



BCG modulation of anaphylactic antibody response, airway inflammation and lung hyperreactivity in genetically selected mouse strains (Selection IV-A)

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Abstract

The effect of *Bacillus Calmette–Guérin* (BCG) treatment in allergic pulmonary reaction was studied in mice genetically selected accordingly to a High (H-IVA) or Low (L-IVA) antibody responsiveness. Mice were immunized with ovalbumin (OVA) or OVA plus BCG. Two days after nasal antigenic challenge, seric IgE and IgG1 anti-OVA, eosinophils in pulmonary tissue, inflammatory cells in bronchoalveolar lavage and the compliance and conductance of respiratory system were evaluated. H-IVA mice were found more susceptible than L-IVA, and BCG was able to inhibit simultaneously the production of IgE, the bronchopulmonary inflammation and bronchial hyperresponsiveness in these genetically selected mice.

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Introduction

Allergy is a state of hypersensitivity that is mediated by immunoglobulin E (IgE) in response to normally harmless environmental antigens, termed allergens (Coombs and Gell, 1975). In most developed countries, 20% to 30% of the population currently suffers from various forms of atopy such as asthma, rhinitis, atopic dermatitis and food allergy (Wuthrich, 1989).

Recent studies suggest that several cytokines play an important role in the modulation of the immune response to allergens and infectious agents (Romagnani, 1997; Mosmann and Sad, 1996). The majority of allergen-specific T cells in atopic individuals belongs to a Th2 sub-type, associated to high levels of IL-4 or IL-5 and low levels or even undetectable interferon- γ (IFN- γ) titres (Kaufmann, 1993; Robinson et al., 1992). On the other side, a great part of intracellular bacterial infections induce a Th1 type response, associated to high production of IFN- γ (Liu et al., 1994).

Although controversial, several epidemiological data correlate *Mycobacterium tuberculosis* infection with protection against asthma (Walker et al., 2003). Experimentally, mouse models of allergic inflammation have shown that the administration of BCG at the time of sensitization attenuates the antigen induced lung hyperreactivity and eosinophilia.

In these experiments, ovalbumin (OVA) immunized animals produce high levels of specific IgE, associated to Th2 cytokines (IL-4, IL-13, IL-5, IL-10). The choice of the adjuvant used in immunization can interfere significantly in the IgE response and in the cytokine pattern induced after OVA sensitization. Specifically, OVA induces a Th2 response when Al(OH)₃ is used as an adjuvant, whereas Complete Freund Adjuvant (CFA) induces a Th1 response (Wang and Rook, 1998). It is possible that the presence of the mycobacterium in CFA might account for the OVA specific Th2 response inhibition.

The majority of these results was obtained in isogenic mouse lines such as C57BL/6 and BALB/c. In order to access the impact of the host background genetic variability in this modulatory effect exerted by BCG, in this study we used “High” and “Low” antibody responder mouse lines (Biozzi mice) produced by bi-directional genetic selection (Biozzi et al., 1979). The main characteristic considered for the selection experiment (named IV-A), was the quantitative antibody production against heterologous erythrocytes (Cabrera et al., 1995). The breeding of mice chosen for extreme phenotypes, in upward or downward directions, were repeated for successive generations until maximal divergence of the two lines was achieved. This divergence is explained by the progressive accumulation in each line, of multiple genes endowed with coherent additive effects on the selected phenotype. When the limit of response to selection is reached, these relevant genes can be considered to be fixed in homozygosis in each line in a heterogeneous genetic background. The final “High” and “Low” phenotypes resulting from the selection of the lines appear quite exceptional not only for the range of their quantitative differences but also for the generalized alteration of their antibody responsiveness to a large spectrum of immunogens (multi-specific effect). Importantly, these selected mice represent the extreme phenotypes found in natural heterogeneous populations and therefore, they resemble the heterogeneity found in humans.

Herein we investigated the degree of OVA-induced allergic pulmonary reaction in “high” (H-IVA) and “low” (L-IVA) antibody responder mice, and the efficacy of BCG to inhibit this response. Lung inflammation was assessed by airway and distal tissue hyperreactivity and histological parameters; Th1/Th2 modulation was analyzed through anaphylactic antibodies titration.

Materials and methods

Animals

Two-month-old High (H-IVA) and Low (L-IVA) antibody responder mice from Selection IV-A (Cabrera et al., 1995) were obtained from a colony at the Institute Butantan, São Paulo, Brazil, and maintained in our own animal facilities.

Our study was approved by the Institutional Review Board of the School of Medicine of the University of São Paulo, Brazil. Adult male Wistar Furth rats (120–200 g) were used in the passive cutaneous anaphylaxis (PCA) tests for IgE evaluation, and BALB/c mice (18–20 g) in the PCA reaction for IgG₁ titration (Ovary, 1958; Mota and Wong, 1969).

Antigens and reagents

Ovalbumin (OVA) five times crystallized (Calbiochem, La Jolla, CA, USA), Alumen Al(OH)₃ (Pepsamar, Sanofi Winthrop Farmaceutica Ltda, Rio de Janeiro, Brazil), methacholine chloride (Sigma Chemical Co., St. Louis, MO, USA), Optimum Cutting Temperature (OCT) (BDH-Poole, France) and lyophilized Bacillus Calmette–Guérin (BCG) (Butantan Institute-Brazil) were used in the experiments. Rat anti-mouse IgG1 (LO-MG1-2; LO-MG1-3; LO-MG1-10) and mouse IgE (LO-ME-3) monoclonal antibodies were purchased from Imex, UCL, Brussels. B8401H5 is a mouse IgG1 specific for DNP and LB-4 is a mouse IgE anti-TNP (kind gift of J. Van Snick, ICP, UCL, Brussels).

Immunization and challenge protocols

Immunization

Mice were divided into four groups (6 animals H-IVA or L-IVA in each group) and sensitized with: OVA 50 µg adsorbed in 7 mg Alumen plus BCG 2×10^6 s.c. (group 1); OVA 50 µg adsorbed in 7 mg Alumen (s.c.) (group 2); saline (group 3); BCG 2×10^6 adsorbed in 7 mg Alumen s.c. (group 4). The second injection was performed within a period of 21 days with the same schedule. All mice were bled by ophthalmic plexus 1 day before the onset of the experiment and at day 27 after the first immunization. The experiments were repeated twice.

Antigen challenge

One week after the second injection all mice received three daily intranasal challenges (day 28, 29 and 30) with 5 µg OVA in 10 µL (one-tenth of the OVA concentration used in the immunization) (Kim et al., 1990; Henderson et al., 2002). At day 32 the mice were submitted to pulmonary mechanics evaluation and BAL fluid collection.

IgG₁ and IgE anti-OVA antibody titration by PCA and ELISA

PCA

PCA was performed in Wistar Furth rats and in BALB/c mice for anti-OVA IgE and IgG₁ measurement, respectively (De Vries, 1994; Foster et al., 1996). The back of the animals was shaved and

injected intradermally (i.d.) with different plasma dilutions obtained from mice submitted to the different protocols of immunization. The animals were challenged intravenously with 0.5 mg of OVA in 0.25% Evans Blue solution, after a sensitization period of 18–24 h in rats for IgE, and of 2 h in mice for IgG1 titration. The PCA titer was expressed as the reciprocal of the highest dilution that gave a lesion greater than 5 mm in diameter in triplicate of tests. The detection of threshold of the technique was established at 1:5 dilution.

ELISA

Specific anti-OVA IgG₁ was quantified by indirect ELISA; anti-OVA IgE and total IgG1 by capture ELISA. Microplates were coated overnight at 4 °C with 100 µL OVA (for OVA specific IgG1) or rat MoAb anti-mouse IgE (for IgE) or rat anti-mouse IgG (for total IgG1) in 0.2 M borate buffer pH 8.6. Plates were washed five times with PBS supplemented with 0.1% Tween 20 (Sigma Chemical Co., St. Louis, MO, USA) at each time of assay. Plates were blocked with protein from skimmed milk in PBS for 1.5 h at 37 °C. 100 µL of serial dilutions of plasma were then added and incubated for 2 h. For IgE titration the plates were incubated with biotin conjugated OVA for 1 h at 37 °C. Then peroxidase labeled rat anti-IgG1 (LO-MG1-2) (10 µg/mL) or peroxidase-conjugated streptavidin (1:1000) (Sigma, USA) was added for 2 h. Finally, 0.4 mg/mL orthophenyldiamine (OPD) (Sigma, USA) in citrate/phosphate buffer pH 5.6 and H₂O₂ was used. The reaction was stopped with H₂SO₄ and read at 492 nm as described (Itami et al., 2003). Plasma titer was given in OD units (492 nm) or converted to antibody concentration by comparison with the titer of purified standard monoclonal immunoglobulins (IgG1: B8401H5 and IgE: LB-4).

Respiratory responsiveness to methacholine

Pulmonary function measurements: Pulmonary conductance (Grs) and pulmonary dynamic compliance (Crs) changes in response do methacholine, administered via a jugular venous catheter to anesthetized and mechanically ventilated mice in a Harvard apparatus, were measured as previously described (Lima et al., 2002; Itami et al., 2003). The output signals from 16 consecutive breaths were analyzed using a computerized cross-correlation method (Janeway et al., 1987). The methacholine doses that resulted in 70% and in 50% of Crs and Grs basal values, respectively, were then calculated as indexes of the pulmonary responsiveness to methacholine.

Bronchoalveolar lavage (BAL)

BAL fluid was collected in all animals submitted to mechanical measurements. Immediately after the dose response curve with intravenous methacholine, the lungs were rinsed three times through the tracheal cannula with 1 mL saline aliquots. For differential cell counts, Cytospin preparations were stained with Leishman. All individual Cytospin preparations were evaluated by three observers using oil immersion at ×1000 magnification in an optic microscope and 300 cells were counted per mouse.

Eosinophil peroxidase (EPO) assay

A histochemical method for identification of cyanide-resistant EPO activity in frozen lung tissue employing diaminobenzidine (DAB) (Sigma) and H₂O₂ and potassium cyanide (Merck) was used to

stain eosinophils as previously described (Lima et al., 2002). Counterstaining with hematoxinilin was employed to evidence cellular nuclei. The nuclei of EPO-positive cells were enumerated in airway wall (between bronchial epithelium and adventitia) employing an integrating eyepiece ($10^4 \mu\text{m}^2$ of total area). We analyzed 10 to 20 fields per lung at $\times 1000$ magnification and the number of positive cells was expressed per unit of area. The staining that was not clearly cell-associated or appeared to be extra-cellular was not considered.

Statistical analysis

All values are expressed as mean $\pm$ S.E.M. Parametric data were evaluated using one-way analysis of variance followed by Tukey test for multiple comparisons (Zar, 1984); $p \leq 0.05$ was accepted as statistically significant.

Results

Anti-OVA specific IgE and IgG1 anaphylactic antibodies measured by PCA

The animals were bled at day 27 after immunization, and the plasmas were analyzed by PCA. Significantly higher titers of IgE anti-OVA were found in H-IVA mice (1:400) when compared to L-IVA (1:100). In both lines, the production of IgE anti-OVA was inhibited by BCG (Fig. 1A). On the other side, IgG1 anti-OVA synthesis was similar in H-IVA and L-IVA and BCG treatment did not modulate IgG1 levels in the two lines (Fig. 1B). The plasmas from animals immunized with BCG alone were negative.

Anti-OVA IgE and IgG1 measured by ELISA

Significantly greater concentrations of anti-OVA IgE were found in OVA immunized H-IVA mice ($63.4 \mu\text{g/mL}$) compared to L ($6.2 \mu\text{g/mL}$). In H-IVA mice there was a significant inhibition (about 3-fold in protein concentration) of IgE production by BCG treatment, whereas low IgE levels were found in all conditions in L-IVA mice (Fig. 2A). High titers of anti-OVA IgG1 were found in all OVA immunized groups, irrespective of BCG treatment (Fig. 2B). On the other side, total IgG1 concentrations following immunization were similar to those observed in saline treated mice and higher IgG1 levels were found in H-IVA compared to L-IVA mice of the several groups (Fig. 2C).

Lung mechanics

A decrease in respiratory system conductance in OVA immunized mice was observed when compared to saline treated controls ($p < 0.05$). On the other hand, after BCG administration, conductance values returned to normal and were found similar to controls ($p < 0.05$) (Fig. 3A). The respiratory system compliance of both mouse strains sensitized with OVA was lower when compared to control animals treated with saline ($p < 0.05$). Again, BCG treatment induced an increment of compliance levels similar to those of H-IVA and L-IVA control groups treated with saline or BCG alone (Fig. 3B). There was no difference between the strains in these two parameters.

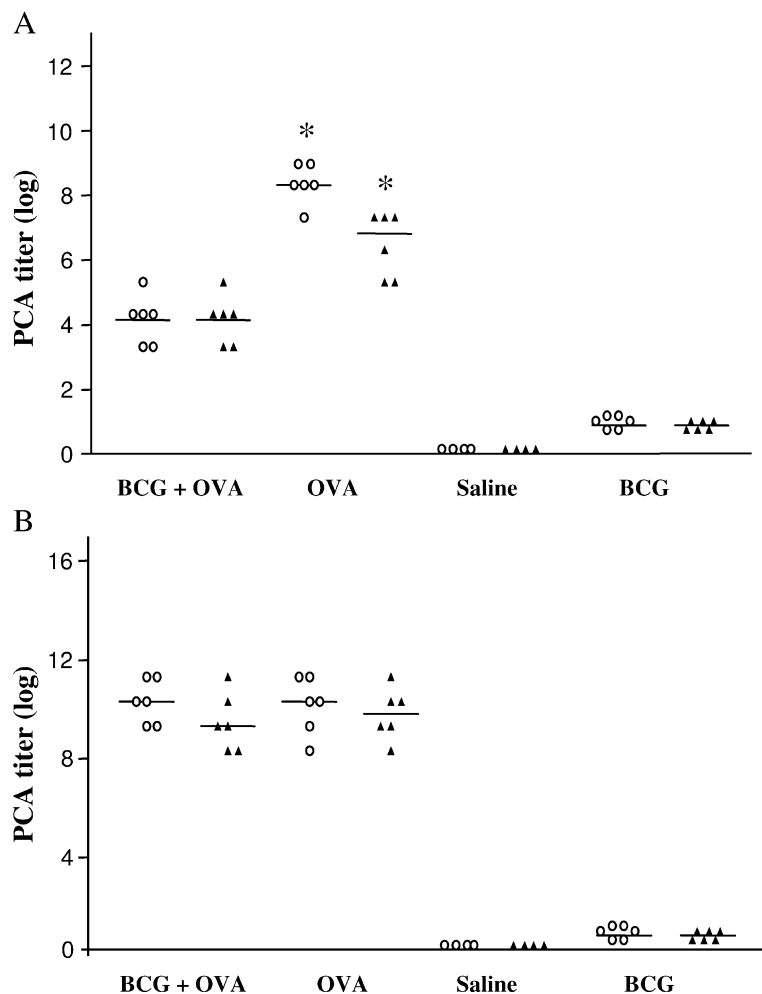


Fig. 1. Effect of BCG on anti-OVA IgE (A) and IgG1 (B) antibody levels quantified by PCA in individual plasma of H-IVA (○) and L-IVA (▲) mice immunized with OVA (s.c.). * $p < 0.001$ compared to BCG plus OVA immunized group.

Eosinophils in lung tissue (EPO)

OVA immunization and challenge induced an increment in eosinophils in lung tissue of both mouse lines compared to controls ($p = 0.05$). The treatment with BCG abolished almost completely the eosinophil infiltration in both lines (Fig. 4).

Leukocyte migration in bronchoalveolar lavage

There was an increment in eosinophils in BAL from OVA sensitized H-IVA and L-IVA mice when compared to the groups treated with saline, BCG or BCG+OVA ($p < 0.001$). The difference in eosinophil numbers between the OVA immunized H-IVA and L-IVA mice was found significant ($p < 0.05$) (Table 1). There was also an increase in the influx of neutrophils due to OVA

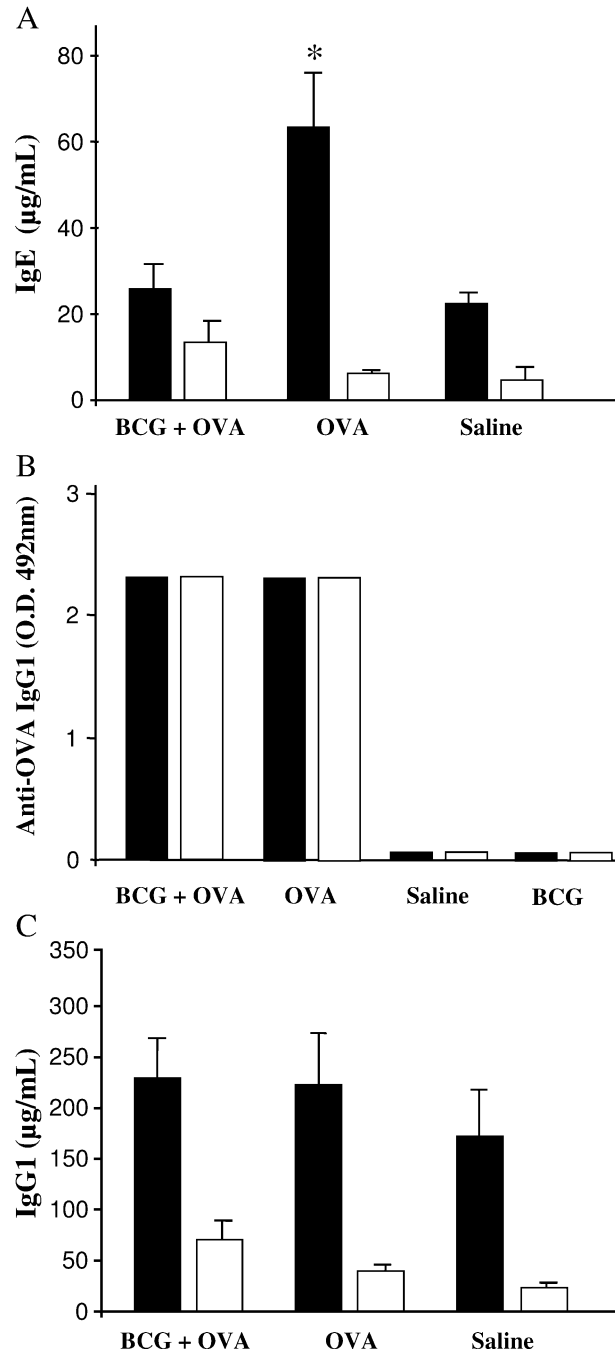


Fig. 2. Effect of BCG on IgE (A), anti-OVA IgG1 (B) and total IgG1 (C) quantified by ELISA in individual plasma of H-IVA (■) and L-IVA (□) mice immunized with OVA (s.c.). Ab levels expressed as mean $\pm$ S.E.M. * $p < 0.05$ compared to BCG plus OVA immunized group.

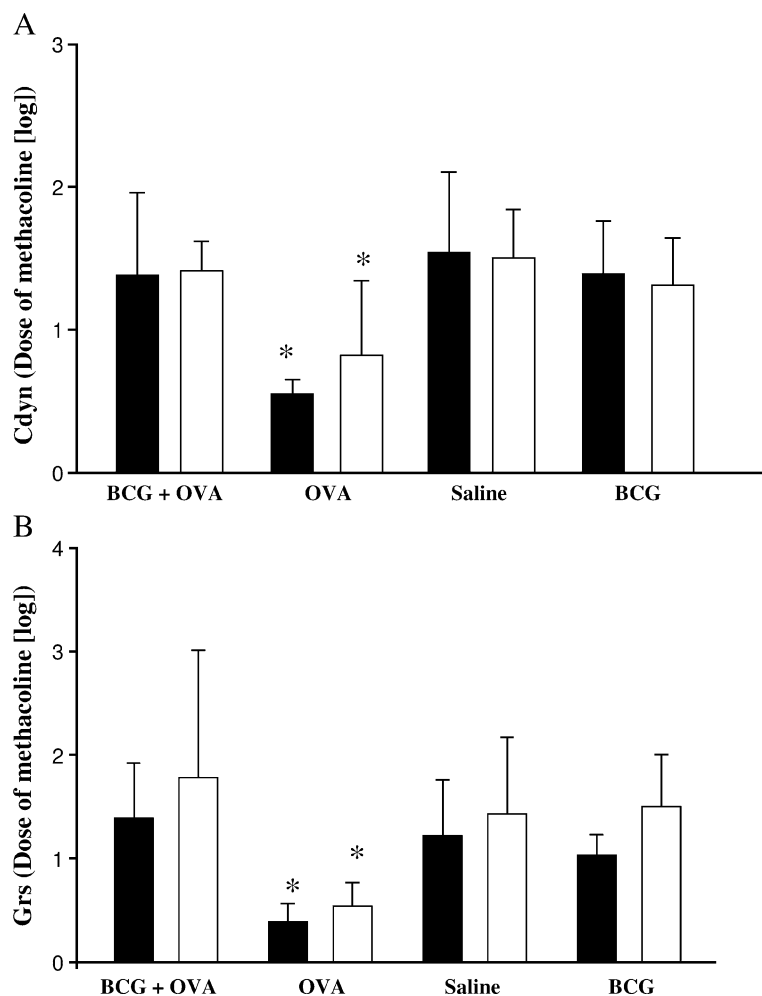


Fig. 3. Effect of BCG on respiratory system conductance (A) and compliance (B) during dose response curve to methacholine i.v. in H-IVA (■) and L-IVA (□) mice immunized with OVA (s.c.) and challenged with OVA i.n. Data expressed as mean indexes $\pm$ S.E.M. * $p < 0.05$ compared to BCG plus OVA immunized group.

immunization which was found similar in H-IVA and L-IVA mice and equally inhibited by BCG treatment.

Discussion

Mice genetically selected on the basis of the high antibody production against heterologous erythrocytes (H-IVA) produce higher levels of Ig-E anti-OVA after immunization with OVA adsorbed in alumen, compared to the low responders (L-IVA) counterparts. This is in agreement with the multi-specific effect of the bi-directional selection process in which the modification in antibody responsiveness of the selected mouse lines is not restricted to the antigens used during the selective

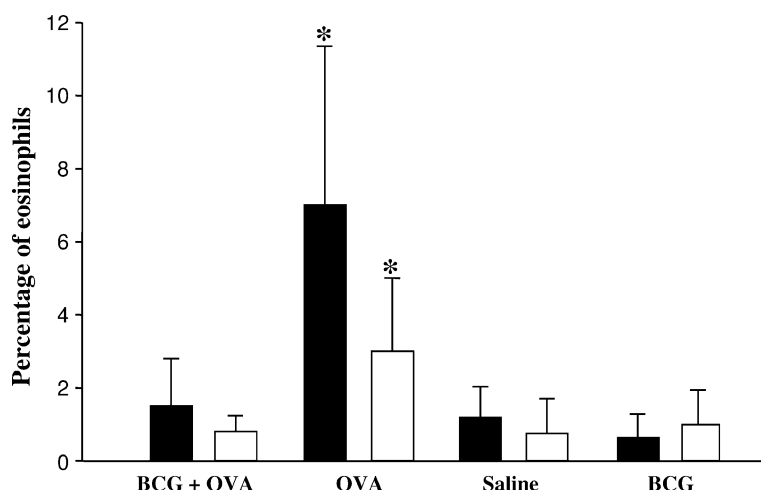


Fig. 4. Percentage of eosinophils in lung tissue of H-IVA (■) ($n=6$) and L-IVA (□) ($n=6$) mice sensitized with OVA alone or OVA plus BCG and challenged with OVA. Results expressed as mean $\pm$ S.E.M. of % EPO positive cells in 300 analyzed cells/microscopic field under 1000 $\times$ magnification. * $p<0.05$ compared to BCG plus OVA immunized group.

breedings but extend to many unrelated antigens and to the several immunoglobulin classes. The main regulatory mechanism that differentiates H-IVA and L-IVA strains seems to be related to a greater macrophage catabolic activity in Low responders, limiting the amount of antigen to an efficient Th lymphocyte stimulation and antibody synthesis (Mouton et al., 1976; Cabrera et al., 1995). This T lymphocyte inhibition might be secondary to the generation of low affinity peptides in TCR–peptide–MHC-class II complex, associated to variations in TCR repertoire between the two strains (Ibanez et al., 1988; Cabrera et al., 1995).

Herein the biological activity of OVA-induced anaphylactic IgE and IgG1 antibodies through mast cell degranulation, was assayed *in vivo* by PCA reaction. When BCG was administered with OVA, there was a suppressive effect on the specific IgE production in both mouse strains. On the other side, OVA specific IgG1 antibodies were found in similar levels in both lines, and in the several experimental groups. The IgE levels measured by ELISA method were also greater in H-IVA than in L-IVA mice, and consistently, H-IVA IgE production was significantly suppressed by BCG. Again, there was no difference between the lines in IgG1 anti-OVA production. Nevertheless, the levels of total IgG1 were

Table 1
Effect of BCG on cell content of BAL from H-IVA and L-IVA mice immunized and challenged with OVA

Treatment	Eosinophils		Neutrophils		Lymphocytes	
	H-IVA	L-IVA	H-IVA	L-IVA	H-IVA	L-IVA
BCG+OVA	7.2 $\pm$ 3.7	4.8 $\pm$ 1.4	8.4 $\pm$ 2.1	9.4 $\pm$ 2.8	7.8 $\pm$ 3.3	8.2 $\pm$ 3.9
OVA	34.2 $\pm$ 6.6***	24.2 $\pm$ 2.2*	34.6 $\pm$ 8.7*	33.0 $\pm$ 4.4*	11.4 $\pm$ 4.2*	13.4 $\pm$ 4.2*
Saline	8.0 $\pm$ 2.3	5.2 $\pm$ 1.9	9.4 $\pm$ 3.1	7.2 $\pm$ 4.9	4.4 $\pm$ 2.3	4.6 $\pm$ 3.7
BCG	0	0	15.3 $\pm$ 2.9	7.2 $\pm$ 4.5	4.7 $\pm$ 2.6	10.2 $\pm$ 4.3

Results are expressed as % of each cell type in a total of 300 analyzed cells.

Monocytes/macrophages which represent the main population in controls were not included.

* $p<0.05$ compared to saline treated controls.

** $p<0.05$ compared to L-IVA.

larger in H-IVA compared to L-IVA, even in control mice, in accordance to previous findings for IgG1 as well as for other Ig isotypes. This phenotype was ascribed to the selection for high or low antibody responsiveness (Sant'Anna et al., 1985).

The inflammatory reaction in the lungs was assessed by histology and BAL analysis. OVA immunization and challenge induced an influx of eosinophils in lung parenchyma of both lines. Furthermore, an increment of neutrophils and lymphocytes was found in the broncho-alveolar lavage (BAL) of these mice compared to control groups. This reaction was also inhibited by BCG given at the time of OVA sensitization.

The immediate hypersensitivity late phase response (LPR) is characterized by an inflammatory infiltration mediated by leukocytes (eosinophils, neutrophils, basophils, T lymphocytes and monocytes) secondary to mast cells degranulation through IgE, and the liberation of cytokines and other mediators, which are closely related to the pathogenesis of several allergic diseases, as asthma (Corrigan and Kay, 1992). Metzger et al. (1989) demonstrated that asthmatic patients presented an increment in TCD4⁺ lymphocytes in BAL 48 h after bronchial challenge. Concomitantly, Dupuis and colleagues (1992) showed that at 18 to 24 h after antigen challenge there is an accumulation of neutrophils, lymphocytes and specially eosinophils.

Several mediators released by mast cells and other kinds of inflammatory cells present in this allergic process, including eosinophils, have direct or indirect effect on airways and lung tissue, increasing pulmonary reactivity. The intensity of the inflammatory process thus determines the degree of the respiratory function alterations (Erb et al., 1998).

In the present study, OVA sensitization promoted an impairment of respiratory system functions in vivo, demonstrated by an increase in airway resistance (low conductance) and a higher rigidity of lung tissue (low compliance) after methacholine challenge in both strains. These data can reflect the intensity of the inflammatory process, as conductance evaluates airways and compliance evaluates the distal lung tissue and small airways, pointing out to a diffuse process affecting airways and also alveolar spaces. There was no difference on these mechanical parameters between the strains. Probably these measurements were not sufficiently sensitive to evidence the different levels of inflammation as evaluated by histology in the two selected mouse strains.

When BCG was administered at the time of OVA immunization, this lung hyperreactivity was inhibited in both strains of mice, in accordance to the decreased inflammatory cell influx, represented mainly by eosinophils and neutrophils in respiratory system.

Early childhood vaccination (early months of age) is an effective way of protection to infectious diseases, and perhaps, also allergic asthma. This proposal is based on the observation made by Shirakawa et al. (1997) that BCG vaccinated children in Japan presented lower incidence of atopic diseases. The *M. tuberculosis* and BCG constitute potent stimulators of dendritic cells (DCs) (Thurnher et al., 1997). Recently, Nahori and colleagues (2001) showed that BCG intranasal instillation in mice was able to induce airway recruitment of antigen presenting cells, macrophages and DCs. Chambers and colleagues (1997) demonstrated that BCG was able to induce the production of IFN- γ at the local of injection during 7 days, as well as in lymph nodes during some weeks after immunization.

In this study, s.c. BCG administration increased from about 20% (in OVA treated groups) to 70% (in BCG+OVA groups) the relative proportion of recruited monocytes/macrophages in BAL. Since we are dealing with an active process, two alternative mechanisms could be proposed: 1) an increment of IFN- γ produced by activated cells with a consequent Th1 response stimulation, or 2) the generation of T regulatory cells. Considering the first hypothesis, an impairment of the IgE production in both strains

was expected, accordingly to the consensus that IgE synthesis is induced by IL-4 and suppressed by IFN- γ . Several studies based mainly on bronchial biopsies and BAL analysis have implicated eosinophils and its mediators in the inflammatory and the bronchial hyperreactivity mechanisms in asthma in humans. Accordingly, IL-5 deficient mice did not react with eosinophilia or bronchial hyperreactivity after antigen stimuli (Foster et al., 1996). Nahori et al. (2001) observed that BCG immunization reduced the lung eosinophil infiltration and suppressed bronchial hyperreactivity. The IFN- γ production during the Th1 response might act straightly through eosinophils, blocking these cells' influx into the airways of BCG vaccinated mice. In reference to the second hypothesis, recent studies demonstrated that the inhibitory effect of *Mycobacterium vaccae* on allergen-induced eosinophilic lung inflammation was IFN- γ independent. The treatment with heat killed *M. vaccae* before OVA immunization confers long-term protection against airway inflammation and gives rise to CD4 regulatory T cells (Treg) which express the Il-2 α chain (CD25⁺) and are CD45RB^{low}. Transfer of these cells to OVA-immunized mice before antigen challenge specifically reduced airway eosinophilia. This inhibition was mediated through IL-10 and TGF- β which potently suppress IgE production (Akdis et al., 2004; Walker et al., 2003; Zuani-Amorim et al., 2002).

The results of the present study showed that OVA sensitization and challenge of HIV-A and LIV-A selected mice resulted in IgE anaphylactic antibody production, eosinophil infiltration in lung tissue and increment in airways cellular influx, associated to a respiratory system hyperreactivity in vivo, similar to the late phase response induced by allergens in bronchial asthma.

The simultaneous s.c. administration of BCG was able to modulate the allergic response secondary to OVA immunization by inhibiting anti-OVA IgE antibody production, the inflammatory process in lung tissue and airways and bronchial hyperreactivity. The modulation was evident even in the High responder mice, which present higher eosinophilia in the lungs and serum IgE levels than the low responders. The mechanisms underlying this regulation remain to be clarified.

These data demonstrate the efficacy of BCG inhibition of the allergic response in the particular genetic backgrounds of H-IVA and L-IVA mice and corroborate the previous findings in other isogenic laboratory mouse lines.

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