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Inhibition of an established allergic response to ovalbumin in BALB/c mice by killed *Mycobacterium vaccae*

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SUMMARY

Allergic disorders are mediated by T lymphocytes secreting T helper 2 (Th2) cytokines, interleukin-4 (IL-4) and interleukin-5 (IL-5), resulting in high levels of serum immunoglobulin E (IgE) and recruitment of eosinophils. One of the treatment strategies is to downregulate the Th2 component by inducing a T helper 1 (Th1) response to the relevant allergen, because Th1 and Th2 cytokines are thought to be mutually antagonistic. In this study, we examined the effects of *Mycobacterium vaccae*, a potent inducer of Th1 immunity, on allergic responses in a murine model. A single injection of *M. vaccae* into ovalbumin (OVA)-preimmunized BALB/c mice suppressed serum IgE over a wide dose range (10^7, 10^8 or 10^9 *M. vaccae*). Further experiments, using 10^7 *M. vaccae* injected twice, showed that this treatment inhibited not only serum IgE, but also the potential for ovalbumin-induced IL-5 production by spleen cells. This non-specific ability of a mycobacterium to decrease Th2 activity, even when not presented together with the allergen, is in agreement with recent epidemiological studies on the impact of bacillus Calmette–Guérin (BCG) vaccination, and of other potent Th1 stimuli, on the incidence of atopy. The suppression of serum IgE and allergen-specific IL-5 synthesis by *M. vaccae* suggest that this organism is likely to have clinical application in the immunotherapy of allergy.

INTRODUCTION

Allergic disorders affect at least 20% of the population of developed countries. They include hayfever, asthma, atopic dermatitis and food allergies. These symptoms are associated with high levels of serum immunoglobulin E (IgE) and allergen-specific IgE[1–3] and eosinophilia,[4–6] and are dependent upon interleukin-4 (IL-4) and interleukin-5 (IL-5) released from allergen-specific CD4 T cells expressing the T helper 2 (Th2) cytokine profile.[7–9] Current specific allergen immunotherapy involves a series of injections of allergen extracts with the aim of reducing the patient's sensitivity to the allergen. However, this form of immunotherapy has been increasingly questioned because of limited efficacy and the danger of anaphylaxis. Moreover, when it works the mode of action is unclear.

More recent strategies aim to switch allergen-specific Th2 responses to T helper 1 (Th1), with predominant production of interferon-γ (IFN-γ) and interleukin-2 (IL-2). The basis of this strategy is the supposed mutual opposition between Th1 and Th2, where Th1 cytokines downregulate Th2 function, and so would be expected to reduce IgE.[10,11] Most authors attempt to switch the cytokine profile in an allergen-specific manner. However, a recent report of an inverse association between atopy and delayed-type-hypersensitivity (DTH) responsiveness to tuberculin suggests that exposure to immunogenic mycobacteria (whether *Mycobacterium tuberculosis* itself, bacillus Calmette–Guérin (BCG), or immunogenic environmental species) can inhibit atopic disorders without any link between allergen epitopes and the mycobacteria concerned.[12]

The purpose of this study was to examine whether a killed mycobacterial preparation derived from *Mycobacterium vaccae*, which in other contexts has undergone extensive toxicological studies and safety assessment in humans, can downregulate an ongoing allergen-specific Th2 immune response.[13–15] The results suggest that killed *M. vaccae* can suppress serum IgE and allergen-specific IL-5 synthesis. These findings suggest that killed *M. vaccae* is effective in modulating allergic responses, and may provide a novel therapeutic approach for allergic diseases.

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MATERIALS AND METHODS

Animals

Female BALB/c and C57Bl/6 J mice were obtained from OLAC (Bicester, Oxfordshire, UK) and maintained under standard conditions. These mice were used between 6 and 8 weeks of age.

Mycobacterium vaccae *(NCTC11659)*

The material used had been manufactured to Good Manufacturing Practice at the Centre for Applied

Microbiology and Research (CAMR, Porton Down, UK), for use in human trials. The manufacturing process involves growth on Sauton's medium solidified with 1·5% agar, followed by autoclaving. Organisms were stored in glass vials at 4°, suspended in borate-buffered saline (pH 8·0) at 10^{10} organisms/ml. Mice were immunized by injection of 100 µl of the appropriate dilution in pyrogen-free normal saline. All immunizations were subcutaneous at the base of the tail.

Immunization and treatment of mice

Ovalbumin (OVA) was dissolved in sterile water at 1 mg/ml and emulsified with an equal volume of Incomplete Freund's Adjuvant (IFA, DIFCO, Detriot, MI) just prior to injection. One-hundred microlitres of antigen–adjuvant mixture was injected subcutaneously (s.c.) at the base of the tail. Each animal received 50 µg OVA on day 0 and again on day 21. *M. vaccae* was administered as 10^7, 10^8 or 10^9 organisms in 100 µl saline given s.c. on day 42; on the same days, controls received saline only. There were eight mice in each group. Sera were collected on day 32 (i.e. after immunization with OVA but before treatment with *M. vaccae*) and again on days 52 and 70.

In further experiments the immunization schedule was identical, but treatment with *M. vaccae* preparations (10^7) was continued for a further two doses, given on days 53 and 81. Controls received saline only. There were four or five mice in each group. Sera were collected on days 46, 67, 91 and 109.

All animals were earmarked so that longitudinal studies were possible in each individual animal, and data could be normalized by calculating changes in IgE level relative to the reading in the sample taken after immunization, but before treatment.

Total IgE levels in serum samples

Total IgE levels were determined by a sandwich enzyme-linked immunosorbent assay (ELISA) using purified antimouse IgE monoclonal antibody (mAb) (Pharmingen, 02 111D, via Cambridge Bioscience, Cambridge, UK) as capture antibody to coat the microtitre plate, and biotinylated antimouse IgE mAb (Pharmingen, 02 122D) was used as detecting antibody. The protocol was as recommended by the manufacturer. Purified antimouse IgE mAb was diluted in coating buffer (0·1 M $NaHCO_3$, pH 8·2) at a concentration of 2 µg/ml and adsorbed to the wells of a 96-well microtitre plate (Corning, NY) at 4°, overnight. Uncoated sites in the wells were blocked with blocking buffer (2% bovine serum albumin (BSA) in phosphate-buffered saline (PBS)), for 2 hr at 37°, and the wells were washed three times with PBST (PBS–0·05% Tween). The serum samples were diluted (1 : 25) in blocking buffer and incubated with the bound anti-IgE mAb for 1 hr at 37°; the wells were then washed three times with PBST. Biotinylated anti-mouse IgE mAb was added to each well, at a dilution of 2 µg/ml in blocking buffer, and incubated at 37° for 1 hr. After the wells had been washed three times with PBST, ExtrAvidin conjugated to alkaline phosphatase (Sigma, Poole, Dorset, UK) was added at a dilution of 1 : 1000 and incubated at 37° for 1 hr. The wells were washed three times with PBST and the substrate solution added (pNPP; Sigma, N-2770). The absorbance values of the solutions in each well were recorded by using an ELISA plate reader at 405 nm. IgE levels were calculated by reference to standard curves of known amounts of purified mouse IgE (Pharmingen, 03 121D).

Anti-OVA-specific IgE levels in serum samples

Specific antibodies to OVA in the immune sera were determined by an ELISA described in detail elsewhere.[16] Briefly, 100 µl of OVA (20 µg/ml) was used to coat the microtitre plate, mouse sera were incubated in the antigen-coated wells at a final dilution of 1 : 15, and bound IgE was detected with a biotinylated anti-mouse IgE (Pharmingen; 02 112D). Diluted ExtrAvidin-Alkaline Phosphatase Conjugate (Sigma) was added, the bound enzyme was detected with pNPP (Sigma) and the absorbance read at 405 nm.

Cell culture and supernatant collection

The medium used was bicarbonate-buffered RPMI 1640 medium (Imperial Laboratories, Andover, Hampshire, UK) with added glutamine and gentamicin, containing 1% Nutridoma SR (Boehringer, Mannheim, Germany), referred to henceforth as RPMI/SR. Spleens were aseptically removed and placed in bacteriological Petri dishes. Suspensions of splenic cells were obtained by inserting a syringe needle through the capsule, clamping the spleen round the needle with forceps and then forcing RPMI/SR through the spleen, as described previously.[17] This burst the capsule, and forced out the unattached cells. The procedure was continued until the spleen was transparent. Cell suspensions were collected and washed once and resuspended in ammonium chloride/potassium bicarbonate for 7 min to lyse erythrocytes. They were then washed twice and resuspended in RPMI/SR. Viable cells were counted using trypan blue exclusion and plated in 24-well flat-bottomed tissue culture plates at 10^7 cells/ml/well. Concanavalin A (Con A) (2 µg/ml), OVA (40 µg/ml), or autoclaved *M. vaccae* (2×10^7/ml) were added as antigen. Control wells contained no antigen, in order to check for spontaneous cytokine secretion. Cultures were maintained at 37° in a humidified atmosphere with 5% CO_2. After various intervals (24, 48, 72 hr), lymphocyte suspensions were collected and centrifuged (400 ***g***, 5 min) and supernatants were collected and stored at −20° until used.

Cytokine assays

Concentrations of IFN-γ, IL-2, IL-4 and IL-5 in the culture supernatants were determined by a sandwich ELISA. The assays were performed as suggested by the manufacturer, using purified antimouse cytokine mAbs (Pharmingen 18 181D, 18 161D, 18 031D and 18 051D) to coat the microtitre plate and biotinylated antimouse cytokine mAbs (Pharmingen 18 112D, 18 172D, 18 042D and 18 062D) as detecting antibodies. Briefly, the plates (Nunc, Roskilde, Denmark) were coated overnight at 4° with 2 µg/ml purified antimouse cytokine mAb in coating buffer (0·1 M $NaHCO_3$, pH 8·2). The plates were then washed with PBST three times and blocked with blocking buffer (PBS–2% BSA) at 37° for 2 hr. The supernatant samples were incubated with the bound anticytokine mAb for 1 hr at 37° and the wells were then washed three times with PBST. Biotinylated antimouse cytokine mAb was added to each well, at a dilution of 2 µg/ml in blocking buffer, and incubated at 37° for 1 hr. Streptavidin conjugated to horseradish peroxidase (DAKO, P0397, High Wycombe, Bucks, UK) was added at a final dilution of 1 : 1000 and incubated at 37° for 1 hr. The colour reaction was developed by 2, 2′-diameno-benthiazoline sulphoric acid ABTS; Sigma) and the absorbance values of the solutions in each well were recorded by using an ELISA plate reader at 405 nm. Cytokine

levels were calculated by reference to standard curves of known amounts of rIFN-γ, rIL-2, rIL-4 and rIL-5 (Pharmingen 19301T, 19211T, 19231 W and 19241 W).

Statistical analysis

Data from cytokine and antibody assays were expressed as the mean ± standard deviation (SD) or standard error (SE). Two-tailed Student's *t*-test and Mann–Whitney *U*-test were used to determine significant differences between control and experimental groups. Differences were considered statistically significant when the *P*-value was less than 0·05.

RESULTS

The effect of different doses of *M. vaccae* on serum IgE

To determine the effect of *M. vaccae* on total serum IgE, BALB/c mice were immunized twice with OVA in IFA (on days 0 and 21) to induce IgE responses. They were bled on day 32, and then treated with saline or with 10^7, 10^8 or 10^9 *M. vaccae* on day 42.

On day 32 the OVA-immunized mice had 117·09 ± 35·81 (SD) ng/ml IgE, compared to 69·27 ± 6·09 (SD) ng/ml in unimmunized animals ($P < 0·001$). For each mouse, the day 32 value was used to normalize data to a starting (i.e. day 32) value of 0, and subtracted from each subsequent value. Therefore, the values plotted are the changes in ng/ml relative to day 32.

The IgE response in control mice (treated with saline) had increased further by days 52 and 70 when further samples were taken (i.e. 10 and 28 days after treatment on day 42) (Fig. 1). In contrast, the increase in IgE level was suppressed in mice treated with *M. vaccae* at 10^7, 10^8 and 10^9 (Fig. 1). All *P*-values are less than 0·01 between mice treated with saline and different doses of *M. vaccae*.

The effect of *M. vaccae* treatment on cytokine production by spleen cells

BALB/c mice were subjected to the same protocol used to provide the data for Fig. 1. Then, on day 82, their spleen cells were harvested and cultured *in vitro* with OVA, *M. vaccae* and Con A. In response to OVA, spleen cells from the saline-treated group produced IL-4 but no IL-2 (Fig. 2(a), 2(b)). Splenic cells from OVA-immunized mice that had been treated with 10^7 autoclaved *M. vaccae* showed IL-2 synthesis and decreased IL-4 production in response to OVA. IL-2 synthesis in response to OVA was also seen using spleen cells from mice treated with 10^8 or 10^9 *M. vaccae*.

The effect of two doses of *M. vaccae* on serum IgE

As 10^7 *M. vaccae* had been shown previously to be the optimal dose for evoking Th1 responses to its own antigens,[17] and had been the most effective dose in the pilot experiments (Fig. 2), this dose was selected for further studies. BALB/c mice were immunized with OVA twice on days 0 and 24 to induce IgE responses and bled on day 46, and then treated with saline, or with 10^7 *M. vaccae* twice on days 53 and 81. On day 46 the OVA-immunized mice had 112·9 ± 10 (SE) ng/ml IgE, compared to 55·4 ± 1·7 (SE) ng/ml in unimmunized animals ($P < 0·01$). For each mouse the day 46 value was used to normalize data to a starting (i.e. day 46) value of 0, and subtracted from each subsequent value. Therefore, the values plotted are the changes in ng/ml relative to day 46.

The total serum IgE response in control mice (treated with saline) increased steadily for the duration of the experiment. In contrast, the increase in IgE was suppressed in mice treated with 10^7 autoclaved *M. vaccae* (Fig. 3(a). Meanwhile, the suppression of anti-OVA IgE by autoclaving *M. vaccae* was not significant by this assay (Fig. 3(b)). However, further experiments show that OVA-sensitized BALB/c mice that received *M. vaccae* four times had significantly lower OVA-specific IgE titres (unpublished observations).

IgG1 and IgG2a antibodies to OVA were not affected by *M. vaccae* treatment (data not shown).

The effect of *M. vaccae* treatment on IFN-γ, IL-2 and IL-5 production by spleen cells

BALB/c mice were subjected to the same protocol used to generate the data presented in Fig. 3. On day 109, the spleen

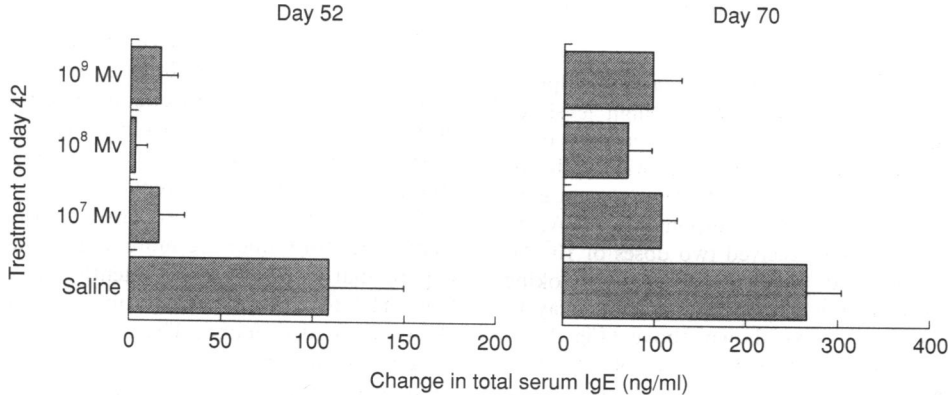

Figure 1. The effect of treatment with single s.c. injections of different doses of *M. vaccae* compared with saline on serum IgE levels in BALB/c mice previously immunized with OVA. Serum IgE is expressed as the change relative to readings obtained with the samples taken on day 32, before the treatment with *M. vaccae* or saline (mean ± SE). Post-treatment serum samples were collected on days 52 and 70 (i.e. 10 days and 28 days after the treatment with saline or *M. vaccae* on day 42). Immunization and treatment schedules are detailed in the Materials and methods. Comparison between different groups was performed by the Mann–Whitney *U*-test.

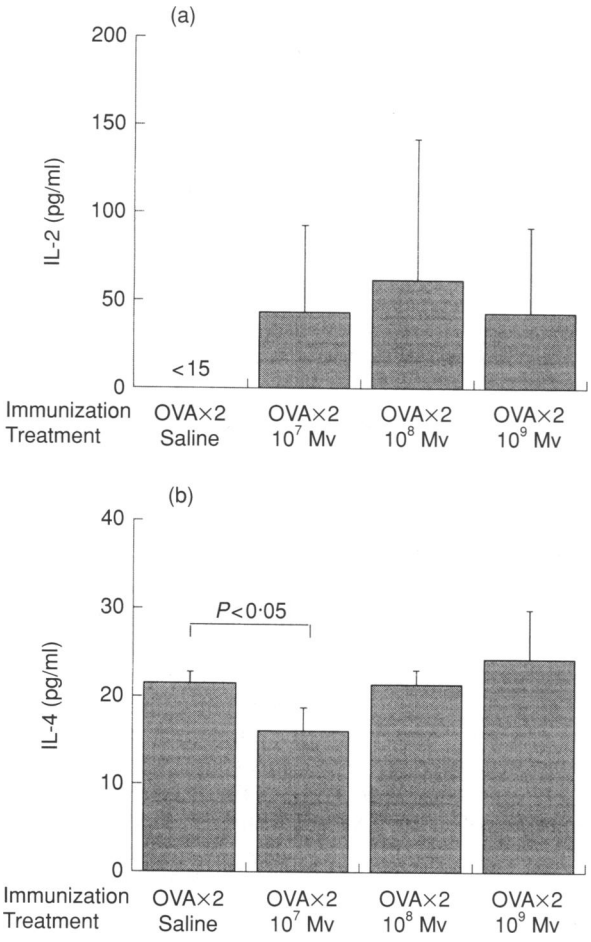

Figure 2. Production of IL-2 (a) and IL-4 (b) by splenocytes cultured with OVA. BALB/c mice were given OVA twice prior to the different doses of *M. vaccae*: 10^7, 10^8 or 10^9 Splenocytes were harvested and cultured with OVA on day 82. Immunization and treatment schedules are detailed in the Materials and methods. Culture supernatants were collected for IL-4 estimation after 48 hr and for IL-2 after 24 hr. The data are presented as the mean levels of cytokine ± SD. Comparison between different groups was performed by the Student's *t*-test. Saline, immunization twice with OVA prior to saline treatment; 10^7, 10^8, 10^9 Mv, immunization twice with OVA prior to treatment with 10^7, 10^8 or 10^9 *M. vaccae*.

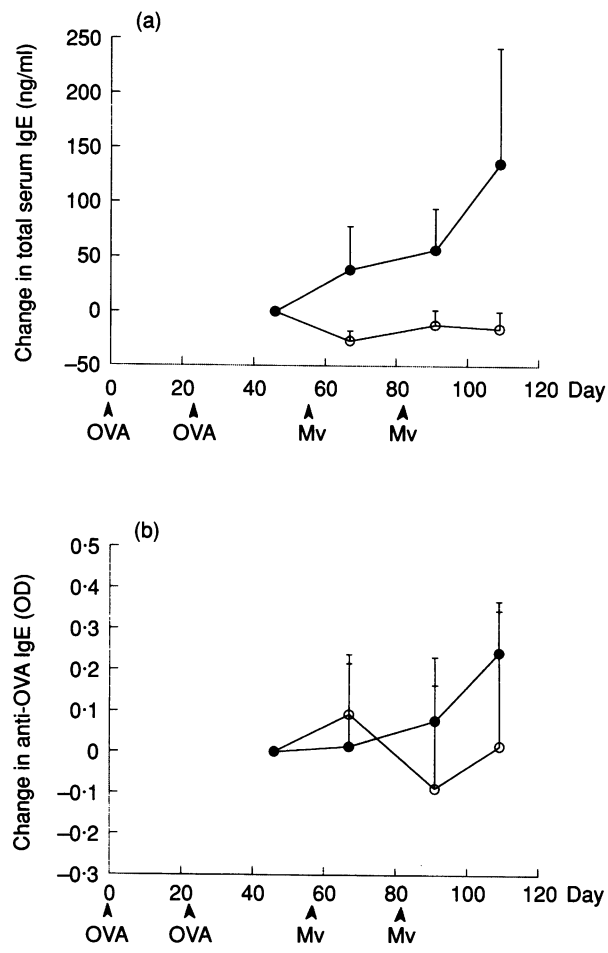

Figure 3. The effect of treatment with two s.c. injections (days 53 and 81) of 10^7 *M. vaccae* (○), compared to saline (●), on total serum IgE (a) and OVA-specific IgE (b). Immunization and treatment schedules are indicated on the Figure. Serum IgE is expressed as the change ± SD relative to readings obtained with the samples taken on day 46. Comparison between different groups was performed by the Mann–Whitney *U*-test. OVA, ovalbumin immunization; Mv, *M. vaccae* immunization)

cells were harvested and cultured with OVA *in vitro*. Spleen cells from the saline-treated group produced high levels of IL-5, but no IL-2, in response to OVA. However, cells from OVA-immunized mice that had been treated twice with 10^7 autoclaved *M. vaccae* failed to release IL-5 in response to OVA (Fig. 4(a)). IL-4 production, in response to OVA, by spleen cells from mice that had received two doses of 10^7 *M. vaccae* appeared to be reduced, but the levels of this cytokine were too close to the detection limit of the immunoassay to be reliable (data not shown). As shown above (Fig. 2(a)), OVA-induced IL-2 production was again detectable in supernatants from spleen cells of mice treated with *M. vaccae* (Fig. 4(b)). There was no difference in OVA-induced IFN-γ synthesis by spleen cells from the different groups (Fig. 4(c)).

DISCUSSION

BALB/c mice that received two immunizations with OVA in IFA developed a typical Th2-type response. There were rising levels of serum IgE, and spleen cells from these mice released IL-5 but not IL-2 in response to OVA *in vitro*. The most striking finding is that this ongoing allergen-specific response in BALB/c mice was downregulated by treatment with a low dose of killed *M. vaccae* without any need for OVA or OVA epitopes in the *M. vaccae* preparation.

BALB/c mice have a propensity towards Th2 responses, whereas C57Bl/6 mice are more Th1 prone.[18,19] Moreover, it is particularly difficult to persistently alter responses to OVA from Th2 to Th1 in BALB/c mice.[11,20] When polymerized OVA was used to induce Th1-like responses in mice injected with OVA, BALB/c mice were resistant to long-term IgE suppression.[11] In contrast, our experiments showed prolonged suppression of total serum IgE response in BALB/c mice injected with *M. vaccae* after OVA sensitization. This effect is not restricted to BALB/c mice. Although C57Bl/6 mice failed to give an IgE response to OVA using the protocol described here (data not shown), parallel experiments using house dust mite allergen in this mouse strain showed a similar depression

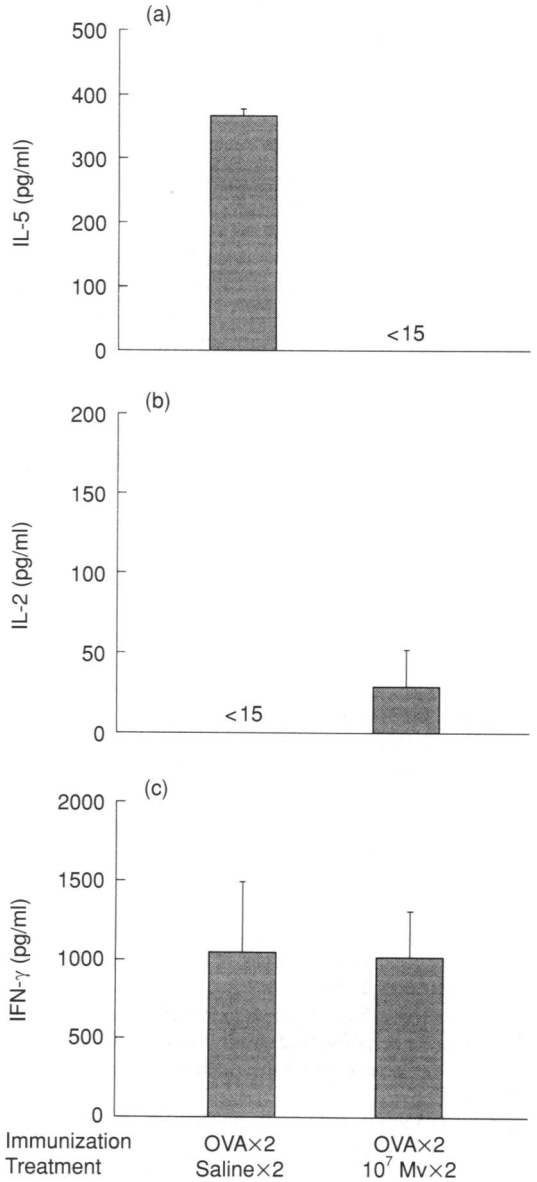

Figure 4. Production of IL-5 (a), IL-2 (b), and IFN-γ (c) by splenocytes cultured with OVA. Splenocytes were harvested and cultured with OVA on day 109. Immunization and treatment schedules are detailed in the legend to Fig. 3. Culture supernatants were collected for IL-5 estimation after 48 hr and for IFN-γ and IL-2 after 24 hr. The data are presented as the mean levels of cytokine ± SD. Comparison between different groups was performed by Student's *t*-test. Saline, immunization twice with OVA prior to saline treatment twice; 10^7 Mv, immunization twice with OVA prior to treatment twice with 10^7 *M. vaccae*.

of IL-5 production following treatment with *M. vaccae* (R. Janssen, J. Thole and D. B. Young, personal communication).

Most recent studies have concentrated on allergen-specific or epitope-specific effects designed to tolerize responses to specific epitopes recognized by Th2 lymphocytes,[21] or to switch those responses to Th1. Downregulation of pre-existing Th2 response has been achieved recently by therapeutic immunization with a DNA vaccine[22,23] and with an allergen–IL-12 fusion protein.[24] Such allergen-specific strategies are very attractive, and suitable preparations may be safe to administer because they do not need to contain the IgE-binding epitopes. However, the allergen-specific approach has three disadvantages. First, there are limited experimental data to suggest that treatment with one epitope within a complex allergen can downregulate the response to other epitopes in the same allergen[25,26] and there are no data to demonstrate this in humans. Second, suppression (or diversion to Th1) of the response to a single allergen may merely encourage development of allergic responses to other allergens, if the individual is left with a tendency towards a Th2 bias. Finally, it may not be desirable to leave the patient with a Th1 response to the allergen.

An alternative strategy, which may avoid these problems, is to use potent immunogens that can have systemic long-lasting non-specific effects on the nature of the immune response to unrelated antigens. There is now no doubt that this can happen. For instance, measles infection reduces the incidence of atopy and of allergic reactions to house dust mite.[27] Measles vaccination is another example. This vaccine, when used at the standard low dose, reduces mortality by considerably more than can be accounted for by the incidence of measles in the unvaccinated population. Diphtheria, tetanus and pertussis vaccines (Th2-inducing) do not show this non-specific protective effect.[28] Similarly, Japanese children that are tuberculin skin-test positive are less likely to be atopic than are tuberculin-negative children, and their ratio of circulating Th1/Th2 cytokines is higher. Moroever, after repeated injection of BCG, those in whom tuberculin conversion occurs have an increased probability of losing their atopic symptoms.[12]

As there is considerable evidence for reciprocal inhibition of Th2 by Th1 and vice versa,[29,30] the data of Shirakawa *et al.*[12] may have been caused by a non-specific systemic suppressive effect of Th1 cytokines on the generation of Th2 responses. This is compatible with the finding that the level of Th1 cytokines was increased relative to Th2 cytokines in the serum of the tuberculin-positive children. Such a mechanism may also account for the observations that repeated injections of Complete Freund's Adjuvant (CFA) can block subsequent attempts to evoke IgE responses[31] and that serum from animals immunized with CFA will suppress IgE formation after passive transfer.[32]

However, the downregulation of Th2 by Th1 or Type 1 cytokines has recently been questioned, and there are considerable complexities. For instance, under some circumstances interleukin-12 (IL-12) can enhance rather than inhibit development of the Th2 cytokine pattern[33,34] and it may be clinically important that reversibility of the Th2 phenotype by exposure to Type 1 cytokines is eventually lost.[35] In view of these conflicting findings, it is interesting that recent data from further studies with *M. vaccae* in the BALB/c model presented here (C.-C. Wang, R. Janssen, J. Thole & G. Rook in preparation) have revealed that conjugated *M. vaccae* preparations, which contain OVA proteins, drive strong Th1 responses to OVA; this does not enhance suppression of Th2 and can abrogate the ability, that we demonstrate here, of *M. vaccae* to inhibit IL-5.

Similarly, our experiments revealed that high levels of serum IgE induced by OVA were suppressed by a wide range

of doses of *M. vaccae*. This is unexpected because previous dose–response studies in mice identified 10^7 as the optimum dose for inducing a Th1 response to the mycobacterial antigens contained within *M. vaccae*, with no detectable Th2 component,[17] while 10^9 evokes a mixed Th1 + Th2 response. For all these reasons it is likely that the effects of *M. vaccae* in this model may not operate via the suppressive effects of Th1 cytokines. A further level of regulation of IgE levels may be attributable to the induction of IgE-binding factors.[36] It has been revealed that IgE can be suppressed by the unglycosylated form of an IgE-binding factor, production of which is regulated by the recently cloned cytokine, glycosylation-inhibiting factor (GIF).[37]

In summary, several mechanisms may be responsible for the suppression of allergen-induced IL-5 secretion and serum IgE in OVA-sensitized mice treated with killed *M. vaccae*. Although these are at present unidentified, the availability of extensive safety data in humans suggested that killed *M. vaccae* should be tested as an immunotherapeutic for allergic diseases, and it is currently undergoing clinical trials for this purpose.

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