Human seroreactivity to gut microbiota antigens

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Background: Although immune responses directed against antigens from the intestinal microbiota are observed in certain diseases, the normal human adaptive immune response to intestinal microbiota is poorly defined.
Objective: Our goal was to assess the adaptive immune response to the intestinal microbiota present in 143 healthy adults and compare this response with the response observed in 52 children and their mothers at risk of having allergic disease.
Methods: Human serum was collected from adults and children followed from birth to 7 years of age, and the serum IgG response to a panel of intestinal microbiota antigens was assessed by using a novel protein microarray.
Results: Nearly every subject tested, regardless of health status, had serum IgG that recognized a common set of antigens. Seroreactivity to the panel of antigens was significantly lower in atopic adults. Healthy infants expressed the highest level of IgG seroreactivity to intestinal microbiota antigens. This adaptive response developed between 6 and 12 months of age and peaked around 2 years of age. Low IgG responses to certain clusters of microbiota antigens during infancy were associated with allergy development during childhood.
Conclusions: There is an observed perturbation of the adaptive response to antigens from the microbiota in allergic subjects. These perturbations are observable even in childhood, suggesting that optimal stimulation of the adaptive immune system by the microbiota might be needed to prevent certain immune-mediated diseases. (J Allergy Clin Immunol 2015;136:1378-86.)

Key words: *Adaptive, atopy, allergy, childhood, IgG, microarray, microbiota, bacterial antigens, bacterial antibodies*

The intestinal microbiota has become a major focal point in the study of many immunologic diseases, and advances in the characterization of the gut microbiota have identified patterns of colonization associated with disease severity and pathogenesis. Multiple autoimmune and inflammatory diseases have been linked to alterations in the gut microbiota.[1,2] The symbiotic relationship between the microbiota and the human host begins at birth.[3] The microbiota rapidly expands and changes before converging to a stable colonization pattern.[4-6] The developing microbiota informs the immune system by modulating inflammatory gene expression,[7] and microbial colonization is necessary for the development of normal immune structures.[8,9] Even in the mature immune system the microbiota exerts a powerful influence by maintaining immune homeostasis through the regulation of various T-cell lineages.[10-14]

Despite the overall stability of gut microbiota colonization in individual subjects,[15] the species composition appears to vary among subjects.[16] This variation might be beneficial because a less diverse gut microbiota is present during the first month of life in infants with later atopic eczema[17] and asthma.[18] The diversity of the microbiota in healthy subjects, coupled with the known influence of the microbiota on immune homeostasis, suggests that the specific makeup of the microbiota might be of less importance than the body's adaptive immune response to the microbiota itself.

Much effort has been expended to characterize the microbiota in healthy adults,[19,20] including the evolution of microbial colonization in a healthy infant from birth to 3 years of age,[21] but development of the normal human adaptive immune response to the human microbiota is less understood. To this end, we developed a novel protein microarray to investigate the interplay between the adaptive immune system and the gut microbiota and categorized the IgG seroreactivity of subjects from the United States, Canada, and Sweden to a panel of antigens from the gut microbiota.

METHODS

Serum samples

Serum samples were collected with parental consent from 52 Swedish children and their mothers 1 week postpartum, as well as from 70 healthy

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Supported by National Institutes of Health grant DK071176; the Swedish Research Council (K2011-56X-21854-01-06); the Swedish Heart-Lung Foundation (20050514); the Ekhaga Foundation (210-53); the Research Council for the South-East Sweden; the Olle Engqvist Foundation; the Swedish Asthma and Allergy Association; the Vårdal Foundation for Health Care Science and Allergy Research, Sweden (B2007 042); and the University Hospital of Linköping, Sweden.
Disclosure of potential conflict of interest: This study was supported by a National Institutes of Health grant (DK071176), the Swedish Research Council (K2011-56X-21854-01-06), the Swedish Heart-Lung Foundation (20050514), the Ekhaga Foundation (210-53), the Research Council for the South-East Sweden, the Olle Engqvist Foundation, the Swedish Asthma and Allergy Association, the Vårdal Foundation for Health Care Science and Allergy Research, Sweden (B2007 042), and the University Hospital of Linköping, Sweden. T. R. Abrahamsson has received consultancy fees from BioGaia, and his institution has received or has grants pending from BioGaia AB. C. N. Bernstein's institution has received funding from the Canadian Institutes of Health Research; has received or has grants pending from AbbVie, Takeda, Shire, Janssen; has received compensation for board membership from AbbVie, Shire, Takeda, Pfizer, Cubist Pharmaceuticals, and Theradiag; and has received payment for delivering lectures from AbbVie and Shire. M. C. Jenmalm has received consultancy fees from BioGaia. C. O. Elson has received consultancy fees from NovoNordisk, Ferring Research Institute, Theravance, ImmusanT, Ironwood Pharmaceuticals, Baxter Healthcare, Lumira Capital, and Bristol Myers Squibb; has received payment for delivering lectures from Janssen; and receives royalties from Prometheus. The rest of the authors declare that they have no relevant conflicts of interest.
Received for publication November 19, 2014; revised March 11, 2015; accepted for publication March 23, 2015.
Available online May 23, 2015.
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0091-6749/$36.00

http://dx.doi.org/10.1016/j.jaci.2015.03.036

Abbreviations used
ARC: Allergic rhinoconjunctivitis
NCBI: National Center for Biotechnology Information

adults in Linköping, Sweden; 43 in Birmingham, Alabama; and 30 in Winnipeg, Manitoba, Canada. The mothers and children participated in an allergy prevention study in which *Lactobacillus reuteri* (ATCC 55730; 1×10^8 colony-forming units/d; BioGaia AB, Stockholm, Sweden) or placebo was administered to the mother from gestational week 36 and to the infant through the first year of life.[22] At least 1 family member of the child had an allergic disease. The background factors and allergic manifestations in these children until 7 years of age are described in Table I. Nonatopic control subjects participated in an investigation of immune responses to paternal antigens during pregnancy.[23] For Swedish mothers, the median age was 29 years (range, 21-44 years). For Birmingham adults, the median age was 32 years (range, 20-76 years; 56% male/44% female). Samples were obtained with consent. For Winnipeg adults, the median age was 43 years (range, 17-75 years; 41% male/59% female). Sera collected from patients with Crohn disease in Birmingham (n = 10) and Winnipeg (n = 30) were used in some experiments for comparison with sera from healthy and allergic subjects for reactivity to flagellin antigens.

Microbiota antigen microarray

Proteins were diluted in TRIS buffer (pH 8.0) with 0.5% SDS at 0.2 mg/mL. The proteins were printed onto FAST 16 nitrocellulose pad slides (Whatman; GE Healthcare Life Sciences, Pittsburgh, Pa) using a MicroGrid II robot (Genomic Solutions, Ann Arbor, Mich) in duplicate in 2 different parts of the pad. Thus each antigen is present in quadruplicate. The printed slides were allowed to air-dry overnight. Slides were blocked (Protein Array Blocking Buffer; Whatman), probed with human sera at 1:100 dilution, washed, and incubated with Alexa Fluor 647– or Alexa Fluor 546–labeled goat anti-human IgG or IgA (KPL, Gaithersburg, Md). The proteins included in the microarray are listed in Table I.

Analysis of microarray data

Software programs that were developed for analysis of DNA microarrays were used to analyze the data from the microbiota antigen array. The slides are read in an Axon GenePix 4000B dual laser microarray reader (Molecular Devices, Sunnyvale, Calif). The accompanying GenePix Pro 6.0 software determines the net median pixel intensities for each feature (antigen spots) from a set of 10 measurements per feature. The instrument and software automatically subtracted the pixel intensities of the background area surrounding the feature. The median net digital fluorescence unit value for each feature represents the median value from 4 replicate antigen features on each array. Statistical analysis of data was performed with the R Statistical Package or GraphPad Prism software (GraphPad Software, La Jolla, Calif) by using appropriate tests to compare values between groups. Data analysis was done without and with a Bonferroni correction for multiple comparisons; *P* values were highly significant with both approaches. *P* values in the text and figures are analyses uncorrected for multiple comparisons.

Sequences from the antigens obtained from murine cecum were compared with human microbiota sequences present in the following databases: the National Institutes of Health Human Microbiome Project (http://www.hmpdacc.org), the National Center for Biotechnology Information (NCBI) Gene Bank (http://blast.ncbi.nlm.nih.gov/Blast.cgi), and the metagenome gene catalog.[20]

Clinical features and definitions of allergic children

Allergic manifestations included eczema, recurrent wheeze, allergic rhinoconjunctivitis (ARC), allergic urticaria, gastrointestinal allergy, and IgE sensitization against food or other allergens. A diagnosis of eczema was defined as a pruritic, chronic, or chronically relapsing noninfectious dermatitis with typical features and distribution. An asthma diagnosis required at least one of the following 2 criteria: (1) a doctor's diagnosis and asthma symptoms and/or medication during the last 12 months or (2) wheeze or nocturnal cough and a positive reversibility test result and/or pathologic fraction of exhaled nitric oxide value. In Sweden most asthmatic children are asymptomatic when visiting the doctor because they are efficiently treated with inhaled corticosteroids. If the asthma diagnosis was based on a doctor's diagnosis, the child's medical records were always reviewed to confirm that the diagnosis was consistent with Global Initiative for Asthma criteria (http://www.ginasthma.com). The diagnosis of ARC was based on standard International Study of Asthma and Allergies in Childhood questions (http://isaac.auckland.ac.nz/Index.html) and required watery discharge at least twice in contact with the same allergen and no signs of infection. The diagnosis of gastrointestinal allergy required vomiting, diarrhea, or a systemic reaction after ingestion of a potentially allergenic food and a confirmation by means of challenge, unless there was a clear history of a severe systemic reaction. Urticaria was defined as allergic when appearing at least twice in conjunction with a certain food. Infants were regarded as sensitized if they had at least 1 positive skin prick test response, detectable circulating allergen-specific IgE antibodies, or both. Skin prick tests were done on the volar aspects of the forearm with

TABLE I. Background factors and other allergic manifestations in children with and without allergic manifestation until 7 years of age

	Allergic disease until age 7 y		
	Yes, % (n/N)	No, % (n/N)	*P* value*
Probiotic group	38 (8/21)	42 (13/31)	.78
Boys	52 (11/21)	52 (16/31)	.96
Older sibling	43 (9/21)	45 (14/31)	.87
Maternal allergic disease	71 (15/21)	90 (28/31)	.13
Asthma	14 (3/21)	27 (7/31)	.72
ARC	19 (4/21)	45 (14/31)	.05
Eczema	27 (6/21)	26 (8/31)	.83
Food allergy	24 (5/21)	10 (3/31)	.24
Allergic urticaria	24 (5/21)	7 (2/31)	.10
Atopic (sensitized to allergens)	38 (8/21)	65 (20/31)	.06
Cesarean section	14 (3/21)	10 (3/31)	.68
Breast-feeding (exclusive) at 3 mo	76 (16/21)	72 (22/31)	.68
Breast-feeding (any) at 6 mo	76 (16/21)	84 (26/31)	.50
Breast-feeding (any) at 12 mo	10 (2/21)	26 (8/31)	.17
Parental smoking (prebirth)	5 (1/21)	10 (3/31)	.64
Furred pets at birth	10 (2/21)	13 (4/31)	1.00
Antibiotics at 0-6 mo	5 (1/21)	16 (5/31)	.38
Antibiotics at 6-12 mo	14 (3/21)	26 (8/31)	.49
Antibiotics at 12-24 mo	33 (7/21)	48 (15/31)	.28
Infections at 0-12 mo, mean (SD)	5.4 (2.9)	5.3 (3.0)	.90
Infections at 12-24 mo, mean (SD)	5.5 (3.8)	5.4 (4.2)	.91
Day care at age 12 months	5 (1/21)	7 (2/31)	1.00
Day care at age 24 months	71 (15/21)	81 (25/31)	.51
Asthma until age 7 y	43 (9/21)	0 (0/31)	<.001
Allergic rhinitis until age 7 y	29 (6/21)	0 (0/31)	.003
Eczema until age 7 y	91 (19/21)	0 (0/31)	<.001
Allergic urticaria until age 7 y	14 (3/21)	0 (0/31)	.06
Sensitization until age 7 y	100 (21/21)	0 (0/31)	<.001

Follow-up was performed by research nurses at 1, 3, 6, 12, and 24 months of age and by structured telephone interviews with parents at 2, 4, 5, 8, 10, and 18 months. Parents were asked about infections at each contact. Upper respiratory tract infections dominated. As indicated, the mean of infections was 5.4 and 5.5 during the first and second years of life, respectively. The mean of gastrointestinal infections was 0.3 (SD, 0.5) and 0.3 (SD, 0.5) in the allergic and nonallergic children, respectively ($P = .78$, *t* test).

*The χ^2 test was used for categorical variables. The Fisher exact test was used when the expected frequency for any cell was less than 5. The Student *t* test was used for continuous variables.