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Influence of immunisation with *Mycobacterium bovis* bacillus Calmette-Guérin on the sensitisation to inhaled allergens after infection with respiratory syncytial virus

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Abstract Respiratory syncytial virus (RSV) may play an important role in allergic diathesis by creating a Th2-type immune response. *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) is known to induce a Th1-type immune response, but the association of BCG vaccination and the suppression of allergy development remain controversial. We investigated the influence of BCG vaccination on the immune response to RSV in a mouse model. Balb/c mice were BCG vaccinated, RSV infected and ovalbumin (OVA) challenged. Mice were sacrificed one, two and four weeks after allergen exposure. Bronchoalveolar lavage was performed. Alveolar macrophages and lymphocytes from spleens and lung-associated lymph nodes were investigated for cytokine production and cell proliferation. Serum was tested for allergen-specific immunoglobulin-E (IgE). Lung eosinophilia was diminished by BCG immunisation. OVA-specific serum IgE was increased regardless of prior BCG

vaccination. Interleukin-4 secretion of spleen lymphocytes increased in BCG-vaccinated mice only one week after allergen exposure but was comparable to non-vaccinated mice at four weeks. The reactivity of spleen lymphocytes towards concanavalin-A to secrete interferon- γ was increased in the vaccinated group at the end of the observation period. Interleukin-6 and tumour necrosis factor- α secretion of alveolar macrophages as well as proliferation of stimulated thoracic lymph node cells were increased and prolonged in vaccinated mice. BCG immunisation led to a local suppression of the allergic reaction within the lung. No reduction of systemic IgE production was observed. Further studies are necessary to determine a possible time dependence of BCG immunisation.

Key words BCG • Vaccination • RSV • Infection • Allergy

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Introduction

In recent years atopic disorders have been noticed more frequently, especially in industrialised countries [1]. Differences in lifestyle and environmental factors as well as changes in exposure to infectious agents early in life have been discussed as contributors to this increase [2, 3]. Exposure to mycobacteria such as *M. bovis* bacillus Calmett-Guérin (BCG) vaccination is known to result in a shift towards a Th1-type cytokine profile leading to increased levels of interferon- γ (IFN- γ) and a reduction of lung eosinophilia in humans or mice [4–7], which, in consequence, might decrease the risk for subsequent Th2-dependent atopic diseases [8].

Several epidemiological studies addressed this question with contradictory results. The International Study of Asthma and Allergy in Childhood (ISAAC) noted a significant inverse association between prevalence of asthma and tuberculosis notification [9]. An inverse association

between purified protein derivative (PPD) skin test reactivity and subsequent development of atopic disorders has been reported from Japan [10]. In contrast, investigations from Scandinavia failed to confirm these observations but showed a tendency to a lower prevalence of allergy among immigrant children born in non-western countries only [11].

Respiratory syncytial virus (RSV) is the most common cause of lower respiratory tract infection during infancy with annual recurrence in winter outbreaks without long term immunity [12]. In contrast to several other viral infections RSV induces a typical Th2-type immune response in humans and mice [13, 14] and increases the uptake of inhaled allergens [15]. RSV infection early in life seems to play an important role in the development of an allergic diathesis and has been associated with recurrent wheezing or symptoms of asthma later on [16, 17].

Balb/c mice are known to be susceptible to RSV infection [14] and to respond to mycobacterial antigens [6]. We established a model to show the relationship between BCG immunisation and subsequent contact with the allergen ovalbumin (OVA) in the RSV-infected mouse.

Materials and methods

Mice

Pathogen-free female Balb/c mice were purchased from Charles River (Sulzfeld, Germany). All animals used in this experiment were between 6 and 8 weeks of age.

Experimental protocol

As shown in Figure 1, mice were immunised with 2×10^6 colony-forming-units (CFU) BCG Copenhagen strain (BCG-Vaccine Behring, Chiron Behring, Marburg) intraperitoneally. Successful immunisation was verified 4 weeks later by measuring delayed type hypersensitivity (DTH) against PPD of *Mycobacterium tuberculosis*. Three additional weeks later the animals received an intranasal application of 1×10^6 plaque-forming units (PFU) of RSV (long strain) and a daily intranasal OVA challenge from day 5 to 9 after RSV infection. Animals were sacrificed 1, 2 and 4 weeks after the last application of allergen by an intraperitoneal overdose of pentobarbital (Nembutal, Sanofi CEVA). This group of animals was then named "BCG+RSV+OVA". To evaluate the influence of BCG immunisation, a second group of mice

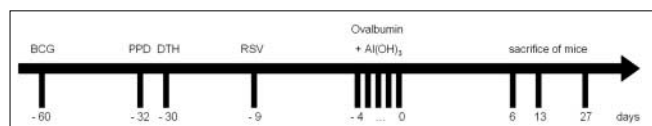


Fig. 1 At days 6, 13 and 27 after the end of allergen exposure mice were sacrificed and samples from different sites were obtained. BCG immunisation had been performed 60 days and RSV infection 9 days in advance

("RSV+OVA") received RSV infection and OVA challenge as described, but without prior BCG vaccination. A third group of mice ("control") never came into contact with BCG, RSV or OVA but became sham infected with sterile saline instead. Forty-five mice were used in each group.

RSV purification and infection

RSV was isolated and grown on HEp-2-cell monolayers in the Department of Virology in Medical School Hannover. Stock virus had a titre of 3.8×10^8 PFU/ml and was stored in aliquots at -80°C . Respiratory infection was established by intranasal administration of 50 μl PBS containing 10^6 PFU RSV after narcosis by an intraperitoneal injection of 20 μl ketamin 10% (WDT, Garbsen) and 2 μl xylazin (Rompun, Bayer, Leverkusen) in 200 μl 0.9% NaCl. "Control" animals received a volume of 50 μl sterile saline only.

OVA-induced airway inflammation

Five to nine days after viral infection a daily intranasal administration of 20 μg OVA with 250 μg Al(OH)₃ as an adjuvant dissolved in 50 μl PBS was performed in ketamin-xylazin-narcosis.

Isolation of alveolar macrophages

After insertion of an 18-G teflon catheter (Abbocath) into the trachea the lungs were then flushed with 1 ml of ice-cold Mg^{2+} - and Ca^{2+} -free PBS containing 0.6 mM EDTA. The lavage procedure was repeated 10 times. Lavaged cells were then washed by centrifugation at 1200 rpm for 10 min at 4°C twice with RPMI 1640. Differential cell count was performed. Cytospots of lavage fluid from untreated mice consisted of $>98\%$ alveolar macrophages after centrifugation of 1×10^5 cells at 600 rpm for 5 min (Cytospin, Shandon Southern, UK) and Pappenheim staining.

Isolation of lymphocytes

Lung-draining lymph nodes were collected and homogenised into a single cell suspension using a glass homogenisator. The spleens of animals were removed aseptically and homogenised until a single cell suspension was achieved. Erythrocytes were lysed by haemolytic buffer containing 37 mg dinatrium-EDTA-2-hydrate, 8.3 g NH_4Cl and 839 mg NaHCO_3 in 1 l aqua dest (pH 7.6). The remaining cell suspension was washed twice in RPMI 1640 culture medium.

Blood samples and serum immunoglobulin-E (IgE)

Blood was obtained by puncture of the vena cava and additional cardiac puncture. Serum was stored in aliquots at -80°C for

OVA-specific IgE determination by ELISA. Results were expressed as optical density (OD).

Culture conditions of cells

Macrophages were cultured in RPMI 1640 culture medium supplemented with 10% foetal calf serum (FCS) and 100 U of penicillin and streptomycin each. Lymphocytes from lymph nodes and spleens were cultured in RPMI 1640 culture medium containing 5% FCS, 0.5% HEPES-mercaptoethanol and antibiotics as mentioned above. Concentrations of stimulants were: 1 µg/ml LPS, 5 µg/ml concanavalin-A (ConA), 25 µg/ml or 40 µg/ml OVA and 10 µg/ml or 20 µg/ml PPD. Cells were stimulated for 18 h. After that time period the supernatants were removed and stored in aliquots at -20°C for cytokine determination by ELISA or bioassay.

Lymphocyte proliferation assay

Lymphocyte proliferation was stimulated by 5 µg/ml ConA or 1 µg/ml LPS. ³H-thymidine was added and blastogenesis was determined by ³H-thymidine incorporation and measured by β-scintillation counting. Results were expressed as multiplicity of medium proliferation (serving as negative control).

Bioassays

Interleukin-6 (IL-6) levels in supernatants of macrophage cultures were determined by proliferation of the IL-6-dependent hybridoma 7TD1 B-cells using MTT. Macrophage supernatant dilutions and 1x10⁵ 7TD1 cells per well were incubated for 72 h. Ten microlitres of MTT were added for 2 h. The reaction was ended by adding 100 µl aqua dest. containing 50% N,N-dimethylformamide and 5% natriumdodecylsulfate. Cytokine levels were measured by a microwell-reader (Dynatech) using recombinant IL-6 to determine a standard curve. Tumour necrosis factor-α (TNF-α) activities of macrophage supernatants were measured using a bioassay on actinomycin D-treated (Sigma Chemical Co.) L929 cells. Briefly, 1x10⁵ murine L929 cells in RPMI with 10% FCS were added to each well of a 96-well flat-bottom microtitre plate, incubated at 37°C in 5% CO₂ for 24 h. Serial dilutions of recombinant murine TNF-α were used for standard curve determination. After an incubation period of 18 h at 37°C in 5% CO₂ the cytotoxic effect of TNF-α was determined by diphenyltetrazolium reduction assay as described.

ELISA for the detection of cytokines and IgE

Culture supernatants of lymphocytes were screened for levels of IFN-γ and IL-4, alveolar macrophage supernatants for levels of IL-1 and serum for OVA-specific IgE levels with a double-sandwich enzyme-linked immunosorbent assay with 2 specific monoclonal antibodies (Pharmingen) or biotin-conjugated OVA respectively. Concentrations were calculated with ELISA-Software Mikrowin.

Statistical analysis

Statistical significance ($P < 0.05$) was analysed by Student's *t*-test. Figures show the mean of results and the standard error of the mean (SEM).

Results

To assess local allergic effects within the lung, eosinophilia of bronchoalveolar lavage was determined. Reactivity of alveolar macrophages towards secretion of cytokines was measured to evaluate local inflammation. Systemic immune response was measured by total allergen-specific serum IgE titre. Furthermore, lymphocytes from the spleen and from thoracic lymph nodes were tested for an increased reactivity towards cytokine secretion and proliferation. Levels of Th1-type cytokines such as IFN-γ as well as Th2-type cytokines such as IL-4 were measured.

Control of successful BCG vaccination

Intraperitoneal BCG immunisation was performed 51 days before intranasal RSV infection to ensure a clearance of the mycobacteria and a well established immunisation status in the animals. Seven weeks after BCG injection no viable bacteria were detectable in liver, spleen, lung, lung-associated lymph nodes and the peritoneal cavity of treated mice (data not shown). To determine the T-cell-mediated response, DTH reaction was measured at day 28 after immunisation. A positive response in the BCG-treated group was shown by a significantly increased footpad thickness after PPD injection. Animals that received PBS only did not show any footpad swelling (data not shown).

Control of successful RSV infection

In RSV-infected mice clinical symptoms of respiratory tract infection such as tachypnoea and a reduced general condition were observed for 48 h after infection when compared to non-infected controls (data not shown).

Organ weight

The weights of lungs and spleens were determined. Lung weights slightly increased in both RSV-infected groups at all time points without any influence of prior BCG immunisation, whereas spleen weights were increased in BCG-

immunised mice only. These data failed to reach statistical significance (data not shown).

Eosinophilia in bronchoalveolar lavage

Eosinophilia was induced by RSV infection and subsequent OVA challenge. To determine the influence of BCG immunisation on the sensitisation reaction, the proportion of eosinophilic granulocytes in the bronchoalveolar lavage was determined. As shown in Figure 2, an increased number of eosinophils in the RSV-infected and allergen-challenged animals were found, whereas previous BCG vaccination led to a lower proportion of eosinophil granulocytes in comparison to the non-immunised group. Lung eosinophilia was significantly reduced by BCG at 2 weeks after allergen exposure. At the end of the observation time period the proportion of eosinophils in both groups was equivalent to the control group level (<1%).

Serum IgE titre

Only animals being exposed to OVA showed OVA-specific serum antibodies. No significant differences were

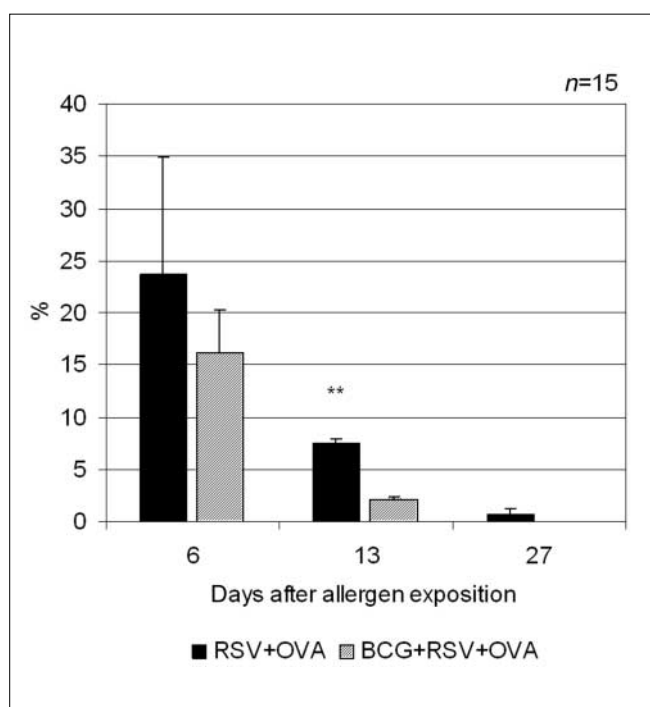


Fig. 2 The proportion of eosinophil granulocytes of all granulocytes in the bronchoalveolar lavage fluid was determined by light microscopy at different time points. A statistical significant difference between immunised and non-immunised mice was shown at day 13 (** $P < 0.01$)

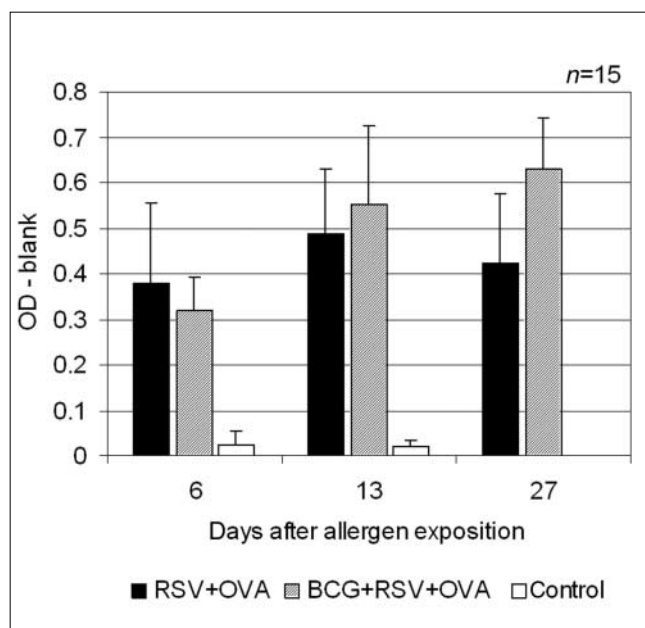


Fig. 3 The allergen-specific immunoglobulin-E serum titre was determined by ELISA at different time points. The figure shows the calculated optical density without the blank. No significant differences were observed at either time point

observed, but IgE titres continued to rise in the vaccinated group but did not in mice without previous mycobacterial contact (Fig. 3).

Functional analysis of alveolar macrophages (Table 1)

Alveolar macrophages represent an important population of the immune system of the lung, and induce several immunologic reactions via soluble mediators, especially pro-inflammatory cytokines. To determine the role of the macrophage system in the induction of allergy, we performed bronchoalveolar lavage. Cell populations were then quantified and differentiated. After a BCG vaccination the LPS-stimulated alveolar macrophages secreted higher amounts of IL-6 and TNF- α compared to the non-vaccinated group. No difference with respect to the IL-1 production was observed.

Functional analysis of thoracic lymph node cells and spleen lymphocytes (Table 1)

No differences in IL-4 secretion of ConA-stimulated lung draining lymph node cells were noticed (data not shown), but stimulated spleen lymphocytes showed an increased IL-4 production over time in the non-vaccinated group while IL-4 production decreased in the BCG-vaccinated

Table 1 Functional analysis of alveolar macrophages (IL-6, TNF- α , IL-1) stimulated by 1 μ g/ml LPS, of spleen lymphocytes (IL-4, IFN- γ) stimulated by 5 μ g/ml ConA, and of lymphocytes from lung draining lymph nodes (proliferation assay) stimulated by 5 μ g/ml ConA; $n=15$; n.s., not significant

	Days after allergen exposure			<i>P</i> -value
	6	13	27	
IL-6, U/ml				
RSV+OVA	224.4	227.6	96.1	n.s.
BCG+RSV+OVA	384.5	368.4*	218.1*	$P<0.05^*$
Control	39.4	39.8	53.8	n.s.
TNF- α , U/ml				
RSV+OVA	1,286.1	574.6	568.9	n.s.
BCG+RSV+OVA	1,034.1	1,162.8*	1,147.7*	$P<0.05^*$
control	137.8	99.5	117.9	n.s.
IL-1, mg/ml				
RSV+OVA	5.6	3.6	3.8	n.s.
BCG+RSV+OVA	4.8	3.6	3.3	n.s.
Control	1.1	1.4	1.1	n.s.
IL-4, U/ml				
RSV+OVA	6.8	14.8	21.1	n.s.
BCG+RSV+OVA	28.7	21.6	22.8	n.s.
Control	7.6	6.7	7.7	n.s.
IFN- γ , U/ml				
RSV+OVA	8.7	19.7	13.1	n.s.
BCG+RSV+OVA	9.9	21.1	29.1*	$P<0.05^*$
Control	18.0	18.9	19.5	n.s.
Proliferation (multiplication of medium control)				
RSV+OVA	3.5	3.4	9.4	n.s.
BCG+RSV+OVA	3.8	8.6*	11.9	$P<0.01^*$
Control	3.8	3.0	3.1	n.s.

group reaching similar levels at the end of the experiment. IFN- γ secretion of ConA-stimulated spleen lymphocytes continued to rise in the immunised group reaching a statistic significant difference on day 27 after allergen contact compared to non-vaccinated mice. Concerning thoracic lymph node cells no differences could be found in the experimental groups (data not shown). Proliferation of ConA-stimulated lymphocytes from lung draining lymph nodes determined by ^3H -thymidine incorporation was also elevated after BCG immunisation. Spleen lymphocytes did not show differences in proliferation (data not shown).

Discussion

The study presented here was based on the findings that there was an inverse association between tuberculin response and atopic disorder in Japanese school children [10], indicating a protective effect by a Th1-type immune response after contact with mycobacteria. However, several other studies worldwide failed to detect a relationship between a positive tuberculin reaction and atopic disorder later in life. The findings of these studies are hardly comparable for several reasons:

First, different results of epidemiological studies may be due to different environmental factors. The prevalence of tuberculosis or atypical mycobacteria as well as differences in BCG strains used for vaccination varies worldwide and may have influenced the reaction to a consecutive tuberculin testing [18, 19]. Second, the age at which BCG vaccination is administered also varies in different countries and immunological reactions to such antigens might be age dependent, although Bager et al. [20] failed to show a relationship between the age of BCG vaccination and the onset of allergic symptoms. Third, there have been regional differences in the diagnostic approach of BCG vaccination assessment. Some studies are based on questionnaires only while others documented the size of BCG scars [21, 22]. Furthermore, different skin test antigens and dosages are in use and the dimension of induration itself that would be considered as a positive skin reaction varies in different countries [23, 24]. This may have led to a selection of strong Th1-responding individuals in some studies, and these individuals are then less likely to develop subsequent allergic diseases.

In our view, concordant with a recently published review on this topic [25], the most reasonable explanation is a genetic predisposition towards atopy that influences tuberculin response too. Minor reactions to PPD after

mycobacterial contact by affected individuals would then serve as a confounder that is associated with a higher prevalence of allergic diseases also. A different association of BCG vaccination and allergic disease depending upon the genetic origin of screened individuals has been described [11]. Adams et al. [26] showed an inverse correlation of tuberculin indurations (Mantoux size) and IgE response. Sarinho et al. [27] have published similar findings: large BCG scars were significantly more common in non-asthmatic control children than in children with a history of three or more episodes of dyspnoea in the previous two years. Recently, a potential genetic risk factor for atopy in relation to BCG vaccination has been described [28].

We developed a mouse model in order to test the influence of BCG immunisation to the allergic reaction to inhaled OVA after RSV lung infection when allergen uptake is known to be increased. Successful BCG vaccination of Balb/c mice was shown by DTH reaction four weeks after immunisation. Consecutive RSV infection and OVA challenge were performed. We were able to show a significant reduction of eosinophilia in bronchoalveolar lavage fluid from intraperitoneally immunised mice compared to non-immunised mice two weeks after allergen exposure as it is known after intranasal application of BCG [5]. Secretion of pro-inflammatory cytokines such as IL-6 or TNF- α from stimulated alveolar macrophages was significantly lowered in the vaccinated group. However, no significant differences in allergen-specific serum IgE titres were observed.

There was no difference in IL-4 secretion of stimulated lung draining lymph node cells. Stimulated spleen cells of vaccinated mice showed even increased IL-4 production at six days after allergen exposure. However, spleen cells' IL-4 secretion in the vaccinated group decreased over time while non-vaccinated animals started at low levels of IL-4 that increased over time instead. Herz et al. [29] also observed normalisation of IL-4 production by splenic mononuclear cells after BCG immunisation in a mouse model. The initial increased secretion of IL-4 in our study might be due to the fact that Balb/c mice are not a strongly Th1-inducible animal compared to e.g., Balb.B10 or C57BL/6 mice, but might be partly counterbalanced by a Th2-type immune response [7]. Orme et al. [30] also described a transient elevated secretion of IL-4 in murine CD4-positive T lymphocytes some weeks after a mycobacterial infection. Whether a suppression of IL-4 secretion after BCG contact can be achieved after longer time periods remains unknown and is subject to further studies.

IFN- γ secretion as a typical Th1-type cytokine of spleen lymphocytes of BCG-vaccinated mice after ConA stimulation increased continuously over the observation time period. The difference compared to non-vaccinated mice was significant at the end of the experiment. Similar findings are reported by Herz et al. [29] who showed increased reactivity of spleen lymphocytes of Balb/c mice

towards IFN- γ secretion after prior BCG immunisation. We did not find differences in IFN- γ secretion by lung draining lymph node cells after intraperitoneal BCG application (data not shown) while for intranasal BCG vaccination of Balb/c mice rapid production of IFN- γ has been described [31]. However, as shown in Table 1, proliferation of these cells after BCG vaccination was elevated in our study indicating an increased level of stimulation caused by the immunisation process.

Our data show that intraperitoneal BCG vaccination reduces lung eosinophilia in Balb/c mice, as has been shown for other forms of BCG application [6, 29]. A systemic anti-inflammatory response in terms of allergen-specific IgE reduction by Th1-inducing BCG vaccination could not be found in our experiments. Although IFN- γ production of spleen lymphocytes was elevated by BCG it remains to be determined whether IL-4 secretion of spleen lymphocytes would have been reduced after longer time periods. Further studies are under investigation to understand the complex interaction of Th1- and Th2-type response in the mouse model.

In addition, we agree with Krishna and Salvi [32] that a well controlled double-blind placebo-controlled multicentre study is necessary to assess reliable epidemiological data.

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