

Effect of BCG accination on cytokine mRNA expression in atopic children with asthma

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Abstract

Background: To investigate whether a preexisting TH2-type immune response could be suppressed by BCG immunization in atopic children with asthma. **Methods and results:** We have used PCR to amplify reverse transcribed RT IFN- and IL-5 mRNA expressed by peripheral blood mononuclear cells PBMCs in response to in vitro phytohemagglutinin A, purified protein derivative and Dermatophagoides pteronyssinus II stimulation from nine atopic children, both before and 8 weeks after BCG accination. We have demonstrated that IFN- expression was induced in response to all stimulants IFN-actin after the accination, whereas there was no expression before $P < 0.001$. Although there was a tendency to diminish in the expression of IL-5 mRNA in response to the stimulants, only PHA rendered a statistically significant decrease after the accination. **Conclusions:** These results provide some evidence of TH1 dominance after BCG administration in atopic children. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: BCG; Atopy; Asthma

1. Introduction

Bronchial asthma is characterized by a TH2 type immune response, altered airway reactivity to inhaled allergens and chronic inflammation of the airways [1]. The prevalence of atopic diseases has been increasing in many parts of the world over the last 30 years [2]. A recently advanced hypothesis suggests that changes in the general range of infections may account for the increased prevalence of atopic diseases. The reason for the suggested inhibitory effect of infections on the development of atopy could be that the characteristic cytokine product of TH1- and TH2-type T cells is mutually inhibitory for the differentiation and effector function of the reciprocal phenotype.

Indeed, epidemiologic studies showed an inverse correlation between the frequency and intensity of the response to viral and bacterial infections and the develop-

ment and prevalence of allergic diseases [35]. Recovery from natural measles infection reduces the incidence of atopy and of allergic reactions to house dust mite, to half the incidence seen in vaccinated children, suggesting a systemic and nonspecific switch to TH1 activity [3]. Studies by Shirakawa et al. on children in Japan point to the role of tuberculosis and the use of BCG vaccine in influencing asthma prevalence [5]. They demonstrated a negative relation between delayed hypersensitivity to tuberculin response and the presence of asthma. They observed that children with a positive delayed type hypersensitivity test response had serum cytokine concentrations suggestive of predominant TH1 responses, in contrast to TH2 profile seen in children with negative tuberculin test.

BCG vaccine stimulates a TH1 response with increased IFN- and IL-12, which in turn inhibits cytokines from TH2-like cells and also inhibits proliferation of TH2-like cells [6]. By stimulating the TH1 response with BCG vaccine and thus increasing IFN-, it may be possible to decrease the TH2 profile seen in allergic persons.

To investigate the effect of BCG accination in atopic children, we used reverse transcribed RT PCR to show

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IFN-, and IL-5 mRNA expression in peripheral blood mononuclear cells PBMCs in response to phytohemagglutinin A PHA, purified protein derivate PPD and Dermatophagoides pteronyssinus II Der p II stimulation both before and 8 weeks after BCG accination.

2. Materials and methods

2.1. Study population

Nine patients with atopic asthma were selected among the patients followed at Marmara University Hospital Pediatric AllergyImmunology Outpatient Clinic. Eligibility for enrollment in the study specified that all patients had to have asthma according to criteria established by American Thoracic Society [7]; have high IgE levels for age and positive response to Der p II on skin prick testing. A test response was considered positive if the wheal had a mean diameter of at least 3 mm or greater than that of the saline control. In addition, all patients had to have a negative response to PPD skin testing. All subjects had scars due to BCG accination at birth.

The hospital's Ethical Committee approved the study and parents of the participating children signed the informed consent forms.

2.2. Study design

After heparinized blood was collected from all children, BCG was administered intradermally. We then investigated the gene expression of 2 cytokines IL-5 and IFN- by RT-PCR in PBMC in response to *in vitro* PHA, PPD and Der p II stimulation in all cases. Later 8 weeks, after the documentation of PPD conversion in all subjects, the same procedure was repeated.

According to the practice in our country, BCG accination is performed routinely at birth. Our study design is in compliance with the recommendations of Ministry of Health since according to their recommendation, documentation of a negative PPD response at least 5 years after the first accination warrants another BCG accination.

2.3. PBMCs isolation and cell culture conditions

PBMCs were isolated by FicollHypaque density gradient centrifugation and then suspended in RPMI 1640 with 2 mmol/l l-glutamine, 100 U/ml penicillin/streptomycin Sigma Chemical Co., St Louis, MA supplemented with 10% FCS JRH Biosciences, S-00016. Cells were incubated with 1.2 g/ml Der p II, 30 g/ml PHA and 5 g/ml PPD at 37°C with 5% CO₂ for 5 days. After centrifugation at 1500g for 15 min at room temperature,

PBMC pellets were frozen and stored at 80°C until RNA isolation.

2.4. Total RNA extraction

Frozen PBMC pellets were lysed with 800 µl of a denaturing solution containing 4.0 M guanidium thiocyanate, 25 mM sodium citrate pH 7.0, 0.1 M 2-mercaptoethanol and 0.5% sarcosyl. Total cellular RNA was isolated by using the acid-guanidium thiocyanate method [8] and quantified by absorbency spectrophotometry at 260 nm. The OD_{260/280} ratios of all samples were found between 1.7 and 2.0.

2.5. RT-PCR analysis

Three micrograms of total cellular RNA in 33 µl of diethylpyrocabonate DEPC treated water from each sample were incubated at 65°C for 5 min. After cooling the samples on ice, a mixture containing 125 ng oligo-dT1218 Promega, USA, 50 ng bovine serum albumin Sigma, 1.25 mM each dNTPs Promega, USA, 0.5 U RNasin and 15 U M-MLV reverse transcriptase Promega were added in a total volume of 40 µl buffer 50 mM TrisHCl pH 8.3, 50 mM KCl, 5 mM MgCl₂. The samples were incubated for 10 min at room temperature followed by 60 min at 42°C and 10 min at 72°C and stored at 80°C. Five microliters of the reverse transcribed sample was used for amplification. PCR was performed in the presence of β -actin and cytokine-specific primers 1.25 M, 2 mM MgCl₂, 200 M dNTPs and 2.5 U Taq polymerase Promega in a total volume of 50 µl of PCR buffer 500 mM KCl, 100 mM TrisHCl pH 9.0, 1.0% Triton X-100. β -actin was amplified as a control for the RNA isolation and transcription. After heating to 94°C for 5 min, samples were subjected to 30 repeated cycles of denaturation 94°C, 1 min, annealing 60°C, 2 min and extension 72°C, 3 min with the thermal cycler Biometra, Germany. To verify the size and purity of PCR products, 5 µl aliquots of the PCR product reaction mixtures were electrophoresed on 2% agarose gels containing 0.5 µl ethidium bromide in TBE buffer. The gels were photographed under ultraviolet illumination. The total areas under the scanned photography peaks each cytokine band were expressed as a percent of those of the corresponding β -actin.

The following primers were used:

β -actin F 5'-ATGGATGATGATATCGCCGCG-3',
 β -actin R 5'-CTAGAAGCATTTCGCGGTGGACGATGGAGGGGCC-3',
 IFN- F 5'-ATGAAATATACAAGTTATATCTTGTTT-3',
 IFN- R 5'-GATGCTCTTCGACCTCGAAACAGCAT-3',
 IL-5 F 5'-GCTTCTGCATTTGAGTTTGCTAGCT-3',

IL-5 R 5-TGGCCGTC AATGTATTTCTTTATT-AAG-3.

2.6. Dot-blot hybridization

We have also verified our data by dot-blot analysis with β -actin and IFN- specific probes. Ten micrograms of PCR amplification mixture from all subjects were simultaneously denatured by making up to 100 μ l with TE buffer 10 mM TrisHCl pH 8.8, 0.1 mM EDTA, adding 6 μ l 0.5 M EDTA and 8 μ l 6 M sodium hydroxide and incubating on ice for 10 min. After neutralization with 115 μ l 2 M ammonium acetate, the samples were applied with suction to a single membrane Gene-Screen Plus, Dupont, USA using a dot blotting apparatus Bio-Rad and the cDNAs crosslinked to the membrane by incubating at room temperature. The membranes were sequentially hybridized with ^{32} P ATP-labeled oligonucleotide probes specific for each cytokine and β -actin. β -Actin was used as an internal control probe. The membranes were then exposed to X-ray film for a time period sufficient to allow visualization of the blots. For each cytokine and β -actin, exposed films were scanned using a flat bed scanner and relative amount of each cytokine mRNA in each sample was calculated as the ratio of cytokine-actin. The following probes were used:

β -actin 5-GCGCGGCTACAGCTTCACCACC-3,
IFN- 5-GCCAAATTGTCTCCTTTTACTTC-3,
IL-5 5-GGCACTGCTTTCTACTCATCG-3.

2.7. Statistics

Relative amount of each cytokine mRNA in each sample was calculated as the ratio of cytokine-actin. Non-parametric statistical comparisons of IFN- and IL-5 levels in each patient before and after BCG accination for various stimulants were made by using Wilcoxon signed-rank test. A P value <0.05 was considered to be statistically significant.

3. Results

The median range age for subjects was 8.3–12 years. Serum IgE values of patients did not show any significant change after the accination data not shown. All subjects developed a positive PPD reaction with a duration of at least 55 mm 8 weeks after BCG accination.

3.1. Expression of cytokine mRNA

The expression of cytokines mRNAs obtained by RT-PCR and dot-blot analysis in PBMC of one patient are shown in Figs. 1 and 2, respectively.

3.1.1. IFN-

We did not detect any expression of IFN- mRNA in any patients in response to unstimulated medium, Der p II, PPD stimulation, but in one in response to PHA Fig. 3. An induction of IFN- mRNA expression was detected in six of nine patients in unstimulated medium P0.02, in all nine patients with PHA P0.007, seven of nine with PPD stimulation P0.01 and five of seven patients with Der p II P0.04.

3.1.2. IL-5

We detected variable degrees of expression of IL-5 mRNA in eight of nine patients in unstimulated medium with PHA and PPD stimulation, whereas in response to Der p II, six of seven patients showed IL-5 mRNA expression Fig. 4. A decrease of IL-5 mRNA expression was shown in seven and an increase in two of nine patients in unstimulated medium P0.26. In eight of nine patients, a decrease was detected in response to PHA stimulation, whereas one patient showed an increase P0.02. A decrease of IL-5 mRNA expression was shown in five and an increase in two and no change in two of nine patients in response to PPD stimulation P0.31. In response to Der p II stimulation, we detected a decrease in four patients, an increase in two patients and no change in one patient P0.83.

4. Discussion

This study demonstrates an increased induction in the gene expression of IFN- in PBMCs of atopic children after the administration of BCG vaccine, while there was no expression before. Of the Th2 cytokines, IL-5 expression showed a trend to diminish after the accination which was not statistically significant except for PHA stimulation.

Mycobacteria, especially *M. tuberculosis*, are known to be very potent inducers of Th1 immune responses [9]. Sander et al. demonstrated that there was a marked production of Th1 lymphokines, such as IFN-, IL-2 and TNF- at days 45 after BCG stimulation in PBMC from healthy individuals [10]. Another human study by Tsicopoulos et al. had shown that cells at the tuberculin injected sites expressing mRNA for IFN- and IL-2 increased at 24 h and reached a peak at 48 h [11]. It has been demonstrated by Cooper et al. that IL-12 mRNA expression is induced by *M. tuberculosis* infection both in vivo and in vitro in mice [12]. They showed that IL-12 increases the production of IFN- and both of these

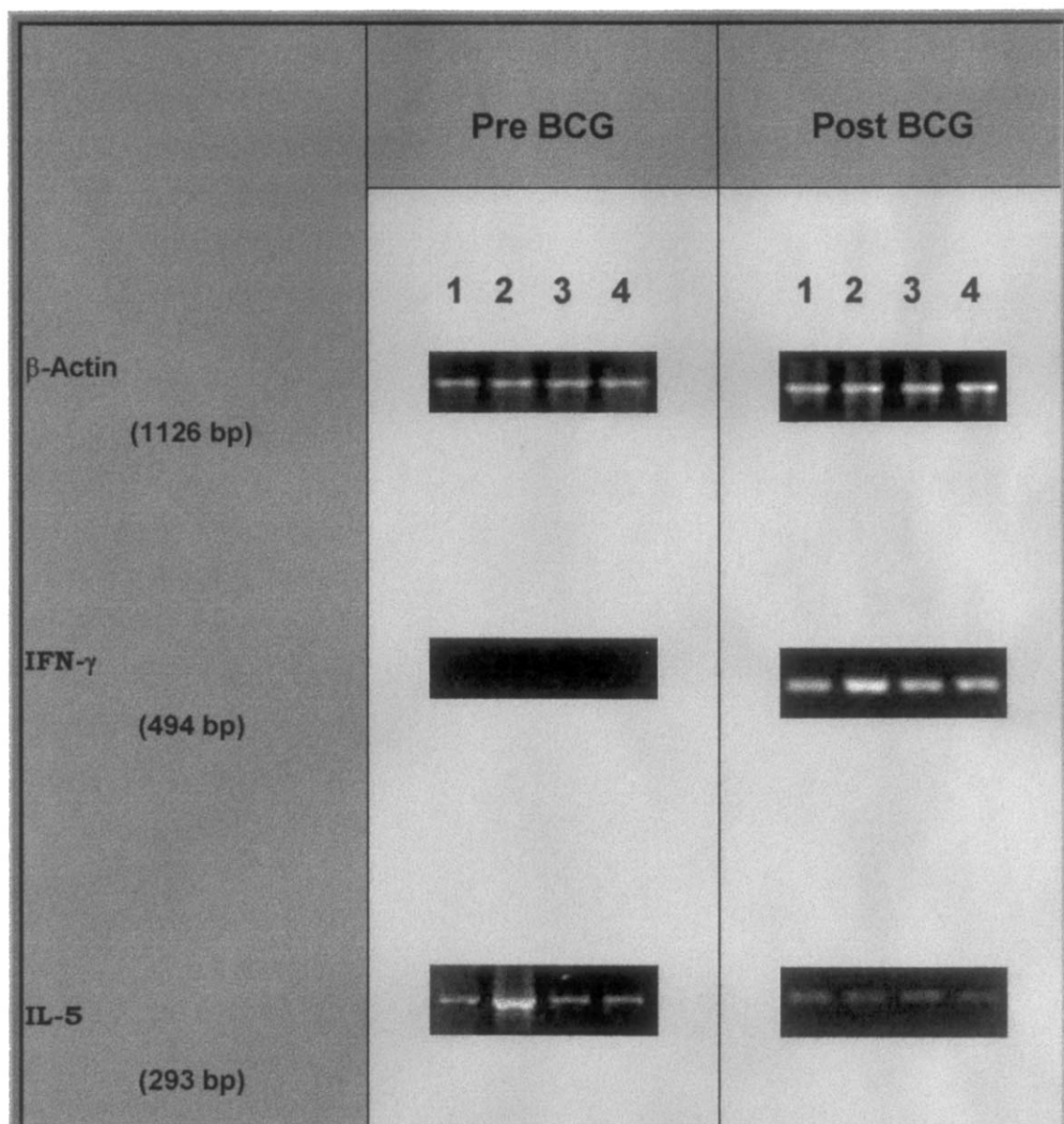


Fig. 1. Cytokine mRNA expression in PBMC of a patient. RNA extracted from PBMCs both pre and 8 weeks post BCG vaccination was subjected to reverse transcription and amplified by PCR by using primers specific for each cytokine (IFN- γ , IL-5). Lane 1: PBMCs without stimulation; lane 2: PBMCs stimulated with PHA; lane 3: PBMCs stimulated with Der p II; lane 4: PBMCs stimulated with PPD.

cytokines have been able to skew the immune response to TH1. Whether BCG vaccine can accomplish this shift in atopic children is examined for the first time in our study. Our data provided further evidence of IFN- γ as the major cytokine induced by BCG stimulation even in atopic children. Using Der p II, a major allergen of the house dust mite HDM, we have demonstrated that cells from HDM-sensitive atopic patients can be induced to express IFN- γ gene.

This data challenges the view that low tuberculin responder rate among atopic children reported in Shirakawa's paper is due to the fact that those children

cannot mount an optimal delayed type hypersensitivity DTH to *M. tuberculosis* PPD, therefore the findings of the study should be interpreted as a surrogate rather than a cause. According to our data, nine atopic and previously tuberculin negative children were able to develop an appropriate DTH response to PPD 99 and showed an increased gene expression of IFN- γ in response to in vivo BCG stimulation 99. Therefore, our results contradict the suggestions that atopic children have a decreased ability to mount a strong TH1-cell-mediated delayed hypersensitivity response [13,14].

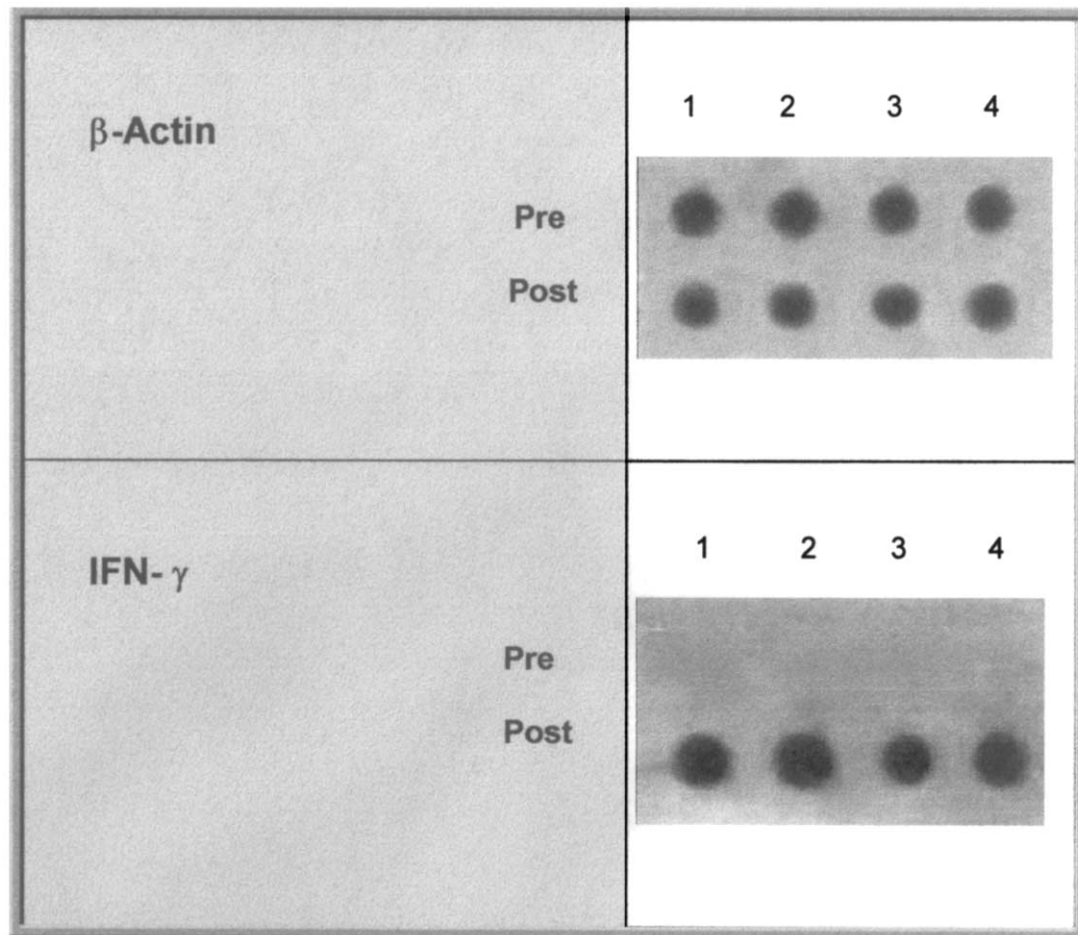


Fig. 2. Dot-blot analysis of β -actin specific and IFN- γ specific cDNA obtained by reverse transcription of mRNA from PBMC of a patient amplified by PCR using primers specific for β -actin and IFN- γ . Lane 1: PBMCs without stimulation; lane 2: PBMCs stimulated with PHA; lane 3: PBMCs stimulated with Der p II; lane 4: PBMCs stimulated with PPD.

Although we acknowledge the limitations of RT-PCR technique which is certainly not a quantitative assay, we preferred to measure mRNA in this study rather than

the corresponding translated protein products because the technique is more sensitive and also because of the possibility that culture of PBMC in vitro might be

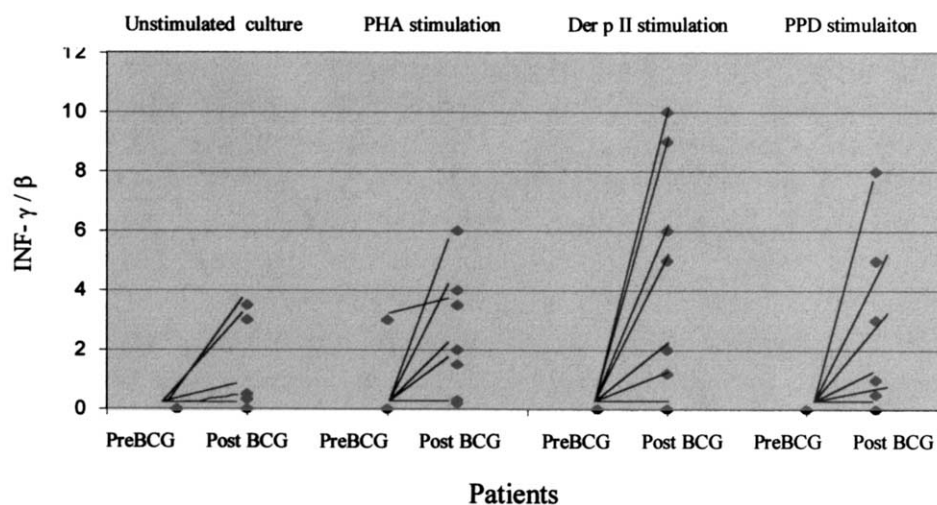


Fig. 3. IFN- γ mRNA level in response to unstimulated, PHA, Der p II and PPD stimulated medium both pre- and post-BCG administration in all patients.

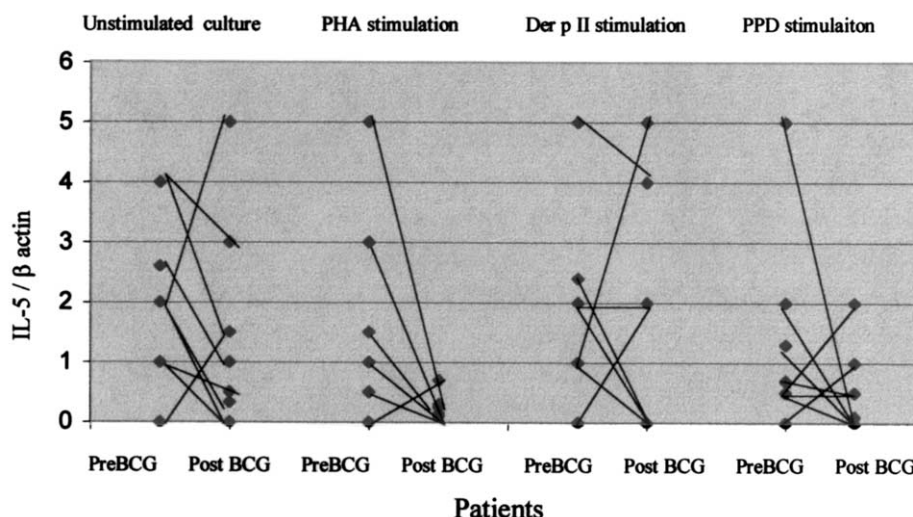


Fig. 4. IL-5 mRNA level in response to unstimulated medium pre- and post-BCG administration in all patients.

associated with rapid alterations in their profiles of cytokine synthesis.

Of the Th2 cytokines, although IL-5 gene expression was generally downregulated by BCG accination, it reached a statistically significant decrease only with PHA stimulation. This could be due to the limitation of the size of the study population. For a conclusive result, more subjects need to be studied in this respect. In mice, it has been shown that certain mycobacterial strains can switch off a pre-existing TH2 response to unrelated molecules. The data from Rook et al. [15] showed that ongoing allergen-specific response in BALBc mice was downregulated by treatment with a low dose of killed *M. vaccae* without any need for ovalbumin OVA or OVA epitopes in the *M. vaccae* preparation. This treatment inhibited not only serum IgE, but also the potential for ovalbumin-induced IL-5 production by spleen cells. In a recent study from our group [16], we showed that preimmunization with killed *M. bovis* alone or in addition to *M. vaccae* resulted in downregulation of serum IgE response in ovalbumin sensitized newborn mice. Herz et al. [17] recently demonstrated that systemic BCG immunization hinders the development of allergic sensitization and prevents the development of increased airway responsiveness in BALBc mice [18,19].

It could be speculated that in children with an atopic heredity, early infections may skew the TH1/TH2 balance towards a TH1-cytokine pattern, thereby may have a suppressive effect on the development and the severity of allergic diseases. In countries where the prevalence of *M. tuberculosis* is high, BCG, which is often performed during the early first days of life, might have such an effect, as well.

In conclusion, this study demonstrated the induction of IFN- γ mRNA expression in PBMCs of atopic children in response to BCG accination, while there was no

expression before accination. IL-5 expression showed a trend to decrease after the accination that reached to statistical significance only in response to PHA stimulation. Since all our atopic patients developed an appropriate PPD reaction after the administration of BCG, our results contradicted the suggestions that atopic children have a decreased ability to mount a delayed hypersensitivity response.

These results provide some evidence of Th1 dominance after BCG administration in atopic children. Suppression of TH2 response needs to be further evaluated in a larger number of subjects.

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