

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;■■■:■■■-■■■.)

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

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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 eczema¹⁷ 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

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 Micro-Grid 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

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)	<i>P</i> 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.

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 II. Antigens represented on the protein microarray

Phylum	Class	Name	Protein ID	Function	Primary species	Identity (%)	Positive results (%)
Bacteroidetes	Bacteroidia	B-theta	CBir28 - hypothetical protein	Other/unknown	<i>Bacteroides</i> species	70	87
		CBir19	ABC transporter ATP-binding protein	Other/unknown	<i>Bacteroides vulgatus</i>	78	93
		CBir23	Elongation factor 1A	Transc/transl	<i>Alistepes shahii</i>	85	95
		Cbir45	Glycosyl hydrolase	Metabolism	<i>Bacteroides eggerthii</i>	59	75
		CBir8	Elongation factor thermo unstable	Transc/transl	<i>Tannerella</i> species	90	94
		Keto	Transketolase	Metabolism	<i>Bacteroides fragilis</i>	100	100
Firmicutes flagellin	Clostridia	P3	HSP90	Other/unknown	<i>Bacteroides fragilis</i>	80	80
		14-2	Flagellin from 14-2 isolate	Motility	<i>Roseburia intestinalis</i>	80	87
		3_1_57	Flagellin	Motility	Lachnospiraceae	100	100
		CBir1	Flagellin	Motility	<i>Butyrivibrio fibrisolvens</i>	83	89
		CBir11	Flagellin	Motility	<i>Roseburia inulinivorans</i>	46	58
		CBir66	Flagellin	Motility	<i>Roseburia intestinalis</i>	66	83
		Fla 2	Flagellin 2 from A4 isolate	Motility	<i>Roseburia intestinalis</i>	72	80
		Fla 3	Flagellin 3 from A4 isolate	Motility	<i>Roseburia inulinivorans</i>	81	91
		Fla X	Flagellin	Motility	<i>Roseburia inulinivorans</i>	56	70
		MDR254	Flagellin	Motility	<i>Flavonifractor plautii</i>	73	78
Firmicutes	Bacilli	CBir14	Glyceraldehyde-3-phosphate dehydrogenase	Metabolism	<i>Lactobacillus salivarius</i>	90	93
		EF20	Sal A	Other/unknown	<i>Enterococcus faecalis</i>	100	100
	Clostridia	CBir63	Ig-like surface protein	Other/unknown	<i>Roseburia intestinalis</i>	47	64
		MDR247	Adenine deaminase	Metabolism	<i>Clostridium bolteae</i>	54	76
		MDR90	Collagen adhesion protein	Other/unknown	<i>Roseburia intestinalis</i>	66	77
		rIB12	Homoserine dehydrogenase	Metabolism	<i>Flavonifractor plautii</i>	60	76
		rIB16	Pyruvate synthase	Metabolism	<i>Flavonifractor plautii</i>	74	86
		rIB17	NlpC/P60	Other/unknown	<i>Flavonifractor plautii</i>	39	56
		rIB4	Relaxase	Transc/transl	<i>Clostridium asparagiforme</i>	63	72
		rIB8	Glycosyltransferase	Metabolism	Lachnospiraceae 3-1-57	59	75
		rIB9	Methyl-accepting chemotaxis protein	Motility	<i>Roseburia intestinalis</i>	45	63
Proteobacteria	Delta/epsilon	CBir5	Methyl-accepting chemotaxis protein	Motility	<i>Helicobacter cinaedi</i>	63	74
		CBir56	Methyl-accepting chemotaxis protein	Motility	<i>Helicobacter canadensis</i>	60	71
	Gamma	FtsZ	FtsZ protein - homologue of tubulin (putative pANCA)	Other/unknown	<i>Escherichia coli</i>	100	100
		OmpC	OmpC from UNC <i>E coli</i>	Other/unknown	<i>Escherichia coli</i>	100	100
	Epsilon	SalFlhC	<i>Salmonella dublin</i> flagellin	Motility	<i>Salmonella dublin</i>	100	100
		rIB18	Surface array protein	Other/unknown	<i>Campylobacter showae</i>	30	53
Firmicutes and Proteobacteria		rIB5	Hypothetical protein - cytoplasmic	Other/unknown	Erysipelotrichaceae 3-1-53	37	60
Firmicutes	Clostridia	rIB1	RecN	Transc/transl	<i>Clostridium citroniae</i>	80	82
		rIB10	Nucleotidyltransferase/hypothetical	Transc/transl	<i>Roseburia intestinalis</i>	48	70
Proteobacteria	Beta	rIB20	ABC transporter	Other/unknown	<i>Verminephrobacter eiseniae</i>	<50	<50
Firmicutes and Bacteroidetes		rIB2	SAM domain protein	Transc/transl	<i>Blautia hanseni</i>	61	77

Thirty-eight antigens cloned from the murine cecal microbiota^{24,25} were searched against sequences from the described human microbiota in the MetaHit, HMB, and NCBI databases, and the percentages of exact amino acid matches (percentage identity) and percentages of exact and similar (percentage positive) amino acid matches are listed, along with putative protein ID and function. Each sequence was also matched to the phyla, class, and primary species in the human microbiota to which it was tracked.

Transc/transl, Transcription/translation.

egg white, fresh skimmed cow's milk (lipid concentration, 0.5%), and standardized cat, birch, and timothy extracts (Soluprick; ALK-Abelló, Hørsholm, Denmark) at 6, 12, and 24 months and 7 years of age. Histamine hydrochloride (10 mg/mL) was used as a positive control, and albumin diluents were used as negative controls. The test result was regarded as positive if the mean diameter of the wheal was 3 mm or greater. Circulating IgE antibodies to egg white and cow's milk were analyzed at 6, 12, and 24 months of age in venous blood (UniCAP Pharmacia CAP System; Pharmacia Diagnostics, Uppsala, Sweden). The cutoff level was 0.35 kU/L, according to the manufacturer's protocol. In addition, circulating IgE to a mixture of food allergens, including egg white, cow's milk, cod, wheat, peanut, and soybean, was analyzed at 6, 12, and 24 months of age (UniCAP Pharmacia CAP System, fx5, Pharmacia Diagnostics). All of the 21 children with allergy were sensitized, whereas none of the 31 healthy

children were sensitized (Table I). Nineteen of the allergic children had eczema, 9 had asthma, 6 had ARC, and 3 had urticaria during the first 7 years of life. Several children had more than 1 allergic symptom.

RESULTS

Healthy adults exhibit circulating antibodies to antigens of the intestinal microbiota

To investigate the IgG-adaptive immune response to the intestinal microbiota in human subjects, we used a novel protein microarray containing recombinant protein antigens and cloned from the murine microbiota.^{24,25} The antigens were chosen

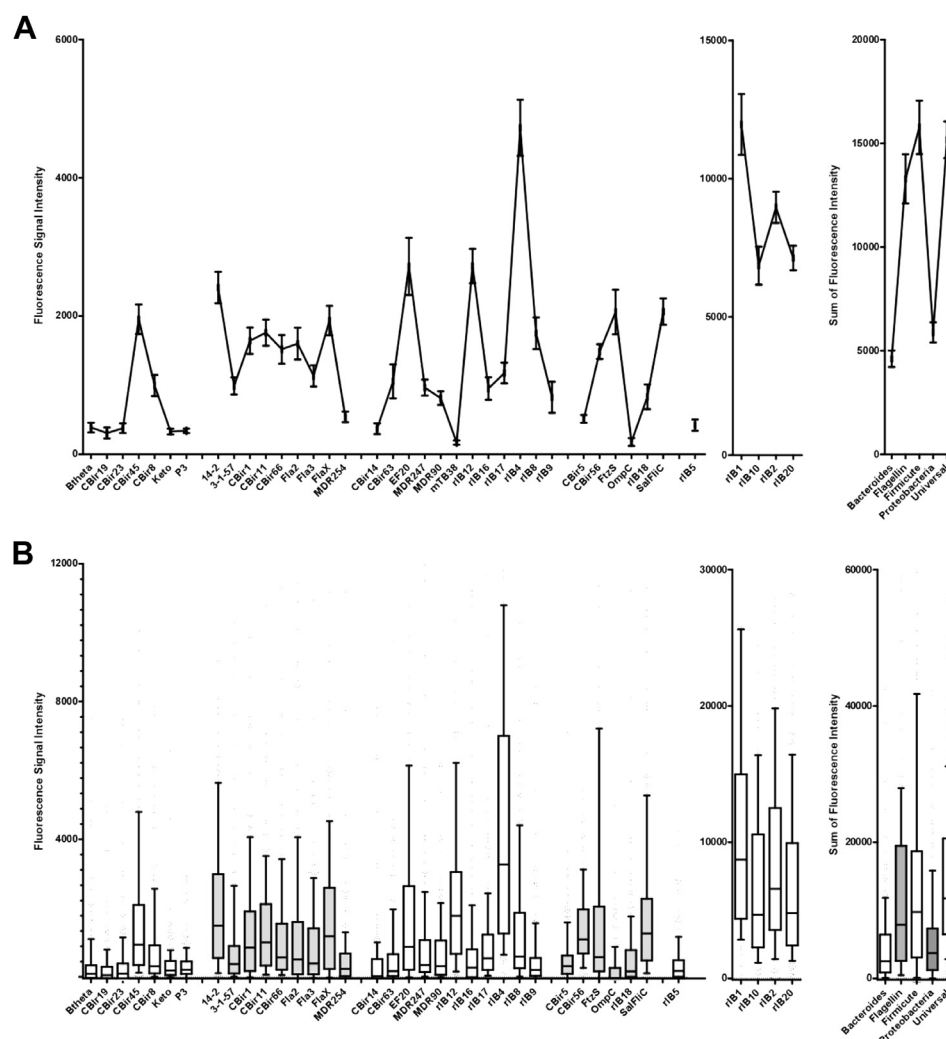


FIG 1. There is a normal human adaptive immune response to antigens from the gut microbiota. Sera from 143 healthy white adults in 3 countries (Canada, Sweden, and the United States) was analyzed on the microarray, and IgG reactivity to the antigens was determined. **A**, Data are expressed as means $\pm$ SEMs to illustrate the pattern of response. **B**, Data are expressed as boxes and whiskers (10% to 90%) of fluorescence intensity for each antigen to illustrate the variance of the response among subjects. Four antigens, rIB1, rIB2, rIB10, and rIB20, were found to be universally recognized among nearly all subjects.

because all had been found previously to be immunogenic in mice^{24,25} and because IgG seroreactivity to most of them was found in normal human serum in pilot studies. The individual protein and DNA sequences of these antigens were searched against sequences from the described human microbiota in the MetaHit,²⁰ Human Microbiome Project (www.hmpdacc.org), and NCBI (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) databases, and percentages of exact amino acid matches (percentage identity) and percentages of exact and similar (percentage positive) amino acid matches are listed, along with a putative protein ID and function (Table II).^{24,25} Each sequence was also matched to the phylum, class, and primary species to which it was tracked. Of the antigens selected for the array, 31 of 38 had greater than 70% homology to sequences from the human gut microbiota, whereas 37 of 38 had greater than 50% homology, suggesting that there are evolutionarily conserved antigens or shared microbial colonization between mice and human subjects. One antigen, rIB20, appears to be a unique sequence because it had no greater than 50% homology

to any known protein sequence in the NCBI database. Two of the antigens, rIB2 and rIB5, had high homology sequence matches to highly conserved sequences found in multiple phyla. Counting these overlapping sequences, the antigens on our array represent 8 Bacteroidetes antigens, 24 Firmicutes antigens, and 8 Proteobacteria antigens: 7 are involved in metabolic functions, 13 are flagellin/motility proteins, 6 are transcription/translation machinery, and 12 others are cell-surface proteins of various kinds. Thus the array represents a diverse set of antigens from the 3 most prominent phyla of the human gut microbiota (Table II). Several of these antigens, particularly Firmicutes flagellins, are known immunodominant antigens in patients with Crohn disease.²⁴

Sera from healthy adults from Canada, Sweden, and the United States were tested against this panel, and the immune response to antigens from the intestinal microbiota was evidenced by the presence of serum IgG reactivity (Fig 1). Despite individual differences in magnitude of response, a common pattern of response to particular antigens emerged (Fig 1, A). Although not all antigens

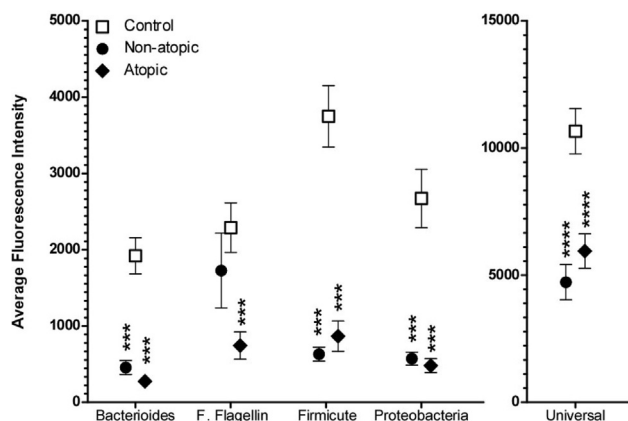


FIG 2. The human adaptive immune response to antigens from the gut microbiota differs between mothers of at-risk children and control subjects. Swedish mothers were separated by health status (24 allergic and 30 nonallergic), and the average reactivity to antigens from different phyla or universal antigens was compared with that of healthy control subjects ($n = 40$). Data are expressed as means $\pm$ SEMs. *** $P < .0005$ and **** $P < .0001$, Mann-Whitney test.

were recognized by individual sera, there was a significant correlation in reactivity between specific antigens, particularly among Firmicutes flagellins and 4 universal antigens (see Fig E1 in this article's Online Repository at www.jacionline.org). In regard to the latter, nearly every adult had a strong response to these 4 antigens: rIB1, rIB10, rIB2, and rIB20 (Fig 1, B, and see Fig E2 in this article's Online Repository at www.jacionline.org). Because IgG responses to peptide antigens require $CD4^+$ T_H cells to stimulate isotype switching, these data reflect the participation of both T- and B-cell immunity in generating a response to these antigens. Furthermore, these data indicate that even in healthy subjects there is a normal adaptive immune response to antigens present in the commensal microbiota, including antigens known to be targets in patients with inflammatory disease.

Perturbation of the seroresponse to antigens from the intestinal microbiota in patients with allergic disease

We next studied allergic subjects because childhood allergy is associated with alterations of the intestinal microbiota.^{1,17,26} Serum collected 1 week postpartum from 53 Swedish mothers, 30 of whom had allergic disease and 23 who did not, were compared with that from 40 nonallergic Swedish adults. In contrast to the seroresponse seen in healthy subjects, IgG reactivity in women with allergic disease was significantly lower to each cluster of antigens, including the 4 universal antigens (Fig 2). Each of the mothers was recruited based on having allergy or having children at risk for allergy. In postpartum women without allergy, there was still a significant reduction in IgG responses (Fig 3, A) on par with the weak responses seen in atopic subjects, with the single exception of Firmicutes flagellin antigens. The reactivity to this group of antigens is equivalent to the reactivity observed in North American adults (Fig 3, B).

Differences in seroresponses to antigens from the intestinal microbiota are present in infancy

Although the reactivity to universal antigens was perturbed in the mothers, at 2 years of age, the magnitude of reactivity in their

children was significantly greater and compared equivalently with that in adult control subjects (Fig 3, A). In contrast, the seroreactivity to the set of Firmicutes flagellins at 2 years of age in healthy children was significantly greater than the reactivity observed in the unrelated control subjects ($P < .001$). The magnitude of this response to Firmicutes flagellin antigens approaches that seen in adults with Crohn disease (Fig 3, B).

Although allergic adults have a lower adaptive IgG response to microbiota antigens, little is known about the development of this response in children. It is possible that the reduced adaptive immune response that correlates with certain immune-mediated diseases in adults could predispose children to the development of disease. To this end, sera from 52 Swedish children were collected at time points from 6 months to 7 years of age and analyzed through a protein microarray for serum IgG reactivity to antigens from the gut microbiota. These children all had a family history of allergic disease, and during the course of the study, 21 had allergic disease, and 31 remained allergy free.²² Seroresponses to antigens from *Bacteroides* species (Fig 4, A), Firmicutes flagellin (Fig 4, B), other Firmicutes proteins (Fig 4, C), or Proteobacteria (Fig 4, D) or to the 4 universal antigens (Fig 4, E-H) were grouped together and compared with cord blood (labeled as 0 months, representing antibodies transferred to the child during pregnancy) and then results from 6 months to 7 years of age. Allergic children had significantly lower reactivity to Firmicutes (Fig 4, C) and the universal (Fig 4, E-H) antigens at 6 months of age and continued this trend until 7 years. This pattern of lower reactivity is even evident in cord blood (Fig 4, E and G). Although serum antibody levels against the majority of antigens on the array were very low at 6 months, serum antibodies against the 4 universal antigens were detectable at 6 months of age, and for each of these antigens, allergic children, or those who would have allergy, had significantly lower seroreactivity than healthy children. This trend continued throughout early life. Reactivity to nonflagellin antigens from the Firmicutes phyla was also significantly higher at 6 months in healthy children than in those with allergy and continued in this trend, mirroring the pattern observed with reactivity to universal antigens.

Many external factors, such as breast-feeding exclusivity and duration,^{27,28} use of antibiotics,^{29,30} and probiotic therapies, have been proposed to alter the composition of the gut microbiota.³¹ Although they can alter the composition, in our study we found no significant differences when children in the study were grouped by exclusive breast-feeding during the first 3 months of life or probiotic use (see Figs E3 and E4 in this article's Online Repository at www.jacionline.org). Treatment with antibiotics at less than age 2 years increased reactivity selectively to several of the universal antigens at 24 months of age (see Fig E5 in this article's Online Repository at www.jacionline.org); however, antibiotic treatment had no effect on the subsequent development of allergy. Only 3 children were delivered by means of cesarean section in the allergic and nonallergic groups, which is too small a number to make a valid comparison of seroreactivity. Detailed comparisons between the allergic and nonallergic children are provided in Table I.

DISCUSSION

Although the composition and development of the microbiota is beginning to be understood,¹⁹⁻²¹ the interplay of the normal human adaptive immune response and the human microbiota,

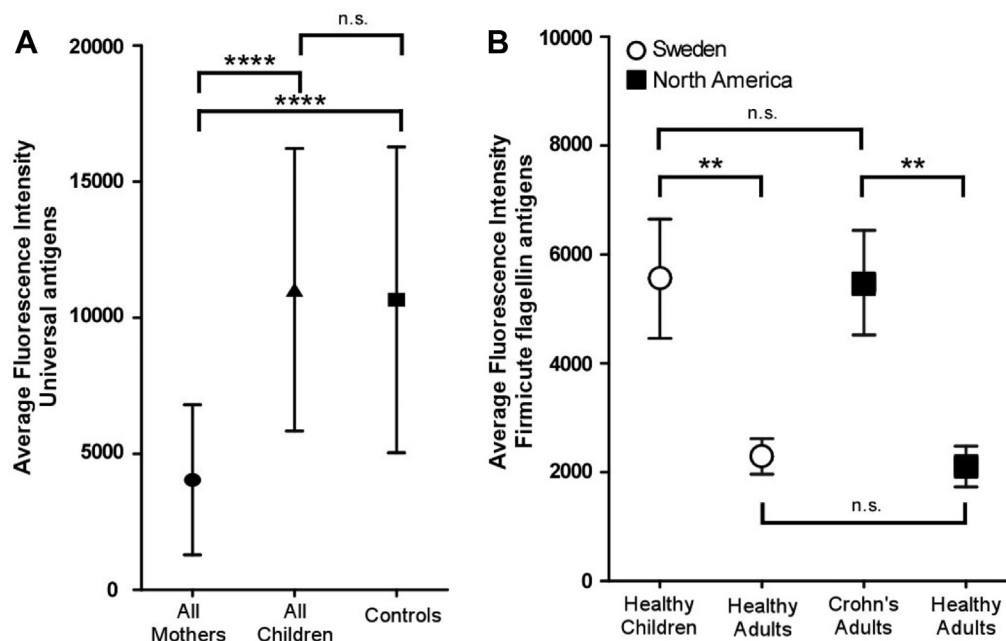


FIG 3. A, IgG reactivity to the universal antigens for Swedish mothers was compared with that of all infants (regardless of allergy) at 24 months or control Swedish adults (means $\pm$ SEMs). B, IgG reactivity to Firmicutes flagellins of healthy 24 month-old Swedish infants ($n = 31$) compared with healthy Swedish adults ($n = 40$), healthy adult North Americans ($n = 54$), or adults with Crohn disease from the United States and Canada ($n = 45$; means $\pm$ SEMs). ** $P < .005$ and **** $P < .0001$, Mann-Whitney test. n.s., Not significant.

especially in healthy subjects, remains to be explored. This protein microarray serves as a unique tool to investigate this interplay and begin to define the normal response to antigens in the microbiota. Each of the antigens present on the array was initially cloned as a result of being immunogenic in mice.^{24,25} The majority of them had very high homology matches to sequences from the characterized human microbiome, suggesting that these are evolutionarily conserved epitopes. Our data clearly indicate that patients with allergic disease have a decreased response to clusters of antigens from the commensal gut microbiota compared with healthy subjects. Furthermore, low IgG responses to certain clusters of microbiota antigens during infancy were associated with allergy development during childhood. To confirm our microarray results, we cross-linked selected antigens to Luminex beads and compared the relative binding signals observed in a fluorescent Luminex bead assay to the values obtained for the same samples from the protein microarray; for each group tested, the same pattern of reactivity and same level of significance were obtained between the protein microarray and the Luminex bead assay (data not shown).

Our results demonstrate that rather than there being a lack of response to the gut microbiota, there is activation of the adaptive immune system evidenced by IgG seroreactivity to antigens derived from the microbiota. It has been known for some time that there is normal IgG autoantibody production in healthy subjects,^{32,33} and these autoantibodies are directed to multiple different cellular components, both intracellular and extracellular. As such, it should be expected that there is also adaptive immune activation directed toward the commensal microbiota. In this study we highlighted 4 particular antigens as being universal in that nearly every subjects tested responded to all 4 of them. Aside from rIB20, the identity of which remains unknown, the other 3

universal antigens all come from proteins involved in transcription and translation. Because naturally occurring autoantibodies are likely to play a role in proper immune function, it appears that an active immune response to the microbiota is also involved in normal immune function.

The reduced reactivity to universal antigens observed in allergic children appears to be present in the mothers of allergic children as well. Although allergic children had reduced reactivity to these universal antigens compared with healthy children, at 2 years of age, the magnitude of reactivity in all children was greater than that of their mothers at parturition, matching the levels observed in the control group (Fig 3, A). Of note, seroreactivity to the set of Firmicutes flagellins at 2 years of age was significantly greater than the reactivity observed in either the mothers ($P < .0001$ for all cases) or unrelated control subjects ($P < .001$ for all cases). The magnitude of this response to Firmicutes flagellin antigens approaches that seen in adults with Crohn disease to these antigens (Fig 3, B). Although the magnitude of response in children was higher than that of their mothers or healthy adults, the pattern of antigens to which they respond was the same. The common pattern of seroreactivity in infants contrasts with the succession of varying microbiota colonization, as assessed by using 16s DNA, which has been observed in African, South American, and North American children before the age of 3 years, when the microbiota begins to resemble that of adults.^{4,6,34} However, this common pattern of reactivity might be coherent with the common functional microbiota that has been revealed by metagenomic studies.²⁰ This succession of bacterial colonization and eventual stabilization might contribute to the heightened response observed from 12 to 24 months of age, which is followed by the reduction to more adult-like levels at 7 years of age.

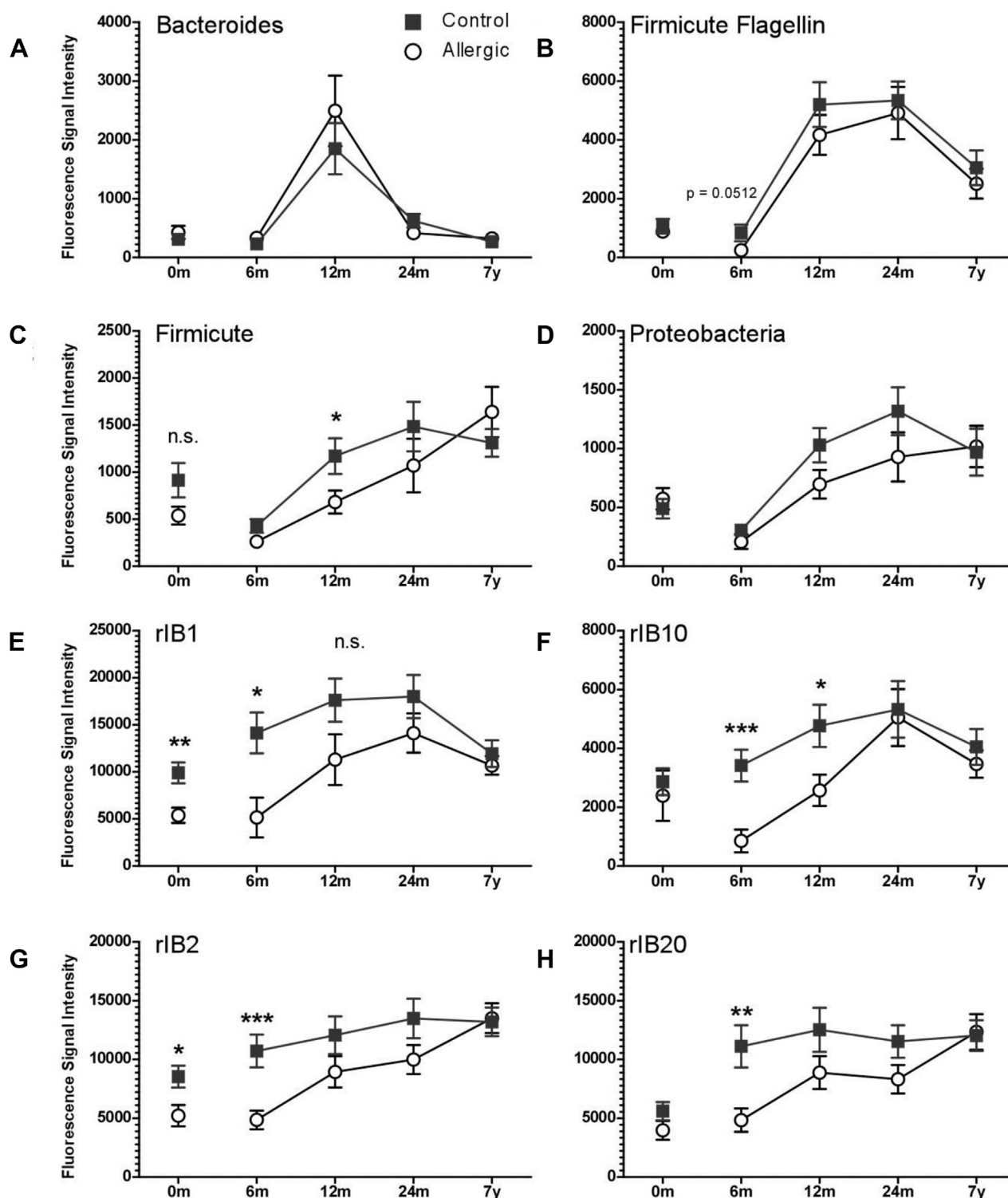


FIG 4. Development of the human adaptive immune response to antigens from the gut microbiota begins in infancy, and reduced seroreactivity is observed in children with allergy. Average fluorescence intensity to antigens from the different clusters (**A-D**) or the 4 universal antigens (**E-H**) was compared in sera from 33 healthy Swedish children (control subjects, *squares*) or 21 subjects who had eczema (allergic subjects, *open circles*) over the first 7 years of life and in the cord blood of their mothers (0-month time point; mothers are grouped by the health status of their child). Data are expressed as means $\pm$ SEMs. * $P < .05$, ** $P < .005$, and *** $P < .0005$ compared with the same time point. *n.s.*, Not significant.

The children in this study came from a study of probiotic effectiveness in which newborns at risk for allergic disease received *L reuteri* from birth in an attempt to reduce the incidence of allergy. This same group of children has been shown to have reduced diversity in the gut microbiota,¹⁷ a circumstance also seen in patients with Crohn disease^{35,36} and type 1 diabetes.³⁷ A lower adaptive immune response to gut microbiota can be suggested as a predisposing factor for development of atopy because all these children were considered at risk for allergy development. Indeed, comparing the seroreactivity of the mothers of these children with that of healthy control subjects (Fig 2) indicated that as a group, these mothers had lower seroreactivity than healthy control subjects. When mothers were separated by their own allergy status, allergic mothers comprise the cohort with the lowest response. Pregnancy profoundly decreases the richness of the microbiota,³⁸ and thus some of the decrease in reactivity in the mothers could be due to these alterations. However, there remain clear differences in reactivity to antigens from the gut microbiota between allergic and nonallergic mothers.

We are colonized during transition out of the birth canal. Newborns are protected during initial exposures to microbes through transplacental passage of maternal IgG but must respond on their own IgG after the mother's IgG is metabolized. This initial response is vigorous and maintained throughout life at lower levels. Perturbed responses to microbiota antigens correlate with development of certain immune-mediated diseases in adults. Taken together, these data are compatible with the concept that a strong adaptive immune response to the microbiota in infancy is protective against immune-mediated disease later in life. An appropriate intensity and diversity of microbial stimulation during infancy might be required for adequate development of the adaptive immune system.³⁹ This concept is consistent with the findings of a reduced gut microbiota diversity during infancy preceding development of atopic eczema^{17,40-42} and asthma.¹⁸

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Key messages

- We describe the development of adaptive immune responses against antigens from the intestinal microbiota and show that immune activation against antigens from the gut microbiota is normal and present from infancy into adulthood.
- In allergic subjects we show that there is a significant decrease in seroreactivity to particular groups of microbiota antigens and that low seroreactivity during infancy associates with allergy development during childhood.

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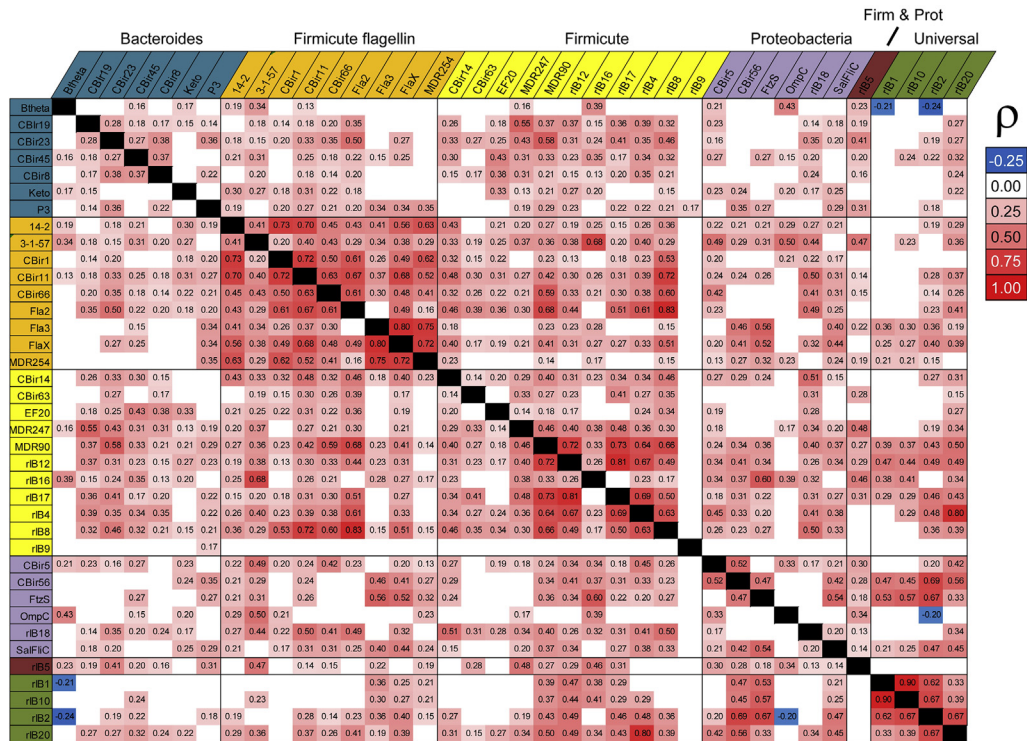


FIG E1. Positive IgG seroreactivity to antigens from the gut microbiota correlates to seroreactivity to other antigens. Sera from 143 healthy white adults in 3 countries (Canada, Sweden, and the United States) were analyzed on the microarray, and IgG reactivity to the antigens was determined (Fig 1). The Spearman rho correlation coefficient for each antigen was calculated against each other antigen on the array, and significant ($P < .05$) correlation coefficients are displayed. Blue coloring indicates negative correlations, red coloring indicates positive correlations, and white coloring indicates no significant correlation. There was a high degree of significant correlation among the Firmicutes flagellins (orange labels) and also among the 4 universal antigens (green labels). Interestingly, there were also strong correlations among sequence and structurally unrelated antigens, such as Fla2 (flagellin from *Lachnospiraceae* A4) and rIB8 (glycosyl-transferase from *Lachnospiraceae*) or rIB20 (unknown) and rIB4 (relaxase from *Clostridia*). Negative correlations exist between some of the universal antigens and B-theta, a Bacteroidetes antigen, or OmpC, a Proteobacteria antigen.

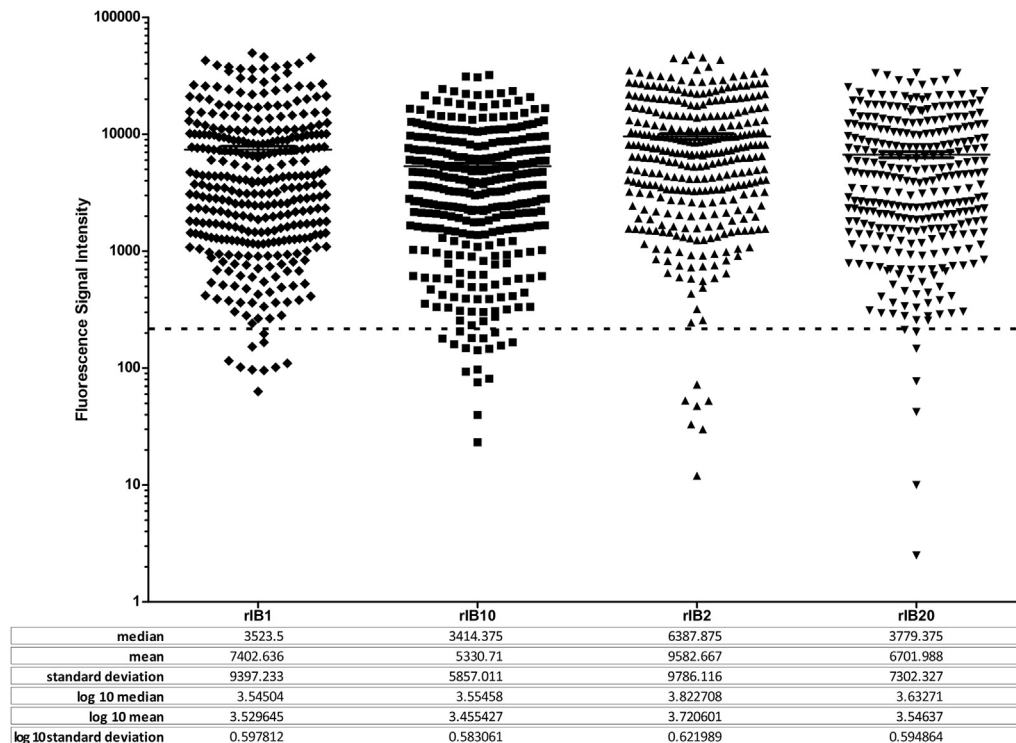


FIG E2. IgG seroreactivity to 4 universal antigens from the gut microbiota in white adults. Sera from all 341 white adults in 3 countries (Canada, Sweden, and the United States) were analyzed on the microarray, and the fluorescent signal intensity for the 4 universal antigens was plotted for each subject. Median, mean, and SD (raw values and $\log_{10}$ values) are listed below each antigen. Ninety-nine percent (336/341) responded to at least 2 of these antigens, 96% (327/341) responded to at least 3 antigens, and 82% (280/341) responded to all 4 antigens. In this case *response* is defined as a fluorescence signal intensity of greater than 2 SDs less than the logarithmic mean (223.6, *dashed horizontal line*). Three hundred twenty-eight subjects recognized rIB1, 312 recognized rIB10, 318 recognized rIB2, and 324 recognized rIB20.

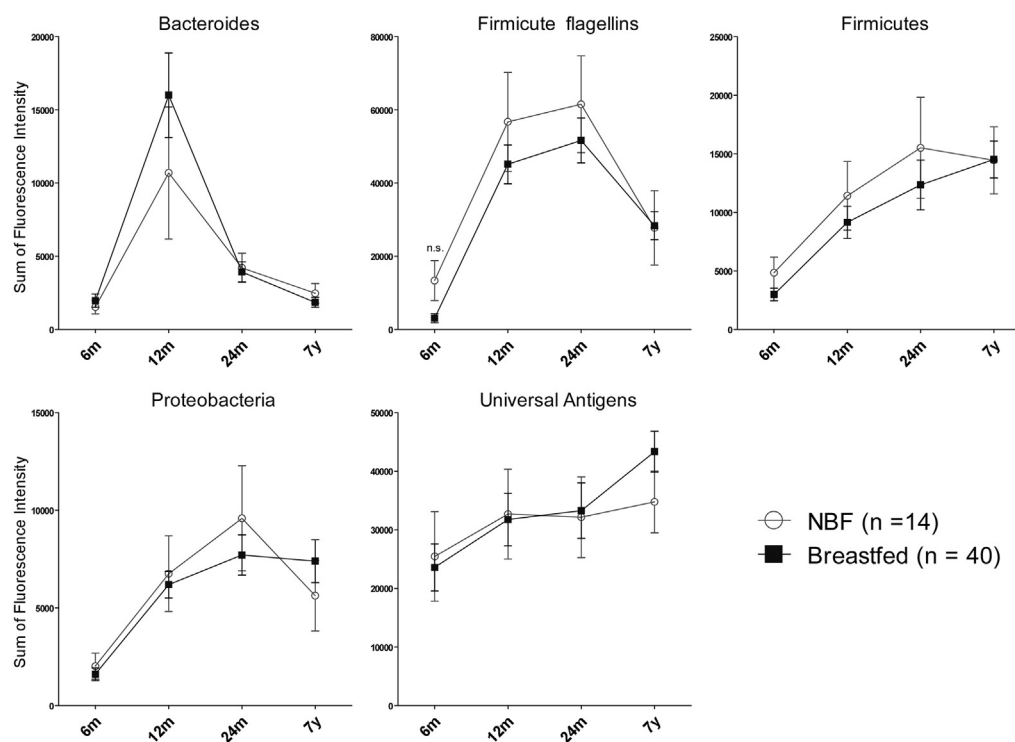


FIG E3. Effect of formula feeding on development of the human adaptive immune response to antigens from the gut microbiota. Sera from Swedish children were grouped according to exclusive breast-feeding at 3 months (*squares*) or introduction of formula (*circles*; NBF), and the sum of fluorescence intensity to antigens from a particular grouping was graphed from 6 months to 7 years of age as means $\pm$ SEMs. No significant differences were observed among any group of antigens at any age, suggesting that the introduction of formula is not affecting the response. *n.s.*, Not significant.

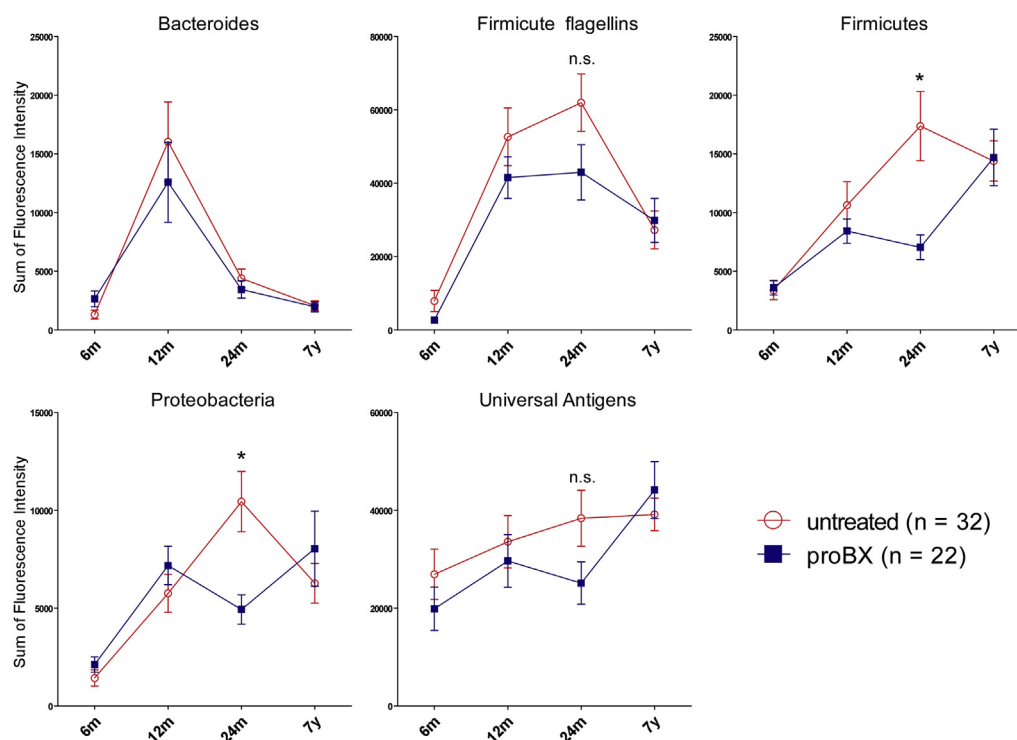


FIG E4. Effect of probiotic treatment on the development of the human adaptive immune response to antigens from the gut microbiota. Sera from Swedish children were grouped according to treatment with probiotics (squares) or lack of treatment (circles), and the sum of fluorescence intensity to antigens from a particular grouping was graphed from 6 months to 7 years of age as means $\pm$ SEMs. Mothers received *Lactobacillus reuteri* before birth, and neonates were supplemented with *L reuteri* through 12 months of age. Interestingly, at 24 months of age, there are significant reductions ($P < .05$) in the probiotic group compared with the control group. The rest of our data would suggest that reduced reactivity to antigens from the gut microbiota is associated with allergy development, yet probiotic treatment reduced IgE-associated eczema.³¹ However, these differences do not correlate with eczema development, the effect appears to be localized to 24 months of age, and the differences are not present at 6 and 12 months. It is at these early time points where there are significant differences in seroreactivity that are associated with allergy development (Fig 2). * $P < .05$. n.s., Not significant.

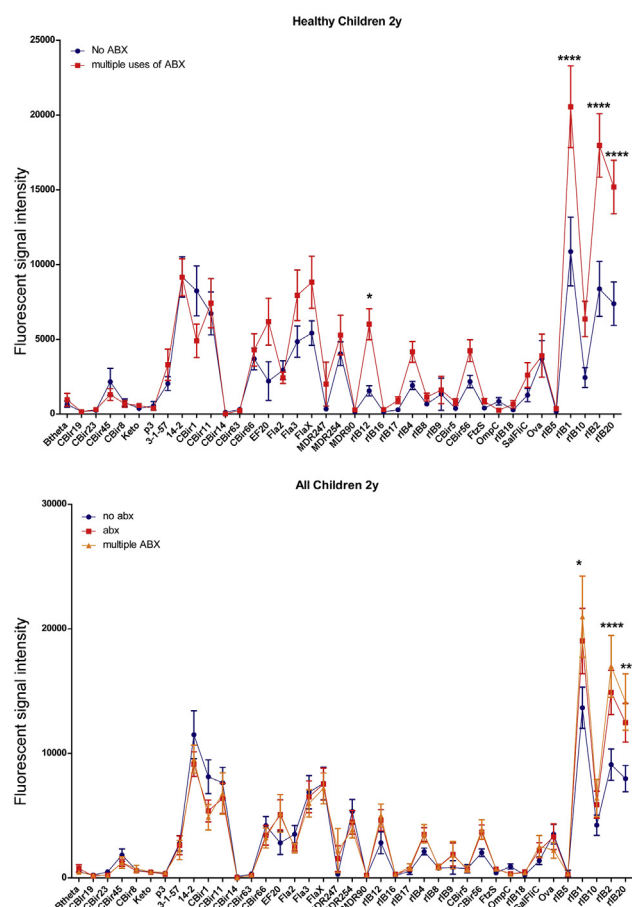


FIG E5. The influence of early antibiotics on seroreactivity to the microbiota. Children who received antibiotics at less than age 2 years were compared with those who did not. The antibiotics used included ampicillin, flucloxacillin, cephalosporin, sulfa, and macrolides. *Top panel*, Children who did not have allergy. At 2 years of age, there are significant differences in healthy children treated with antibiotics ($n = 18$) in seroreactivity to 3 of the universal antigens. Children who did not receive antibiotics ($n = 14$) had a lower response than children who had been treated with some form of antibiotics. *Bottom panel*, All children, regardless of allergy. Children treated with antibiotics ($n = 26$) had a higher response to 3 of the universal antigens than did children not treated with antibiotics ($n = 28$). * $P < .05$, ** $P < .005$, and *** $P < .0005$.