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Sensitive detection of major food allergens in breast milk: first gateway for allergenic contact during breastfeeding.

Short title

Major food allergens in breast milk

Carlos Pastor-Vargas [a]*, Aroa S Maroto [a]*, Araceli Díaz -Perales[b], Mayte Villaba[c], Natalia Casillas Diaz[d], Fernando Vivanco[a,c], Javier Cuesta-Herranz[e].

[a]Department of Immunology, IIS- Fundación Jimenez Diaz, Madrid, Spain

[b]Center for Plant Biotechnology and Genomic (UPM-INIA), Pozuelo de Alarcon. Madrid, Spain.

[c]Department of Biochemistry and Molecular Biology I, Universidad Complutense de Madrid, Spain.

[d]Department of Pediatric, Fundación Jimenez Diaz, Madrid

[e]Department of Allergy, Fundación Jimenez Diaz, Madrid

* Both authors contributed equally to this work.

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Corresponding author

Dr. Carlos Pastor Vargas, Department of Immunology, Hospital Fundación Jiménez Diaz, Avda. Reyes Católicos 2, 28040 - Madrid, Spain

E-mail: cpastor@fjd.es

Tel: +34915504800 ext 2202

Fax: +34915448246

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Abstract

Food allergy is recognized as a major public health issue, especially in early childhood. It has been hypothesized that early sensitization to food allergens maybe due to their ingestion as components dissolved in the milk during the breastfeeding, explaining reaction to a food which has never been taken before.

Thus, the aim of this work has been to detect the presence of the food allergens in breast milk by microarray technology. We produced a homemade microarray with antibodies produced against major food allergens. The antibody microarray was incubated with breast milk from 14 women collected from Fundación Jiménez Díaz Hospital. In this way, we demonstrated the presence of major foods allergens in breast milk. The analysis of allergens presented in breast milk could be a useful tool in allergy prevention and provide us a key data on the role of this feeding in tolerance induction or sensitization in children.

Abbreviations used

FU: Fluorescence Units

LTP: lipid transfer protein

RT: room temperature

Key words: antibody array, breast milk, food allergens, food allergy.

Food allergy is recognized as major public health issue, and its prevalence has been increasing over the last years (1). The incidence of food allergy is especially serious in early childhood. Except for milk, egg, and peanut, no treatment has been described at present and nowadays, strict avoidance of allergen-containing foods is the way to these avoid allergic reactions. It has been hypothesized that early sensitization to food allergens is probably due to their ingestion as components dissolved in the milk during the breastfeeding. However, only peanut, egg and milk allergens have been described in breast milk (2-4). To our knowledge, the limited number of allergens detected in breast milk is more likely a technical problem that a scarce presence of allergens and so, other allergens has not been detected in breast milk. This work attempts to explain why a child has an allergic reaction to a food which has never eaten before or that the adverse reactions to breast milk during lactation could be due to the presence of these allergens.

In this work, we have developed an antibody array to detect different food allergens and we studied their presence in breast milk samples. Microarray technology is a sensitive relevant technique in the allergy field that allows us testing simultaneously multiple allergens. We selected ten major food allergens from the most frequent allergenic foods, some of them, panallergens (such as lipid transfer protein (nsLTP), profilin, thaumatin or parvalbumin), in order to have a wide range available. Positive results could explain the sensitization during the breastfeeding to other foods, such as nuts or fruits that can subsequently trigger cross-reactivity.

Antibodies were purchased or produced in our laboratory using purified allergens (table I). Rabbit antisera were produced following the technique described by Albar et al. (5). 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 (6).

Microarrays were printed by Raybiotech (Norcross, GA, USA) that provides us all reagents. Thirty-five ~~µl~~microliters from samples were biotinilated according manufacture protocol. The glass slide containing arrays was incubated for 1 hour at room temperature with blocking solution (provided by Raybiotech), and the biotin-labeled reagent was added onto the glass slide, which is pre-printed with capture antibodies, and incubated overnight at 4°C to allow for the interaction of target proteins. After washing, streptavidin-conjugated fluorescent dye (Cy3 equivalent) is then added to the array and incubated for two hours at room temperature. Finally, the glass slide is dried, and laser fluorescence scanning is used to visualize the signals, that was digitized with Axon ~~Genepix~~ GenePix 4200A Profesional (Molecular Devices, Sunnyvale, CA, U.S.A.) and analyzed with GenePix™ software (Genomics Solutions, PE, USA). Only those spots having two replicates fulfilling the analysis criteria and provided that signals are well above background (Mean background + 3 standard deviation, accuracy ≈ 95%) were considered for analysis. Fluorescence Units (FU) were defined as mean signal intensity for spot minus mean signal intensity for background.

We checked the selectivity and sensitivity of microarrays using decreasing amount of purified allergens or allergic food. Antibody microarray were able to detect nanograms of each allergen checked (Supplementary fig 1). Breast milk from 14 women was collected from Fundación Jiménez Díaz Hospital (Madrid, Spain) and protein content was quantified. The study was approved by the hospital ethics committee, and all women gave their written consent to participate in the study. Milk samples were analyzed showing the presence of different allergens. Due to the wide variation of protein concentration in the different breast milks, ranging from 30 to 70 mg/ml, results were normalized to the amount of protein (FU/mg protein loaded in the

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array). Interestingly, as selectivity internal control, the amount of cow milk allergens varied in the different samples, ~~no~~ without detecting these allergens in some human breast milks. ~~An unspecific~~ Unspecific signal in milk allergens should be in all milk samples if microarray could recognize human caseins instead of Bos d 8, but, as you can see in Fig 1, the Normalized FU measure for milk allergens is low in some sample, even there is a sample we do not detect milk allergen (BM9). Therefore, these data ~~-~~provide the high selectivity of the antibody array..

Antibody array was able to detect all allergens studied (Fig 1). Some proteins such as profilins, thaumatins or ovalbumin are degraded by gastric enzymes but have been detected in breast milk, probably due to the detection method by polyclonal antibodies: proteins are degraded but could maintain large peptides or protein fragments, enough to be recognized by polyclonal antibodies. The high number of allergens detected and the great variety between the different samples might due to the allergens content depends on several factor: the mother diet or the allergen structure features as the intrinsic resistance to proteolytic degradation. Also, the sensitization to allergens with a high structural stability and present in very consumed foods during the breastfeeding could explain the episodes of allergy to foods previously never eaten by the child.

Human milk is a complex substance that contains, among others, antibodies (mainly secretory immunoglobulin A), antioxidants, fatty acids, lactoferrin, hormones, growth factors, anti- and pro-inflammatory cytokines and nutrients (7). The benefits of breastfeeding are widely accepted due to promote stimulation of the immune system and the correct development of gut mucosal barrier (8, 9). But there is still lot of debate regarding on human milk constituents and its role in the development of allergic disease. Thus, some factors in human milk have been described with protective effects

against atopy (10-12) while others increase the risk of atopy and subsequent allergy development (13-16)

We demonstrated the presence of allergens in breast milk probably due to the food ingestion from the mother diet. In this sense, a common practice has been to recommend mothers to avoid the "allergenic foods" from their diet during lactation as a prevention measure (17). Although in 1994, Canadian Task Force on the Periodic Health Examination suggested that dietary measure results unsuccessful due to the variation on amount of antigens in breast milk, data confirmed by us with the antibody microarrays assay (Fig. 1). Although inflammatory responses or tolerance induction of the newborn depends on more factor as for instance the immaturity of the intestinal barrier (18), the analysis of allergens in breast milk could be a useful tool in allergy prevention. A study with an increasing number of allergens and quantifying their concentration, as well as following the development of allergic diseases in the children that have taken these breast milks, could provide key data on the role of this feeding in tolerance induction or sensitization in children.

Conflict of interest

The authors declare that no conflicts of interest exist.

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FIGURE LEGENDS

Table I: Antibody list printed in microarray. HMA: Homemade polyclonal antibody. Affinity Purified Sheep anti-Bovine Casein recognize: alphaS1-Casein, alphaS2-Casein, beta-Casein, kappa-Casein.

Figure 1: Allergens detection in breast milk samples by antibody microarray. Measures were normalized to the amount of protein; Normalized fluorescence unit= Fluorescence Unit/mg protein loaded in the array.

Supplementary Figure 1: Panel A: Antibody Array Map. Example of two specificity tests in antibody arrays: incubation with 100 ng of fish extract (Panel B) and incubation with 100 ng of egg extract (Panel C). The microarrays were overexposed to confirm the specificity of the antibodies.

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Antibody	Supplier	Reference	Bibliography
Affinity Purified Sheep anti-Bovine Casein	Biorbyt	MBS560643	
Sheep anti bovine beta-lactoglobulin	Raybiotech	DS-PB-00261	
lyclonal Rabbit anti-Watermelon profilin (Cit la 2)	HMA		Int Arch Allergy Immunol. 2010;153(3):215-22
Polyclonal Rabbit anti-Ovalbumin (Gal d 2)	HMA		Eur J Immunol. 1994;24(3):599-604
Rabbit anti wheat Gliadin	Abcam	ab50602	
Monoclonal Anti-Parvalbumin	Sigma Aldrich	P3088	
olyclonal Rabbit anti-Peach thaumatin (Pru p 2)	HMA		PLoS One. 2012;7(9):e44088.
Polyclonal Rabbit anti-Peach LTP (Pru p 3)	HMA		PLoS One. 2012;7(12):e50799
olyclonal Rabbit anti-Mustard 11S globulin (Sin a 2)	HMA		Clin Transl Allergy. 2012 Dec 11;2(1):23.
Polyclonal Rabbit anti-Wheat LTP (Tri a 14)	HMA		PLoS One. 2012;7(12):e50799

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206 **Table I**: Antibody list printed in microarray. HMA: Homemade polyclonal antibody.
207 Affinity Purified Sheep anti-Bovine Casein recognize: alphaS1-Casein, alphaS2-
208 Casein, beta-Casein, kappa-Casein.

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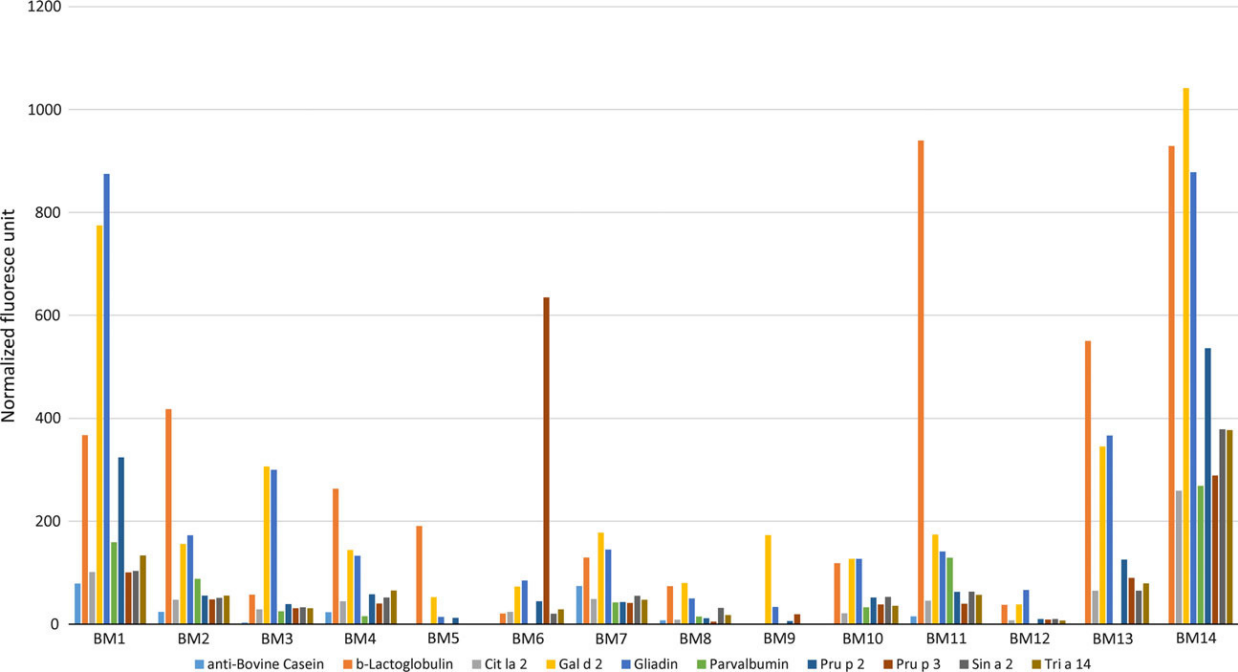
Normalized fluoresce unit
1200
1000
800
600
400
200
0
BM1
BM2
BM3
BM4
BM5
BM6
BM7
BM8
BM9
BM10
BM11
BM12
BM13
BM14
anti-Bovine Casein
b-Lactoglobulin
Cit la 2
Gal d 2
Gliadin
Parvalbumin
Pru p 2
Pru p 3
Sin a 2
Tri a 14