

Effect of BCG vaccination in asthmatic schoolchildren

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Recent studies have explored the effect of Bacilli Calmette-Guerin (BCG) or *Mycobacterium vaccae* vaccination in asthmatic patients, yielding conflicting results. We investigated the effect of BCG vaccination in asthmatic schoolchildren, especially focusing on the cytokine pattern released by mononuclear cells. After a 1-yr run-in period, 67 asthmatic schoolchildren received intradermal immunization with BCG (33 patients) or placebo (34 patients). Both groups were followed during 1 yr. Serum immunoglobulin E (IgE) levels did not change after BCG (407.1 ± 86.6 vs. 415.1 ± 86.7 IU/ml, mean $\pm$ s.e.m.), but increased after placebo (406.7 ± 67.0 vs. 619.7 ± 90.7 IU/ml, $p = 0.001$) administration. Interleukin (IL)-4 and interferon (IFN)- γ measured in the supernatant of stimulated cultured blood mononuclear cells did not change in the BCG group (10.8 ± 2.3 vs. 17.9 ± 5.7 pg/ml, and 348.6 ± 118.0 vs. 354.8 ± 139.0 pg/ml, respectively), while in the control group IL-4 increased (from 6.7 ± 1.3 to 16.1 ± 6.0 pg/ml, $p < 0.05$), and IFN- γ decreased (from 279.9 ± 82.1 to 232.1 ± 109.6 pg/ml, $p = 0.01$). In comparison with their initial status, most patients maintained the same asthma severity and the same proportion of emergency room visits at the end of the study. The proportion of those in whom asthma improved or worsened was the same in both groups. We concluded that, contrary to the common hypothesis, BCG vaccination in asthmatic children was unable to cause a long-term reinforcement of Th₁ response, although it could avoid the increased Th₂ response observed in control patients.

**Mario H. Vargas¹,
Demetrio A. Bernal-Alcántara²,
Miguel A. Vaca^{2*},
Francisco Franco-Marina² and
Ricardo Lascurain³**

¹Departamento de Investigación en Asma, Instituto Nacional de Enfermedades Respiratorias, México DF,

²Subdirección de Investigación Sociomédica, Instituto Nacional de Enfermedades Respiratorias, México DF and ³Departamento de Investigación en Bioquímica, Instituto Nacional de Enfermedades Respiratorias, México DF, Mexico

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Dr Mario H. Vargas, Instituto Nacional de Enfermedades Respiratorias, Tlalpan 4502, CP 14080, México DF, Mexico

Tel.: (52-55) 5665-6696

Fax: (52-55) 5665-4623

E-mail: mhvargasb@yahoo.com.mx

*Present address: Unidad de Inteligencia Epidemiológica y Sanitaria, Dirección General de Epidemiología, Mexico

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Major advances in the knowledge of asthma pathogenesis have been gained in last decades. A number of studies have conclusively demonstrated the key role of activated Th₂ lymphocytes in the induction, amplification and maintenance of the inflammatory components in asthmatic airways (1). The fact that the Th₁- and Th₂-type lymphocyte subsets are mutually inhibitory has attracted much interest because of the speculation that a naturally or artificially induced enhancement of Th₁ responses would reduce the Th₂ activity occurring in the asthmatic condition. This speculation was first supported by the work of Shirakawa et al. (2) who found an inverse relationship between atopy and infection by

mycobacteria, a microorganism known to induce strong Th₁ responses (3). Several subsequent reports have confirmed this type of epidemiologic association, although others did not (4–11). *In vivo* and *in vitro* studies have also provided some support to this speculation because they showed that allergic immunization might be partially prevented, or even reversed, by bacilli Calmette-Guerin (BCG) or *Mycobacterium vaccae* administration (12, 13). A few recent studies have explored the effect of intradermal immunization with BCG or *M. vaccae* in asthmatic patients, yielding conflicting results (14–18).

In the present study, we investigated the long-term (12-month) effect of BCG vaccination in a

cohort population of schoolchildren, with special focus on its effect over the pattern of cytokines released by peripheral blood mononuclear cells (PBMC).

Materials and methods

Patients and study design

The study was carried out in primary schoolchildren of either gender, from October 1998 to October 2000. After a questionnaire survey was carried out in several schools of the South of Mexico City, children with clinical symptoms suggestive of asthma were selected for entering the study. Parents were asked to bring their children to a clinic, where they were submitted to an in-depth clinical examination by a pediatrician. Asthma diagnosis was confirmed because of the presence of symptoms and a positive exercise test, consisting by a 20% decrement in the peak expiratory flow (PEF) after execution of repetitive squats during 10 min. The study was carried out in those children whose parents accepted the participation of the child in the study by signing an informed consent letter. The study was approved by the Institutional Scientific and Bioethics Committees.

In order to standardize the management of these patients, they entered in a run-in period of 12-months, during which all children received anti-asthmatic treatment according to international guidelines (19). In addition, during this period all parents received a 30-min educational talk about the main concepts on the pathogenesis of asthma, its treatment and the non-pharmacologic measures to control it. These concepts were reinforced in every visit, which occurred at least 3 months apart. During this period, intradermal purified protein derivative (PPD; 2 IU, Institute of Copenhagen, Denmark) was applied in order to evaluate their skin reactivity to mycobacteria.

At the end of the run-in year, children were alternately allocated to the experimental or the control groups. Patients from the experimental group were vaccinated through intradermic inoculation of one standard dose (1 ml, containing 200,000 cfu) of BCG vaccine (Dirección de Biológicos y Reactivos, Mexico) in the deltoid area. Control group received 1 ml saline solution using the same procedure. Before BCG or placebo administration, a specific questionnaire was applied and samples for blood leukocyte count, serum immunoglobulin E (IgE) level, eosinophil count in nasal mucus,

and parasite search in stool were obtained. In a subset of children (first 17 from the experimental group and first 18 from the control group), determination of interleukin (IL)-4 and interferon (IFN)- γ in stimulated PBMC was also performed. After a 12-month follow up, the questionnaire and laboratory tests were performed again.

IL-4 and IFN- γ

Whole peripheral blood (5 ml) was drawn from the asthmatic patients by venous puncture into heparinized tubes and diluted 1:2 in RPMI-1640 culture medium (Sigma Chemical, St Louis, MO, USA) layered onto 1.077 density Histopaque (Sigma) by centrifugation at 800 *g* for 30 min at 18°C (20). The interface containing PBMC was collected, twice washed, and counted in a Neubauer hemocytometer, assessing the cell viability by the trypan blue dye exclusion technique. PBMC (1×10^6 /well) were stimulated with 10 ng/ml phorbol myristate acetate and 500 ng/ml ionomycin in a 24-well flat bottomed tissue culture plates (Falcon, Franklin Lakes, NJ, USA) in RPMI-1640 supplemented with 1 mM sodium pyruvate, 0.5 mM 2-mercaptoethanol, 2 mM L-glutamine and 10% fetal calf serum (Gibco BRL, Rockville, MD, USA) at 37°C in an atmosphere of 5% CO₂ in air. Cell-free supernatants were collected after 24 h culture and stored at -20°C until their use.

The quantitative measurement of the cytokines in the supernatants was performed using commercially available enzyme-linked immunosorbent assay (ELISA) kits for human IL-4 and IFN- γ (Quantikine, R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. These kits use the sandwich technique in which a polyclonal antibody to IL-4 or IFN- γ has been pre-coated onto a microplate. Samples were put into the wells and any IL-4 or IFN- γ , are bound by the immobilized antibody. After washing away any unbound cytokine, an enzyme-linked polyclonal antibody for the cytokine is added to each well. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution was added to the wells and color developed in proportion to the amount of cytokine deposited. After 30 min incubation, optical density of each well was determined using an ELISA reader (LabSystem Multiskan MS, Finland) set to 450 nm. Cytokine levels were calculated by reference to standard curves using recombinant cytokines provided by the manufacturer.

Statistical analysis

Changes observed at the end of the study were compared with initial values through Student's t-test for paired observations. In the case of IL-4, IFN- γ and IgE, concentrations were first log-transformed before statistical analysis. Dichotomous variables were compared by using Fisher's exact test, while 2×2 tables were assessed by the chi-squared test. Statistical significance was set at $p < 0.05$ bimariginally. Data in the text and illustrations correspond to mean $\pm$ s.e.m.

Results

A total of 82 asthmatic patients were initially recruited into the study. Fifteen patients abandoned the study during the run-in period because eight moved from the neighborhood, six initiated social security services, and one had the diagnosis of a congenital heart disease. Thus, 67 patients (between 6 and 13 yr of age) entered the second-phase of the study and all of them completed the 12-month follow up. A total of 33 children received BCG, while 34 children were included as control group. As can be seen in Table 1, the main demographic variables were homogenously distributed between both groups, as it was also for the family or personal history of atopy, passive exposure to tobacco smoke, breast-feeding, house conditions and triggering factors for asthma attacks. One child showed a positive PPD response (12 mm) without demonstration of active tuberculosis. The remaining children had a tuberculin skin test < 5 mm. The majority of patients in both groups had mild asthma and has had four emergency room visits in the last year at the initiation of the study, without differences between groups.

As can be seen in Fig. 1a, serum IgE levels measured before and 1-yr after the BCG administration did not change (407.1 ± 86.6 vs. 415.1 ± 86.7 IU/ml, respectively, $p = 0.90$), whereas in the control group a statistically significant increment was observed at the end of the study (406.7 ± 67.0 vs. 619.7 ± 90.7 IU/ml, respectively, $p = 0.001$).

The IL-4 and IFN- γ were determined in the supernatant of mononuclear cells culture obtained from 17 and 18 children in experimental and control groups, respectively. In the BCG group, IL-4 and IFN- γ levels at the end of the study (17.9 ± 5.7 pg/ml and 354.8 ± 139.0 pg/ml, respectively) were not statistically different from initial values (10.8 ± 2.3 pg/ml, $p = 0.14$, and 348.6 ± 118.0 pg/ml, $p = 0.52$, respectively,

Table 1. Baseline characteristics of patients included in the study

Feature	BCG group (n = 33)	Control group (n = 34)	p-Value
Male/female (n)	15/18	18/16	0.63
Age (yr)	10.4 ± 0.3	10.3 ± 0.3	0.83
Weight (kg)	38.9 ± 2.2	39.2 ± 2.2	0.94
Height (cm)	141.1 ± 2.1	141.2 ± 2.3	0.97
BCG in the first month of life (n)	33	34	1.00
PPD result, mm (n)			
0-4	29	31	0.71
5-9	3	3	1.00
≥ 10	1	0	0.49
Family history of atopy (n)			
Asthma	19	21	0.81
Rhinitis	4	1	0.20
Eczema	3	4	1.00
Passive tobacco exposure (n)	23	26	0.59
Breast-feeding (n)	25	29	0.37
Home moisture (n)	24	22	0.60
Severity of chronic asthma (n)*			
Intermittent	20	24	0.45
Mild	7	5	0.54
Moderate	5	5	1.00
Severe	1	0	0.49
Emergency room visits in the last year (n)			0.11
4	28	33	
> 4	5	1	
Triggering factor (n)			
House dust	33	32	0.49
Pets (mainly dogs and cats)	24	27	0.58

*Severity of asthma was classified according to the Global Initiative for Asthma (19).

BCG, bacilli Calmette-Guerin; PPD, purified protein derivative.

Fig. 1b,c). By contrast, in the control group IL-4 increased from 6.7 ± 1.3 pg/ml at the beginning of the study to 16.1 ± 6.0 pg/ml after 1 yr ($p < 0.05$, Fig. 1b), while IFN- γ values decreased from 279.9 ± 82.1 to 232.1 ± 109.6 pg/ml ($p = 0.01$, Fig. 1c).

Blood leukocyte counts at the beginning of the study were into normal values in both experimental and control groups (Table 2). At the end of the study, a statistically significant increase in the blood monocyte count was observed in both groups. Although a mild decrease was found in blood eosinophil counts in both groups, it only achieved statistical significance in the control group. Eosinophils in nasal secretion diminished at the end of the study in both groups.

Finally, from the clinical point of view at the end of the study most patients had maintained the same asthma severity status and the same proportion of emergency room visits, when compared with their initial status. The proportion of those in whom asthma improved or worsened, either in the asthma severity classification or in the number of emergency room visits, was the same in both groups (Table 3).

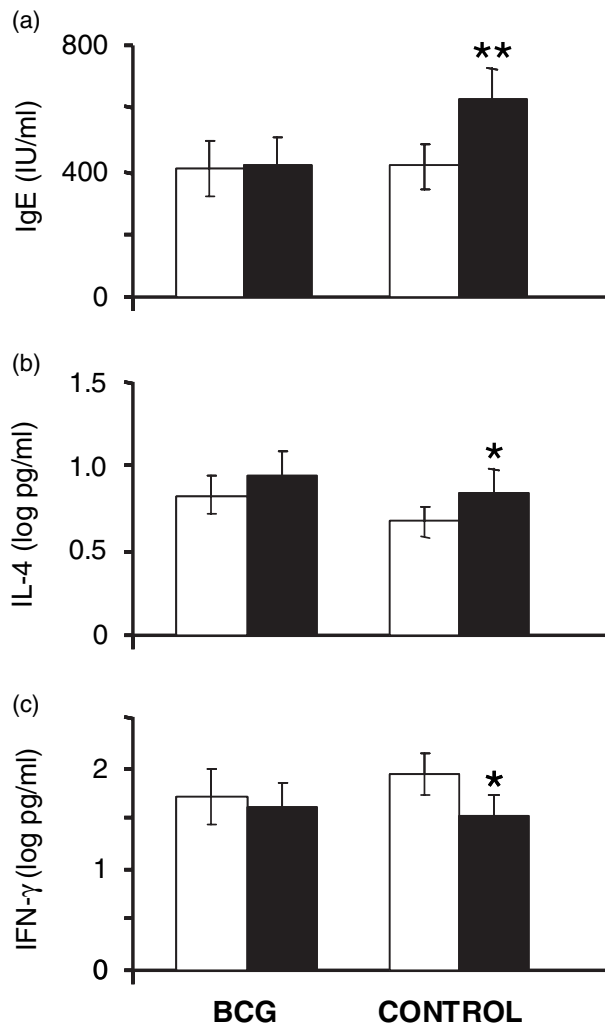


Fig. 1. Effect of bacilli Calmette-Guerin (BCG) vaccination on serum immunoglobulin E (IgE) (a), and on interleukin (IL)-4 (b) and interferon (IFN)- γ (c) released from stimulated mononuclear cells, cultured from peripheral blood. Bars correspond to the initial measurement before BCG or placebo administration (open bars) and to the final measurement made 1 yr later (closed bars). Numbers of patients were: for IgE measurement, $n = 33$ (BCG) and $n = 34$ (control). For IL-4 and IFN- γ determination, $n = 17$ (BCG) and $n = 18$ (control). * $p < 0.05$ and ** $p < 0.002$ when compared with its respective basal value.

Table 3. Changes in asthma severity at the end of the study

	BCG group ($n = 33$)	Control group ($n = 34$)	p-Value*
Asthma severity			0.59
Increased	4	2	
Unchanged	25	26	
Decreased	4	6	
Emergency room visits in the last year			0.29
Increased	6	2	
Unchanged	16	20	
Decreased	11	12	

*Chi-squared test in 2×3 table.

BCG, bacilli Calmette-Guerin.

Discussion

The main finding of our study was that BCG vaccination curtailed the ongoing increment of the Th₂ response observed in control asthmatics, although this was not accompanied by differences in clinical manifestations.

At the end of the 12-month post-vaccination period, peripheral leukocytes from children of the control group displayed a stronger Th₂ response, when compared with their initial status. This increment was observed even after 2 yr of anti-asthmatic treatment supervised by a pediatrician and based on the international guidelines for asthma treatment (19). This result is in full agreement with the study by Choi *et al.* (15) performed in adult asthmatic patients. Like us, these authors found that the IL-4/IFN- γ ratio increased in the control group, while it was maintained unchanged in the BCG-vaccinated subjects. Thus, this phenomenon seems to indistinctly occur among children and adult asthmatic subjects. This is a puzzling finding that, if confirmed in subsequent studies, might indicate that current asthma therapy is not enough to avoid the ongoing immunologic abnormalities taking place in asthma, which continue to be reinforced toward a Th₂ response. Although

Table 2. Effect of bacilli Calmette-Guerin (BCG) vaccination on the cell counts from peripheral blood and nasal mucous smear

	BCG group ($n = 33$)		Control group ($n = 34$)	
	Initial	Final	Initial	Final
Peripheral blood (cells/mm ³)				
Leukocytes	6779 $\pm$ 342	7048 $\pm$ 246	7238 $\pm$ 370	7350 $\pm$ 284
Neutrophils	3027 $\pm$ 258	3032 $\pm$ 190	3493 $\pm$ 355	3518 $\pm$ 262
Lymphocytes	2709 $\pm$ 149	2951 $\pm$ 113	2606 $\pm$ 102	2722 $\pm$ 93
Monocytes	464 $\pm$ 38	583 $\pm$ 31**	494 $\pm$ 34	573 $\pm$ 31*
Eosinophils	561 $\pm$ 78	449 $\pm$ 49	627 $\pm$ 84	498 $\pm$ 65*
Eosinophils in nasal cytology (%)	28.6 $\pm$ 5.2	15.0 $\pm$ 3.4*	31.9 $\pm$ 5.3	15.1 $\pm$ 3.1**

* $p < 0.05$ and ** $p < 0.01$, compared with its respective initial value.

parents of all children received an introductory talk explaining the need to avoid allergens suspected to be involved in the disease, we did not monitor the exposure to such allergens. In this sense, the possibility that children remained exposed to these allergenic factors cannot be discarded. Interestingly, some *in vitro* studies suggested that corticosteroids *per se* induce Th₂ responses (21, 22). Thus, an additional factor that could be partially involved in the increased Th₂ responses observed in control children might be the corticosteroid treatment.

Notably, a single administration of intradermal BCG was able to restrain the modifications of the Th₂ cytokine pattern observed 1 yr later. This fact demonstrates that the beneficial effect over the immune status offered by BCG is long-lasting. Contrasting with this finding, however, we were unable to demonstrate changes in the clinical measurements of asthma severity between the BCG and control group, suggesting that the possible clinical benefits obtained in shorter periods could be lost after 1 yr.

A further difference between the study by Choi et al. (15) and our own study was the site where the cytokine pattern was assessed. In the former study, cytokines concentrations were measured in sputum samples, while we analyzed them using polyclonally stimulated PBMC. In spite of this difference, the results of both studies concerning the IL-4/IFN- γ ratio were in full agreement, indicating that mononuclear cells obtained from blood probably reflect the changes observed in those immune cells recruited in airways.

In a similar fashion as with IL-4, the serum IgE levels significantly increased in the control group but not in the BCG-vaccinated subjects. Since IgE switching and production is partly dependent on IL-4, the changes observed in IgE concentrations are in agreement with the modifications observed in IL-4 levels. Although not totally comparable, our results are in the same line than those obtained by Barlan et al. (23). These authors cultured PBMC from six children with atopic asthma and five healthy children, before and 8 wk after BCG vaccination. They found that stimulated PBMC produced lower levels of IgE after BCG administration in both groups. They did not find BCG-induced changes in the release of IL-2, IL-4, IL-5 and IFN- γ .

On the contrary, at least two recent studies have tested the effect of a different mycobacterium (*M. vaccae*) on the evolution of asthma in adult patients. Shirlcliffe et al. (14) studied 43 adult patients with allergic asthma who received intradermal injections of placebo or either heat-killed or delipidated deglycolipidated *M. vaccae*.

After a 3-month follow up, authors found that clinical and laboratory parameters (including IFN- γ , IL-5 and IgE determinations in peripheral blood) did not change in vaccinated groups, when compared with controls. Camporota et al. (17) studied 24 adults with atopic asthma who received an intradermal injection of *M. vaccae* and were followed up for 3 wk. In this work there was a trend for improvement of functional parameters and a marginal diminution of IgE and IL-5.

We concluded that, contrary to the common hypothesis, BCG vaccination in asthmatic children was unable to cause a long-term reinforcement of Th₁ response, although it could avoid the increased Th₂ response observed in control patients.

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