Aberrant IgA responses to the gut microbiota during infancy precede asthma and allergy development

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Background: Although a reduced gut microbiota diversity and low mucosal total IgA levels in infancy have been associated with allergy development, IgA responses to the gut microbiota have not yet been studied.
Objective: We sought to determine the proportions of IgA coating together with the characterization of the dominant bacteria, bound to IgA or not, in infant stool samples in relation to allergy development.
Methods: A combination of flow cytometric cell sorting and deep sequencing of the 16S rDNA gene was used to characterize the bacterial recognition patterns by IgA in stool samples collected at 1 and 12 months of age from children staying healthy or having allergic symptoms up to 7 years of age.
Results: The children with allergic manifestations, particularly asthma, during childhood had a lower proportion of IgA bound to fecal bacteria at 12 months of age compared with healthy children. These alterations cannot be attributed to differences in IgA levels or bacterial load between the 2 groups. Moreover, the bacterial targets of early IgA responses (including coating of the *Bacteroides* genus), as well as IgA recognition patterns, differed between healthy children and children with allergic manifestations. Altered IgA recognition patterns in children with allergy were observed already at 1 month of age, when the IgA antibodies are predominantly maternally derived in breast-fed children.
Conclusion: An aberrant IgA responsiveness to the gut microbiota during infancy precedes asthma and allergy development, possibly indicating an impaired mucosal barrier function in allergic children. (J Allergy Clin Immunol 2017;139:1017-25.)

Key words: *Allergic disease, asthma, secretory IgA, IgA index, IgA recognition patterns, microbiome composition, gut microbiota, childhood*

Allergic diseases have become a major public health problem in affluent societies.[1] Reduced microbial exposure, both prenatally and postnatally, has been proposed to underlie the increase in allergy development.[2-5] The gut microbiota, which hosts a complex bacterial community, is quantitatively the most important source of microbial stimulation and might provide a primary signal for appropriate immune development.[4] The gut microbiota differs in composition and diversity during the first months of life in children who later do or do not have allergic disease,[2,6-17] although no specific microbes with consistently harmful or allergy-protective roles have yet been identified. Also, we observed that differences in the gut microbiota diversity during infancy between healthy children and children with allergies were mainly related to asthma and not allergic rhinoconjunctivitis (ARC) development.[16] Early establishment of a diverse gut microbiota, with repeated exposure to new bacterial antigens, might be more important than the distribution of specific microbial species in shaping a normal immune mucosal and systemic maturation.[4]

A reduced mucosal barrier function might increase the risk for allergy development,[1] and IgA is the primary mediator of humoral mucosal immunity.[18] IgA is the most abundantly produced antibody in human subjects, with the highest amount of secretion in the intestinal tract.[18,19] Secretory IgA (SIgA) has a crucial role in the gut through its binding to bacterial antigens, thus preventing their direct interaction with the host through immune exclusion and maintaining mucosal homeostasis.[18,20] SIgA can also limit overgrowth of select species, thus stimulating diversity.[18,21] Therefore this antibody represents a key host mechanism in regulation of the commensal community, and innate receptor signaling in T cells seems to decide the specificity of IgA to constrain the composition of the intestinal bacteria, ensuring a benign symbiotic relationship.[19] However, in contrast to IgG and IgM levels, generation of this anti-inflammatory antibody is limited during early infancy, and delayed development of mucosal IgA production, for instance in the absence of breast-feeding, might lead to infectious disease in young infants.[22,23] Studies and clinical reports suggest that SIgA that originates from the mother's breast milk is important for

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Abbreviations used
ARC: Allergic rhinoconjunctivitis
FITC: Fluorescein isothiocyanate
IgA+: IgA-coated bacterial cells
IgA−: IgA-noncoated bacterial cells
LEfSe: Linear discriminant analysis effect size
qPCR: Quantitative PCR
PCA: Principal components analysis
RDP: Ribosomal Database Project
SIgA: Secretory IgA

immune regulation and protection against bacterial, viral, and parasitic infections in suckling infants.[23-27]

Although total SIgA levels in saliva and fecal samples have been investigated in children with allergy before, little is known about the identities of the bacterial taxa targeted by IgA in the infant gut and what role mucosal immune responses to the gut microbiota play in childhood allergy development. However, earlier studies have shown that low levels of salivary and intestinal SIgA are associated with an increased risk for allergic manifestations during early life.[28-30] Recent advances in flow cytometry[31] and next-generation sequencing[32] now allow study of the complex interactions between human antibodies and microbiota. In this study we used flow cytometry–based cell sorting and barcoded 16S rDNA 454-pyrosequencing to characterize the dominant gut bacteria, coated or noncoated with IgA, and determined total SIgA levels and bacterial load in stool samples collected during the first year of life in infants who either had allergic manifestations or stayed healthy up to 7 years of age.

METHODS

For detailed methods, experimental protocols and statistical analyses, see the Methods section in this article's Online Repository at www.jacionline.org.

Study design

The infants included in this study were part of a larger randomized double-blind trial in southeastern Sweden between 2001 and 2003 evaluating the potential allergy prevention effect of probiotic *Lactobacillus reuteri* ATCC 55730 until 2[33] and 7[34] years of age. The recruited children had a family history of allergic disease (≥1 family members with eczema, asthma, gastrointestinal allergy, allergic urticaria, or ARC), and more detailed inclusion and exclusion criteria are explained in the study of Abrahamsson et al.[33] Among the 188 infants completing the original study, infant stool samples collected at 1 and 12 months of life in 20 children with allergy (see Table E1 in this article's Online Repository at www.jacionline.org) and 28 children staying healthy up to 7 years of age (see Table E2 in this article's Online Repository at www.jacionline.org) were randomly selected for this study (Fig 1). Ten of the allergic children had asthma. Other allergic diseases included eczema (n = 9 at 7 years of age and n = 17 at 2 years of age; no infants had eczema before 1 month of age), ARC (n = 10), and allergic urticaria (n = 1), with symptoms defined as described in detail previously.[33,34] The samples were immediately frozen at −20°C after collection and later stored at −70°C until use.

There were no differences regarding potential confounders, such as sex, mode of delivery, birth order, maternal atopy, breast-feeding, antibiotics, and probiotic supplementation, between the infants who did or did not have allergic manifestations (Table I). All included infants were exclusively breast-fed for at least 1 month, and no infant received antibiotics before 1 month of age.[12] The Regional Ethics Committee for Human Research at Linköping University approved the study. Informed consent was obtained from both parents before inclusion. The study is registered at ClinicalTrials.gov (ID NCT01285830).

Sample labeling and flow cytometry protocol

Stool samples were suspended in sterile saline solution (autoclaved H_2O; NaCl Sodium Chloride 99.5% PA-ACS-ISO; Panreac, Barcelona, Spain; reference 131689.1211) with 5% BSA (Sigma-Aldrich, St Louis, Mo; reference A7030-100gr) to prevent nonspecific antibody binding. The samples were stained with goat anti-mouse IgA labeled with fluorescein isothiocyanate (FITC) used as an isotype control corresponding to unspecific binding (Invitrogen, Frederick, Md; reference M31001) or with goat anti-human IgA labeled with FITC (Invitrogen; reference H14001), according to the manufacturer's instructions (see Fig E1 in this article's Online Repository at www.jacionline.org). The sorting of the bacterial cells according to whether they were IgA coated (IgA+) or IgA noncoated (IgA−) was performed with the MoFlo XDP Cell Sorter (Beckman Coulter, Brea, Calif), according to the procedures of Simon-Soro et al.[32]

DNA extraction

DNA from sorted fecal bacteria, both IgA+ and IgA−, was isolated by using the MasterPure complete DNA and RNA Purification Kit (Epicentre Biotechnologies, Madison, Wis), according to the manufacturer's instructions, with a previous glass bead beating (0.17 mm in diameter) and an additional enzymatic lysis step with lysozyme (20 mg/mL, 37°C, 30 minutes; Thermomixer comfort, Eppendorf, Hamburg, Germany).

16S rDNA gene amplification and sequencing

DNA from 192 samples in total was used for PCR amplification and pyrosequencing to describe the bacterial composition of the sorted populations. A region of approximately 650 bp of the 16S rDNA gene was amplified by using universal bacterial degenerate primers,[35] which encompass the hypervariable regions V3 to V5 of the gene. A secondary amplification was performed with the purified PCR product as a template.[36]

Sequence processing and taxonomic classification

The resulting 16S rDNA read ends were trimmed in 10-bp sliding windows, with an average value of 20 or greater, by using the Galaxy tool,[37] and only reads of longer than 250 bp were considered. The sequences were assigned to each sample by using the 8-bp barcode through the Ribosomal Database Project (RDP) pipeline, version 11.3,[38] and chimeric sequences were filtered out with UCHIME.[39]

Taxonomic assignment was performed by using the RDP classifier,[38] in which the reads were assigned a phylum, class, family, and genus, and phylogenetic ranks were allocated when scores exceeded the 0.8 confidence threshold. Shannon indices, based on a randomly selected 700 reads per sample, was used to estimate the samples' diversity on the gene and phylum level.

For analyzing IgA coating patterns, the threshold used for including the genera was 1% or greater relative abundance in either the IgA+ or IgA− fractions. The abundance proportions of a given genera were used to calculate the ratio between the IgA+ and IgA− fractions, providing the IgA index.[40] Thus this score was based on proportional representation for every given genus within the IgA+ (positive IgA index values) and IgA− fractions (negative IgA index values), reflecting the degree of mucosal immune responsiveness to the microbiota. Linear discriminant analysis effect size (LEfSe)[41] was then used for high-dimensional biomarker discovery, comparing the IgA indices between healthy infants and infants with allergic manifestations. Furthermore, principal components analysis (PCA) was performed with the R software ade4 package.[42]

Bacterial load analysis with quantitative PCR

Quantitative PCR (qPCR) amplifications were performed to measure the bacterial load (number of bacterial cells normalized by number of human cells) by using primers targeting the single-copy housekeeping bacterial gene