

Effect of pre-immunization by killed *Mycobacterium bovis* and *vaccae* on immunoglobulin E response in ovalbumin-sensitized newborn mice

Tükenmez F, Bahçeciler NN, Barlan IB, Başaran MM. Effect of pre-immunization by killed *Mycobacterium bovis* and *vaccae* on immunoglobulin E response in ovalbumin-sensitized newborn mice. *Pediatr Allergy Immunol* 1999; 10: 107–111. © Munksgaard, 1999

A recently advanced hypothesis suggests that decreased exposure to T-helper (Th) 1-inducing agents causes Th2-biased differentiation in response to concomitant allergens. We therefore examined the effect of pre-immunization with killed *Mycobacterium bovis* and killed *M. vaccae* which are known to be very potent inducers of Th1 immune response, on serum IgE response in ovalbumin (OVA)-sensitized newborn mice. Eighty-four newborn Balb/c mice were divided into four groups and were immunized intraperitoneally 24 h after birth with 50 µl of 5×10^4 colony-forming units (c.f.u.) of killed *M. bovis* in group I (*M. bovis* group, n = 19), with 25 µl of 2.5×10^8 c.f.u. of killed *M. vaccae* plus 25 µl of 5×10^4 c.f.u. of killed *M. bovis* in group II (*M. vaccae* + *M. bovis* group, n = 28) and with 50 µl of only phosphate-buffered saline (PBS) in group III (no mycobacterial immunization, n = 18). No injection was applied to mice in group IV (control group, n = 19). Starting from 8 weeks of age, all mice except the control group were sensitized with 0.5 ml of 20 mg/ml OVA administered intraperitoneally 7 times every other day. Thirty days after the final injection, all animals except those in the control group were challenged with an aerosol of 2 mg/ml OVA. Forty-eight hours later, blood was collected from all mice for determination of serum IgE levels. A statistically significant difference was observed in the serum total IgE levels between groups III and IV ($p = 0.0099$), indicating that the mice were successfully sensitized with OVA. Serum total IgE values of the female mice in *M. bovis* group were found to be significantly lower than group III ($p = 0.009$), while no difference was observed in males. Serum total IgE levels of the *M. vaccae* + *M. bovis* group were found to be significantly lower than group III both in male and female mice ($p < 0.0001$ and $p = 0.0001$, respectively). Female values were even lower than controls ($p = 0.0092$). Pre-immunization in the newborn period with killed *M. bovis* alone or in addition to *M. vaccae* may potentially be helpful in down-regulating an IgE response.

Faruk Tükenmez,
Nerin Nadir Bahçeciler,
Işıl Berat Barlan,
Müjdat Mustafa Başaran

Marmara University Hospital, Department of
Pediatrics, Division of Allergy/Immunology,
Istanbul, Turkey

Key words: atopy; IgE; *Mycobacterium vaccae*; Balb; c mice; newborn

I B Barlan, MD, Kantarcı Rıza sok., Şekerciler
apt. 5/8, Erenköy 81070, Istanbul, Turkey
Tel./fax: +90 216 326 8030
E-mail: aonder@ibm.net

Accepted 12 March 1999

Development of atopy depends on the interaction of heterogeneous environmental factors and is controlled predominantly by the Th2 (T-helper) subset of T lymphocytes which secrete interleukins 4 and 5 (IL-4 and IL-5) leading to enhanced production of IgE by B cells and to eosinophilia,

respectively (1–5). The prevalence of atopic diseases including hay fever, asthma, atopic dermatitis and food allergy has been increasing in developed countries in recent decades, now affecting more than 30% of the population in these countries (6). The reasons for this increase

in prevalence are largely unknown. Suggested causes include environmental changes associated with increased indoor exposure to dust mite, changed indoor climate, exposure to cigarette smoke and increased air pollution (7–10). A recently advanced hypothesis suggests that the increased prevalence of atopy in developed countries could be associated with the decline of some infectious diseases such as tuberculosis that induce Th1 immune responses as a result of improved living standards (11,12).

Induction of interferon- γ (IFN- γ) especially occurs during infections by viruses, intracellular bacteria, fungi and certain protozoa (1,13). Mycobacteria are known to be very potent inducers of the Th1 immune response (13,14). Shirakawa et al. demonstrated a strong inverse relationship between atopy and infection with, or exposure to, *M. tuberculosis* (15). In a recent study, infection of mice with *M. bovis* suppressed allergen-induced airway eosinophilia and IL-5 production, dependent on IFN- γ signalling (16). Wang & Rook also recently reported that a single injection of killed *M. vaccae* into OVA-pre-immunized mice suppressed serum IgE and allergen-specific IL-5 synthesis (17). A growing body of evidence suggests that *M. vaccae*, one of the saprophytic mycobacterium species, is a more potent inducer of Th1 immune responses than *M. bovis* (18).

In this study we addressed the question of whether immunization of newborn Balb/c mice with heat-killed *M. vaccae* and/or killed *M. bovis* in the newborn period prior to sensitization with OVA can suppress serum IgE production.

Materials and methods

One-year-old pregnant Balb/c mice were obtained, bred and housed until labor. After labor, the newborn mice were fed with breast milk up to 1 month of age and then with an OVA-free diet. At 1 month of age, they were separated according to gender (8–9 mice per cage). The experimental procedures were approved by the Animal Ethics Committee of Marmara University, and maintenance of animals was in accordance with institutional guidelines.

Eighty-four newborn mice were divided into four groups. Group I consisted of 19 mice (10 females, 9 males), group II had 28 mice (17 females, 11 males), group III had 18 mice (7 females, 11 males), and group IV had 19 mice (9 females, 10 males). Mice in group I were immunized intraperitoneally 24 h after birth with 5×10^4 colony-forming units (c.f.u.) of *M. bovis* (Pasteur Merieux Serum & Vaccine; strain

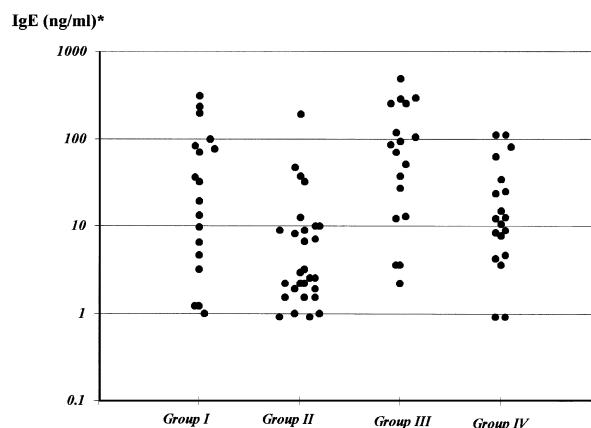


Fig. 1. The distribution of serum total IgE values of all groups. *Logarithmic representations of the serum total IgE values. Group I, *M. bovis*; group II, *M. bovis* + *M. vaccae*; group III, mice undergoing OVA sensitization but no mycobacterial immunization; group IV, control. The median (range) of IgE values (ng/ml) for groups I–IV are 19.3 (1–312), 2.7 (0.9–193), 77.6 (2.2–494) and 12.2 (0.9–109.3), respectively.

1077) in 50 μ l of PBS autoclaved at 120°C for 15 min (group I: *M. bovis*). Group II were immunized intraperitoneally 24 h after birth with 2.5×10^8 c.f.u. of *M. vaccae* (North Carolina State University strain ATCC 29678) in 25 μ l PBS autoclaved at 120°C for 15 min in addition to 25 μ l of the same *M. bovis* strain as in group I (group II: *M. bovis* + *M. vaccae*). Intraperitoneal injection of 50 μ l of PBS was performed at the same time in the mice in group III (group III: no mycobacterial immunization). No injection was performed to mice in group IV (group IV: control).

Starting from 8 weeks of age, 0.5 ml of 20 mg/ml OVA (Sigma) in 0.9% saline was administered intraperitoneally 7 times every other day to all mice except the control group. Thirty days after the final injection (14 weeks of age), all animals except for those in the control group were exposed to an aerosol of 2 mg/ml OVA in 0.9% saline for 5 min with a Devilbiss 2000 ultrasonic nebulizer. Forty-eight hours after the aerosol OVA challenge, blood was collected from all mice. Sera were stored at –30°C until assay. The serum total IgE levels were determined by sandwich enzyme-linked immunosorbent assay (purified anti-mouse IgE monoclonal antibody (Pharmingen catalogue number: 02111D) and biotinylated anti-mouse IgE monoclonal antibody (Pharmingen catalogue number: 02122D)).

Statistical analysis

The Kruskal–Wallis test was used for comparison of the serum total IgE values of all groups. Since standard deviations of the IgE values of the

groups were not equal, the logarithmic values were calculated for further comparisons. The significance of pair differences was determined by unpaired *t* test.

Results

The distribution and median (range) of serum total IgE values for all groups, and for males and females separately, are shown in Figs 1–3, respectively.

A statistically significant difference was observed in the serum total IgE levels between groups III and IV ($p = 0.0099$), indicating that the mice were successfully sensitized with OVA. Log IgE values of the *M. bovis* + *M. vaccae* group were found to be significantly lower than groups I, III and IV ($p = 0.0084$, $p < 0.0001$, $p = 0.015$, respectively). A statistically significant difference was also seen in the comparison of log IgE values between the male mice in groups III and IV. When log IgE values of male mice in the *M. bovis* + *M. vaccae* group were compared with the values of male mice in the other three groups, the differences were found to be statistically significant (group II versus group I ($p = 0.023$), III ($p = 0.0001$) and IV ($p = 0.026$). No significant difference was observed between the log IgE levels of males of group I versus group III and group I versus group IV ($p = 0.326$, $p = 0.39$). These results indicate that although *M. bovis* + *M. vaccae* suppressed IgE response in OVA-sensitized male mice, *M. bovis* alone did not.

The evaluation of serum total IgE levels of female mice revealed that they were successfully sensitized with OVA ($p = 0.0031$). In females, both group I and group II showed significantly different log IgE values when compared with group III ($p = 0.009$, $p < 0.0001$, respectively). While immunization with *M. bovis* prior to OVA sensitization suppressed the IgE response in female mice, this suppression was even more evident in the female mice immunized with *M. bovis* + *M. vaccae* ($p = 0.05$). Finally log IgE values of female mice in group II were found to be significantly lower than the female controls ($p = 0.0092$). The differences for all mice groups

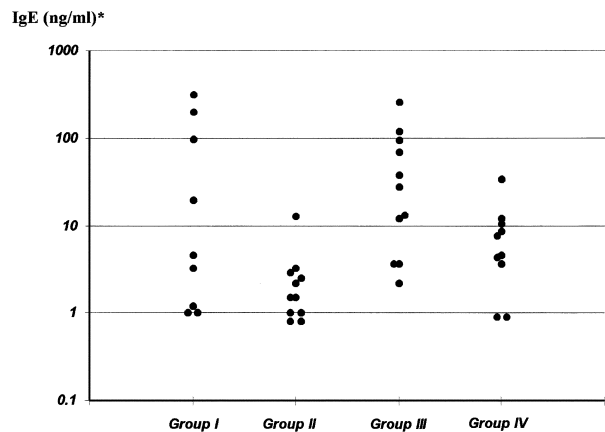


Fig. 2. The distribution of serum total IgE values of males in all groups.*Logarithmic representations of the serum total IgE values. Group I, *M. bovis*; group II, *M. bovis* + *M. vaccae*; group III, mice undergoing OVA sensitization but no mycobacterial immunization; group IV, control. The median (range) of IgE values (ng/ml) for groups I–IV are 4.6 (1–312), 1.5 (0.9–12.6), 27.2 (2.2–252) and 6.15 (0.9–34), respectively.

independent of and according to gender are shown in Table 1.

Discussion

Development of atopy is controlled predominantly by the Th2 subset of T lymphocytes which secrete IL-4, leading to enhanced production of IgE by B cells (1–5). Thus, using immunomodulators which induce IFN- γ production may suppress Th2 and/or stimulate Th1 cells resulting in decreased IgE synthesis. In a number of studies, mycobacterium species such as *M. bovis*, *M. tuberculosis* and *M. vaccae* have been shown to be very potent inducers of the Th1-type immune response, leading to secretion of Th1-like cytokines (13,18–23).

In the study by Lin & Zhu, improvement was observed in 80% of the asthmatic patients who received killed bacillus Calmette–Guérin (BCG) by the oral route (24). Shirakawa et al. showed that there was an inverse association between atopy and positive tuberculin responses (15). Positive tuberculin responses predicted a lower incidence of asthma, lower serum IgE levels, and serum cytokine profiles shifted toward Th1 type (15). On the other hand, in a study conducted in

Table 1. Comparison of pair differences of all mice groups independently of and according to gender

	Group I vs group II	Group I vs group III	Group I vs group IV	Group II vs group III	Group II vs group IV	Group III vs group IV
Independent of gender	0.0084	0.0774*	0.5992*	< 0.0001	0.015	0.0099
Male	0.0230	0.3255*	0.3904*	0.0001	0.0257	0.0254
Female	0.050	0.0090	0.6437*	< 0.0001	0.0092	0.0031

*Not significant. The numbers shown in the table are *p* values. Group I, *M. bovis*; group II, *M. bovis* + *M. vaccae*; group III, mice undergoing OVA sensitization but no mycobacterial immunization; group IV, control.

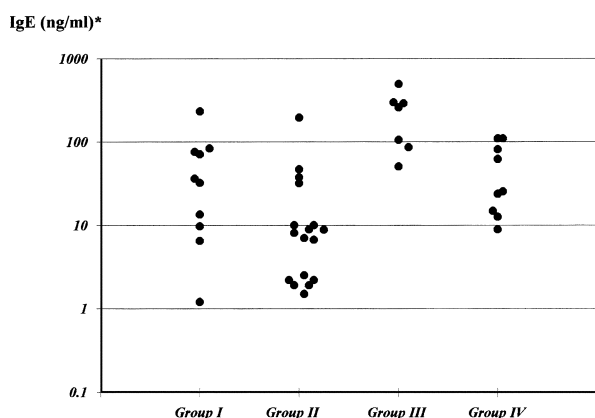


Fig. 3. The distribution of serum total IgE values of females in all groups.*Logarithmic representations of the serum total IgE values. Group I, *M. bovis*; group II, *M. bovis* + *M. vaccae*; group III, mice undergoing OVA sensitization but no mycobacterial immunization; group IV, control. The median (range) of IgE values (ng/ml) for groups I–IV are 34.05 (1.2–230), 8.1 (1.5–193), 256 (50.4–494) and 25.3 (8.9–109.3), respectively.

Sweden, BCG-vaccinated children did not show a statistically significant lower frequency of allergy than unvaccinated ones. However, a low prevalence was observed only in immigrant children, suggesting that other factors besides BCG, attributable to the environmental influences, could play role in the prevention of allergy (25). We hypothesized that, in addition to BCG vaccination, environmental influences occurring very early in life such as exposure to saprophytic mycobacterium species may have a protective effect on development of atopic disease later in life.

Intradermal injection of killed *M. vaccae* has been demonstrated to promote cell-mediated responses to antigens common to all mycobacteria. Studies of vaccination in children showed that those receiving BCG plus killed *M. vaccae* were more likely to have an enhanced delayed-type hypersensitivity reaction to leprosin A than those who were given BCG alone (26). Skinner et al. (18,27) demonstrated that heat-killed *M. vaccae*, unlike other mycobacteria species, contains both adjuvant and antigen components capable of stimulating CD8⁺ T-cell responses, thus after immunization with killed *M. vaccae* CD8⁺ cells are generated. Furthermore, following *in vitro* stimulation with mycobacteria species, cytotoxic CD8⁺ T cells are revealed, consistent with the activation of memory cells. They suggested that these CD8⁺ T cells secrete Th1 class cytokines maintaining the dominance of the Th1-type immune response (18,27).

Our group recently demonstrated that pre-immunization of Balb/c mice by heat-killed BCG prior to OVA sensitization significantly decreased

serum total IgE values, whereas live BCG had no such effect (28).

In the light of these data, we attempted to determine the effect of killed BCG alone and in addition to *M. vaccae* on serum IgE response. We hypothesized that, in order to be able to demonstrate the protective effect of *M. vaccae* and/or *M. bovis*, a newborn mice model would be more successful than the inhibition of an already established allergic response in older mice.

Due to the difficulty in distinguishing gender in the newborn period, both genders of mice were included initially in our study. Our results demonstrated that female mice gave a better IgE response, in accordance with the findings of Renz et al. (29).

Although both immunization with *M. bovis* and *M. bovis* + *M. vaccae* resulted in a lower serum total IgE value in the female mice when compared with those undergoing OVA sensitization but no mycobacterial immunization, the latter resulted in a more pronounced decrease, even significantly lower than the healthy control group.

Recently, Erb et al. demonstrated that when OVA-sensitized mice were infected with BCG before allergen airway challenge, airway eosinophilia and IL-5 production by T cells of draining lymph nodes were inhibited (16). Also Wang and Rook recently showed that a single injection of different doses of killed *M. vaccae* into OVA-pre-immunized Balb/c mice suppressed serum IgE response. They observed suppression of serum IgE levels over a wide dose range of *M. vaccae* (10^7 , 10^8 , 10^9) and concluded that higher doses can evoke a mixed Th1 + Th2 response. Twice-injection of 10^7 c.f.u. *M. vaccae* showed not only suppression of the IgE response, but also inhibition of OVA-induced IL-5 production by spleen B cells (17). In our study, we preferred to administer half the dose used by Skinner et al. for immunization of adult mice (27).

In conclusion, immunization within the newborn period with killed *M. bovis* alone or in addition to *M. vaccae* may potentially be helpful in reducing the risk of developing allergic diseases by suppressing the serum IgE response in mice. The relevance of these observations to human atopic disease remains to be elaborated but indicates a potential major prevention strategy.

Acknowledgments

We thank Dr J.J. Perry from North Carolina State University, Department of Microbiology, for supplying *M. vaccae* and Dr James D. Watson from Genesis

Research Center, New Zealand, for sharing their experience in this field with us.

References

- MOSMANN TR, CHERWINSKI H, BOND MW, GIEDLIN MA, COFFMAN RL. Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. *J Immunol* 1986; 136: 2348–57.
- ABBAS AK, MURPHY KM, SHER A. Functional diversity of helper T lymphocytes. *Nature* 1996; 383: 787–793.
- CARRER LL, DUTTON RW. Type 1 and type 2: a fundamental dichotomy for all T-cell subsets? *Curr Opin Immunol* 1996; 8: 336–42.
- SHER A, GAZZINELLI RT, OSWALD LP, et al. Role of T cell derived cytokines in the downregulation of immune responses in parasitic and retroviral infection. *Immunol Rev* 1992; 127: 183–204.
- ROMAGNANI S. Induction of Th1 and Th2 responses: a key role for the 'natural' immune response? *Immunol Today* 1992; 13: 379–81.
- WEIZMAN M, GORTMAKER SL, SOBOL AM, PERRIN JM. Recent trends in the prevalence and severity of childhood asthma. *JAMA* 1992; 268: 2673–7.
- ANDRAE S, AXELSON O, BJÖRKSTEN B, FREDRIKSSON M, KJELLMAN N-IM. Symptoms of bronchial hyperactivity and asthma in relation to environmental factors. *Arch Dis Child* 1988; 63: 473–8.
- STRACHAN DP. Damp housing and childhood asthma: validation of reporting of symptoms. *BMJ* 1988; 297: 1223–6.
- ISHIZAKI T, KOIZUMI K, IKEMORI R, ISHIAMA Y, KUSHIBIKI E. Studies of prevalence of Japanese cedar pollinosis among the residents in a densely cultivated area. *Ann Allergy* 1987; 58: 265–70.
- WICKMAN M, NORDVALL SL, PERSHAGEN G. Risk factors in early childhood for sensitization to airborne allergens. *Pediatr Allergy Immunol* 1992; 3: 128–33.
- WEGMAN TG, LIN H, GILBERT L, MOSMANN TR. Bidirectional cytokine interactions in the maternal-fetal relationship: is successful pregnancy a Th2 phenomenon? *Immunol Today* 1993; 14: 353–56.
- ROMAGNANI S. Regulation of the development of type-2 T helper cells in allergy. *Curr Opin Immunol* 1994; 6: 838–46.
- MOSMANN TR, SAD S. The expanding universe of T-cell subsets. Th1, Th2 and more. *Immunol Today* 1996; 17: 138–46.
- DEL PRETE GF, CARLI MD, MASTOMAURO C, et al. Purified protein derivative of mycobacteria tuberculosis and excretory-secretory antigen(s) of *Toxocara canis* expand *in vitro* human T cells with stable and opposite (Type 1 T helper or type 2 T helper) profile of cytokine production. *J Clin Invest* 1991; 88: 346–50.
- SHIRAKAWA T, ENOMOTO T, SHIMAZU S, HOPKIN JM. The inverse association between tuberculin responses and atopic disorder. *Science* 1997; 275: 77–9.
- ERB KJ, HOLLOWAY JW, SOBECK A, MOLL H, GROS G. Infection of mice with *Mycobacterium bovis*-Bacillus Calmette Guérin (BCG) suppresses allergen induced airway eosinophilia. *J Exp Med* 1998; 187: 561–9.
- WANG CC, ROOK GAW. Inhibition of an established allergic response to ovalbumin in BALB/c mice by killed mycobacterium vaccae. *Immunology* 1998; 93: 307–13.
- SKINNER MA, YUAN S, PRESTIDGE R, CHUK D, WATSON JD, TAN PL. Immunization with heat killed *Mycobacterium vaccae* stimulates CD8+ cytotoxic T cells specific for macrophage infected with mycobacterium tuberculosis. *Infect Immun* 1997; 65: 4525–30.
- STANFORD JL, STANFORD CA. Immunotherapy of tuberculosis with mycobacterium vaccae NCTC 11659. *Immunobiology* 1994; 191: 555–63.
- HERNANDEZ PR, PAVÓN LARR, ARRIAGA K, et al. Pathogenesis of tuberculosis in mice exposed to low and high doses of an environmental mycobacterial saprophyte before infection. *Infect Immun* 1997; 65: 3317–27.
- TSICOPULUS A, HAMID Q. Preferential mRNA expression of Th1 type cells in classical delayed type hypersensitivity reaction in human skin. *J Immunol* 1992; 148: 2058.
- MAGGI E, PARRINCHI P. Reciprocal regulatory effects of IFN gamma and IL-4 on the *in vitro* development of human Th1 and Th2 clones. *J Immunol* 1992; 148: 2142.
- HUYGEN K, LOZES E, GILES B, et al. Mapping of Th1 helper T cell epitopes on major secreted mycobacterial antigen 85A in mice infected with live *Mycobacterium bovis* BCG. *Infect Immun* 1994; 62: 363–370.
- LIN AY, ZHU SZ. 301 cases of bronchial asthma treated by kahusu. *Chin J Integ Trad West Med* 1991; 11: 209–11.
- STRANNEGARD JL, LARSON LO, WENNERGEN G, STRANNEGARD Ö. Prevalence of allergy in children relation to prior BCG vaccination and infection with atypical mycobacteria. *Allergy* 1998; 53: 249–54.
- STANFORD JL, ROOK GAW, BAHR GM, et al. *Mycobacterium vaccae* in immunoprophylaxis and immunotherapy of leprosy and tuberculosis. *Vaccine* 1990; 8: 525–30.
- SKINNER M, YUAN S, PRESTIDGE R, WATSON JD, TAN PLJ. Heat killed *Mycobacterium vaccae* as an adjuvant and vaccine for tuberculosis. Stimulation of CD8+ cytotoxic T cells. *Vaccine Weekly* 1997; 6: 23.
- BAKIR M, TÜKENMEZ F, GÜREL A, et al. Heat killed BCG decreased serum IgE level in ovalbumin sensitized mice. *J Allergy Clin Immunol* 1997; 99: 1770.
- RENZ H, SMITH HR, HENSON JE, et al. Aerosolized antigen exposure without adjuvant causes increased IgE production and increased airway responsiveness in the mouse. *J Allergy Clin Immunol* 1992; 89: 1127–38.