

In Vitro Production of Interleukin-4 and Interferon- γ after PHA and PMA Stimulation, and Mononuclear Cell Proliferative Response to Betalactoglobulin and Ovalbumin in Neonates with Different Family History of Atopy. A Follow-up of Five Years

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ABSTRACT

We have performed the lymphocyte stimulation test (LST) with β -lactoglobulin (BLG) and ovalbumin (OVA) and the measurement of interleukin-4 (IL4) and interferon- γ (IFN γ) *in vitro* production after phytohemagglutinin (PHA) and phorbol myristate acetate (PMA) stimulation by cord blood mononuclear cells (CBMC) in neonates with biparental heredity (group A), with uniparental heredity (group B), and without atopic disease heredity (group C). At follow-up, 18 of 55 neonates with a positive family history of atopic disease (9 of 25 neonates of group A and 9 of 30 of group B) versus 3 of 30 neonates of group C showed atopic disease ($p < .05$). Significant differences were not seen in the percentage of neonates with positive (stimulation index [SI] ≥ 3) proliferative responses of CBMC to BLG or OVA, neither among the three groups of neonates with different family history of atopy nor, at follow-up, among children with evidence of atopic disease versus those who were nonatopic. The association of biparental family history of atopy with an SI of 3 or greater to BLG or OVA after 3 days of culture had a 100% positive predictivity and specificity for the development of atopic disease. On the other hand, the same test after 3 or 8 days of culture, when associated with absent family history of atopy, had a negative predictivity of approximately 90%. Significant differences were not found in the percentage of CBMC samples with detectable levels of IL4 and undetectable levels of IFN γ and in the medians and centiles of IL4 and IFN γ *in vitro* production among the three groups of neonates with different family history of atopy. Also between the atopic and nonatopic groups, we did not observe significant differences in percentage of positive responders or medians of cytokine production. The association between the IL4 production and absent family history of atopy had 100% negative

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This work was partly presented at the VII Congresso Nazionale di Immunologia ed Allergologia pediatrica in Riva del Garda (Trento) Italy, April 16–19, 1997.

This work was in part supported by a grant from the Scientific Research Ministry of Italy.

predictivity and sensitivity for the development of atopic disease. According to our results, the test performed at birth did not show sufficient validity to be proposed as screening test to define better the neonate at "high risk for atopic disease." (Pediatr Asthma Allergy Immunol 1998;12[4]:259–270.)

INTRODUCTION

ATOPIC DISEASES, which account for a number of illnesses in infants and children, are associated with considerable health costs.⁽¹⁾ This has prompted attempts at disease prevention by dietary and environmental measures.^(2,3) It might be reasonable to apply these measures only to infants recognized as having an increased risk of developing allergic manifestations. However, it is not sufficient to predict "high allergy-prone newborn" on the basis of family history of atopy alone, because 30% to 40% of neonates with biparental and 60% to 70% of neonates with uniparental history of family atopy will not develop atopic disease. Recent studies suggest that cord IgE may not be a useful screening test because of its poor sensitivity, despite a good specificity.^(4,5) Additional genetic, biochemical, or immunologic markers such as platelet and eosinophil counts, HLA antigen, eosinophil cationic protein, and suppressor T-cell percentage have been investigated.^(6–8) However, none of these can yet help predict with sufficient certainty which neonate will develop atopic disease. In recent years, other laboratory tests have been proposed. It has been reported⁽⁹⁾ that proliferative response of cord blood mononuclear cells (CBMC) to bovine serum albumin (BSA) and ovalbumin (OVA) may be a useful test in the prediction of atopy. Some authors have found an association between low levels of *in vitro* interferon- γ (IFN γ) production by CBMC and a positive family history of atopy^(10–12) or with the development of atopic disease.^(13,14)

We performed the lymphocyte stimulation test (LST) with β -lactoglobulin (BLG) and OVA and the measurement of interleukin-4 (IL4) and IFN γ *in vitro* production after phytohemagglutinin (PHA) and phorbol myristate acetate (PMA) stimulation by CBMC in neonates with biparental heredity (both parents with atopic disease), with uniparental heredity (one parent with atopic disease), and without atopic disease heredity. Then, we followed these neonates for a mean duration of 5 years, 9 months, to evaluate the development of atopic disease.

PATIENTS AND METHODS

Subjects

Parents of 85 neonates (40 males, 45 females) born at term (70 by natural delivery, 15 by cesarean section) between April 1991 and February 1994 were contacted before the birth of their child. The study was fully explained to them and informed consent obtained. Approval from the hospital's ethical committee was obtained. The infants were regarded as having positive family history when one or both parents suffered from one or more atopic disorder. The mothers did not take any special precaution regarding their own diet during the pregnancy. None of the parents was given any special advice about allergy prevention. Of those in the study, 25 neonates had biparental (group A), 30 uniparental (group B), and 30 absent family history of atopy (group C). The study groups were comparable in family size, parental education, and exposure to environmental pollutant. We enrolled 14 healthy (history for atopic disease and prick test were negative) adult subjects as controls (group D) to compare the results obtained in our neonates with a mature immune response.

Blood sampling

Cord blood samples were aseptically collected in heparinized tubes (Falcon 2070, Becton Dickinson, NJ) immediately after delivery with the usual procedures including careful cleaning of the cord and strict puncture of the umbilical vein to avoid maternal contamination. However, total serum IgA was measured in all cord samples by a standard nephelometric technique (Beckman IgA test kit; Beckman Instruments Inc., Brea, CA). When the IgA level was greater than 1.8 mg/dL, the sample was considered contaminated.⁽¹⁵⁾ An aliquot (1 mL) of every blood sample was centrifuged to obtain autologous serum.

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Mononuclear cell isolation

A blood sample (5 mL) was placed into heparinized sterile tubes for leukocyte isolation. CBMC and peripheral blood mononuclear cells (PBMC) of adult controls were obtained by Ficoll-Hypaque gradient centrifugation and then were washed three times with Ca^{2+} and Mg^{2+} free phosphate-buffered saline (PBS). Thereafter, CBMC and PBMC were resuspended at a 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), L-glutamine (2 mmol/L, Gibco Laboratories, Grand Island, NY), and 10% autologous human serum.

LST and lymphokine production

Into 96-well, flat-bottom tissue plates (Falcon 3070, Beckton Dickinson) were placed 2.5×10^5 CBMC or PBMC in 250 μL of culture medium. The cells were incubated at 37°C in a humidified atmosphere with 5% CO_2 for 3 or 8 days, in the presence of one of the following mitogens or antigens added to triplicate wells: PHA (100 $\mu\text{g}/\text{mL}$, M form, Gibco Laboratories, Grand Island, NY) plus phorbol myristate acetate (PMA, 1 ng/mL, SIGMA, Milan, Italy); BLG (10 $\mu\text{g}/\text{mL}$, kindly donated by SARM Allergeni, Pomezia, Italy); or OVA (100 $\mu\text{g}/\text{mL}$, kindly donated by SARM Allergeni). Negative control wells received culture medium alone. The antigen doses were selected as being optimal from the results of stimulation of 10 CBMC samples with concentrations ranging from 50 ng/mL to 200 $\mu\text{g}/\text{mL}$. During the last 16 hours 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, MA). The incorporation of thymidine into DNA was determined by liquid scintillation counter (Beckmann LS 7000) and radioactivity expressed as mean of triplicate cultures of counts per minute (cpm) and stimulation index (SI: mean cpm of stimulated cultures divided by mean cpm of unstimulated cultures). Other PHA and PMA stimulated cultures were prepared and their supernatants were harvested after 24 or 48 hours of incubation and stored at -70°C until testing for IL4 and $\text{IFN}\gamma$ production, respectively.

Determination of IL4 and $\text{IFN}\gamma$

In the culture supernatants, IL4 and $\text{IFN}\gamma$ were quantified by enzyme-linked immunosorbent assay (ELISA) kits (Intertest-4, Genzyme Corporation, Cambridge, MA, minimal sensitivity 45 pg/mL IL4 and Intertest- γ , Genzyme, minimal sensitivity 100 pg/mL $\text{IFN}\gamma$). Briefly, 96-well microtiter plates, coated overnight with monoclonal antibody specific for human IL4 (or $\text{IFN}\gamma$), were used to capture IL4 (or $\text{IFN}\gamma$) present in standard samples and unknown specimens. A goat polyclonal antibody, which binds to multiple epitopes on the IL4 (or $\text{IFN}\gamma$), contained on the solid phase was added for 2 hours. A third antibody, biotin-labeled donkey-anti-goat immunoglobulin, which binds to the goat polyclonal antibody already linked to IL4 (or $\text{IFN}\gamma$), was then applied for 45 minutes. Streptavidin-peroxidase, which binds to the biotin on the third antibody, was added for 40 minutes. Finally, the substrate reagent (OPD chromagen dissolved in peroxide reagent solution) was dispensed into each well. The peroxidase enzyme acts with substrate reagent and increased absorbances due to bound, immunoreactive IL4 (or $\text{IFN}\gamma$) were quantified by an ELISA reader. The IL4 (or $\text{IFN}\gamma$) concentrations in studied samples were determined by comparison of their absorbances with those obtained from the known amounts of human IL4 (or $\text{IFN}\gamma$) in the standard dilutions. To determine $\text{IFN}\gamma$, on the basis of the results of previous reports,⁽¹⁰⁻¹⁴⁾ we considered an undetectable level of this cytokine as a positive response. We speculated that the atopy-prone neonate could be identified because of a high IL4 production and/or a low $\text{IFN}\gamma$ production.

Clinical follow-up

A follow-up of all the neonates was performed every 6 months by consulting room examinations by a staff physician (S.M.S). Clinical criteria that defined allergy were chosen according to those of Orgel et al.⁽¹⁶⁾ and Kjellman.⁽¹⁷⁾ Atopic disease was diagnosed according to Hanifin's criteria.⁽¹⁸⁾ The children with evidence of atopic disease were skin tested and the food challenges were conducted using the double-blind, placebo-controlled method.⁽¹⁹⁾ To avoid any unwanted interference in the child's evaluation neither the parents nor the examining physician were told the results of the test before the clinical follow-up. Any change

in house allergen exposure and food regimens (breast-feeding, subsequent introduction of cow's milk and other foods) were also recorded. Atopic and nonatopic subjects were compared for their results (percentage of positive responders, medians, and centiles) in the test performed at birth (mononuclear cell proliferative response to BLG and OVA, *in vitro* production of IL4 and IFN γ after PHA and PMA stimulation).

Statistical analysis

In addition to general descriptive statistics, groups were compared, when required, with Kruskal–Wallis test, Dunn test, Mann–Whitney test, Student's *t*-test for independent samples, chi quadro test, and Fisher exact test. Differences were considered significant at $p < .05$. The data were analyzed by the program in Glanz's *Primer of Biostatistics*.

RESULTS

We examined our results by comparing neonates according to their family history of atopic disease (biparental, uniparental and absent) or to the development of atopy (atopics and nonatopics). First we show the results of clinical follow-up and later on the results of the laboratory tests performed at birth. None of the cord blood samples was excluded, because serum IgA levels were always less than 1.8 mg/dL.

TABLE 1. POSITIVE TESTS OF THE NEONATES WHO SHOWED ATOPIC DISEASE AT FOLLOW-UP

Subjects	Group	Atopic disease	Follow-up (months)	SI BLG 3rd day	SI BLG 8th day	SI OVA 3rd day	SI OVA 8th day	IL4 <i>in vitro</i> production (pg/mL)	IFN γ <i>in vitro</i> production (pg/mL)
S.A.	A	CMA	50	9.5		7.2	10.6		nd
D.P.	A	Asthma	46						nd
T.G.	A	Asthma	46					48	nd
C.C.	A	Conjunctivitis	44			4.5		92	nd
D.F.P.	A	Fish allergy	42				6.09		nd
S.M.	A	Conjunctivitis	37					90	nd
M.D.	A	Asthma	36	6.2		4.4			nd
M.A.	A	AD	29						
N.I.	A	Asthma	26			5.1			
B.R.	B	Egg allergy	48				7.6		nd
R.T.	B	AD	47					70	nd
D.L.A.	B	Rhinitis	46					90	nd
P.R.	B	Rhinitis	44						nd
C.G.	B	Conjunctivitis	36					100	nd
M.G.	B	CMA	33						
P.M.	B	Asthma	33		3.5				
E.L.	B	Asthma	32				4.1		nd
C.S.	B	Asthma	26	3.1					nd
B.C.	C	CMA	42		5.5	4.2		82	nd
F.A.	C	CMA	38					45	nd
T.A.	C	Asthma	37					86	nd

SI, stimulation index; BLG, beta-lactoglobulin; OVA, ovalbumin; IL, interleukin; IFN γ , interferon- γ ; AD, atopic dermatitis; CMA, cow milk allergy; nd, not detectable; positive SI = ≥ 3 ; 3°d = 3 day.

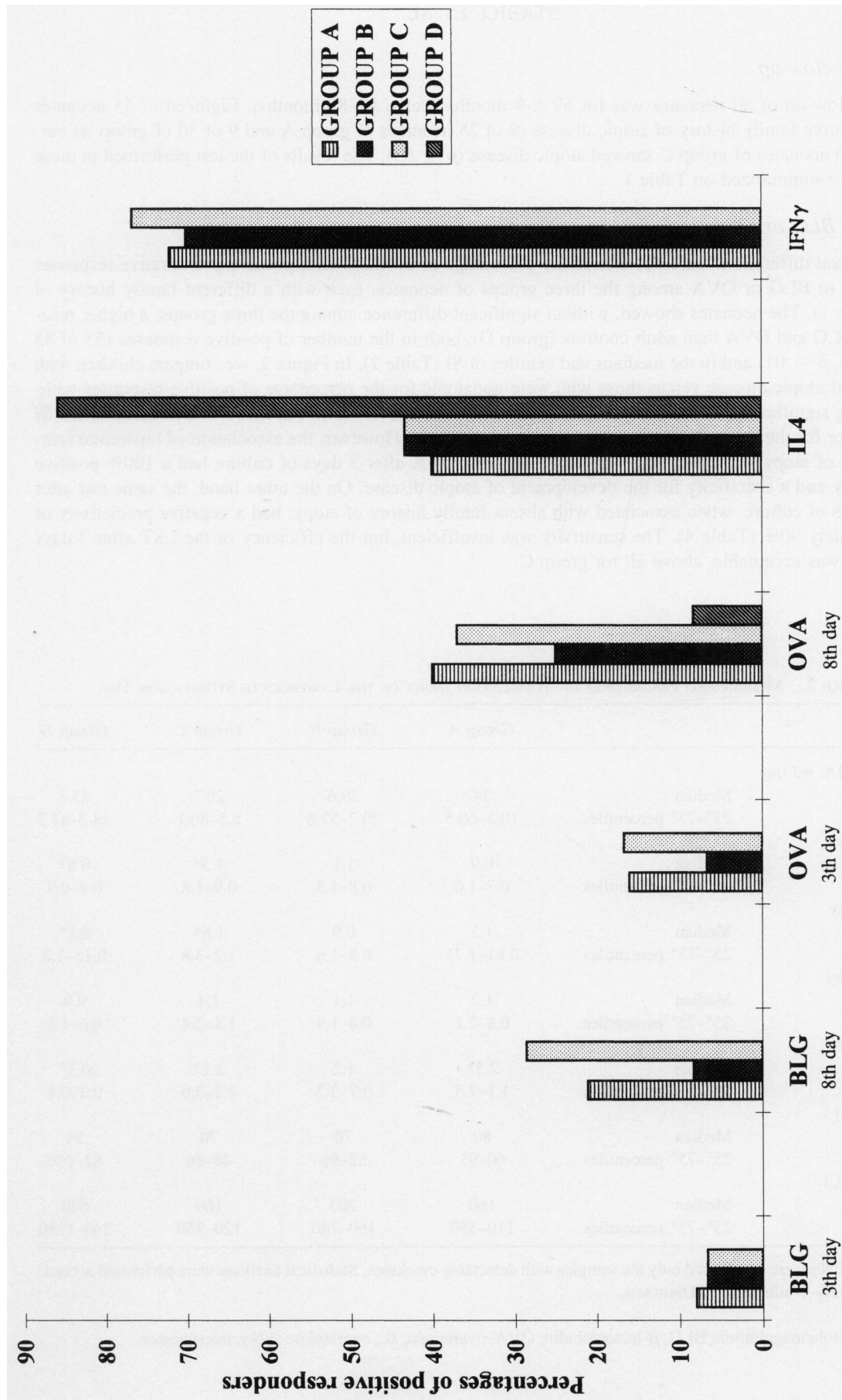


FIG. 1. Percentage of positive responders after BLG and OVA stimulation and after measurement of IL4 and IFN γ in vitro production, and of neonates having atopic disease at follow-up. Results were analyzed with chi-square test and Fisher exact test.

Clinical follow-up

The follow-up of all neonates was for 69 ± 9 months (range 52–87 months). Eighteen of 55 neonates with a positive family history of atopic disease (9 of 25 neonates of group A and 9 of 30 of group B) versus 3 of 30 neonates of group C showed atopic disease ($p < .05$). The results of the test performed in these subjects are summarized on Table 1.

LST with BLG or OVA

Significant differences were not seen in the percentage of neonates with positive proliferative responses of CBMC to BLG or OVA among the three groups of neonates, each with a different family history of atopy (Fig. 1). The neonates showed, without significant difference among the three groups, a higher reactivity to BLG and OVA than adult controls (group D), both in the number of positive responses (55 of 85 vs. 1 of 14, $p < .01$) and in the medians and centiles of SI (Table 2). In Figure 2, we compare children with evidence of atopic disease versus those who were nonatopic for the percentage of positive responses without finding significant differences. Atopic and nonatopic subjects did not differ for the medians of the SI (Table 3) or for the percentages of positive responses (Fig. 2). However, the association of biparental family history of atopy with an SI 3 or greater to BLG or OVA after 3 days of culture had a 100% positive predictivity and a specificity for the development of atopic disease. On the other hand, the same test after 3 or 8 days of culture, when associated with absent family history of atopy, had a negative predictivity of approximately 90% (Table 4). The sensitivity was insufficient, but the efficiency of the LST after 3 days of culture was acceptable, above all for group C.

TABLE 2. MEDIAN AND PERCENTILES OF STIMULATION INDEX OF THE LYMPHOCYTE STIMULATION TEST

		Group A	Group B	Group C	Group D
PHA + PMA 3rd day	Median	24	29.6	28.7	23.3
	25°–75° percentiles	10.5–60.5	20.2–57.6	8.5–50.1	18.3–61.2
BLG 3rd day	Median	0.9	1.1	1.3*	0.8*
	25°–75° percentiles	0.7–1.6	0.8–1.5	0.9–1.8	0.4–0.9
BLG 8th day	Median	1.3	0.9	1.8*	0.2*
	25°–75° percentiles	0.81–1.71	0.8–1.6	1.2–3.8	0.15–1.2
OVA 3rd day	Median	1.2	1.1	1.4	0.9
	25°–75° percentiles	0.8–2.1	0.8–1.9	1.1–2.4	0.6–1.3
OVA 8th day	Median	2.5*	1.2	2.2*	0.3*
	25°–75° percentiles	1.1–7.3	0.7–3.2	1.2–3.6	0.2–0.8
IL4 (pg/mL)	Median	80	70	70	94
	25°–75° percentiles	60–91	52–95	48–86	82–250
IFN γ (pg/mL)	Median	160	200	160	620
	25°–75° percentiles	110–350	160–780	120–350	240–1350

IL4 and IFN γ were considered only the samples with detectable cytokines. Statistical analyses were performed according to Kruskal–Wallis test and Dunn test.

* $p < .05$.

PHA, phytohemagglutinin; BLG, β -lactoglobulin; OVA, ovalbumin; IL, interleukin; IFN γ , interferon- γ .

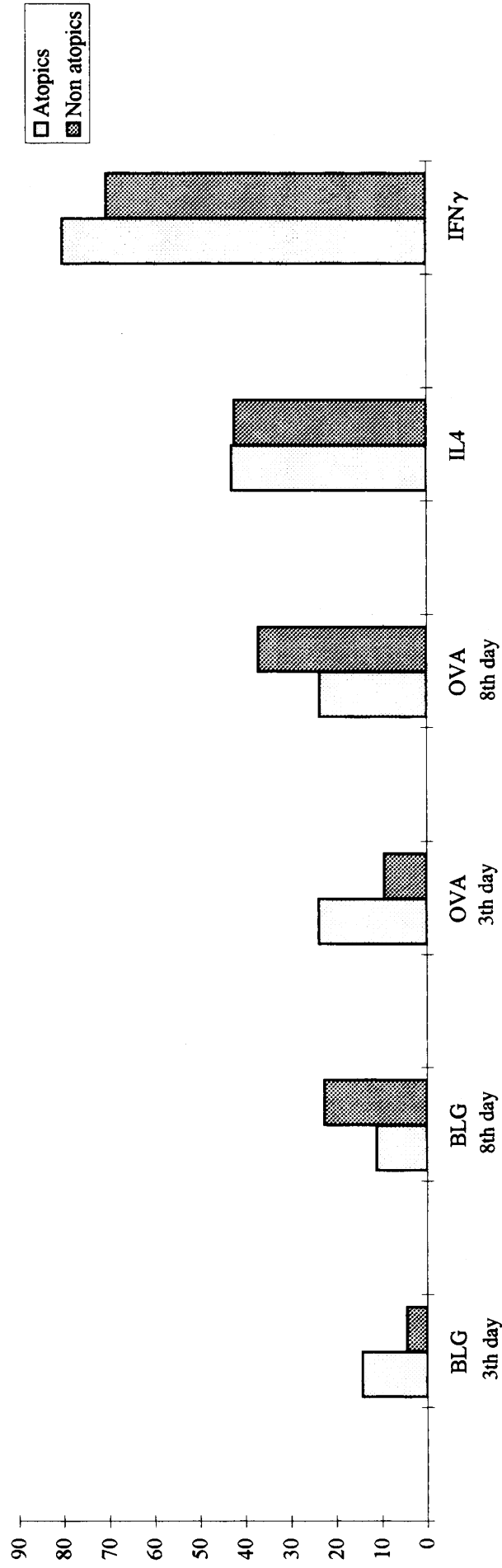


FIG. 2. Percentages of positive responders after BLG and OVA stimulation and after measurement of IL4 and IFN γ in vitro production between atopic and nonatopic neonates. Results were analyzed with chi-quadro test and Fisher exact test.

BLG, beta-lactoglobulin; OVA, ovalbumin; IL, interleukin; IFN γ , interferon- γ .

TABLE 3. MEDIAN AND RANGES OF THE TESTS, PERFORMED AT BIRTH, BETWEEN ATOPIC AND NONATOPIC NEONATES

	BLG 3rd day	BLG 8th day	OVA 3rd day	OVA 8th day	IL4 (pg/mL)	IFN γ (pg/mL)
Atopic	1.07 0.2–9.5	1.13 0.4–5.5	1.3 0.18–7.2	1.4 0.6–10.5	86 45–100	117.5 100–200
Nonatopic	1.12 0.15–12.6	1.4 0.08–29.6	1.15 0.05–6.6	2.3 0.2–22.3	69 45–160	190 100–2600

IL4 and IFN γ were considered only the samples with detectable cytokines. Statistical analyses were performed according to Kruskal-Wallis test and Dunn test.

BLG, β -lactoglobulin; OVA, ovalbumin; IL, interleukin; IFN γ ; interferon- γ .

CBMC in vitro production of detectable levels of IL4 and IFN γ after in vitro activation by PHA and PMA

Significant differences were not found in the percentage of CBMC samples with detectable levels of IL4 and undetectable levels of IFN γ among the three groups of neonates with different family history of atopy (Fig. 1). In the CBMC samples, 36 showed a detectable production of IL4 and 62 CBMC samples did not produce IFN γ . Eight (2 of group A, 4 of group B, 2 of group C) CBMC samples produced both IL4 and IFN γ ; 34 (11 of group A, 11 of group B, 12 of group C) failed to produce detectable levels both of IL4 and of IFN γ . All of the adult samples showed detectable levels of both IL4 (except for one) and IFN γ . Significant differences were not seen in the medians and centiles of IL4 and IFN γ production among the three groups of neonates. The overall levels of CBMC IL4 and IFN γ production were lower than those produced by PBMC, even if the differences were not significant (Table 2).

Also, between atopic and nonatopic subjects we found no significant differences in percentage of positive responses (Fig. 2) or medians of cytokine production in picogram per milliliter (Table 3). The association between the IL4 production and absent family history of atopy (group C) had a 100% negative predictivity and sensitivity for the development of atopic disease (Table 4).

DISCUSSION

It is thought that the immune responses underlying the expression of allergic disease are initiated in infancy. Intervention at an early stage in this activation process may prove to be the best approach if we are to manipulate this key element of the immune response and alter the course of allergic sensitization with any degree of success. Precise identification of at risk children is then an inevitable challenge.

In this study, we looked for a correlation among some *in vitro* tests performed at birth, the family history of atopic disease, and the development of atopic disease. The choice of the LST with food antigens is justified by the occurrence of the antenatal sensitization to food antigens.^(20,21) We used BLG and OVA to stimulate CBMC, both because these antigens are frequently involved in the atopic manifestations of the first year of life and because of their wide presence in the mother's diet. In our hand, this test did not show significant differences for the percentage of positive subjects and for the medians among the neonates with different family history of atopy and between atopic and nonatopic subjects. However, an SI of 3 or more after 3 days of culture to BLG or to OVA had a very good positive predictivity when associated with biparental history of atopy. On the other hand, the same test had a good negative predictivity when associated with absent family history of atopy. Also the specificity was good, whereas the sensitivity was very poor. When the cell cultures were prolonged until the eighth day, we found that 36% of CBMC samples showed positive responses to BLG or OVA without any significant difference among the three groups of neonates or between atopic and nonatopic subjects. In keeping with our results, Szepefalusi et al.⁽²¹⁾ recently reported that in adopting a long culture time (7 days), the positive responses after BLG stimulation that were observed in 59% of neonates were unrelated to the history of atopy or IgE level of the mother. In the past year, other authors have also observed a high frequency of positive proliferative responses to specific antigens (ragweed, *Parietaria judaica*, *Dermatophagoides pharinae*) using a long culture time in nonatopic

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as well as atopic adult subjects^(22,23) or in neonates.⁽²⁴⁾ On the contrary, Kobayashi et al.⁽⁹⁾ reported that the proliferative responses of CBMC to food antigens (OVA or BSA) after double stimulation times of 5 and 3 days are useful in the prediction of allergic disorder development with a sensitivity of 53% and a specificity of 82%. Also, Warner et al.⁽¹⁴⁾ reported that children with a milk-associated eczema at 1 year of age have shown, at birth, a significantly raised response to BLG compared with the no eczema group and with the non-food-associated eczema group.

The results, then, of the various studies on the reliability of the LST with food allergens as a marker of atopy are conflicting. On the basis of our results, we think that the positive response to BLG or OVA is an unreliable marker of atopy despite its good positive predictivity after 3 days of culture, because of its poor sensitivity. Only in theory could this test be used to detect the neonates with absent family history of atopy who will not develop atopic disease (most neonates). After 8 days of culture, the LST maintains a good negative predictivity when associated with absent family history of atopy. It loses in positive predictivity and specificity, however, because of the increase of positive response with respect to the 3-day cultures. Because of this finding, the possibility of an *in vitro* sensitization seems unlikely; in fact, it has been shown that T cells alone did not proliferate in response to the antigens, presumably indicating a requirement for antigen-presenting cells and therefore excluding a mitogenic effect of the tested antigen, which suggests that allergen priming must occur prenatally.⁽²¹⁾ Perhaps this test can detect only those neonates who have been sensitized *in utero* to the same allergen used in the LST. This hypothesis is in keeping with the results of Warner et al.⁽¹⁴⁾ and, more recently, with the two case reports of Kondo et al.⁽²⁵⁾ The consequence is that the LST needs a wide panel of allergens to be an effective screening test, which makes it too complicated and expensive.

TABLE 4. RESULTS OF THE TEST PERFORMED AT BIRTH IN THE THREE GROUPS OF NEONATES AND IN ALL NEONATES IN RELATION TO THE DEVELOPMENT OF ATOPIC DISEASE

	BLG 3rd day	BLG 8th day	OVA 3rd day	OVA 8th day	IL4	IFN γ
Positive predictivity						
-Group A	100	0	100	25	30	39
-Group B	50	50	0	33	23	33
-Group C	0	12	20	0	23	14
-All neonates	50	14	45	16	25	27
Negative predictivity						
-Group A	69	40	76	41	60	71
-Group B	71	63	67	61	64	78
-Group C	89	90	92	82	100	57
-All neonates	77	66	78	63	75	82
Sensitivity						
-Group A	22	0	44	22	33	78
-Group B	11	11	0	22	33	78
-Group C	0	33	33	0	100	50
-All neonates	14	9	23	19	42	81
Specificity						
-Group A	100	60	100	45	56	31
-Group B	95	93	90	73	52	33
-Group C	92	72	85	58	62	18
-All neonates	95	76	90	60	57	30
Efficiency						
-Group A	72	31	80	35	48	48
-Group B	70	62	63	54	46	47
-Group C	83	67	80	51	66	23
-All neonates	75	56	74	47	54	42

BLG, β -lactoglobulin; OVA, ovalbumin; IL, interleukin; IFN γ , interferon- γ

The other laboratory finding that we evaluated as a marker of atopy was the *in vitro* production of cytokines IL4 and IFN γ after PHA and PMA stimulation. Our results show no significant differences, either for the percentage of positive subjects or for the medians among the neonates with different family history of atopy, or between atopic and nonatopic subjects. To our knowledge, the *in vitro* IL4 production after stimulation with PHA and PMA by CBMC has been reported only by our group,⁽²⁶⁾ whereas the reports on the CBMC *in vitro* IFN γ production are more numerous. Holt et al.⁽¹⁰⁾ reported that the IFN γ producing capacity of CD4+ T-cell clones from normal children and from those with high genetic risk for atopy (spanning the age range from 1 month to 4 years) was markedly reduced by comparison with adults, especially in the high risk group. Rinas et al.⁽¹¹⁾ reported that peripheral mononuclear cells from newborns with a familial risk for atopic disease showed a significant decrease in PHA-induced IFN γ production compared with those from neonates with no identifiable risk. More recently, these results were confirmed by Liao et al.⁽¹²⁾ Tang et al.⁽¹³⁾ found a significant association between reduced PHA-induced IFN γ secretion by CBMC and the development of atopic disease, and Warner et al.⁽¹⁴⁾ showed that the neonates (all with positive family history of atopic disease) with low *in vitro* production of IFN γ after BLG or OVA stimulation at clinical follow-up, had developed atopic dermatitis caused by milk or by egg, respectively.

Our findings are in agreement with the results of Martinez et al.⁽²⁷⁾ showing that *in vitro* IFN γ production of CBMC is unrelated to maternal or paternal skin test reactivity, to the development of eczema or rhinitis in the first year of life, to total serum IgE levels, and to skin test reactivity at aged 6 years. Also, Szepefalusi et al.⁽²¹⁾ found no significant differences between atopy-prone versus non-atopy-prone neonates in IFN γ production after PHA stimulation. In this study, BLG stimulation led to only one subject with measurable IFN γ levels. In our experiments, approximately 40% of CBMC samples failed to produce detectable levels of IL4 or IFN γ . These findings may be related to a later development of the capacity to produce normal levels of these cytokines (it has been demonstrated that commitment to a TH2 phenotype in atopic patients appears to occur between ages of 2 and 5 years,⁽²⁸⁾ to a production of factors suppressing their synthesis, to the lack of a CD45R negative T-cell population as proposed by other authors,^(29,30) or to the absence or very low levels, of IL4 and IFN γ). As to IFN γ , the commercial ELISA kit we used had a minimal sensitivity higher than that adopted in other works. However, it must be borne in mind that in 1991, when the work was initiated, this was the minimal sensitivity offered and we wanted to maintain the homogeneity of the experimental conditions.

On the basis of our results, neither the positive family history for atopy and the development of atopic disease relate to increased IL4 production or reduced IFN γ production by CBMC. The measurement of IL4 *in vitro* had good sensitivity and negative predictivity when associated with absent family history of atopy. As for LST with food allergens, the test could be used to identify the neonate with absent family history of atopy who will not become atopic, but only theoretically. In fact, we conclude that there are no sufficient demonstrations found in the literature regarding the validity of these tests as markers of atopy. Moreover, these tests are very expensive and require a qualified laboratory for their performance.

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