ORIGINAL ARTICLE EPID

Detection of major food allergens in amniotic fluid: initial allergenic encounter during pregnancy

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Abstract

Background: Ingestion of food allergens present in maternal milk during breastfeeding has been hypothesized as a gateway to sensitization to food; however, this process could develop during pregnancy, as the maternal–fetal interface develops a Th2- and Treg-mediated environment to protect the fetus. We hypothesized that in these surroundings, unborn children are exposed to food allergens contained in the mother's diet, possibly giving rise to first sensitization.
Methods: The presence of allergens *in utero* was studied by analyzing amniotic fluid (AF) samples in two different stages of pregnancy: at 15–20 weeks and after delivery at term. An antibody microarray was developed to test for the most common food allergens. The array detects the presence of ten allergens from milk, fruit, egg, fish, nuts, and wheat.
Results: AF from 20 pregnant women was collected: eight after delivery at term and 12 from women who underwent diagnostic amniocentesis between weeks 15 and 20 of gestation. The presence of allergens was detected in all samples. Samples from amniocentesis had a higher allergen concentration than samples after delivery at term.
Conclusions: We demonstrated the presence of intact major food allergens in AF samples. This early contact could explain subsequent sensitization to foods never eaten before.

The increasing incidence of food allergy seen nowadays is recognized as a major public health problem, especially in early childhood (1). Breastfeeding has been hypothesized as a gateway to IgE sensitization to foods due to the ingestion of food allergens dissolved in maternal milk (2). However, it has been previously argued that sensitization may occur during pregnancy (3).

In pregnancy, the maternal–fetal interface develops a Th2 and Treg environment rich in cytokines such as IL4, IL10, IL13, and TGF-β, thus protecting the fetus from rejection by the maternal immune system against paternal–fetal antigens (4). Other immune system modifications leading to maternal tolerance include the following: (i) the inhibition of complement system by sobreexpresions of complement regulatory proteins such as CD46, CD55, and CD59; (ii) generation of tolerance by immune cells, such as CD4+ CD25+ regulatory T cells, natural killer (NK) T cells, and immature dendritic cells; (iii) suppression of T_H17-mediated local inflammation by NK cells; and (iv) expression of non-classical truncated class Ib HLA molecules named HLA-G and HLA-E by trophoblast cells (5–7). An immune response can be triggered in newborns, and this capacity can only have been developed *in utero*. Peripheral blood mononuclear cell sensitivity to allergens has been detected after birth (8), and specific allergen-induced responses have been seen as early as week 22 of gestation (9). Food allergens from a mother's diet, which are in general very stable proteins, cross the placenta and the fetus are exposed *in utero*, constituting the first route of sensitization. Similarly,

Abbreviations
AF, amniotic fluid; FU, fluorescence units; LTP, lipid transfer protein; RT, room temperature.

mite allergens have been detected in samples of AF (10). Also, the egg allergen ovalbumin was detected in AF of rats after maternal intake (11). However, there are no studies about the presence of food allergens in the human uterine environment.

We hypothesized that in these surroundings, unborn children are exposed to food allergens contained in the mother's diet, possibly giving rise to early sensitization *in utero* that could explain childhood allergic reactions to foods never eaten before. The aim of this work was to detect the presence of different food allergens – mainly panallergens – in AF samples using an antibody array.

Methods

Amniotic fluid samples

AF at 15 to 20 weeks of gestation was obtained from remnants of the samples collected from women undergoing diagnostic amniocentesis (fetal karyotyping) by standard procedures. Medical ultrasound or ultrasonography was performed prior to amniocentesis to confirm fetal viability, gestational age, number of fetuses, placental location, and AF volume; to perform a fetal anatomic survey; and to scan for uterine cavity abnormalities or the presence of fibroids. The needle insertion site was identified by ultrasound, and the skin was cleaned with an antiseptic, prepped, and draped. The ultrasound probe was covered with a sterile glove or bag. The procedure was performed with a 20- to 22-gauge spinal needle. A 20-cc syringe was used to aspirate the AF following removal of the needle stylet. The first two cc were discarded and then, using another syringe, we removed 15–20 cc of AF. The AF of pregnant women who delivered at term was collected in a 20-cc pipette at the time of birth through artificial amniorrhexis (rupture of membrane). Total protein content was determined by Bradford Assay (Pierce, IL, USA).

The study was approved by the hospital ethics committees, and all women gave their written consent to participate in the study. All the women who participated in the study have not allergic disorders.

Antibodies

Ten major food allergens from the most common allergenic foods were selected as previously described (2): bovine casein (Bos d 8), bovine beta-lactoglobulin (Bos d 5), watermelon profilin (Cit la 2), ovalbumin (Gal d 2), frog parvalbumin (Ran e 1), peach thaumatin (Pru p 2), lipid transfer protein (Pru p 3), mustard 11S globulin (Sin a 2), wheat lipid transfer protein (Tri a 14), and wheat gliadin (Tri a 20). To have a wide range available, some panallergens were included (i.e., lipid transfer protein (nsLTP), profilin, thaumatin, and parvalbumin), thereby allowing sensitization to other foods (nuts or fruits) to be explained by cross-reactivity.

Antibodies were purchased or produced in our laboratory using purified allergens as previously described (2). Ovalbumin was provided by Sigma. Sin a 2, Pru p 3, Pru p 2, Cit la 2, and Tri a 14 were purified as previously described (12). Affinity-purified sheep anti-bovine casein recognized alphaS1-casein, alphaS2-casein, beta-casein, and kappa-casein.

Protein microarrays

Briefly, microarrays were printed with specific antibodies; these and all other reagents were provided by Raybiotech (Norcross, GA, USA). Thirty-five microliters of proteins was biotinilated according the manufacturer's protocol. The glass slide containing arrays, which is pre-printed with capture antibodies, was incubated for 1 h at RT with blocking solution, and then, the biotin-labeled reagent was added and incubated overnight at 4°C. After washing, streptavidin-conjugated fluorescent dye (Cy3 equivalent) was incubated with the array for two hours at RT. Finally, the glass slide was dried, and laser fluorescence scanning was used to visualize the signals; these signals were digitized with Axon GenePix 4200A Professional (Molecular Devices, Sunnyvale, CA, USA) and analyzed with GenePix™ software (Genomics Solutions, PE, USA). Provided that signals were well above background (mean background +3 standard deviation, accuracy ≈95%), only those spots having two replicates that fulfilled the analysis criteria were considered for analysis. Fluorescence units (FU) were defined as mean signal intensity for spot minus mean signal intensity for background.

Results

The selectivity and sensitivity of allergen microarrays have been evaluated previously (2). AF from 20 women was collected in the Fundación Jiménez Díaz Hospital (Madrid, Spain), and protein content was quantified. Eight AF samples were collected from women after delivery at term (AL1FT-AL8FT) and 12 from women undergoing diagnostic amniocentesis between weeks 15 and 20 of gestation (AL9-AL20).

Protein concentrations in the AF samples ranged from 2.3 to 11.6 mg/ml. Therefore, the fluorescence measurements were normalized with respect to the amount of protein loaded into the array and called normalized fluorescence units (normalized FU = FU/mg protein loaded into the array). Antibody microarray was able to detect nanograms of each allergen checked as previously described (2).

AF samples were analyzed, and the presence of all allergens was detected in samples AL9–AL20 (data are shown in Table 1, and graphic representation is shown in Fig. 1). Allergens derived from the most commonly consumed foods were detected with higher normalized FUs: beta-lactoglobulin and casein (milk), gliadin (wheat), and Gal d 2 (egg).

AF samples collected from women after delivery at term (AL1FT-AL8FT) showed a lower allergen presence – even undetectable in some samples (AL8) – compared with the allergen concentration in samples from amniocentesis. Allergens from milk, egg, and wheat were also detected in most samples.

Table 1 Normalized FU measurement in AF samples

Sample ID	Bovine Casein	b-Lactoglobulin	Cit la 2	Gal d 2	Gliadin	Parvalbumin	Pru p 2	Pru p 3	Sin a 2	Tri a 14
AL1 FT	0.00	96.95	0.00	0.00	44.28	0.00	0.00	15.21	0.00	0.00
AL2 FT	82.89	421.11	0.00	263.30	248.56	120.28	45.90	35.61	36.62	35.98
AL3 FT	8.25	310.98	0.00	87.66	177.81	135.77	0.00	0.00	17.47	0.00
AL4 FT	0.00	95.53	0.00	29.39	52.99	34.66	0.00	0.00	0.00	0.00
AL5 FT	0.00	72.87	0.00	0.00	69.62	0.00	323.80	0.00	17.30	0.00
AL6 FT	0.00	82.94	0.00	43.90	54.35	30.81	0.00	1.53	0.00	0.00
AL7 FT	0.79	43.15	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
AL8FT	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
AL9	199.57	464.18	35.83	414.57	448.69	185.02	72.09	27.23	36.41	39.07
AL10	129.09	309.65	25.77	294.01	269.21	125.17	79.29	22.46	25.61	36.02
AL11	231.53	363.40	8.57	358.23	347.12	140.65	102.33	20.76	13.24	28.77
AL12	241.96	298.04	52.91	303.37	300.59	177.01	67.60	27.84	48.40	49.99
AL13	187.18	292.09	14.34	299.50	302.24	163.28	94.02	22.10	30.62	25.87
AL14	166.38	318.75	19.30	340.06	306.29	69.97	72.81	29.17	23.73	23.32
AL15	29.91	540.92	33.06	297.29	355.07	81.00	64.64	42.04	33.10	51.91
AL16	364.62	786.95	77.95	913.32	924.67	181.26	296.40	121.34	82.24	94.97
AL17	75.93	840.38	64.59	668.33	725.70	85.53	101.78	290.31	62.69	89.15
AL18	171.34	969.78	41.87	737.77	805.44	85.95	84.92	58.80	55.48	54.45
AL19	40.25	168.12	8.11	158.88	125.35	26.38	18.75	10.60	6.36	10.81
AL20	28.81	105.91	7.03	131.34	104.05	19.33	16.17	7.55	7.07	8.65

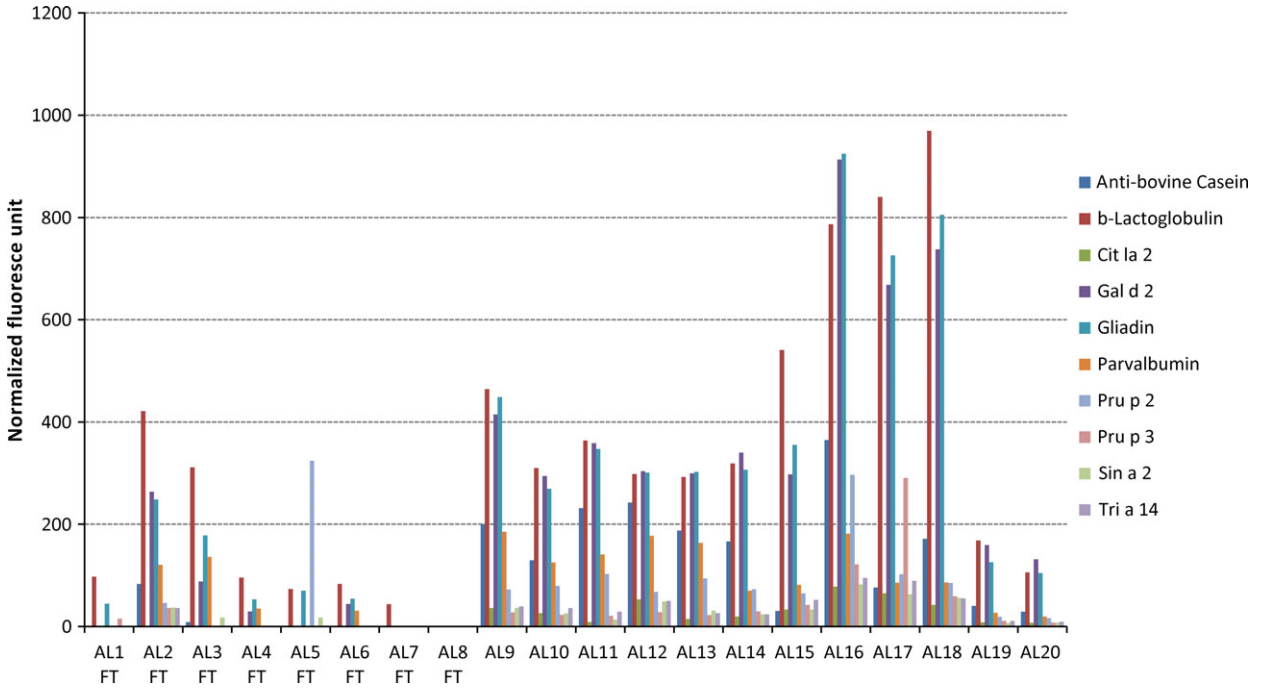

Figure 1 Graphic representation of allergen detection in amniotic samples is shown in Table 1. Normalized FU measurement for each allergen is represented by a different color.

Discussion

In this study, presence of major food allergens in AF has been measured, revealing stable allergens in AF. These results are important as the first allergenic contact might condition the future of the allergic state of the child following birth as concerns both induction of sensitization/allergy and tolerance, that is, LEAP study (13). Also, to check the presence of allergens is a first step to investigate when and how the initial prevention has to be initiated.

The design of the present study did not allow for detection of changes in allergen levels over time, although it is clear that higher levels of allergen appear at the beginning than at the end of gestation. A larger number of studies are needed to detect and quantify the presence of allergens as well as to follow the development of allergic disease in children. Future investigations should address how these changes take place.

Antigen-specific reactivity in the gastrointestinal tract of the human fetus is mature from early as 16 weeks of gestation (14), and thus, intrauterine sensitization has been studied as regards its relation to allergy development in offspring. Increased levels of Th2-like chemokines such as CCL17, CCL18, and CCL22 in cord blood have been related to the development of allergic symptoms during the first years of life (15, 16). Moreover, high levels of long-chain polyunsaturated fatty acids in cord blood have been considered a factor predisposing to allergy (17). Amniotic fluid contains intact maternal IgE that might bind to local IgE receptors within the lymphoid follicles of the fetal gastrointestinal tract and may induce the phenomenon known as antigen focusing, a process that has been postulated to be associated with the development of atopic disease (18).

However, in light of the fact that the fetus could be able to produce IgE from 11 weeks of gestation, numerous studies have focused on the presence of IgE in both placenta and cord blood (19).

Although the presence of specific maternal IgEs has been detected in the placenta (20) but not in cord blood, the relevant issue here is whether the fetus is able to produce allergen-specific IgE. While some investigations have postulated that there is no sensitization *in utero* (21), other studies have demonstrated the existence of allergen-specific IgE in cord blood from fetal origin (22). The prerequisite for sensitization is the presence of allergens in the uterine environment, and this is precisely one of the findings of this article; from this, it can be concluded that these allergens interact with the fetus. In this sense, AF is the first fluid available for study of the fetal environment *in utero*, making it an ideal material to detect the presence of allergens and the possibility of interaction with the fetus in the early stages of pregnancy. AF is a liquid that circulates around the fetus during pregnancy and is constantly inhaled and released by the fetus. AF favors proper bone growth and development and maintains a constant temperature around the baby, protecting the fetus from heat loss and outside injury. At first, AF is composed of mainly water and electrolytes; however, by week 12–14 of gestation, the liquid also contains proteins, carbohydrates, lipids, and metabolites. Several studies have reported absorption of dietary antigens and the detection of circulating intact food proteins in human serum (23). These allergens pass from the mother's bloodstream through the placenta and into the AF and the fetal respiratory tract and gut by suction, constituting the first route of sensitization. Until week 20, maternal contribution is essential in the formation of AF. After 20 weeks, the fetus participates in the formation of AF, primarily through the kidneys and lungs (24). At term, the fetus is more involved in AF formation, causing the influence of the maternal diet on level of food allergens to decrease. This fact would explain the higher concentration of food allergens in the samples at 15–20 weeks (Fig 1) due to the entrance of food allergens via the maternal diet.

Pollen-food syndromes result from the cross-reactivity between pollen-specific IgE and homologous proteins found in plant-derived foods and more precisely panallergens. Clinical utility of panallergen detection in allergy diagnosis is its ability to reveal markers of cross-reactivity. In the array used here, some antibodies recognized panallergens such as profilin and LTP. A previous study detected mite allergens in AF (7), thereby suggesting that other aeroallergens may be present in the AF, such as allergens from pollens. For this reason, it is possible that some antibodies can recognize inhalant allergens. For instance, watermelon profilin (Cit la 2) shares a high sequence similarity (around 96%) with some pollen profilins such as that of *Phleum pratense* (Phl p 12) (25). Therefore, it may be possible that part of positive signals obtained from the array may be sourced in cross-reactivity reactions.

This study demonstrates exposure to food allergens *in utero* from the mother's diet through AF. The exposure route could direct the immune system toward allergy or tolerance depends on various factors such as genetic disposition or the diet and environment of the born child.

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