

## Vaccination with a multi-epitopic recombinant allergen induces specific immune deviation via T-cell anergy

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### SUMMARY

Prophylactic vaccination has recently emerged as a major paradigm toward the prevention and therapy of allergies and asthma; however, the immunological basis of this approach remains to be elucidated. We examined the potential and mechanism of prophylaxis of allergic response in B6D2F1 mice with a multi-epitopic recombinant allergen, rKBG8·3 (MERA-8·3), which represents a major group of allergens of grass pollens, used herein as a model of MERA vaccine. Vaccination (subcutaneous) with soluble MERA-8·3, prior to immunization with the MERA-8·3 in alum, led to suppression of the IgE antibody response and a concomitant increase in IgG2a antibody response specific to the MERA-8·3 in a dose-dependent manner. Analysis of cytokine patterns in spleen and lymph node cells revealed a marked decrease of interleukin-2 (IL-2) and IL-4 production and to a lesser extent a decrease of interferon- $\gamma$  (IFN- $\gamma$ ) synthesis, resulting in an increased ratio of IFN- $\gamma$ :IL-4 in vaccinated-immunized mice compared with untreated-immunized control mice. Furthermore, splenocytes of mice treated with the MERA-8·3 alone proliferated to MERA-8·3 *in vitro* with reduced capacity compared with the splenocytes of MERA-8·3-alum immunized mice, owing to a markedly reduced level of IL-2 production in the former. Collectively, these results suggest that vaccination with the MERA-8·3 induces T-cell anergy, which is pivotal to deviation of specific immunity from Th2- to Th1-like, and may serve as an important approach to prevention and therapy of allergic disorders.

### INTRODUCTION

Helper T-cell responses are often polarized in disease states resulting in a predominantly Th1- or Th2-like immune responses.<sup>1–3</sup> Studies in both mouse and human systems demonstrated that IgE-mediated allergic disorders are controlled primarily by Th2-type cytokines.<sup>2</sup> Th1- and Th2-like cells reciprocally regulate each other through the cytokines they secrete. Thus interferon- $\gamma$  (IFN- $\gamma$ ) and interleukin-12 (IL-12) inhibit Th2-like cells, whereas IL-4 and IL-10 indirectly inhibiting functions of Th1-like cells by affecting antigen-presenting cells.<sup>2,4</sup> Association of allergic disorders with the antigen-specific Th2-like cytokine pattern raises the possibility to design prophylactic and therapeutic modalities in a way that would promote the development of Th1-like immune responses, which might prevent disease induction.<sup>4,5</sup> Because

selection of the appropriate T-cell population occurs during the early stages of immune response in a naive host, it was hypothesized that a deliberate administration of allergens to stimulate development of Th1 immunity may control the T-cell selection process.<sup>6</sup>

Specific T-cell responses in the periphery can be modulated by three principal mechanisms: antigen-specific T-cell elimination, antigen-induced T-cell unresponsiveness or anergy,<sup>7</sup> and antigen-induced T-cell response in a form that differs from the default response, referred to as immune deviation.<sup>5</sup> However, these mechanisms are not mutually exclusive. Both *in vitro* and *in vivo* studies have shown that T cells can be rendered anergic by antigen stimulation in the absence of costimulatory signals, or by supraimmunogenic doses of antigens and antigenic peptides.<sup>7,8</sup> Peptides containing T-cell epitopes induced antigen-specific T-cell tolerance measured by reduced proliferation and IL-2 production in naive and primed mice,<sup>8</sup> thus suggesting that T-cell peptides may be utilized for allergen-specific immunotherapy of allergic patients.<sup>9,10</sup> A potential drawback of the T-cell peptide immunotherapy is the difficulty in designing therapeutic cocktails because of multiplicity and diversity of epitopes in allergens to which allergic patients may be sensitized.<sup>10</sup>

We have reported previously on molecular cloning and expression in *Escherichia coli* of a group of Kentucky Blue

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Abbreviations: KBG, Kentucky Blue Grass; MERA, multi-epitopic recombinant allergen; RT-PCR, reverse transcriptase polymerase chain reaction.

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Grass (KBG) pollen allergens, which were designated as Poa p V allergens, according to the new nomenclature.<sup>11,12</sup> The C-terminal fragment, rKBG8·3 (containing 201 amino acids), constituted the conserved domain of Poa p V allergens and included the majority of T- and B-cell epitopes of this group of allergens.<sup>13,14</sup> In the present study we demonstrated that vaccination of mice with rKBG8·3, as a model multi-epitopic recombinant allergen (NERA) vaccine, induced changes in specific immunity to this NERA-8·3, which were manifested by a marked decrease of IgE and a concomitant increase in IgG2a antibodies. These changes paralleled T-cell cytokine production reflected by an increase in IFN- $\gamma$ :IL-4 ratio, and a shift from Th2-like to Th1-like immune response.

## MATERIALS AND METHODS

### Animals

Female, 6–8-week-old B6D2F1 (C57BL/6J  $\times$  DBA/2J) mice were purchased from Charles River (St Constant, PQ, Canada), male Sprague-Dawley (S-D) rats were bred at the University of Manitoba breeding facility. All animals were maintained and used in accordance with guidelines issued by the Canadian Council on Animal Care.

### Preparation of NERA-8·3 protein

Glutathione S-transferase (GST)-NERA-8·3 fusion protein was expressed in JM105 bacterial cells, and was purified with glutathione agarose beads as described previously.<sup>15</sup> Thrombin (Sigma Chemical Co., St Louis, MO) was used to cleave NERA-8·3 from the fusion protein when it was bound to glutathione agarose beads (Sigma Chemical Co.). Prior to its use, purity of NERA-8·3 was assessed by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) followed by staining with Coomassie Blue and silver stain as described.<sup>16</sup>

### In vivo treatment and immunization

Groups of four B6D2F1 mice were injected subcutaneously (s.c.) with either different doses of purified NERA-8·3 in phosphate-buffered saline (PBS; 0·1 M, pH 7·2), or with PBS only as control, on day 1 and 2. Mice were immunized intraperitoneally (i.p.) with 2  $\mu$ g NERA-8·3 in 1 mg alum on day 7.

### Antibody assays

Mice were bled on day 20 after primary immunization. The sera of individual mice were evaluated for levels of antigen-specific IgE antibody by passive cutaneous anaphylaxis (PCA) as described.<sup>17</sup> Briefly, hooded S-D rats were sensitized with serially diluted mouse sera by intradermal (i.d.) injection on the back. Forty-eight hours later, the rats were challenged intravenously (i.v.) with 200  $\mu$ g NERA-8·3 in 1 ml PBS containing 0·5% Evan's blue. PCA titre was calculated as the highest dilution giving a clearly visible skin reaction (>0·5 cm in diameter). Antigen-specific IgG1 and IgG2a antibodies were determined by enzyme-linked immunosorbent assay (ELISA).<sup>18</sup> Briefly, microtitre plates (Corning Science Products, Rochester, NY) were coated overnight with 0·1  $\mu$ g NERA-8·3 per well in bicarbonate buffer (0·05 M, pH 9·6). The wells were incubated with diluted sera overnight at 4°, washed and then the antibody binding was detected with

alkaline phosphatase conjugated rabbit anti-mouse IgG1 or IgG2a antibodies (Zymed, South San Francisco, CA). Antibody titres were determined by 0·5 units of OD<sub>405</sub> value.

### Cytokine protein measurements

Spleens and mesenteric lymph nodes of mice were collected aseptically on day 6 after first immunization. Single cell suspensions were prepared and cultured at  $10 \times 10^6$ /ml in presence of 200  $\mu$ g/ml of NERA-8·3 as described.<sup>18</sup> Production of IL-2, IFN- $\gamma$  and IL-4 from 24-hr culture supernatants was measured by using sandwich ELISA reagents (Pharmingen, San Diego, CA) according to the manufacturer's instructions. Briefly, 96-well ELISA plates were coated with 0·5  $\mu$ g/well of purified rat monoclonal antibodies (mAb) to mouse IL-2, IFN- $\gamma$ , or IL-4 as capture mAb overnight at 4° and blocked with 3% bovine serum albumin (BSA) at room temperature for 2 h. Then serially diluted recombinant cytokine standards of IL-2, IFN- $\gamma$ , or IL-4, and supernatants collected from cultured spleen and lymph node cells in the presence or absence of specific antigens, were added and incubated overnight at 4°. Cytokines were detected by incubation with biotinylated rat mAb to corresponding mouse cytokine (1  $\mu$ g/well), followed by substrate reaction using alkaline phosphatase-conjugated streptavidin (Sigma Chemical Co.).

### Cytokine mRNA measurements by reverse transcriptase–polymerase chain reaction (RT–PCR)

Total RNA was isolated from spleen cells of control and treated mice after 24-h culture *in vitro* in the presence of NERA-8·3. RNA was reverse transcribed into cDNA and the first strand cDNA was subject to polymerase chain reaction (PCR), for amplification of  $\beta$ -actin and cytokines using the following primers:  $\beta$ -actin, 5'-GTGGGCCGCTCTAG-GCACCAA and 5'-CTCTTTGATGTCACGCACGATT-TTC; IL-2, 5'-GAGCAGGATGGAGAATTACAGG and 5'-TCCTCAGAAAGTCCACCACAGT; IL-4, 5'-GTCAT-CCTGCTCTTCTTTCTCG and 5'-GATGCTCTTTAG-GCTTTCCAGG, and IFN- $\gamma$ , 5'-TGGCTGTTTCTGG-CTGTTACTG and 5'-AATCAGCAGCGACTCCTTTTCC. An annealing temperature of 58° was used for the primer sets of  $\beta$ -actin and IFN- $\gamma$ , and 60° for IL-2 and IL-4. Two microlitres of the cDNA mixture was added to 0·2 mM dNTP, 0·25  $\mu$ M each of sense and anti-sense primers in PCR buffer (50 mM KCl, 20 mM Tris–HCl, pH 8·4, 2·5 mM MgCl<sub>2</sub>, 0·1 mg BSA) in a total volume of 23·5  $\mu$ l and 0·75 U (0·5 U/ $\mu$ l) of Taq DNA polymerase (BRL, Life Technologies Inc., Gaithersburg, MD). This mixture was then cycled through 95° for 1 min, annealing temperature for 2 min and 72° for 3 min (40 cycles, 10 min extension time was used in the last cycle). PCR products were assessed by electrophoresis through 1% agarose gel containing ethidium bromide (0·5  $\mu$ g/ml) in TAE buffer [0·04 M Tris–acetate and 0·002 M ethylenediamine tetra-acetic acid (EDTA)]. Gels were photographed and the amplified products were converted to OD value by densitometry.

### Proliferation assay

Eleven days after immunization or treatment, spleens were aseptically removed and individual spleen cells ( $1 \times 10^6$  cells/well of 96-well plates) were cultured in triplicate in complete RPMI-1640 medium containing 10% fetal calf serum in the presence or absence of 50  $\mu$ g/ml of rKBG8·3 and/or

50 U/ml of rmIL-2 at 37° for 48 h. Cells were pulsed with 1  $\mu$ Ci [ $^3$ H]thymidine ([ $^3$ H]TdR, ICN Radiochemicals, Irvine, CA) per well for 16 hr. Cells were then harvested and the amount of [ $^3$ H]TdR incorporation was counted by a liquid scintillation counter (Packard Tri-Carb 2200CA, Packard Instrument Co., Downer Grove, IL).

## RESULTS

### Vaccination with MERA inhibited production of IgE antibody and enhanced production of IgG2a antibody

Groups of B6D2F1 mice were treated s.c. with soluble MERA-8·3, prior to immunization with the MERA-8·3 in alum as adjuvant. Sera were collected on day 20 after the first immunization for antibody assays. As shown in Fig. 1(a), treatment with soluble MERA-8·3 led to dose-dependent suppression of MERA-8·3-specific IgE antibody production. In contrast, specific IgG2a antibody synthesis was elevated following treatment. However, specific IgG1 antibody level remained unaffected. IgE, IgG1 and IgG2a antibody patterns were similar even after secondary immunization in treated

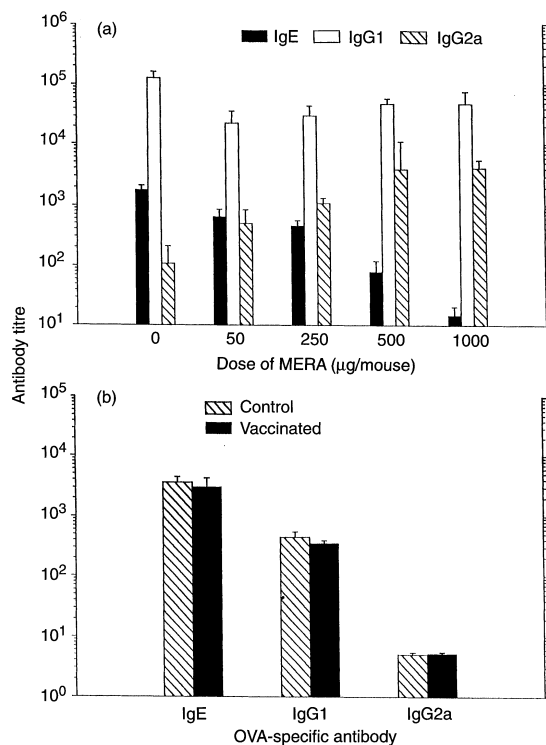


**Figure 1.** (a) Vaccination with MERA-8·3 followed by immunization suppressed MERA-specific IgE antibody production. Groups of four B6D2F1 mice were treated s.c. with 25–500  $\mu$ g of MERA-8·3 per injection in PBS or with PBS alone as control on day 1 and 2, prior to i.p. immunization with 2  $\mu$ g of MERA-8·3 in 1 mg alum on day 7. Antibody titres of sera collected on day 20 after immunization are presented as geometric mean  $\pm$  SEM. Similar results were obtained in two separate experiments. (b) Antigenic specificity of MERA-8·3 treatment on antibody response to unrelated antigen, OVA. Mice were treated s.c. with 250  $\mu$ g of MERA-8·3 per injection in PBS on day 1 and 2, and then immunized i.p. with 2  $\mu$ g of OVA in alum on day 7. Antibody titres of sera collected on day 20 after immunization are presented as geometric mean  $\pm$  SEM.

mice (data not shown). Moreover, this pattern of antibody production was antigen-specific because immunization with ovalbumin (OVA) following treatment with MERA-8·3 did not alter OVA-specific antibody production (Fig. 1b). These results indicated that vaccination with the MERA-8·3 resulted in shifting of MERA-8·3-specific antibody production from IgE to IgG2a type.

### Vaccination with MERA altered the expression of cytokine genes

In order to investigate mechanisms underlying the shift in antibody repertoires, we examined profiles of T-cell cytokines, such as, IL-2, IFN- $\gamma$  (Th1-like) and IL-4 (Th2-like) and the results are shown in Fig. 2(a). Twenty-four-hour bulk cultures of spleen cells of individual mice yielded readily detectable IL-2 (detection level was 10–12 pg/ml), IFN- $\gamma$  (detection level was around 20 pg/ml) and IL-4 (detection level was 2–4 pg/ml) responses. In control (PBS-injected) mice, immunization with the MERA-8·3 in alum elicited strong cytokine responses. In marked contrast, in the supernatants of cultured spleen cells established from the MERA-8·3-vaccinated mice, synthesis of both IL-2 and IL-4 was drastically reduced, production of IFN- $\gamma$  was also reduced, albeit to a lesser degree. A similar pattern of cytokine expression was obtained in cultured lymph node cells of treated mice (data not shown).

To determine the effect of the *in vivo* treatment on antigen-specific Th1- and Th2-like responses more directly, we compared IL-2, IFN- $\gamma$  and IL-4 mRNA expression in cultured spleen cells of treated and control mice by RT-PCR. As shown in Fig. 2(b), mRNA expression of IL-2, IFN- $\gamma$  and IL-4 was induced in spleen cells of control mice in response to *in vitro* restimulation with the MERA-8·3. However, in spleen cells from the MERA-8·3-vaccinated mice, mRNA expression of IL-2, IFN- $\gamma$  and IL-4 was decreased, even totally abrogated in some mice. The expression of cytokine mRNA in spleen cells of vaccinated and control mice was quantified by comparison of relative OD of RT-PCR products of cytokine mRNA standardized with  $\beta$ -actin. The lymph node cells of control and vaccinated mice showed similar profiles of cytokine mRNA expression (data not shown). These data indicated that vaccination with MERA-8·3 down-regulated cytokine mRNA expression of antigen-specific T cells.

To examine the dominant pattern of cytokine responses, we compared the IFN- $\gamma$ :IL-4 and IL-2:IL-4 protein ratios in control and treated mice and the results are summarized in Fig. 2(c). About a 10-fold increase in the ratio of IFN- $\gamma$ :IL-4 was found in both splenocyte and lymph node cultures of mice vaccinated with MERA-8·3 as opposed to control mice. Moreover, the ratio of IFN- $\gamma$ :IL-4 mRNA levels as measured by RT-PCR and densitometry, was also elevated in spleen and lymph node cells in treated mice (data not shown). However, the ratio of IL-2:IL-4 was decreased in vaccinated mice. These results indicated that the net cytokine balance shifted in favour of IFN- $\gamma$  dominance following vaccination with MERA.

### Administration of MERA-8·3 alone decreased IL-2 synthesis

To establish the mechanism underlying the early phase of vaccination with MERA-8·3, we further compared the cytokine profiles following administration of MERA-8·3 with or with-

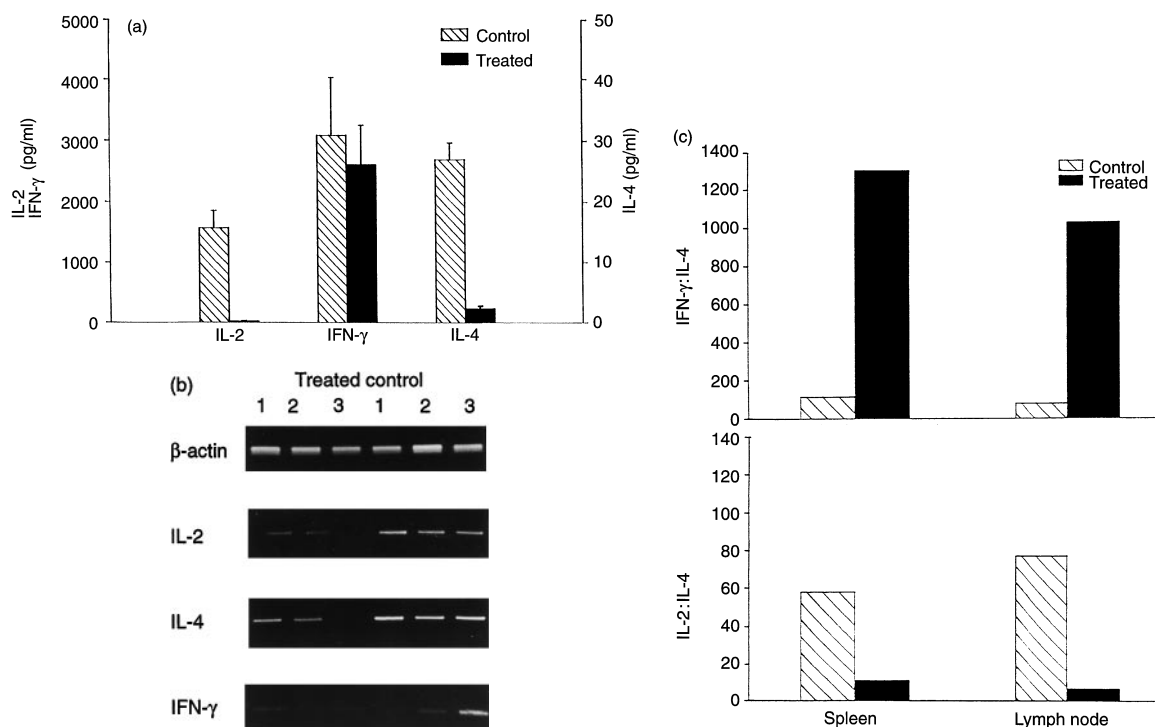


**Figure 2.** (a) Vaccination with MERA-8·3 followed by immunization decreased production of Th1-like (IL-2 and IFN-γ) and Th2-like (IL-4) cytokines. Groups of four mice were vaccinated s.c. with either 250 μg of MERA-8·3 (vaccinated) or with PBS (control) on day 1 and 2, and then immunized with 2 μg of MERA-8·3 in 1 mg of alum. Six days after immunization, cytokine synthesis was determined in the supernatants from 24 hr, MERA-8·3-stimulated (200 μg/ml) bulk cultures of individual mouse spleen cells as described in the Materials and Methods. Bars represent arithmetic mean ± SEM. (b) Cytokine mRNA levels assessed by RT-PCR in vaccinated (lanes 1–3) and control (lanes 4–6) groups. (c) Folds increase in IFN-γ:IL-4 and folds decrease in IL-2:IL-4 in spleen and lymph node cell cultures of vaccinated group compared to the control. Similar results were obtained in two separate experiments.

out adjuvant. As shown in Fig. 3(a), bulk cultures of spleen cells of mice after immunization with the MERA-8·3 in alum elicited significant levels of IL-2, IL-4 and IFN-γ, whereas administration of the MERA-8·3 in the absence of alum stimulated significant IFN-γ and IL-4, but markedly reduced level of IL-2. Moreover, the later culture induced much less proliferation *in vitro* in response to rKBG8·3, the proliferative capacity of these cells was reconstituted by addition of exogenous IL-2 to the culture (Fig. 3b). These results suggested that the treatment with MERA-8·3 in the absence of alum, suppressed IL-2 production and anergized MERA-specific T cells. Moreover, MERA-8·3-treated non-immunized mice induced detectable IgG1 and IgG2a but no IgE antibodies compared to the immunized mice (data not shown). It is inferred from these experiments that vaccination with the MERA resulted in decreased IL-2 synthesis and consequently immune deviation.

## DISCUSSION

Recent demonstrations that first, development of sensitization to allergens occurs within the first 2 years of life,<sup>19</sup> second, a significant proportion (20–30%) of individuals are sensitized to a few major offending aeroallergens,<sup>20</sup> and third, specific immunotherapy with one aeroallergen prevented general sensitization, support the notion of specific prophylaxis against major aeroallergenic components.<sup>21</sup> We demonstrate here the

potential and mechanism of prophylactic vaccination with a multi-epitopic recombinant allergen, MERA-8·3 in a mouse model. A major finding of this study is that vaccination with MERA-8·3 via the s.c. route suppressed IgE antibody production with a concomitant increase of IgG2a antibody production in a dose-dependent manner. Essentially, similar results were also obtained by i.v. injection, in which case the inhibition of IgE antibody response was noted for up to 87 days (Cao and Mohapatra, unpublished data). Our results are consistent with earlier findings using OVA, however, in the latter case the antigen was polymerized.<sup>22</sup> It appears, therefore, that synthetic antigen with multiple epitopes, at an appropriate dose, can induce immune deviation as manifested by inhibition of the IgE antibody response and elevation of IgG2a antibody response. In contrast to the tightly reciprocal control of IgE and IgG2a antibody responses, the specific IgG1 antibody level showed little change. This observation is in agreement with our previous study<sup>17</sup> and is presumably due to multiple factors controlling the isotype-specific responses *in vivo*. However, our results differ from the general reduction of IgG antibody levels found in another study.<sup>8</sup> Use of different adjuvants, experimental protocols and antigens may account for the different outcomes.

Previous studies reported that Th1, Th2 and Th0 cells were capable of anergy induction, however, these T-cell subsets differed in their potential to anergy induction because of differences in their lymphokine requirement, lymphokine

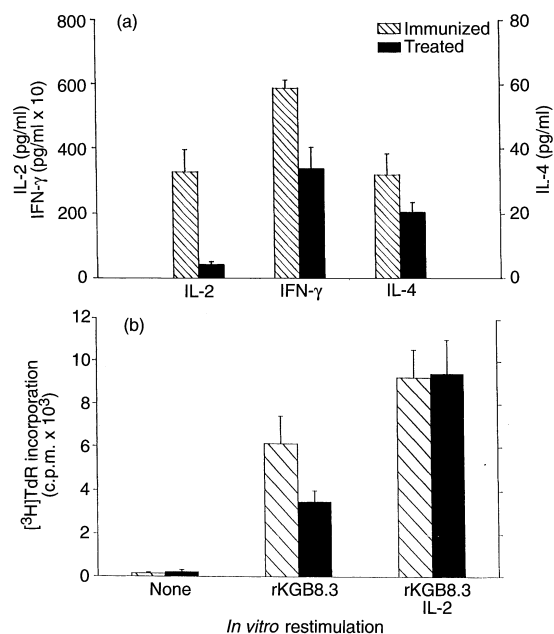


**Figure 3.** (a) Administration of MERA-8.3 itself induced anergy by suppressing IL-2 production. Groups of three B6D2F1 mice were either immunized s.c. with 2 µg MERA-8.3 in alum, or treated s.c. with 250 µg of MERA-8.3 in PBS for 2 days. Five days later, spleen cells were cultured *in vitro* in the presence of 200 µg MERA-8.3. Cytokine productions in 24-hr culture supernatants were measured as described in Material and Methods. Bars represent arithmetic mean  $\pm$  SEM. (b) Recombinant IL-2 reconstituted anergy induced by MERA-8.3 treatment. Mice were treated and immunized as in Fig. 3(a). Eleven days later, spleen cells were cultured in triplicates in presence of 50 µg/ml rKGB8.3 and/or 50 u/ml of rIL-2 for 3 days. Proliferation was correlated with [<sup>3</sup>H]thymidine incorporation which was expressed as arithmetic mean of c.p.m.  $\pm$  SEM. Similar results were obtained in two separate experiments.

secretion, early activation events, use of APC, the route and dose of the specific antigen.<sup>8,23–26</sup> Our data clearly indicate that vaccination with MERA-8.3 resulted in a decrease of expression of IL-2, IL-4 and IFN-γ at the level of both mRNA and protein, suggesting that perhaps all the MERA-8.3-specific T cells, i.e. Th0-, Th1- and Th2-like cells were anergized by vaccination. The RT-PCR results suggested that the MERA-8.3 treatment down-regulated cytokine mRNA expression at the transcription or post-transcription level. Furthermore, reduction in the level of these cytokines in vaccinated-immunized mice differed inasmuch as IFN-γ production was least affected, which led to an increase in the ratio of IFN-γ:IL-4 in the MERA-8.3-vaccinated mice in comparison with the control mice. This result is in agreement with a previous report that the anergized T cells could synthesize considerable amounts of IFN-γ. Presumably IFN-γ expression requires multiple signalling pathways, only some of which are inhibited in the anergic state.<sup>26</sup>

It has been suggested that a stable ratio of antigen-specific CD4<sup>+</sup> T cells committed to IL-4 or IFN-γ production is established within the first 4 days after immunization with antigen.<sup>27</sup> Indeed, comparison of cytokine profiles at day 6 after the administration of MERA-8.3 with or without adjuvant demonstrated that IL-4 and IFN-γ production by spleen cells were largely unaffected in these mice. In contrast, IL-2

levels were drastically decreased in mice treated with MERA-8.3 alone, suggesting a crucial role of this cytokine in further maturation of T-cell response. Because vaccination with MERA-8.3 followed by immunization of mice with MERA-8.3 plus alum resulted in an increased IFN-γ:IL-4 ratio and decreased IL-2:IL-4 ratio, we infer that lack of IL-2 limited the proliferation and helper activity of Th2-like cells. This notion is consistent with earlier findings of an *in vitro* model that anergy induction prevents clonal expansion of T cells producing both IL-2 and IL-4.<sup>28</sup>

Although the precise molecular mechanism of antigen-mediated immune deviation remains unknown at present, our results clearly establish that immune deviation occurs essentially in two sequential phases. Firstly, treatment with the soluble antigen without adjuvant results in drastic reduction in IL-2 production, which induces T-cell anergy. Secondly, subsequent immunization with the same antigen in adjuvant leads to selective proliferation of Th1-like cells and an increase in IFN-γ:IL-4 ratio. Together, these events result in a shift in the T-cell repertoires at the population level that balances T-cell response in favour of developing a Th1-like response instead of the default Th2-like response to the MERA-8.3, and ultimately leads to a shift from IgE to IgG2a isotype of antibody production. Our demonstration that a major recombinant allergen is capable of mediating immune deviation suggests the potential of major recombinant allergen(s) as preventive and therapeutic modality against allergic diseases.

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