressive Responses**

Inflammation found in an antibody-mediated hypersensitivity reaction is characterized by the production of allergen-specific IgE antibodies and the influx of activated T cells and other effector cells, e.g. eosinophils and mast cells, to the site of allergen exposure [8]. The inflammatory response is orchestrated by Th2 cells. The Th2 cells produce cytokines such as IL-4, IL-5, IL-9, and IL-13 that regulate both the production of IgE and the influx of eosinophils, mast cells and activated CD4+ T cells to the inflamed tissue.

Th2 cells are under the control of regulatory T cells [8, 9]. The proper balance between Th1, Th2 and regulatory T cells is critical for the development of tolerance against harmless self and foreign antigens, and suppression of Th2-type allergic inflammation. The role of the newly found IL-17-producing Th17 cells in these inflammatory responses is still largely unknown [10]. Oral tolerance is the specific suppression of immune responses to an antigen by means of prior administration of the antigen through the oral route [for review see, 11, 12]. Three types of regulatory T cells have been shown to be important for tolerance: Th3 (suppression mainly by secretion of transforming growth factor, TGF-β), Tr1 (suppression mainly by secretion of IL-10) and CD4+CD25+ T cells (suppression mainly by surface-bound TGF-β). In a case of food allergy these suppressive mechanism have not developed properly or they have broken down. In children with milk-induced gastrointestinal disorders (allergic eosinophilic gastroenteritis or food protein-induced enteropathy), milk-specific duodenal mucosal lymphocytes were found to release in vitro Th2-associated cytokines and very low amounts of TGF-β and IL-10, suggesting a defective oral tolerance induction [13]. No control group was included in these analyses due to technical difficulties. In a subsequent study with controls, no difference in the production of Th2 cytokines (or in Th1 cytokines) was found in duodenal mucosal lymphocytes of children with multiple food allergies [14]. However, these children showed a reduced amount of TGF-β-producing T cells in the duodenal mucosa as compared with those in children with no eventual clinicopathological diagnosis [14]. Again, children who had outgrown their non-IgE-mediated allergy to cow’s milk were shown to have higher numbers of circulating CD4+CD25+ T cells than those children still allergic to cow’s milk [15]. The development of tolerance to cow’s milk was associated with decreased in vitro proliferative responses to bovine β-lactoglobulin, a major protein found in cow’s milk [15]. These studies imply that proper function of regulatory T cells is important in the induction and maintenance of oral tolerance to dietary antigens in human beings.

There is mounting evidence that the gut microbiota acquired during the early postnatal period is required for the development of oral tolerance. Oral tolerance induction to dietary antigen was not possible in germ-free (GF) mice. However, a reconstitution of the gut microbiota of GF mice with *Bifidobacterium infantis* during the neonatal period, but not later, restored the susceptibility to oral tolerance induction [16]. Regulatory CD4+CD25+ T cells obtained from the mesenteric lymph nodes of GF mice
were not as potent suppressors as those from convention-
al mice [17], suggesting a plausible explanation for defec-
tive oral tolerance induction in GF mice. Indeed, Ishikawa
et al. [18] have recently further strengthen the hypothesis.
They demonstrated that the frequencies and absolute
numbers of CD4+CD25+ T cells in the mesenteric lymph
nodes and Peyer’s patches of GF mice were significantly
lower than those in conventional mice. Moreover, ade-
quate production of IL-10 and especially that of TGF-β in
CD4+CD25+ T cells was crucial for oral tolerance induc-
tion to dietary antigen in conventional mice [18]. These
findings indicate that gut microbiota is mandatory for
normal expression and function of regulatory T cells as
well as for oral tolerance to dietary antigens. On the con-
trary, a recent experimental study demonstrated that gut
microbiota may not be required for tolerance induction
for pollinic antigens [19], suggesting different pathogen-
eses for dietary and respiratory allergies.

New Culture-Independent Molecular Methods
Have Revolutionized Microbiological Research
of the Bowel

Each human adult has approximately 10 times more
bacteria in the gut than eukaryotic cells in the whole body
[20]. The number of different bacterial species in the
bowel is estimated to be approximately 800, less than half
of them being culturable. The colon is heavily packed
with bacteria since feces contain about $10^{11}$ bacteria/g,
whereas intestinal contents and stomach hold less than
$10^8$ and $10^9$ bacteria, respectively, per milliliter of luminal
content. Streptococci, lactobacilli, clostrids, enterococci
and Bacteroides are typical inhabitants of the ileum,
whereas the colon harbors clostridia, Bacteroides, pre-
votella, fusobacteria, faecalibacteria, butyrvibrio, eubac-
teria, ruminococcus, roseburia, coprococcus, and nu-
merous still unknown bacteria [for review see, 21]. The
development of new sophisticated molecular techniques
has provided a totally new approach to explore the gut
ecosystem, metagenomics being an elegant example [for
review see, 22]. Metagenomics refers to culture-indepen-
dent studies of the structures and functions of microbial
communities, as well as their interactions with the habi-
tats they occupy. According to this approach, humans are
actually ‘supraorganisms’ whose genome is the sum of
genomes of our own genome and the genomes of our micro-
bial inhabitants (microbiome). Moreover, our metabolic
features are also the sum of our own and microbial char-
acteristics [22].

New culture-independent methods for bacterial de-
tection have so far targeted 16S ribosomal RNA (rRNA)
gene sequences containing regions of both highly con-
served and hypervariable nucleotide sequences [23]. Primers are targeted either for conserved or hypervari-
able regions depending on the purpose of the analysis. In
fluorescent in situ hybridization, fluorescent oligonucle-
otide probes are targeting 16S rRNA sequences of intact
bacterial cells. The method is rather insensitive and labo-
rious. In polymerase chain reaction (PCR) combined
with denaturing- or temperature-gradient gel electro-
phoreses (PCR-DGGE/PCR-TGGE), bacterial DNA is ex-
tracted from the sample (e.g. feces) and fragments of the
16S rRNA gene are amplified by PCR. Subsequently, the
double-stranded 16S fragments are separated by DGGE/
TGGE. Although the method provides a bacterial finger-
print of the major bacteria in the sample, it is very labori-
ous, rather insensitive and not quantitative. In the quan-
titative real-time PCR, target DNA/RNA is amplified by
the use of fluorescent oligonucleotide probes. The initial
concentration of the target can be calculated on the basis
of the change in PCR product concentration throughout
the amplification cycles [23]. Recently, a combination of
microarray technology with new molecular knowledge
about 16S rRNA gene sequences has provided technical
innovations that allow analysis of thousands of genes si-
multaneously from one sample [24]. The kinds of mi-
crobies present in the sample can be identified by com-
parison of their small-subunit rRNA gene sequences to
previously analyzed sequences from well-characterized
isolates [24].

Studies utilizing new molecular techniques have re-
vealed that members of only 10 of over 100 described phy-
la have been found in the human bowel. Actually, the
majority of bacteria identified belong to two phyla: the
Firmicutes and the Bacteroidetes. However, at lower tax-
onomic levels there is great diversity and stable interper-
sonal variation (approximately 30%). These studies fur-
ther indicate that fecal samples can be used to define in-
terpersonal differences in gut microbiota [21, 22].

Early Colonization of the Gut Differs Greatly from
That of Older Children and Adults

Exposure to maternal vaginal and fecal microbiota
during delivery and soon to other environmental micro-
biota has a great impact on early colonization patterns of
the infant, thus offering a potential opportunity for early
intervention, too [24, 25]. Infants born by cesarean deliv-
ery have microbiota resembling even more that of the surrounding environment with lower levels of *Bifidobacterium*, *Bacteroides* and higher levels of *Clostridium* [24, 25]. Several culture-dependent and -independent studies have found that bifidobacteria dominate in the first months of life in the bowel, especially in breastfed infants [21]. In view of this it is surprising that a recent study demonstrated relatively low frequency and abundance of bifidobacteria in the gut during infancy regardless of the type of feeding [24]. Moreover, gut microbiota varied greatly, even chaotically, during the first months of life, suggesting accidental exposures to environmental microbiota being important determiners. However, by the end of the first year more stable and adult-like gut microbiota was established, probably reflecting adaptation advantage of the taxa typically found in the adult gut [24].

**Overview of Studies on the Association between Gut Microbiota and Allergy**

The steep increase in allergy prevalence during the last decades has been linked epidemiologically to Western-type living conditions – e.g. reduced consumption of fermented food, substantial use of antibiotics and other chemicals. The so-called 'hygiene hypothesis' suggests that a lack of exposure to microbial stimuli early in childhood due to, e.g., a decreased number of older siblings and the factors mentioned above would be a major factor involved in this trend [26–29]. Moreover, certain characteristics of farming, such as farm milk consumption and frequent stay in animal sheds, may be especially protective against the development of allergic diseases [30]. Fecal microbiota of both anthroposophic and farm children diverge significantly from that of children not following those lifestyles, pointing to the importance of the gut microbiota in the development of allergic disorders [31, 32]. The hygiene hypothesis of allergy has thus been extended to gut microbiota (also called the 'microbiota hypothesis of allergy') [33].

There are several important factors that should be kept in mind when interpreting the results of studies where association of gut microbiota with allergy has been evaluated. These factors include, e.g., design of the study (prospective vs. cross-sectional), methods of bacterial analysis (bacterial culture or serology vs. molecular and other non-cultural methods), timing and number of sampling, definitions of allergy and atopic diseases, similarity of the study population, and duration and type of follow-up. In the late 1990s Björksten et al. [34] demonstrated by culture-dependent methods that 27 allergic Estonian and Swedish infants were less often colonized by lactobacilli, whereas they harbored higher counts of facultative aerobic microorganisms such as coliforms and staphylococci than 35 non-allergic 2-year-old children. Since then, there have been published at least 14 reports containing over 2,000 subjects (allergic and control subjects) where the issue has been studied [35–48]. Except for two recent ones [47, 48], the studies have also been reviewed in a recent paper [49]. It should be pointed out that there have been only some tens of subjects in most of the studies.

Except for one small Japanese study [39], all the others have been pediatric studies. Changes in fecal microbiota have been found in 7 of 9 studies, where patients with eczema or atopic eczema have been evaluated [34–36, 38–40, 46–48]. In 6 of these studies bifidobacteria counts have been shown to be altered, that being the most common alteration [34, 36, 38, 40, 46, 47]. Three studies [34, 38, 40] demonstrated lower counts or prevalence of bifidobacteria, whereas Ouwehand et al. [36] demonstrated a higher prevalence of *Bifidobacterium adolescentis* but lower prevalence of *Bifidobacterium bifidum* in children with eczema or atopic eczema than in healthy infants. There are conflicting findings in two recent reports: Gore et al. [47] found that *Bifidobacterium catenulatum/pseudocatenulatum* was the only one of the 6 studied bifidobacterial species that was associated with atopic eczema in a nested case-control study. On the contrary, *B. adolescentis* was shown to prevail in allergic children and *B. catenulatum/pseudocatenulatum* in non-allergic children according to an Estonian report [48]. The children in the study were, however, much older than in the previous one [47] and quite a few of them suffered from allergic rhinitis and/or asthma instead of eczema. All in all, one of the most important drawbacks of the cross-sectional studies is the doubt of causality: the differences found can be reasons or consequences of the disease.

There are 6 reports of prospective studies where early gut microbiota of children was analyzed in relation to later risk of allergy (table 1). The first two of these prospective studies found that a depletion of fecal *Bifidobacterium* spp. preceded later atopic sensitization and manifestation of atopic eczema [50, 51]. In the first study, we also demonstrated that higher counts of clostridia at 3 weeks of age were associated with later atopic sensitization [50]. Again, in a large prospective cohort of 957 newborns, *Clostridium difficile* was found to be associated with later manifestations of eczema, recurrent wheeze and atopic sensitization [52]. *E. coli* was also shown to be
associated with later atopic eczema. A similar finding was obtained in a prospective nested case-control study of atopic eczema [53]. Neither study, however, demonstrated an association of bifidobacteria to later atopic manifestations [52, 53]. A recent prospective study from three European birth cohorts found, however, no differences in gut microbiota by culture-dependent analysis of fecal samples among infants developing or not developing atopic eczema and food allergy [54]. On the contrary, a subgroup analysis of the cohort by cultivation-independent techniques indicated a significantly lower diversity in the gut microbiota of 1-week-old neonates who later manifested atopic eczema than in neonates remaining healthy during the first 18 months of life [55].

Table 1. Overview of prospective studies on the association between gut microbiota and allergy

<table>
<thead>
<tr>
<th>Reference</th>
<th>Study population</th>
<th>Definition of allergy</th>
<th>Fecal analysis</th>
<th>Changes in gut microbiota (allergic vs. healthy)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kalliomäki et al. [50]</td>
<td>76 newborns with a family history of allergy 22 cases and 54 controls</td>
<td>One or more positive skin prick test at 12 months</td>
<td>Gas-liquid chromatography and bacterial culture at 3 weeks and 3 months, FISH at 3 weeks</td>
<td>Different bacterial fatty acid profiles at 3 weeks, no difference in colonization patterns Higher counts of clostridia and tendency to lower counts of bifidobacteria by FISH, lower ratio of bifidobacteria to clostridia by FISH</td>
</tr>
<tr>
<td>Björksten et al. [51]</td>
<td>44 newborns 18 cases and 26 controls</td>
<td>Atopic eczema and/or at least 1 positive skin prick test at 3, 6, 12, or 24 months</td>
<td>Bacteriological culture at 1 week and 1, 3, 6 and 12 months</td>
<td>Lower prevalence of bifidobacteria (during the 1st year) and enterococci (during the 1st month), lower counts of bacteroides (at 12 months), higher prevalence of S. aureus (at 6 months) and higher counts of clostridia (at 3 months)</td>
</tr>
<tr>
<td>Penders et al. [53]</td>
<td>78 newborns (prospective nested case-control) 26 cases and 52 controls</td>
<td>Eczema and specific IgE at least to 1 allergen at 12 months</td>
<td>PCR-DGGE and quantitative real-time PCR at 1 month</td>
<td>E. coli more prevalent in infants later developing atopic eczema, no difference in total bacterial profiles or in bifidobacterial counts or bifidobacterial species composition</td>
</tr>
<tr>
<td>Penders et al. [52]</td>
<td>957 newborns Over 30% developed eczema and approximately 10% recurrent wheeze Over a quarter became sensitized (at least 1 antigen-specific IgE concentration &gt;0.3 IU/ml)</td>
<td>Eczema or atopic eczema or at least 1 positive antigen-specific IgE (&gt;0.3 IU/ml) or recurrent (&gt;3) wheeze during the first 2 years of life</td>
<td>Quantitative real-time PCR at 1 month</td>
<td>Higher counts and prevalence of E. coli in infants later developing eczema, higher prevalence of C. difficile in infants later developing eczema, recurrent wheeze and sensitization</td>
</tr>
<tr>
<td>Adlerberth et al. [54]</td>
<td>324 newborns 23% developed atopic eczema 26% became sensitized</td>
<td>Atopic eczema, total and food-specific IgE levels at 18 months</td>
<td>Rectal cultures at 3 days, stool cultures at 1, 2 and 4 weeks and at 2, 6 and 12 months</td>
<td>Neither atopic eczema nor food-specific IgE by 18 months of age was associated with time of acquisition of any particular bacterial group</td>
</tr>
<tr>
<td>Wang et al. [55]</td>
<td>35 newborns 15 cases and 20 remained healthy</td>
<td>Atopic eczema at 18 months</td>
<td>Terminal restriction fragment length polymorphism and TTGE analysis of amplified 16S rRNA genes at 1 week</td>
<td>A reduced diversity of early gut microbiota of infants with atopic eczema</td>
</tr>
</tbody>
</table>

Conclusions

Both above-mentioned experimental and clinical cross-sectional and prospective studies suggest that gut microbiota plays a crucial role in the development of allergy. This is further supported indirectly by positive effects of probiotics in some intervention studies [56]. However, until now, it is too early to make an exact list of bacterial species or strains that might be especially beneficial in relation to the later development of allergy manifestations. To make that task a little easier in the future, several aspects should be taken into consideration when addressing the issue in the time to come. It is mandatory to use molecular assessment of gut microbiota since the ma-
jority of the microbiota would otherwise be ignored. The diagnosis of atopic disease should also be as accurate as possible, preferably being based on both relevant clinical and immunological parameters (e.g. in food allergy, on the amounts of regulatory T cells in the gut mucosa or those in the peripheral blood). It should also be kept in mind that different immunological disorders may result in a similar clinical phenotype making proper classification of patients even more challenging. The host-related factors, like genotype, should be analyzed when possible. For example, two independent mutations in the gene encoding the epidermal protein filaggrin have been shown to be strong predisposing factors for childhood eczema [57]. More generally, any means to better ‘stratify’ or select defined subpopulations of subjects (e.g. patients with food allergy as a separate group) would be helpful. The mechanisms of the gut microbiota’s action in oral tolerance should be studied more closely. It might be advisable, e.g., to find out how different bacterial strains or combinations of them would work in the development of oral tolerance and regulatory T cells in GF mice. The ultimate goal of future studies should be the development of tailored interventions, such as new probiotics, prebiotics, and synbiotics that would assist in the fight against the tremendous burden of allergic diseases.
53 Penders J, Stobberingh E, Thijss C, et al: Molecular fingerprinting of the intestinal microbiota of infants in whom atopic eczema was or was not developing. Clin Exp Allergy 2006;36:1602–1608.