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CD4 T-Cell Cytokine Response to Mite Recombinant Tropomyosin in Mite, Snail and Shrimp Allergic Patients

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Abstract

Allergies to snails and mites are definitely linked. Snail allergens have been already identified and there is no evidence of cross-reaction between snail and mite tropomyosin. This work is a preliminary study on immune cell function, evaluating possible tropomyosin-triggering in a snail-mite-shrimp cross-reaction. Peripheral blood mononuclear cells

from 6 different phenotype patients in relation to snails, mites and crustaceans, were cultured with mite recombinant tropomyosin (rDer p 10), anti-CD28 and brefeldin A. Phytohemagglutinin mitogen was used as a positive control. CD4 T-cell response was evaluated towards CD69, IFN-Î³, IL-2, IL-4, IL-5 and IL-13, in flow cytometry. rDer p 10 induced different cytokine expression in the six phenotypes. Allergen-specific IFN-Î³ stimulation was suggested by an increase in IFN-Î³, IL-5, and IL-13 only in allergic to crustaceans, with a dose-dependent effect on IL-5 and IL-13. Tropomyosin is probably the main allergen responsible for the mite-shrimp cross-reactivity but may not play a major role in snail-mite-shrimp cross-reactions.

This work was done in the Laboratory of Immunology from Hospital de S. Bernardo, SetÃºbal, Portugal.

Introduction

Snails, a very popular delicacy in several European countries, are also considered as one of the worst causes of food allergy. In fact, some people may develop severe episodes of asthma [1] after ingestion of snails and a connection with house-dust-mite allergy is firmly established [2,3,4,5,6,7,8,9,10,11,12,13,14,15].

Food allergens are frequently glycoproteins with a molecular weight (MW) from 15 to 50 kDa, with the immunogenic effect depending precisely on the MW, which may facilitate the contact and further absorption through the digestive mucosa. The number of epitopes present on the antigen molecule may also play an important role [16], possibly due to their immune triggering potential.

Helix aspersa, *Otala lactea* and *Theba pisana* allergen repertoires were exposed by immunochemical methods, showing a close pattern between them. Cross-reactivity between *Dermatophagoides pteronyssinus*, *H.aspersa* and *Pandalus borealis* shrimp was also evaluated by RAST inhibition. *D. pteronyssinus* was shown to be a strong inhibitor of the *H. aspersa* RAST (72,6%) and even stronger

inhibitor of the *P. borealis* RAST (91,9%) [15]. Despite 65 sequence identities and 79 similarities between *H. aspersa* and *D. pteronyssinus* tropomyosins [17], the 36-kDa protein from *H. aspersa* extract and the natural and recombinant purified tropomyosins were IgE-recognized by only 4 sera out of 22 from patients reporting snail reactivity [18]. Another study also reported a 37-kDa protein from *H. aspersa* extract, has being recognized by only 1 patient out of 21 with specific IgE to *H. aspersa* above class 2 [15]. Extending the previous study, further unpublished data has shown recognition of the 37-kDa protein by only 4 from 36 patients with specific IgE to *H. aspersa* above class 2.

The two major allergens from *H. aspersa* (recognized by 13 and 18 out of 21 patients, respectively) presented MW >208 kDa, probably corresponding to the heavy chains of snailsâ€™ myosin [15].

In view of the presented results, it seemed rather improbable that tropomyosin could be the key culprit in the snail-mite cross-reactivity with allergy. Therefore, it was then important to identify what could happen at the cellular level, as the cytokine response pattern may differ from what is expected by simple observation of the sensitizing spectrum [19]. In fact, atopy was observed in association with allergen-specific T helper (Th) cells, with a Th2 pattern of response dominated by IL-4, IL-5, IL-9 and IL-13, while IL-10, TNF-Î± and IFN-γ were present both in atopics and non-atopics [20]. Mostly in atopics, bronchial hyper-responsiveness revealed to be strongly associated with eosinophilia and IL-5 synthesis in conjunction with IgE production [20].

Food allergy is, in fact, a hypersensitive reaction to frequently harmless antigens involving IgE-producing B lymphocytes and T lymphocyte cell-mediated responses. According to Bohle [21], IgE mediated mechanism triggers a rapid clinical reaction upon ingestion, which can be exemplified by the oral allergy syndrome. However, in delayed reactions involving the digestive tract or the skin, less clear mechanisms were referred, pointing to allergen-specific T-cell cultures as a useful resource to explain the role of these cells, since the majority of allergen-specific CD4 T-cells isolated from food-allergic individuals

were identified as highly IL-4 and IL-13 producers.

Materials And Methods

Patient Selection

For this preliminary study each patient followed two fundamental criteria, as being in a non crisis state and being free of any concomitant pathology or medication. Six individuals presenting different phenotype patterns of atopy and allergy to snails, mites or shrimps were selected for cell stimulation assays. Total and specific IgE levels are also shown (Table 1).

Table 1: Individual profile from each of the 6 individuals/phenotypes (A, B, C, D, E and F) according to the following patterns...
Table 1: Individual profile from each of the 6 individuals/phenotypes (*A*, *B*, *C*, *D*, *E* and *F*) according to the following patterns: Total and specific IgE (kU/L); NS â€“ non-sensitized; S â€“ sensitized; NR â€“ non-reactive (non-allergic); R â€“ clinical reactive (allergic).

Asthma was the clinical reaction to snails, while asthma, rhinitis and conjunctivitis were the allergic symptoms to mites. Shrimp ingestion conduced hives in *phenotype F* and abdominal pain associated with gastralgia in *phenotype E*. Patients were not taking any medicine for a period of, at least, one month.

Blood Collection and Primary Processing

60 mL of blood were collected from each patient. 50. 2 mL of blood-EDTA were used for complete blood count (CBC) in an Advia 120 blood counter (Bayer AG, Germany). In a grade II laminar flow cabinet (Microflow Laminar Flow Cabinet â€“ MDH Ltd., Hampshire, UK), 12,5 mL of heparinized blood were carefully added over 20 mL of density gradient separation medium HistopaqueÂ® â€“ 1077 (Sigma-Aldrich, Inc., St. Louis, MO, USA) in each of four 50 mL sterile tubes (Sarstedt) and centrifuged at 400g for 20 min at +20 Â°C. PBMC rings were aspired with a sterile Pasteur pipette and pooled in one 50 mL

tube for two sequential washes with sterile phosphate-buffered saline (PBS) pH 7,4 (Biochrom, Berlin, Germany). Pellets from the last wash were pooled and re-suspended in a small volume of culture medium â€“ RPMI 1640 (Sigma) supplemented with 20 Î¼g/mL gentamycin (Labesfal, Tondela, Portugal), 2 mM L-glutamine (Biochrom) and 10% human AB serum (Sigma-Aldrich) for cell counting and viability evaluation according to Shapiro HM [22] and to Coligan JE *et al.* [23]. Cell suspension was then diluted to $4x10^6$ viable cells/mL, for stimulation.

Cell Stimulation

A volume of cell suspension containing $4x10^6$ viable cells was added to each of 19 sterile Falcon tubes (BD Biosciences, San Jose, CA, USA) in two replicates. One tube for isotype control, 5 for negative controls, 5 for positive controls with mitogen and 8 for rDer p 10 stimulation. Each of the positive and isotype control tube was supplemented with 10 Âµg of Phytohemagglutinin (PHA) (Biochrom) for non-specific stimulation. Four of the rDer p 10 tubes were supplemented with 5 Âµg and four with 15 Âµg of the recombinant allergen. Monoclonal antibodies (MoAb) anti-CD28 (Sanquin, Holland) were added to each and every tube for co-stimulatory effect, to a final concentration of 10 Âµg/mL. Final volume was completed to 1 mL with the supplemented RPMI 1640 culture medium. Sample tubes were caped with permeable lids and incubated for 2 h on a 20Â° inclination angle at 37 Â°C in a 5% CO_2 atmosphere incubator (Sanyo CO_2 Incubator, Sanyo Electric Biomedical, Japan). Incubation was stopped and 10 Âµg of brefeldin A (BFA) (Sigma-Aldrich) was added to each tube, except to the isotype control, to inhibit the intracellular flux of proteins produced in the rough endoplasmic reticulum for Golgi-mediated export. Incubation proceeded again for 4 h with gentle stirring at one hour intervals.

After six hours of incubation, stimulation was stopped by adding 3 mL of ice-cold PBS followed by 400g centrifugation for 8 min. 3 mL of PBS â€“ 2 mM EDTA was added to each tube and incubated for 10 min in a 37 Â°C water-bath to promote cell detachment. Samples were centrifuged at 400g for 8 min and decanted. Tube walls were washed for ten-times with 0,7 mL PBS to detach adherent cells and centrifuged

at 400g for another 8 min. Tubes were decanted again and blot-down onto absorbing paper.

Immunophenotyping

Monoclonal Antibody Staining

Cell pellets were re-suspended in 100 ÂµL of PBS â€“ 0,5% bovine serum albumin (BSA) (Sigma-Aldrich) â€“ 0,1% NaN_3 (Merck, Darmstadt, Germany) for MoAb surface staining. Isotype control was stained with 20 ÂµL of each of the three fluorochromes: mouse monoclonal IgG2a-proprotein convertase 5 (PC5) (Immunotech, Marseille, France), IgG2a-fluorescein isothiocyanate (FITC)/IgG1-phycoerithrin (PE) (Cytognos, Salamanca, Spain). Each of the remaining tubes was stained with 20 ÂµL of anti-human CD4 mouse MoAb coupled with peridinin chlorophyll protein â€“ CyChrom 5.5 (PerCP Cy 5.5) (BD Biosciences), for the identification of this T-cell subpopulation. Tubes were stirred in vortex and incubated for 15 min in dark at room temperature. Then, isotype control was directly submitted to fixation by sample suspension with PBS â€“ 0,1% paraformaldehyde (PFA) (Merck, Darmstadt, Germany) and kept at +4 Â°C in dark until sample acquisition. The remaining samples were submitted to cell fixing and permeabilizing protocol, using Fix & Perm kit (Caltag Laboratories, Burlingame, CA, USA) for intracellular stain, according to manufacturerâ€™s instructions. Final pellets were re-suspended and permeabilizing solution added simultaneously with intracellular staining anti-human MoAb, as follows: 15 Î¼L of anti-IFN-Î³ FITC (BD Biosciences) were added to each tube and 10 ÂµL of anti-CD69 PE (Caltag Laboratories) to positive and negative activation and intracellular staining controls, with and without PHA. Anti-IL-2 PE MoAb (10 Î¼L) (Caltag Laboratories) and anti-IL-4, IL-5 and IL-13 PE (30 Î¼L) (PharMingen â€“ BD Biosciences) were added to positive and negative respective control tubes, and also to rDer p 10 (5 and 15 Âµg) stimulated samples. Samples were processed according to manufacturers, and the obtained supernatants aspired. Pellet cells were re-suspended in 400 Î¼L PBS â€“ 0,1% PFA and kept at +4 Â°C in dark until sample acquisition.

Flow Cytometer Acquisition

Cell acquisition was performed in a Facscalibur flow cytometer (Becton Dickinson, NJ, USA) establishing a gate of CD4 T-cells by *Dot Plot* combination of low expression of granularity in side scatter (SSC) with a high expression of the CD4 antigen [24] (Fig. 1). A total number of 150.000 gated cells were acquired and stored for CellQuest software (BD Biosciences) analysis.

Figure 1: Acquisition and analysis Dot Plot with CD4+ region defined by gating cells with high expression of this antigen, iden...
Figure 1: Acquisition and analysis *Dot Plot* with $CD4^+$ region defined by gating cells with high expression of this antigen, identified by anti-human CD4 PerCP Cy 5.5 MoAb and low expression in SSC (red spot of events/cells)

Analysis by the Expression of Subpopulations

Analysis techniques used for these protocols are mainly interactive and somehow empirical, based on Boolean gating strategies defined as one or more analysis gates, using a combination of two parameters [25], like those used for data analysis.

Each patient cytokine pattern of production was accessed in order to understand individual response to stimulation. Individual cytokine response after the stimuli was compared to their simultaneous cytokine response in the exact conditions without stimuli, as individual baseline.

Acquired and stored CD4-expressing lymphocytes from each phenotype were analyzed in terms of different cytokine expression, with and without PHA or rDer p 10. CellQuest software was chosen for two parameter *Dot Plot* analysis. Quadrants were defined within Dot Plots for fluorescence (FL) levels of acceptance. Only events falling above the non-specific level of fluorescence, designed by the isotype control, were considered. Comparative approaches were made between negative and positive controls and with each rDer p 10 stimulating dose, towards the different cytokines, using the *Quadrant Statistics*

software function.

Two parameter cross-analysis, were:

SSC with CD4;
CD69 with CD4;
IL-2, IL-4, IL-5 and IL-13 with CD4;
IFN-γ with CD4;
IFN-γ with CD69, gated on CD4 cells;
IFN-γ with IL-2, IL-4, IL-5 and IL-13, gated on CD4 cells.

Expression of CD69 and cytokine was evaluated for significant differences between replicates.

Cocktail staining using different MoAb coupled with different fluorochromes has proven to be a useful tool for identification of rare cells. This approach, especially when in conjunction with an indifferent parameter such as forward scatter (FSC), allows for identification of cells present at frequencies as low as 1:10.000.000 [26;27].

Identification and counting of rare events (less than 0,1% in the acquired populations) [22] for comparative analysis of different samples, requires determination of statistical significance of small differences within large numbers and what is truly important in this pattern of occurrences is the number of counted cells of interest and not the total [28].

According to Zar [29], Wilcoxon test can be used for comparative studies of pared data in ordinal interval measurement scales, not requiring a normal data distribution. The Wilcoxon test determines if there are any significant differences between two sets of data. This test was meant to evaluate if small differences, as observed in cytokine expression by stimulating challenge, are accidental or due to the stimulus. Each difference observed between two values, such as with or without the addition of rDer p 10, was analysed as belonging to one of the two possible hypotheses â€“ *there is* or *there is not* significant difference between them.

Statistical significance was considered for p < 0,05.

Results

In this study the figures of cytokine expression did not present a normal statistical distribution and a non-parametric test, such as Wilcoxon, was chosen.

As data sets were counting events/cells from a larger universe, they were considered to have a Poisson distribution and 95% confidence intervals, allowing the comparison of values [30;31].

Individual responses to PHA and to rDer p 10 stimuli by the six phenotypes are shown in table 2 (*a* and *b*).

Table 2: (a and b) â€“ CD4 T intracellular expression (%) of different antigens (CD69, IFN-γ, IL-2, IL-4, IL-5 and IL-13...
Table 2: (*a* and *b*) â€“ CD4 T intracellular expression (%) of different antigens (CD69, IFN-γ, IL-2, IL-4, IL-5 and IL-13) in six individuals/phenoytpes, With (W) and without (WO) addition of PHA, or 5 or 15 Âµg of rDer p 10. $CD69^+$ and IFN-γ$^+$ represent the percentage of expression within these CD4 subpopulations.

From the figures, it was observed that the expression of CD69 in PHA stimulated cultures was rather superior to cultures without PHA in all six phenotypes. IFN-Î³ and IL-2 expressions also showed a significant rise in all phenotypes following PHA stimulation. IL-2 expression presented a significant increase following PHA stimulation but, like IFN-γ, it was less expressive than CD69. IL-4 expression showed significant increase with PHA only in *phenotypes B*, *C*, *D* and *F*, with clear atopic profiles, although lower than IFN-γ or IL-2. IL-4 and IL-5, also presented a significant increase with PHA, except in *phenotypes A* and *E*. PHA induced an IL-13 increase in all phenotypes but with lower evidence in *phenotypes A* and *E*.

PhenotypesC and *F* presented a lower increase in IL-5 expression following PHA stimulation. With regard to rDer p 10 stimulation, IL-2 expression only increased in *phenotypeA* with the higher concentration (15 Âµg). The antigen only induced a significant, although discrete,

increase in IFN-Î³ expression in *phenotypes D* and *E*.

IL-4 expression did not show significant variation following rDer p 10 stimuli. rDer p 10 showed a dose-dependent stimulation on IL-5 expression in *phenotypesA* and *E* and in IL-13 from *E*, where the increase was even greater in IFNÎ³+ CD4 T-cells. In *phenotypeF*, IL-13 expression by rDer p 10 was less expressive, with no dose-dependent effect. The antigen did not induce an increase of cytokine expression in the allergic to snails and mites individual (*phenotypeB*) neither in the mite allergic (*phenotype F*).

Discussion

Aside from RAST inhibition studies showing significant inhibitions between mollusks, like oyster and crustacean like shrimp, lobster, crab or crayfish, cross-reactivity in correlation with clinical response is not always seen between mollusks and crustaceans, suggesting that the presence of specific IgE to mollusks may not be determinant for a clinical reaction [17]. A rather similar situation is observed between snails and mites, where in most cases sensitization to snails is not enough to predict a reaction after ingestion, even in mite allergic patients [15]. Inhalant challenge was shown to be associated with increased levels of IL-5 in peripheral blood eosinophils and lymphocytes [32], suggesting a possible target organ, other than the contact site. In fact, in snail allergy contact is made at the digestive tract level following ingestion and the target is frequently the bronchial tree.

Tissue profile, in which food allergen-specific T-cells are present seems to determine the target organs for the clinical response [21] and may also stand as a conditioner for other Th2-type responses, since the Th2 response to one allergen seems to act as an environmental promoter to further Th2 responses [33]. Analogue *hypothesis* can be formulated regarding allergy to snails and shrimps, where the contact is made at the digestive mucosa and clinical response may emerge as asthma after snail consumption or as hives (*phenotype F*) and gastralgia with conjunctivitis (*phenotype E*) after the ingestion of shrimp.

Different factors may interact in the evolution from sensitization to clinical allergy. Repeated contact with allergens responsible for a cross-reaction is supposed to be an inducing prerequisite for the priming or immunologic initiation in which the allergen is initially presented to T and B naive cells. Then, the allergen makes contact with memory cells and finally with mastocytes, triggering the clinical response [33]. This three-step mechanism mimics what probably succeed trough the snail-mite sensitization, because mites have a worldwide distribution with the inherent ability to induce early sensitization and snail-sensitized individuals are frequent snail consumers [15]. The risk of an allergic reaction upon ingestion of snails appears to be much higher among mite-allergic subjects and this scenario is extended to several other cross-reactions. In fact, there is already a table of approximate risk to develop a clinical reaction to a specific food in the presence of allergy or even sensitization to another food [34].

In the present study, after PBMC stimulation with rDer p 10, an increase in IL-5 and IL-13 expression was observed in CD4 T-cells simultaneously expressing IFN-γ, from *phenotypeE*, and even in such a small group it was possible to observe that individuals with higher increase in IFN-γ expression following stimulation with PHA were precisely the non-atopic (*phenotype A*) and the one only allergic to shrimp (*phenotype E*). According to this it is considered that allergy control may, in addition to a new Th1/Th2 equilibrium, require the association with mechanisms linked to T regulatory (Tregs) cells [35]. In fact, the role of IFN-Î³ as a promoter of Th1 and an inhibitor of Th2 responses has been controversial. It has been even suggested that the level of IFN-Î³ could be used as the key to a positive or negative effect on the Th2 pathway, namely in what regards to the production of IL-4 [36]. The environment created by the Th (1 or 2) response pattern to a given antigen, may also play an important role in subsequent Th cell differentiation, since the addition of IL-4 and anti-IL-12 to stimulated PBMC was reported to select Th2 cell lines, whereas Th1 lines have been selected by the addition of IL-12 and anti-IL-4 [37].

Proposed suppressor effect of Tregs on allergic inflammation

triggering was associated with IL-5 and IL-13 (two cytokines mostly related to respiratory allergic inflammation) synthesis inhibition, but not with IL-4 (a cytokine in the first line of the IgM to IgE switch mechanism) which is compatible with sensitization in the absence of clinical response, what is extensively observed in practice. However, recent studies point to an intricate relation between cytokines and their receptors, resulting in a highly heterogeneous pattern of effector cells, thus increasing the difficulties in understanding the involved mechanisms [38].

IL-4 is suggested to be implicated in sensitization but not necessarily in clinical response, and IL-5 may not be a secure marker of atopy, but closely follows clinical responses in the course of atopy. In fact, a control mechanism for the synthesis of IL-5 at the genetic transcription level, distinct from those regulating the gene transcription for IL-4 or IL-2 was reported [39]. Data from *in vivo* studies with animals suggests a sequential involvement of IL-4 and IL-5 in allergen-induced airway disturbances [40]. Hence, IL-4 seems to be crucial in the primary sensitization process while IL-5 and IL-13 are more important in the secondary exposure to allergenic aerosols.

An increase in IL-5 expression was reported in PBMC cultures from patients allergic to *Cryptomeria japonica* (Japanese cedar) stimulated with the major allergen Cry j 1, while just sensitized and non-atopic individuals did not show significant variation of IL-5 expression. IL-4 expression did not show any significant variation in the non-atopic group and presented lower frequency of expression than IL-5 in the allergic patients [41]. Our preliminary data seems to follow that cytokine pattern of response, with an increase in IL-5 and IL-13 expressing CD4 T-cells, only observed in *phenotype E* with the highest level of specific IgE to shrimp, which is possibly associated with the clinical response following secondary exposure to the probable trigger allergen, tropomyosin. In fact, over 80 identities and about 90 similarities have been found between mite and shrimp tropomyosins [17], and *phenotype E* also recognized the 37-kDa protein band from *D. pteronyssinus*, probably the tropomyosin (unpublished data). By analogy, in this study, *phenotype B* â€“ just sensitized to *P. borealis* shrimp â€“ did not

show an increase in IL-4, IL-5 or IL-13 expression by CD4 T-cells after stimulation with rDer p 10, and *phenotype F* â€“ sensitized with mild clinical reaction â€“ did not present any increase in IL-4 or IL-5, showing only a slight increase in IL-13 expression. *Phenotype E* â€“ presenting much stronger clinical reaction to shrimp â€“ showed a rather important increase in IL-5 and IL-13 expression after identical stimuli. These facts suggest that *phenotypes B*, *E* and *F* could have been sensitized primarily to mite tropomyosin and secondarily cross-recognized shrimp tropomyosin, triggering the clinical response in *E* and *F*. In *phenotype E*, clinical response was more intense in association with a higher rise in IL-5 and IL-13 expression following rDer p 10 challenge.

CD4 T-cells showed good early activation in all of the six individuals, by a strong increase in CD69 intracellular expression, validating the lymphocyte ability for in vitro activation [42]. However, in what concerns to PHA and rDer p 10 stimulation, different variants of cell cytokine response were identified among these patients. After incubation with PHA, IFN-Î³ expression also revealed a significant increase, mostly in activated cells.

Substantial increase in IFN-γ expression by rDer p 10 stimulation was only observed in *phenotypeE*, possibly associated with an important level of sensitization to shrimp tropomyosin, which presents more than 80 similarities with Der p 10 [17]. Conversely, *phenotype F*, also sensitized and clinical reactive to shrimp, but presenting a lower level of sensitization to its recombinant tropomyosin, did not show an increase in IFN-γ expression after incubation.

Besides the non-allergic states, low levels of IL-4 expression may be due to the absence of regular contact with the inducer allergens for a long period of time, like what happens with pollens out of the pollinic season [43] or by avoiding a certain food, which is a frequent condition associated with food allergies such as this, where clinical reactions may show a taquifilactic pattern through successive ingestions, with a clear worsening tendency. IL-4 expression is also one of the hardest cytokine

to be induced *in vitro*[44]. However, when cytokine production by allergen-specific T-cells is evaluated after *in vivo* challenge another important aspect should be noted. Cytokine detected production from collected cells may be under-evaluated, possibly because of their pattern of expression, along with antigen specific selection mechanisms, which may be associated with the migration of activated lymphocytes into the target organs and subsequent depletion of those subpopulations in blood stream [45]. This biological behaviour is obviously out of an *in vitro* challenge and so we are driven to think that despite the absence of natural pathophysiological environment, the *in vitro* system presents some important complementary advantages for research proposals.

Variation in IL-5 and IL-13 expression following incubation with rDer p 10 revealed to be mostly subtle. However, stimulation with rDer p 10 induced a significant rise in IL-5 and IL-13 expression on CD4 T-cells from *phenotype E*, sensitized to shrimp probably trough the tropomyosin (amount of specific IgE to shrimp very close to specific IgE to tropomyosin). IL-5 and IL-13 expression revealed to be higher in IFN-γ^{+} CD4 T-cells from this patient, suggesting an allergen-specific production of IFN-γ [46]. A dose-dependent effect of rDer p 10-induced expression of IL-5 and IL-13 in *phenotype E* is also suggestive of a specific response.

Tropomyosin is probably the main factor responsible for the mite-shrimp cross-reaction, demonstrated by the 91,9% inhibition of the shrimp RAST by the mite extract [15].

As we can observe in this study, there are different patterns of cytokine response to stimulation, according to each phenotype or even individual. Katial *et al*[43] reported, in addition to IL-2 and IFN-γ responses in the same direction after stimulation with PHA, levels of IL-10 (Th2 pattern) positively correlated with IFN-γ, and of IL-4 with TNF-Î², after stimulation with concanavalin A mitogen. Hence, the proper response to mitogen stimulation may show differences from the classical Th1/Th2 paradigm. In our study, CD4 T-cells from *phenotype E* also presented a

rise in IFN-γ along with IL-5 and IL-13, and the non-atopic *phenotype A* expressed an increase in IL-4 and IL-13 precisely in the IFN-γ^{+} CD4 T-cells, upon stimulation with rDer p 10. Nevertheless, the spectrum of target cells may differ according to the mitogen [44] and the stimulating effect of an allergen may also be distinct from a mitogen [46;47]. A possible evidence of these was also observed in our study, when a higher expression of IL-5 and IL-13 was observed in *phenotype E* after stimulation with rDer p 10 rather than with PHA.

These findings point to the need of further studies, increasing the number of patients filling different phenotypes, to extend the results and clarify our hypothesis.

Further immunochemical and cell studies with more available purified and recombinant antigens, and with a wider studied population for each of the most important phenotypes is crucial for better understanding of the mite-mollusk-crustacean cross-reaction, allowing a better prevention of possible unpleasant reactions.

Identification of each patient allergen repertoire, which is highly dependent on individual variability, demands simultaneous immunochemical allergen recognition over two-dimensional protein separations from different sample extracts, and allows a better immunotherapy fine tune focus. The study of the immunochemical isotype response also plays an important role in the evaluation of the efficiency of possible immunotherapeutic strategies [48] along with a better component-resolved diagnosis.

mRNA studies by quantitative real-time polymerase-chain-reaction (RT-PCR) [49] may reveal to be of great utility for the evaluation of cytokines like IL-4, with a short half-life and a low amount of synthesis upon stimulation, which affects the ability of a single method for the follow-up.

From the economic point of view, the standardization of sub-optimal doses of mitogens as co-stimulators, not interfering with the specificity of the response [50], may speed the response and thus, save time and

financial resources of the costly co-stimulator MoAb.

In the near future, besides the phenotypic study strategies, genomic focus will certainly help the study of polymorphisms associated to immune system triggers, already pointed out as targets for future therapeutics [51]. Genetic mechanisms responsible for the induction of T-cell tolerance should play an important role in this domain, possibly standing as a key to the mechanism of immune recognition of â€œselfâ€ and â€œnot selfâ€ non-infectious antigens, where the majority of allergens are inserted [52].

On the phenotypic side, advances in progress in flow cytometry, allowing the simultaneous detection of more than ten different molecules in each cell or sub-cellular particle [53] in association with techniques, such as the â€œCytometric Bead Arrayâ€ (CBA) (Becton Dickinson) [37], will also be very helpful.

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Endnotes

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