

Mononuclear cell reactivity to food allergens in neonates, children and adults

Miceli Sopo S, Pesaresi MA, Guerrini B, Federico G, Stabile A.
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A model of antigen-specific T-cell proliferative responses based on reciprocal patterns of responses to dietary and inhalant allergens has been suggested, the former being frequent in infancy but rare in adults, whereas the latter are preserved and expand between infancy and adulthood. We have evaluated the age-related variations of mononuclear cell reactivity to food allergens. The cord blood mononuclear cells (CBMC) of 30 neonates without family history of atopy and the peripheral blood mononuclear cells (PBMC) of 20 healthy children and of 40 healthy adults were stimulated *in vitro* with β -lactoglobulin (BLG) or ovalbumin (OVA) and the cultures were harvested after 7 days.

Neonates, children and adults were compared for the percentages of positive responses and for the magnitude of response. Adult subjects showed significantly lower percentages of positive responses and reduced magnitude of response than those observed in neonates and children either in BLG or in OVA cultures. We have not observed a decrease of food allergen mononuclear cell reactivity between neonates and children for the frequency of positive responses. The magnitude of response of neonates was significantly lower than that of children in BLG cultures. Our results seem to confirm the loss of mononuclear cell reactivity to food allergens in adult age. However, other reports show conflicting data. We suggest that a rigorous standardization of the methodological steps of *in vitro* mononuclear cell stimulation with allergen is necessary.

**Stefano Miceli Sopo,
Maria Assunta Pesaresi,
Barbara Guerrini,
Gilda Federico and Achille Stabile**

Department of Pediatrics, Catholic University
of Rome, Italy

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Dr Stefano Miceli Sopo, via Trionfale 8250,
00135 Rome, Italy
Tel: +39 06 3053065
E-mail: stefano.micelisopo@mailcity.com

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It has been suggested in various reports (1–5) that the lymphocyte response to allergens is initiated in infancy and early childhood and occurs in infants both with and without a family history of atopy; moreover, both normal and atopic adult subjects have allergen-specific Th cells (albeit from different functional subsets) in peripheral blood mononuclear cells (PBMC) (6).

Some authors (2) have recently proposed a model for the development of immunity to environmental allergens which involves allergen-driven T-cell selection during early life. This leads to the deletion of food allergen-specific T-cells via the induction of specific anergy, with concomitant selection and ultimately expansion of mutually exclusive Th1-like or Th2-like reactivity to inhalant allergens via immune deviation mechanisms. The model is consistent with a report (7) on antigen-specific T-cell proliferative responses. This showed that the responses to

inhalant allergens increased with age both in frequency and intensity and that the reverse pattern was seen for food allergens, i.e. the responses were more frequent during neonatal life, and those persisting into adulthood remained within the low intensity range.

We have also studied the age-related variations of mononuclear cell reactivity to allergens, limiting our observations to food allergens, particularly β -lactoglobulin (BLG) and ovalbumin (OVA), in neonates without a family history of atopy and in healthy children and adults.

Materials and methods

Subjects and blood sampling

The parents of 30 neonates (19 males, 11 females), without a family history of atopic diseases and born at term by natural delivery, were contacted

before the birth of their child. Cord blood samples were collected in heparinized tubes immediately after delivery with the usual procedures including careful cleaning of the cord and strict puncture of the umbilical vein to avoid maternal contamination. Total serum IgA was measured in all cord samples by a standard nephelometric technique (Beckman IgA test kit; Beckman Instruments Inc., Brea, California, USA). If the IgA level was > 1.8 mg/dl the sample was considered contaminated.

In addition, we have enrolled 20 healthy children (12 males and 8 females, median age 28 months, 12th centile = 14 months, 88th centile = 45 months) and 40 healthy adults (21 males and 19 females, median age 29 years, 13th centile = 23 years, 90th centile = 42 years). We have defined as 'healthy' a child or adult who had no history of allergic disease and did not present signs or symptoms of disease. Moreover, the family history was also negative for allergic disease in all subjects.

Blood samples were collected after obtaining informed consent from subjects or their parents and approval from the hospital ethics committee was obtained. The study groups were comparable in terms of family size, parental education and socio-economic state.

Mononuclear cell stimulation *in vitro*

Cord blood mononuclear cells (CBMC) and PBMC of children and adults were obtained by Ficoll-Hypaque gradient centrifugation and then washed three times with Ca^{2+} - and Mg^{2+} -free phosphate-buffered saline. Thereafter, CBMC and PBMC were resuspended at concentration of 1×10^6 cells/ml in culture medium (RPMI 1640, Flow Laboratories, Irvine, UK), supplemented with antibiotics (100 U/ml of penicillin and 100 mg/ml of streptomycin, Flow Laboratories), l-glutamine (2 mmol/l, Gibco Laboratories, Grand Island, New York, USA) and 10% autologous human serum. Into 96-well, flat-bottomed tissue plates (Falcon 3070, Beckton Dickinson Labware, New Jersey, USA), 2.5×10^5 CBMC or PBMC in 250 μl of culture medium were placed. The cells were incubated at 37°C in a humidified atmosphere with 5% CO_2 in the presence of BLG (100 $\mu\text{g}/\text{ml}$) or OVA (100 $\mu\text{g}/\text{ml}$) (both kindly donated by SARM Allergeni, Pomezia, Italy) added to triplicate wells. The purity of BLG and OVA was warranted by the manufacturer. Negative control wells received culture medium alone. The antigen doses were selected as being optimal on the basis of the results of stimulation of 10

samples from subjects with ages ranging from 0 to 20 years, with concentrations ranging from 50 ng/ml to 200 $\mu\text{g}/\text{ml}$. During the last 16 h of incubation, the cells were pulsed with 0.5 $\mu\text{Ci}/\text{well}$ of methyl- ^3H -thymidine (6.7 Ci/mmol, NEN Products, Boston, Massachusetts, USA) and after 7 days the cells were harvested. The incorporation of thymidine into DNA was determined by liquid scintillation counter (Beckmann LS 7000) and radioactivity expressed as stimulation index (SI; mean counts per minute (cpm) of triplicate stimulated cultures/mean cpm of triplicate unstimulated cultures). We have examined our results in terms of both the usual criterion of $\text{SI} \geq 2$ and the more conservative criterion of $\text{SI} \geq 3$ indicating a positive response.

Statistical analysis

In addition to general descriptive statistics, groups were compared with the non-parametric Kruskal-Wallis test and Dunn test for medians and centiles, and with the χ^2 test and Fisher exact test for percentages. Differences were considered significant when $p < 0.05$. The data were analyzed by the program Primer of Biostatistics of S. A. Glanz.

Results

None of the cord blood samples was excluded because all had an IgA level < 1.8 mg/dl. Each group has been compared with the other two for the parameters we considered.

Percentages of positive responses after the mononuclear cell stimulation test (MCST) with BLG and OVA in the three age groups

When we employed the criterion of $\text{SI} \geq 3$, a reduction of positive responses between neonates and adults (to BLG: 10% versus 0%; to OVA: 26% versus 7.5%) was evident but not significant. The positive responses were more numerous (but differences were not significant) in children than in neonates (to BLG: 25% versus 10%; to OVA: 40% versus 26%). Significant differences were observed between children and adults regarding the frequencies of positive responses to BLG (25% versus 0%, $p = 0.003$) and to OVA (40% versus 7.5%, $p = 0.004$). When we employed the criterion of $\text{SI} \geq 2$ (Fig. 1), this trend was more marked and significantly more numerous responses were observed in neonates and children compared to adults.

Magnitude of proliferative response after MCST with BLG and OVA in the three age groups

As shown in Fig. 2, the MCST responses in adults were of significantly lower magnitude than those of neonates and children. We observed significant differences between neonates and children in the BLG-stimulated cultures. The children showed a wide range of responses, perhaps related to the non-homogeneity of their ages when compared to the other two groups.

Discussion

We have observed a decrease of *in vitro* mononuclear cell reactivity to food allergens as age increases, even though this correlation was not always linear. Adult subjects showed a significantly lower number of positive responses than neonates and children to OVA, and than children to BLG; however, the children and not the neonates showed the highest percentages of positive responses. On the basis of our results and of the recent literature, we make some suggestions about the age-related variations of the lymphocyte proliferative response to specific antigens, especially to food antigens of milk and egg.

Even though the *in vitro* response of mononuclear cells to food and inhalant allergens has been evaluated by many researchers for many years, the method has not been standardized. Differences are present at many steps of the assay system: cell separation and washing, dose of allergen, culture time and assessment of DNA synthesis. This produces a great variability of results, that are often contradictory.

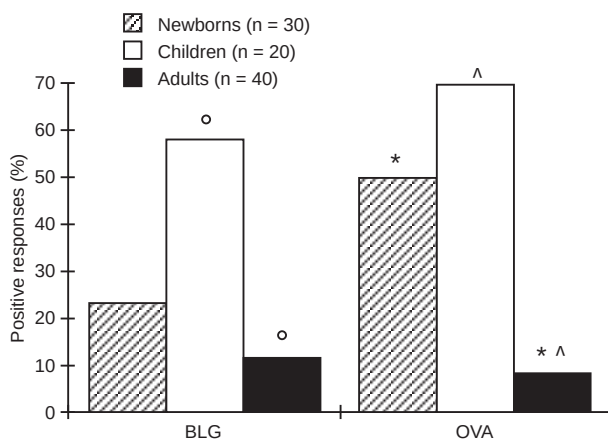


Fig. 1. Percentages of positive responders ($SI \geq 2$) after BLG or OVA stimulation among neonates, children and adults. Data were analyzed with the χ^2 test and Fisher exact test. Significant differences: °p = 0.014; *p = 0.025; ^p = 0.006.

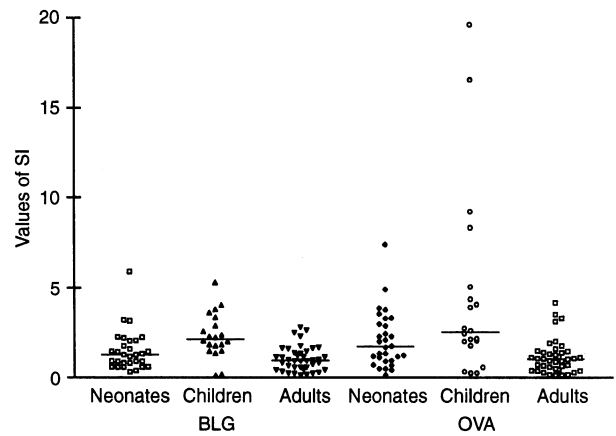


Fig. 2. The SI after BLG and OVA stimulation of PBMC cultures of all studied subjects are represented and subdivided according to the applied stimulus and the age group. Each group was compared against the other two (neonates' BLG SI versus children's and adults' BLG SI, etc.). Medians are represented as bars. The differences were analyzed by the Kruskal-Wallis test and the Dunn test. Significant differences ($p < 0.05$) were observed between neonates and children and between children and adults for SI after BLG stimulation, and between neonates and adults and between children and adults for SI after OVA stimulation.

The presence of age-related variations of allergen T-cell reactivity was first stressed by Holt et al. (7). In their experiments, partially in keeping with our results, the frequency of responses to OVA, but not the magnitude, decreased with increasing age among three groups of subjects (neonates, children and adults). These authors (7) introduced a new variant into the antigen-specific mononuclear cell proliferative methodology, which is a culture system based upon serum-free medium (SFM), which, according to their experience, markedly increases the capacity to detect mononuclear cell responses. Also, Upham et al. (8) have reported that, adopting a SFM and a SI criterion ≥ 3 , the PBMC positive proliferative responses to OVA were fewer, without significant differences, in the adult group than in the infant and children groups. If the SI criterion ≥ 2 was considered, the percentages of positive responses slightly increased with age. Iida et al. (9) have reported that the PBMC positive proliferative responses to food antigens were more numerous for young children and lower for older ones, but the positive responses were all in atopic subjects, suffering from food-sensitive atopic dermatitis. The normal controls and the patients with non food-sensitive atopic dermatitis did not show positive responses. Other reports have not reported similar age-related variations of allergen mononuclear cell reactivity. Yabuhara et al. (10) have observed, using an SFM, percentages of

positive responses to OVA stimulation that were twice as high in the adult samples compared with infant samples.

When considering the magnitude of the response, our results are comparable with those of Holt et al. (7); however, we have observed that in the adult group the medians of SI were significantly lower than those observed in neonates or children. Also, Iida et al. (9) have shown that proliferative responses of PBMC to OVA or bovine serum albumin (BSA) in atopic dermatitis patients more than 6 years old were of significantly ($p < 0.05$) lower magnitude than those in patients aged 1–5 years or < 1 year. By contrast, Yabuhara et al. (10) have shown that the medians of proliferative responses both to house dust mite and to OVA increased with age. Warner et al. (11), after stimulation with BLG and OVA and employing a culture medium supplemented with 10% autologous plasma, have obtained much more elevated SI values than those of the above-mentioned studies.

Each of the above studies report cell-culture techniques which differ from the others in some respect. In the studies by Holt's group (7,8,10), cells were cultivated in a medium without serum (SFM), the doses of OVA and of bovine serum albumin (BSA) were 100 $\mu\text{g/ml}$ (doses that were selected, as also mentioned in the other studies, as being optimal based on the results of stimulation of PBMC with a range of OVA and BSA concentrations), and the culture was performed for 7 days. Warner et al. (11) employed a medium supplemented with 10% plasma, the doses of BLG or OVA were 10 $\mu\text{g/ml}$, and the culture was performed for 7 days. Iida et al. (9) cultivated the PBMC in a medium to which 15% human AB serum had been added, the doses of BSA or OVA were 2.5–25 $\mu\text{g/ml}$, and the duration of the culture was 4 days, chosen on the basis of former experiences. Such variables can influence the cell response and it is not possible, in our opinion, to demonstrate which technique should be considered the best, by analysis of the results. In all the studies, as in ours, PBMC were not further separated, even though in the comments on their results the authors discuss T-cell reactivity. Szepfalusi et al. (12) have reported, studying only CBMC, that T cells alone did not proliferate in response to various milk proteins, indicating the requirement for antigen-presenting cells.

In conclusion, we consider, in keeping with Upham et al. (8), that so-called T-cell culture is

'more of an art than a science'. Thus it will be necessary to confirm results by all groups adopting a method that is identical in every step rather than the current state of affairs with conflicting methods and consequently results.

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