

Is the Betalactoglobulin- and Ovalbumin-Induced *In Vitro* Production of Interferon-Gamma and Interleukin-4 Predictive for Atopy in the Neonate? A Prospective Cohort Study and a Review of the Literature

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ABSTRACT

Some studies suggest that a reduction of interferon-gamma (IFN γ), *in vitro* production by cord blood mononuclear cells (CBMC) could be considered as a marker of atopic predisposition. The aim of our study is to estimate the usefulness of testing interleukin-4 (IL4) and IFN γ in CBMC culture supernatants after stimulus with betalactoglobulin (BLG) and ovalbumin (OVA) in predicting atopic disease. The possible correlation between the *in vitro* production of IL4 and IFN γ and cord blood IgE levels was also investigated. Our sample consisted of 68 neonates; among them, 28 had no atopic family history (group 0), 21 had only one parent suffering from an atopic disease (group 1), and 19 had both parents (group 2). CBMC were stimulated *in vitro* by 100 μ g of betalactoglobulin (BLG) or ovalbumin (OVA) for 24 or 48 h. IL4 and IFN γ in the culture supernatants and cord blood IgE levels were quantified by commercial kits. Detectable production of IL4, undetectable production of IFN γ , and cord blood IgE levels of ≥ 0.7 kU/L were considered as positive responses. The infants attended periodic examination, thus allowing us to follow the development of atopic disease. A total of 16/19 neonates with biparental atopic family history showed undetectable production of IFN γ after stimulus with BLG versus 14/28 without atopic family history ($p = 0.037$) and versus 14/21 with monoparental atopic family history ($p = 0.281$). Similar results were obtained after OVA stimulation. IL4 production was not significantly different among the three groups. IgE cord blood were measured in 55 neonates. Six of seven neonates with cord IgE of ≥ 0.7 kU/L did not produce IFN γ versus 24/48 neonates with IgE of ≤ 0.7 kU/L ($p = 0.112$); 1/7 of the neonates with cord IgE of ≥ 0.7 kU/L produced IL4 versus 23/48 neonates with IgE of ≤ 0.7 kU/L ($p = 0.122$). The clinical follow-up (up to 60 months) involved 56/68 children. Twenty-two neonates developed atopic disease (18 with atopic family history versus 4 without, $p = 0.012$). *In vitro* at birth production of IFN γ and IL4 in atopic and in nonatopic children were compared, and no significant differences were observed. Four of 22 atopic children versus 3/33 nonatopic children ($p = 0.419$) showed cord blood

IgE levels of ≥ 0.7 kU/L. At birth, an association among cord blood IgE of ≥ 0.7 kU/L, undetectable IFN γ production, and atopic family history was shown in 4/22 of the atopic children versus 1/33 nonatopic children ($p = 0.072$). The laboratory tests we examined did not represent a better index of high risk in developing atopic disease than the atopic family history. After the review of the literature, we concluded that the evaluated studies did not suggest that an *in vitro* IFN γ at birth was a reliable marker of atopy. (Pediatr Asthma Allergy Immunol 2003; 16[2]:85–96.)

INTRODUCTION

FAMILY HISTORY OF ATOPY is at present the main indicator for identifying neonates with high, intermediate, and low risk of developing atopic disease. Several laboratory tests have been evaluated, but none of them have shown a sensitivity and a specificity high enough to be used as a screening for atopy. It is well known that subjects suffering from an atopic disease show increased serum levels of total and specific IgE, increased levels of interleukin-4 (IL4), a cytokine that is able to induce IgE production, and reduced levels of interferon-gamma (IFN γ), which inhibits the IgE production.⁽¹⁾ Thus, it was proposed that a lack of balance of IFN γ and IL4 could be present at birth in neonates at risk of atopy. However, very low serum levels of these cytokines in neonates do not allow *in vivo* measurement. Most researchers have induced an amplified production of IL4 and IFN γ through *in vitro* stimulation of mononuclear cells with an aspecific stimulus such as PHA or a specific stimulus, such as betalactoglobulin (BLG). In many studies, only IFN γ levels were detectable and IL4 production was below the sensitivity of the commercial kits. The results of these studies are controversial,^(2–14) and their results are shown as differences among medians or means of IFN γ *in vitro* production. Medians and means are useful to analyze the distribution of a characteristic in a population, but are not reliable indicators to determine sensitivity, specificity, positive and negative predictive value, or the likelihood ratio of a test in the single subject. Specifically, two studies of Warner et al⁽⁵⁾ and Kondo et al⁽¹¹⁾ evaluated the correlation between at birth IFN γ *in vitro* production after stimulus with milk and egg allergens and the development of atopic diseases. Both of them highlighted a lower IFN γ average production at birth in atopic children than in the nonatopic ones. The atopic patients tested in both studies almost exclusively developed atopic dermatitis (AD), indicating a high predictivity of the test for this disease.

We carried out a study similar to the one of Warner et al⁽⁵⁾ and Kondo et al⁽¹¹⁾ in which the results are expressed as “positive” or “negative” test. We searched for an association between the results of the test and the development of atopic diseases. Our results do not show positive associations. We discussed our results and reviewed similar previous studies in the literature, suggesting the reasons for the contrasting results.

MATERIALS AND METHODS

Primary objective of the study and estimate of the sample size

The primary objective of the study was to evaluate the usefulness of the following *in vitro* tests as reliable markers of atopy:

- At birth *in vitro* production of IFN γ after stimulus with BLG and OVA
- At birth *in vitro* production of IL4 after stimulus with BLG and OVA
- Cord blood IgE
- A combination of these tests with atopic family of history

The neonate has parents who are both atopic and has an estimated risk of atopic disease of 60–70%. As a consequence, we assumed that a laboratory test (or a combination of two or more of these tests) to be used as an atopy marker must have at least the same predictive power of the family history of biparental

atopy to be used as an atopy marker. Specifically, the distribution of the subjects tested should be as the one in the following 2×2 table:

	<i>Atopics</i>	<i>Nonatopics</i>
Positive to test	70%	30%
Negative to test	30%	70%

The total number of individuals to be tested according to this estimate is equal to 47 (with an α -error = 0.05 and β -error = 0.20) according to the results obtained using the program Primer of Biostatistics of Stanton Glanz, (version 4.02i). Obviously, a greater discriminatory capability would have required a lower number of individuals tested.

Subjects

Parents of 70 full-term newborn infants (40 males, 30 females) were contacted before the birth of their children. We included the neonates that consecutively delivered at the Obstetric Clinic of the Catholic University of Rome within the same group according to the family history of atopy. The study was fully explained to the parents and informed consent was obtained. The study was approved by the ethical committee of the Department of Pediatrics. Two of the 70 couples we interviewed refused to participate in the study. Therefore one female with and one male without monoparental atopic family history were excluded from the study. The mothers did not take any special precaution regarding their diet during pregnancy. None of the parents was given any special advice about allergy prevention. The infants were regarded as having positive family history when one or both parents had an atopic disorder. The following symptoms were regarded as evidence of atopic disease in the parents: (1) asthma—recurrent episodes of wheezing or dyspnea or medically confirmed asthma; (2) food allergy—weeping, erythematous itching, papular, and vesicular lesions involving primarily the face and the flexural folds; recurrent vomiting, diarrhea, or crampy abdominal pain and/or urticarial skin reactions from a specific food and (3) rhinitis—episodes of nasal itching, paroxysms of sneezing, sniffing with nasal discharge, and/or congestion without infectious components. The allergic diagnosis was confirmed by skin prick tests; when a food allergy was suspected, double-blind placebo-controlled food challenge (DBPCFC) was performed.⁽¹⁵⁾ According to these definitions, 28 neonates had no family history of atopy (group 0), 21 had uniparental (group 1), and 19 biparental (group 2) family history of atopy.

Blood sampling

Cord blood samples from all neonates were aseptically collected in heparinized test tubes (Falcon 2070, Becton Dickinson, Franklin Lakes, NJ) immediately after delivery. Usual procedures were adopted, including careful cleaning of the cord and strict puncture of the umbilical vein, in order to avoid maternal blood contamination. Total serum IgA were measured in all cord samples by a standard nephelometric technique (Beckman IgA test kit; Beckman Instruments Inc., Brea, CA). When IgA level was >1.8 mg/dL, the sample was considered contaminated⁽¹⁶⁾ and rejected. An aliquot (1 mL) of every blood sample was centrifuged to obtain autologous serum.

Mononuclear cell isolation

CBMC were isolated from heparinized cord blood by Ficoll-Hypaque gradient centrifugation (Pharmacia Biotech, Uppsala, Sweden), and washed three times with Ca^{2+} and Mg^{2+} free phosphate-buffered saline (PBS). Thereafter, CBMC were resuspended at concentration of 1×10^6 cells/mL in culture medium (RPMI 1640, Flow Laboratories, Irvine, Scotland), supplemented with antibiotics (100 U/mL of penicillin and 100 mg/mL of streptomycin, Flow Laboratories, Irvine, Scotland), L-glutamine (2 mmol/L, Gibco Laboratories, Grand Island, NY), and 5% heat-inactivated autologous human serum.

Mononuclear cell stimulation assay and cytokine production

CBMC (2.5×10^5) in 250 μL of culture medium were placed into 96-well, sterile flat-bottom tissue plates (Falcon 3070, Beckton Dickinson Labware). Cells were incubated at 37°C in a humidified atmos-

phere with 5% CO₂ at the presence of BLG (100 µg/mL, kindly donated by SARM Allergeni, Pomezia, Italy) or OVA (100 µg/mL, kindly donated by SARM Allergeni) added to triplicate wells. Purity of food allergens was warranted by the manufacturer. The antigen doses were selected as being optimal from the results of stimulation of 10 CBMC samples with concentrations of 50 ng/mL to 200 µg/mL. Supernatants of the stimulated cultures were harvested after 24 or 48 h of incubation and stored at -70°C until testing for IL4 and IFNγ production, respectively.

Determination of IL4 and IFNγ

In the culture supernatants IL4 and IFNγ were quantified by ELISA kits (Intertest-4, Genzyme Corporation, Cambridge, MA, minimum sensitivity 3 pg/mL IL4 and Intertest-gamma, Genzyme Corporation, Cambridge, MA, minimum sensitivity 3 pg/mL IFNγ). Briefly, 96-well microtiter plates, coated overnight with monoclonal antibody specific for human IL4 (or IFNγ), were used to capture IL4 (or IFNγ) present in standard samples and unknown specimens. A goat polyclonal antibody, which is able to bind multiple epitopes on the IL4 (or IFNγ) contained on the solid phase, was added to the culture for 2 h. A third antibody, biotin-labeled donkey-anti-goat IgG, which binds to the goat polyclonal antibody already bound to IL4 (or IFNγ), was then applied for 45 min. Streptavidin-peroxidase, which binds to the biotin on the third antibody, was added for 40 min. Finally, the substrate reagent (OPD chromagen dissolved in peroxide reagent solution) was dispensed into each well. Increased absorbances due to bound, immunoreactive IL4 (or IFNγ) were quantified by an ELISA reader. The IL4 (or IFNγ) concentrations of our samples were determined comparing their absorbances with those obtained from the known amounts of human IL4 (or IFNγ) in the standard dilutions. Regarding the determination of IFNγ, we have considered an undetectable level of this cytokine as a positive response because of its inhibiting role on IL4 and IgE synthesis. On the contrary, detectable levels of IL4 were considered as positive responses.

Cord blood IgE analysis

Cord blood IgE analysis was performed by a non-modified radioimmunologic kit (Pharmacia Diagnostic AB, Uppsala, Sweden). This kit is able to determine IgE in serum or plasma within a range of 0.04–50 KU/L. We considered high a level of IgE of ≥0.7 KU/L.⁽¹⁷⁾

Clinical follow-up

The infants were examined at the ages of 1, 2, 3, 6, 12, 18, 24, 36, 48, and 60 months with consulting room examinations by a staff physician. Parents filled in a questionnaire and carried on a structured interview. Physical examinations were conducted according to standard procedures, and special attention was paid to skin signs. Clinical criteria defining allergy were the same we used to define atopy in the parents. AD was diagnosed according to Hanifin's criteria.⁽¹⁸⁾ Children with evidence of atopic disease were skin tested and food challenges were conducted in a DBPC manner.⁽¹⁵⁾ In order to avoid any unwanted interference in children's evaluation, infants' parents and the examining physician did not know the results of the test before the clinical follow-up.

Statistical analysis

Medians of the at-birth values of IFNγ and IL4 after BLG or OVA *in vitro* stimulation were compared among the subjects of the group 0, 1, and 2 (Kruskal-Wallis test) and between the subjects of the atopic and nonatopic groups (Mann-Whitney test). Non parametric tests were employed as the data did not follow the normal distribution. For a correct analysis of the results, the values under the sensitivity value of the method were considered as half of the sensitivity value, which means 1.5 pg/mL in our case, as suggested by Khalak et al.⁽¹⁹⁾

The χ² test and the Fisher exact test were used to compare the number of subjects with positive tests in the three groups of neonates with different atopic family history and in the two groups of atopic and nonatopic children. Differences were considered significant when $p < 0.05$. Sensitivity, specificity, positive and neg-

ative predictive value, and likelihood ratio (LR) for positive and negative result of the laboratory markers we used were also evaluated. These parameters were calculated as described by Jaeschke et al.⁽²⁰⁾

RESULTS

Clinical follow-up

Fifty-six out of the 68 children enrolled in the study underwent periodic consulting room examinations. Twelve children were lost to the follow-up because a change of address occurred after birth. Follow-up lasted 60 months. Atopic disease developed in 22 of 56 children (39%, 14 males and eight females, 18 with atopic family history versus four without atopic family history, $p = 0.012$). Nine of 22 atopic children had asthma, in one case preceded by AD, and seven rhinoconjunctivitis due to inhalant allergens (dermatophagoides, grass pollen, parietaria judaica), and six AD due to food allergy (four to cow milk, one to egg, and one to fish). Thirty-four children (19 males and 15 females, 15 with positive family history of atopy) did not develop atopic disease during the follow up.

TABLE 1. MEANS, STANDARD DEVIATIONS (SD), STANDARD ERRORS (SE), MEDIANS, AND RANGES OF AT BIRTH *IN VITRO* PRODUCTION OF IFN γ AND IL4 AFTER STIMULUS WITH BLG AND OVA IN THE GROUPS OF NEONATES 0, 1, AND 2 AND IN ATOPIC AND NONATOPIC CHILDREN

	<i>IL4-BLG</i> (pg/mL)	<i>IL4-OVA</i> (pg/mL)	<i>IFNγ-BLG</i> (pg/mL)	<i>IFNγ-OVA</i> (pg/mL)
Group 0				
Mean	57.9	28.1	37.9	30.8
SD	128.9	59.3	57.9	32.4
SE	26.8	12.36	12.1	6.8
Median	1.5	1.5	8*	34
Range	1.5–512	1.5–220	1.5–200	1.5–110
Group 1				
Mean	25.7	17.2	10.7	15.2
SD	66.6	31.4	19.3	27.5
SE	16.2	7.6	4.7	6.7
Median	1.5	1.5	1.5	1.5
Range	1.5–210	1.5–92	1.5–60	1.5–94
Group 2				
Mean	21.7	21.7	6.5	55.1
SD	42.5	38.9	19.1	129.3
SE	10.6	9.7	4.8	32.3
Median	1.5	1.5	1.5*	1.5
Range	1.5–150	1.5–130	1.5–78	1.5–512
Atopics				
Mean	46.34	15.4	18.3	17.9
SD	115.44	27.5	44.5	27.7
SE	24.61	5.9	9.5	5.9
Median	1.5	1.5	1.5	1.5
Range	1.5–512	1.5–90	1.5–200	1.5–100
Nonatopics				
Mean	32.3	27.9	22.2	42.8
SD	76.5	55.9	40.8	91.3
SE	13.1	9.6	7	15.7
Median	1.5	1.5	1.5	1.5
Range	1.5–360	1.5–220	1.5–150	1.5–512

* $p = 0.012$.

Measurement of the in vitro production of IFN γ and IL4

The comparison of medians of IFN γ and IL4 *in vitro* production of groups 0, 1, and 2 are shown in Table 2 while the ones for atopics and nonatopics are in Table 1. No significant differences were found, except for the difference between *in vitro* production of IFN γ after stimulus with BLG in group 2 and in group 0 ($p = 0.012$). IL4/IFN γ ratios did not significantly differ between atopic and nonatopic children. Figure 1 shows the percentages of positive responses according to the stimulus of CBMC *in vitro* and to the atopic risk due to the family history. In group 2, the number of subjects with undetectable IFN γ (positive responses) was significantly higher than the one observed in group 0 after stimulus with BLG ($p = 0.037$) or OVA ($p = 0.031$); no significant differences between the positive responses in neonates of the group 2 and of the group 1 were observed. The number of subjects with detectable IL4 (positive responses) after stimulus with BLG or OVA did not show significant differences among the three groups of neonates. The production of cytokines was then related to the development of atopic disease according to the data of the clinical follow-up. As shown in Figure 2, no significant differences were found between atopic and non atopic children.

Sensitivity, specificity, positive and negative predictive value, and LR of cytokine production after stimulus with BLG or OVA and cord blood IgE measurement are shown in Table 2. A neonate with biparental

TABLE 2. SENSITIVITY, SPECIFICITY, POSITIVE AND NEGATIVE PREDICTIVE VALUE, AND LIKELIHOOD RATIOS OF THE TESTS PERFORMED AT BIRTH IN THE THREE GROUPS OF NEONATES AND IN ALL TOGETHER NEONATES

	IL4-BLG	IL4-OVA	IFNg-BLG	IFNg-OVA	IgE	PAFH	IgE + IFNg	IgE + IFNg + PAFH
Sensitivity								
Group 0	50	50	25	25	0		0	
Group 1	21	21	78	67	33		33	
Group 2	32	21	100	78	11		11	
All neonates	32	26	76	64	18	81	18	18
Specificity								
Group 0	62	74	46	46	88		94	
Group 1	87	62	37	37	100		100	
Group 2	86	70	28	43	88		87	
All neonates	73	70	40	43	85	55	94	97
PPV								
Group 0	21	28	8	8	0		0	
Group 1	67	40	57	54	100		100	
Group 2	75	50	63	64	50		50	
All neonates	44	37	46	41	57	54	67	80
NPV								
Group 0	86	87	75	75	80		81	
Group 1	50	42	60	50	57		57	
Group 2	50	42	100	60	57		47	
All neonates	62	60	74	64	37	82	64	64
LR +								
Group 0	1.4	1.9	0.5	0.5				
Group 1	2	0.7	1.3	1.2				
Group 2	2.3	0.7	1.4	1.4				
LR -								
Group 0	0.8	0.7	1.5	1.5				
Group 1	0.9	1.2	0.5	0.9				
Group 2	0.6	1.1	0	0.5				

These parameters were calculated in relation to the development of atopic disease.

PPV, positive predictive value; NPV, negative predictive value; LR +, likelihood ratio for a positive result; LR -, likelihood ratio for a negative result; PAFH, positive atopic family history.

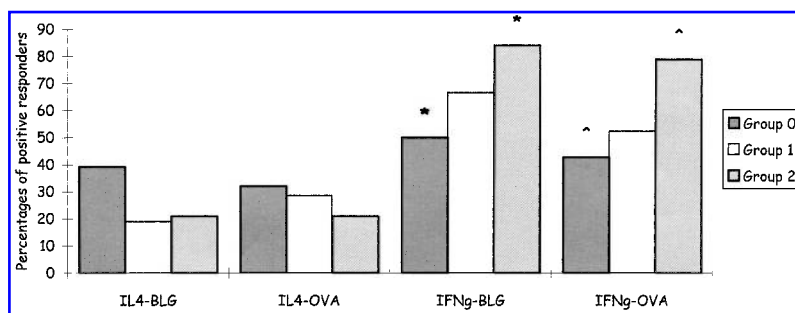


FIG. 1. Percentages of subjects with detectable IL4 and undetectable IFN γ after BLG or OVA stimulation (positive responders) among the three groups of neonates with different family history of atopy. * $p = 0.037$; ^ $p = 0.031$.

atopic family history and detectable production of IFN γ after BLG stimulus was the best combination (sensitivity = 100%; negative predictive value = 100%; LR for a negative result = 0). The other LRs did not modify substantially the pre-test probability of developing an atopic disease.

Cord blood IgE levels

Cord blood IgE levels were analyzed in 55 neonates. Four of 22 atopic children versus 3/33 nonatopic ($p = 0.419$) showed cord blood IgE levels of ≥ 0.7 kU/L (Table 1). We also related the IgE levels to the cytokine production. Six of seven neonates with cord blood IgE of ≥ 0.7 kU/L did not produce IFN γ after stimulus with BLG or OVA versus 24/48 neonates with IgE of < 0.7 kU/L ($p = 0.112$); 1/7 neonates with cord blood IgE of ≥ 0.7 kU/L produced IL4 after stimulus with BLG or OVA versus 23/48 neonates with IgE of < 0.7 kU/L ($p = 0.122$). The only significant difference we observed was that 6/7 neonates with cord blood IgE of ≥ 0.7 kU/L versus 16/48 with cord blood IgE of < 0.7 kU/L ($p = 0.013$), after stimulus with BLG or OVA, did not produce measurable levels either of IFN or of IL4.

Sensitivity, specificity, positive and negative predictive value, and LR of the combination of cord blood IgE levels, in vitro cytokine production, and atopic family history

The combination of cord blood IgE of ≥ 0.7 kU/L, undetectable levels of IFN γ after stimulus of CBMC with BLG or OVA, and atopic family history was found in four (18%) atopic children and in one (3%) nonatopic child ($p = 0.072$). For this combination, the LR for a positive result was 6. Sensitivity, specificity, positive and negative predictive value of the combination of the evaluated parameters are reported in Table 2. When monoparental atopic history, cord blood IgE of ≥ 0.7 kU/L and undetectable *in vitro* lev-

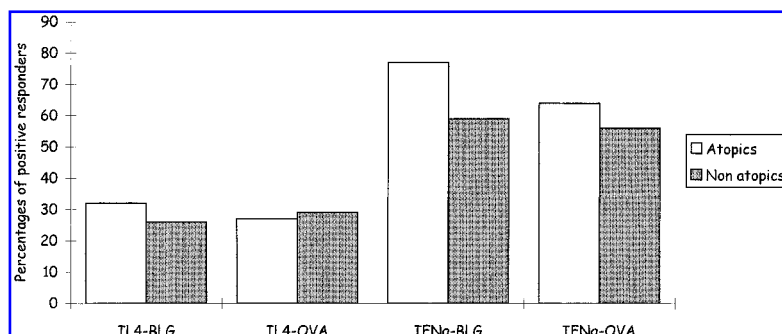


FIG. 2. Percentages of subjects with detectable IL4 and undetectable IFN γ after BLG or OVA stimulation at birth (positive responders) between the atopic and nonatopic children. No significative differences were observed.

els of IFN γ were at the same time positive, specificity of this combination as marker of atopy was 100%, and positive predictive value was 100%; on the contrary, sensitivity and negative predictive value were low (33% and 57%, respectively). We obtained similar results by evaluating the three parameters in the whole group of neonates. The association of cord blood IgE of ≥ 0.7 kU/L, detectable IL4 production, and positive atopic family history did not occur in all studied subjects.

DISCUSSION

The aim of this study was to evaluate the utility of IL4 and IFN γ dosage on supernatants of CBMC cultures stimulated *in vitro* with BLG or OVA as a way of recognizing at birth neonates with high risk of developing atopic disease. We tried to answer the question posed by Jones et al.⁽²²⁾: “Is there a difference in the nature or degree of response to allergens in children at risk of developing atopic disease compared to their low-risk counterparts, and, even more importantly, do those children who develop disease irrespective of their family history have a demonstrably different response to allergen either before or at birth?” The answer to the question of Jones et al.⁽²²⁾ regards many areas of research. We have reviewed only the studies focused on *in vitro* production of IL4 and IFN γ . A synthesis of our review is offered in Table 3.

Studies that employed an aspecific stimulus

Some studies are available in the literature about *in vitro* production of IL4 and IFN γ after nonspecific stimulus (e.g., PHA), but their results are controversial. We briefly summarize them. Some of these studies compare neonates with different atopic family history. Holt et al.⁽²⁾ Rinos,⁽³⁾ and Liao et al.⁽⁷⁾ showed that CBMC of neonates with family history of atopy produced lower levels of IFN γ than neonates without atopic family history. On the contrary, Szepfalusi et al.⁽⁹⁾ and Stabile et al.⁽¹²⁾ did not notice any significant difference. Other investigators^(6,12,14) did not find a similar correlation.

Studies that employed a specific stimulus

The rationale of such methodologic variation is the clinical evidence that the atopic patients develop symptoms only in the presence of the allergen they are sensitized to. Yssel et al.⁽²³⁾ described that cellular

TABLE 3. LIST OF STUDIES THAT HAVE EVALUATED THE *IN VITRO* PRODUCTION OF IFN γ AS A MARKER OF ATOPY

<i>In neonates with versus neonates without family history of atopy</i>		<i>In atopic children versus nonatopic children</i>	
<i>After aspecific stimulus</i>		<i>After aspecific stimulus</i>	
<i>Yes</i>	<i>No</i>	<i>Yes</i>	<i>No</i>
Holt et al. ⁽²⁾	Szepfalusi et al. ⁽⁹⁾	Tang et al. ⁽⁴⁾	Martinez et al. ⁽⁶⁾
Rinas et al. ⁽³⁾	Stabile et al. ⁽¹²⁾	Prescott et al. ⁽¹³⁾	Stabile et al. ⁽¹²⁾
Liao et al. ⁽⁷⁾	Hagendorens et al. ⁽¹⁴⁾		
<i>After specific stimulus</i>		<i>After specific stimulus</i>	
<i>Yes</i>	<i>No</i>	<i>Yes</i>	<i>No</i>
Miceli Sopo et al. (this study)	Prescott et al. ⁽¹⁰⁾	Warner et al. ⁽⁵⁾	Piccinni et al. ⁽⁸⁾
		Kondo et al. ⁽¹¹⁾	Szepfalusi et al. ⁽⁹⁾
			Prescott et al. ⁽¹³⁾
			Miceli Sopo et al. (this study)

The studies are identified with the name of the first author and reference number.

clones CD4⁺ specific for Der p I, belonging to adult subjects with a demonstrated allergy, produced high levels of IL4 and IL5 and low or undetectable levels of IFN γ after stimulus with the specific allergen they were sensitized to. The same cellular clones, on the contrary, produced normal levels of IFN γ after aspecific stimulus with Ca²⁺ ionophorus and PMA. Clinical signs of sensitization are not present at birth, therefore the choice of an allergen to stimulate neonatal lymphocytes cannot be based on clinical evidence. However, cow's milk and egg proteins are plentifully present in a common diet, and it is reasonable to suggest the possibility of an intrauterine sensitization of the lymphocytes, mainly of infants predisposed to allergy. So, in a previous study these proteins have been employed for the lymphocyte proliferation test in neonates.⁽²⁴⁾ Warner et al.⁽⁵⁾ studied 34 neonates with family history of atopy, and IFN γ *in vitro* production by CBMC, after stimulus with BLG, resulted to be lower in the group that developed atopic eczema due to cow's milk allergy at the age of one year than in the group without atopic eczema. The IFN γ production by the same cells after stimulus with OVA followed a similar trend but did not reach a statistically significant difference between the two groups of neonates. Subsequently, Kondo et al.⁽¹¹⁾ reported that the maximal IFN γ concentration in culture supernatants of OVA or bovine serum albumin (BSA) stimulated CBMC in infants who developed allergic disorders (seven infants, all with atopic dermatitis) was significantly lower ($p < 0.01$) than the one of the infants who did not develop any allergic disorders (14 infants). In other studies, the association among low *in vitro* at birth production of IFN γ and atopic family history or development of atopic disease has not been shown.⁽⁸⁻¹⁰⁾

Some observations about our results

We did not observe significant differences between the children who did and did not develop atopic disease during the follow-up, in relation to at birth IL4 and IFN γ *in vitro* production induced by BLG or OVA. We observed the following:

1. Some subjects with dosable IFN γ at birth (23% after stimulus with BLG and 36% after stimulus with OVA) developed atopic disease.
2. Some subjects with undosable levels of IFN γ at birth (59% after stimulus with BLG and 56% after stimulus with OVA) did not develop atopic disease.

Analogous considerations can be made in regard to the IL4 production. Probably the sensitivity could be improved employing a wider variety of the most common food and inhalant allergens that may be responsible of the intrauterine sensitization of the fetus. Warner et al.⁽⁵⁾ reported that IFN γ determination after stimulus with BLG identified just the neonates that developed eczema due to cow's milk protein allergy during the subsequent follow up. Kondo et al.⁽²⁴⁾ observed that neonates having a lymphocyte stimulation index (SI) of >1.5 after stimulus with BSA showed AD caused by milk allergy at the clinical follow up. Similar observations were performed in neonates with SI of >1.5 after stimulus with OVA. Therefore the kind of allergen employed for the lymphocyte stimulation seems to correlate tightly to that allergen responsible for the allergic symptoms. Unfortunately, the number of subjects who developed food allergy due to cow's milk or egg in our study is too small to draw any conclusion. In any case, the study of this subset of individuals did not produce relevant results.

The low specificity may be justified by a delayed development of the capacity of producing these cytokines, particularly IFN γ , as observed by other authors in neonates.^(1,13,25) Therefore, it is possible that our subjects who did not produce IFN γ at birth (and who did not develop atopic disease) could have produced detectable levels of IFN γ after the test performance.

Another feature in our study is that a high cord blood IgE production (≥ 0.7 kU/L) did not relate to reduced IFN γ - or increased IL4-production. Other investigators^(6,14) did not find any correlation between reduced IFN γ and increased cord blood total IgE production. This is not a surprising result once the complex mechanism of IgE synthesis is considered.⁽²⁶⁾ For example, the complex CD40-CD40 ligand, which takes considerable part to the synthesis of IgE,⁽²⁷⁾ is immature in neonates,⁽²⁸⁾ and this may justify the poor sensitivity of the cord blood IgE. The difference between atopic and non atopic subjects in the association of cord blood total IgE of ≥ 0.7 kU/L, undetectable levels of IFN γ *in vitro* by CBMC and positive family history for atopy was very near to significativity ($p = 0.072$). This association showed an excellent speci-

ficity (97%) but a poor sensitivity (18%), probably reflecting the characteristics of one of the three components of the association, that is cord blood IgE.

Methodological considerations

The results of the studies published so far about this argument are controversial. It is possible that the opposite results are explained by the important differences in the methodological steps. For example, among our study and the two other studies^(5,11) that have faced the usefulness of *in vitro* production of IFN γ and IL4 after stimulus with food allergens as markers of future atopic disease, there are differences in all steps: the quantity of allergens, the manufacturer that supplied the allergens, the incubation time, the methods for detecting cytokines, the length of clinical follow up, the statistical analyses and the manner to show the results. In our opinion, the methodological differences among these three studies make impossible a comparison of the respective results. We do not believe that solid biological reasons exist to allow us to judge a method more adequate than the other two. One striking aspect is the fact that, in our study, in many subjects the IFN γ , but also the IL4, resulted undetectable, while in the studies of Warner et al.⁽⁵⁾ and Kondo et al.⁽¹¹⁾ this happened rarely or never. Also, other authors^(9,13) reported the impossibility to measure the IFN γ in supernatants of great part of CBMC's cultures after stimulus with food antigens. This, obviously, affects the final result, and the causes could be several, from the type and duration of the stimulus, to the purity of the used antigen, to the method of detection. Also the time of the handling and stimulation of the isolated CBMC is important. The effect of overnight preservation of the separated CBMC was determined⁽¹⁴⁾ and a considerable decrease in cytokine production was observed when stimulation of the CBMC was postponed. And the cell concentration influences T-cell reactivity in cord blood cells triggered by inhalant and food allergens.⁽²⁹⁾ We have previously stressed the question of methodological variability,⁽³⁰⁾ particularly with regard to the mononuclear cell *in vitro* proliferation after food antigen stimulation. No author reproduced exactly the same methodology of a previous study of another author. Such variables can influence the cell response and it is not possible, on our advice, to demonstrate which technique should be considered the best, by the analysis of the results.

Not only IFN γ

Prescott et al.⁽¹³⁾ added a new result to our comprehension of the atopic predisposition, which throws a new light on the respective roles played by IFN γ and IL4. In this study, the CBMC stimulated with a polyclonal stimulus (PHA) show a reduced production of IFN γ in neonates that will develop atopy, while a specific stimulus, as HDM allergen, does not allow differentiation at birth in atopic and nonatopic subjects by the means of IFN γ measurement. Differently from what one expected, the stimulus with HDM induced a significantly lower production of cytokines Th2 related in the neonates who successively developed atopy if compared with those that did not develop atopic diseases. Therefore, data at birth of the study of Prescott et al.⁽¹³⁾ suggest another possibility of identifying the neonates that will develop atopy, which is the lower levels of Th2 cytokines. The authors explain that the levels of the IL4 and IFN γ proteins were too low to be detected by the ELISA methods, and mRNA measurement was therefore requested, which is a further methodological difference with many other studies.

Also for the IL4 cytokine there are conflicting evidences. In contrast with the results of Prescott et al.,⁽¹³⁾ Spinozzi et al.⁽³¹⁾ found no differences in the number of IL4-producing T cells at birth between newborns from atopic and non-atopic mothers. However, in this study,⁽³¹⁾ the number of CD4⁺ IL13⁺ cord blood T lymphocytes was significantly higher in the group of newborns from atopic mothers, and a greater incidence of wheezing and/or asthma-equivalent symptoms were found in these children at the 1-year follow up. A new cytokine has been proposed as a useful tool for a more accurate identification of the newborn at high risk of atopy.

CONCLUSIONS

Our results and our review of the literature do not allow us to consider the IL4 or IFN γ measurement on supernatants of CBMC stimulated *in vitro* with BLG or OVA, a reliable marker of atopic predisposition.

The performance of the studied tests increases the pre-test probability just in those neonates that have already high probability to develop an atopic disease. The children without a positive family history of atopy did not have a significant increase of the pre-test probability according to our results. Yet, among the atopic children, a great part did not have a positive family history of atopy. Today, these children are unidentifiable, and preventive measure are advisable only for infants with family history of atopy, better if biparental.

A correct standardization of the test (e.g., employing a greater number of allergens and an unique method of measurement for all the laboratories) and the recruitment of a wider population of children (with a percentage of children without atopic history consistent with the one in the general population) than that examined in each single study might definitively state if the cytokine *in vitro* measurement (after specific or nonspecific stimulus) is a marker of future atopy.

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