

Suppression of allergen reactive Th2 mediated responses and pulmonary eosinophilia by intranasal administration of an immunodominant peptide is linked to IL-10 production

Gillian Hall^a, Christian G. Houghton^b, Janne Uldal Rahbek^b,
Jonathan R. Lamb^a, Elizabeth R. Jarman^{a,*}

^a Respiratory Medicine Unit, Immunobiology Group, Rayne Laboratory, MRC Centre for Inflammation Research,
University of Edinburgh Medical School, Teviot Place, Edinburgh EH8 9AG, UK

^b ALK-Abello A/S, Hoersholm, Denmark

Received 29 March 2002; accepted 6 August 2002

Abstract

The potential to induce systemic tolerance following exposure of the airway mucosa to soluble antigen, may be applied therapeutically for the treatment of allergic disease. Since the use of allergen can trigger IgE mediated inflammation, we investigated whether mucosal delivery of a peptide, containing an immunodominant epitope of the Der p1 allergen of house dust mite, can lead to CD4⁺ Th2 cell tolerance and thus protect against airway inflammatory responses to inhalant allergen. The administration of microencapsulated peptide to the nasal mucosa of mice, protected against airway inflammation, with significant reductions in eosinophil infiltration into the airways following allergen challenge. Der p1 specific antibody levels in sera were not modulated. Allergen reactive CD4⁺ T cells expressed a tolerized phenotype, with reduction in levels of the cytokines, IL-5, IL-13 and IFN- γ although IL-10 levels were increased. The mucosal administration of a peptide containing an immunodominant region of an allergen can protect against the induction of systemic and local inflammatory responses to allergen challenge.

© 2002 Published by Elsevier Science Ltd.

Keywords: Allergy; In vivo animal models; Microencapsulated antigen; Immunoregulation

1. Introduction

Allergic asthma is a disease characterized by elevated serum IgE levels and the development of chronic bronchial inflammation, airway hyper-responsiveness and mucus hypersecretion. CD4⁺ T cells expressing the Th2 cytokines IL-4, IL-5, IL-9 and IL-13 have been identified in the airways of asthmatics [1]. These cytokines are primarily responsible for the disease pathology, which is caused by

the influx of mast cells and eosinophils into the airways following exposure to environmental allergens [2].

Specific immunotherapy (SIT), which involves the injection of increasing doses of allergen, can lead to a reduction in clinical symptoms and prevent disease progression. However, the inherent risk of inducing potentially lethal anaphylactic reactions has limited the use of SIT in patients with allergic inhalant asthma. Cross-linking of Fc ϵ R on effector cells by IgE-allergen complexes causes the release of preformed mediators responsible for acute immediate inflammatory responses. This has led to an interest in identifying alternative approaches towards immunotherapy. Successful therapy is associated with increasing levels of IgG4 blocking antibodies and reductions in numbers of mast cells and eosinophils at sites of inflammation [3,4]. The underlying mechanism leading to desensitization is less clearly understood. Recent studies demonstrating augmented levels in IL-10 production, suggest a role for immune regulation and tolerance, rather than the induction of Th1 cytokines.

Abbreviations: AHR: airway hyper-responsiveness; BAL (F): bronchoalveolar lavage (fluids); LN: lymph node; Der p1: *Dermatophagoides pteronyssius* spp.; EPO: eosinophil peroxidase; i.n.: intranasal (ly); PAMP: pathogen associated molecular patterns; SIT: specific immunotherapy

* Corresponding author. Present address: Biological Allergy Research, ALK-Abello A/S, Boege Aue 6-8, DK-2970 Horsholm, Denmark. Tel.: +45-4574-7793; fax: +45-4574-8642.

E-mail addresses: eja@dk.alk-abello.com, eja@dk.alk.abello.com (E.R. Jarman).

Peripheral tolerance would account for reductions in antigen specific lympho-proliferative responses and IL-4 production. This may result in a subsequent shift in the cytokine milieu with a relative increase in levels of the Th1 cytokines, leading to further reductions in Th2 mediated responses [5–7].

In an effort to enhance the efficacy of therapy, studies have attempted to address the use of different routes of allergen administration, which influence the immune response generated. Studies on autoimmune disease have demonstrated that the mucosal administration of antigen via either the oral or intranasal route, in the absence of inflammatory signals, can protect against disease induction or exacerbation following parenteral challenge with antigen [8,9]. The mechanisms underlying mucosal tolerance in Th1 disease models differ depending on the route of administration. Whereas, oral delivery leads to the induction of a regulatory population of Th3 cells secreting TGF- γ and IL-4, administration of antigen via the intranasal mucosa is thought to lead to the induction of a regulatory Tr1 population, which produce IL-10. Clinical studies are currently addressing the efficacy of sublingual (oral) immunotherapy in the treatment of allergies [10]. However, little is understood about the effect of localized intranasal administration of allergen on targeting airway inflammatory responses. In most individuals, soluble proteins delivered to the nasal mucosa do not invoke an immune response, but induce a state of tolerance. This phenomena has been demonstrated by earlier studies which demonstrated that exposure of naive mice to aerosolized allergen protects against induction of asthma [11]. Studies using murine models of asthma have shown that the prophylactic intranasal administration of a recombinant allergen of birch pollen or long peptides covering the major allergen of bee venom, prior to parenteral immunization with allergen, suppressed systemic Th2 responses and IgE production [12,13]. These studies did not investigate airway inflammatory responses, which may be exacerbated following cross-linking of effector cell bound IgE by allergens, and release of inflammatory mediators. To reduce the risk of eliciting IgE mediated inflammatory responses, clinical studies have addressed the use of peptides in therapy. While clinical studies have demonstrated success in the use of this approach, the induction of late phase asthmatic responses in a proportion of patients following intradermal injection of peptides is a concern [14–16].

To understand the mechanism underlying mucosal tolerance, we have investigated whether Th2 mediated airway inflammation can be modulated by the intranasal administration of peptides. We demonstrate, using a murine model of pulmonary allergic inflammation, that the mucosal delivery of an immunodominant peptide of Der p1, can protect against the induction of Th2 mediated airway inflammation and eosinophilia, in mice challenged with the whole Der p1 allergen. Furthermore, we show that IL-10 plays a critical role in suppressing Th2 responses, including production of the cytokines IL-4, IL-5 and IL-13, which are associated with the pathology of asthma.

2. Materials and methods

2.1. Animals

C57BL/6 mice, 6–8 weeks of age, were used. Animals were purchased from Harlan, UK. Animal care and experimental procedures were conducted in accordance with the animal ethics regulations of the Home Office, UK.

2.2. Antigens

The house dust mite allergen Der p1 was affinity purified from spent mite medium using the monoclonal antibody 4C1 (INDOOR biotechnologies Ltd., UK) [17]. The synthetic peptide Der p1; p111–139 (FGISNYCQIYPPNANKIRE-LAQPQRYCR) were synthesized with a free carboxyl terminus and HPLC purified (Advanced Biotechnology Centre, Imperial College School of Medicine at Charing Cross Hospital, London). MEA-p111–139 particles were kindly provided by ALK-Abello (Horsholm, Denmark).

2.3. Immunization and tolerance induction

The following treatment regimes were used. Mice were lightly anaesthetized under ether and treated by the intranasal (i.n.) administration, on five consecutive days, of a 20 μ l volume of endotoxin free saline containing 100 μ g of either peptide Der p1; p111–139, or MEA-Der p1; p111–139 particles (Fig. 1). The control groups received either saline alone or empty MEA particles. In the prophylactic regime, mice were immunized 14 and 16 days after the last i.n. treatment by the intraperitoneal injection of Der p1 (10 μ g) in saline absorbed to an equal volume of aluminum hydroxide adjuvant (Imject Alum; Pierce, Rockford, IL). On days 41 and 43, mice were challenged by intratracheal instillation of Der p1 (20 μ g) into the airways in 50 μ l saline. For the therapeutic regimen, mice were primed by a subcutaneous injection, followed by seven intraperitoneal injections, at 2 day intervals of Der p1 (10 μ g in saline). Eight days after the last injection, groups of mice were treated by the intranasal administration of a 20 μ l volume of endotoxin free saline containing 100 μ g of either peptide Der p1; p111–139, MEA-Der p1; p111–139 particles, Der p1 allergen, or MEA-Der p1 (Fig. 1). The control groups received either saline alone or empty MEA particles. After 2 weeks, the airways were challenged three times, by instillation of Der p1 (20 μ g in 50 μ l saline) at 2 day intervals. Mice were sacrificed after 24 h, using a lethal dose of avertin. To determine antibody levels in sera, mice were bled from the tail vein prior to sacrifice. Blood samples were centrifuged at 2000 $\times$ g, sera collected and stored at -70°C .

2.4. Cytokine production

Single-cell suspensions were prepared from spleen or lymph nodes by teasing the tissue apart and passing it

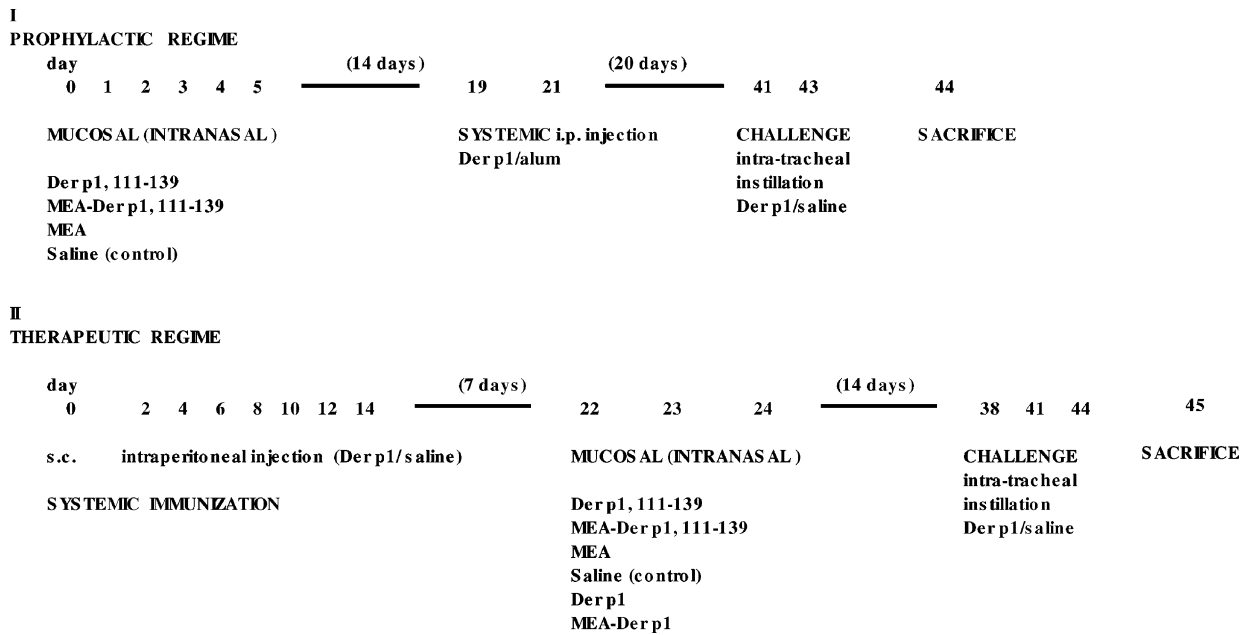


Fig. 1. (I) Prophylactic immunization regimen; groups of naive mice were treated intranasally on five consecutive days with 20 μ l saline containing 100 μ g Der p1; p111–139 peptide either alone, or encapsulated in MEA particles. Control mice received saline or MEA particles alone. On days 19 and 21, mice were immunized with Der p1/alum and after 20 days, challenged twice by instillation of 20 μ g Der p1 into the airways. After 24 h mice were sacrificed. (II) Therapeutic regimen; mice were sensitised as shown by multiple intraperitoneal injections with Der p1 in saline. After 7 days, mice were treated intranasally with either Der p1 or the Der p1; p111–139 peptide either alone or encapsulated in MEA particles. Controls received MEA particles or saline. Mice were then challenged by instillation of Der p1 directly into the airways and sacrificed 24 h later.

through a fine wire mesh to remove cell debris. Cells were cultured in RPMI 1640 medium supplemented with 5% foetal calf serum (FCS; GIBCO), 50 μ M 2-mercaptoethanol, 2 mM L-glutamine and 10 U penicillin/streptomycin (GIBCO, Paisley, UK). Cells (5×10^5 per well) were cultured in 96-well flat-bottomed plates (Costar) with either Der p1 or peptide Der p1; p111–139 (10 μ g/ml). Control wells contained medium alone, OVA (10 μ g/ml) or were coated with anti-CD3 and anti-CD28 (5 μ g/ml). After 72 h supernatants were collected, stored at -70°C and assayed in duplicate for the presence of the cytokines; IL-4, IL-5, IL-10, IL-13 and IFN- γ using ELISA. Antibodies were purchased and used according to the instructions of the manufacturer (IL-4, IL-10, IL-13; R& D Systems, UK, or IL-5, IFN- γ Pharmingen, San Diego, CA).

2.5. Measurement of eosinophil levels in bronchoalveolar lavage

Immediately after sacrifice, the trachea were exposed and cannulated with 27-gauge silicon tubing attached to a needle and syringe. The lungs were instilled through the trachea with 500 μ l of cold PBS and the BAL fluid withdrawn. The volume of BAL fluid collected was measured for each sample. To determine BALF cell numbers and composition, samples were centrifuged ($700 \times g$, 5 min, 4°C), cells were washed (PBS/10% FCS), counted and re-suspended at $1 \times 10^6 \text{ ml}^{-1}$. BALF (1×10^5) cells in 100 μ l were cytospun

onto poly(L-lysine)-coated slides (3 min at 300 rpm). The slides were air-dried, fixed in methanol and stained using Diff/Quick. To determine cytokine levels in BALF, samples were stored at -70°C . All cytokines were analyzed by ELISA.

2.6. Extraction and measurement of eosinophils numbers in lung tissue

Lungs were weighted and homogenized in Hanks balanced salt solution (HBSS; Gibco) containing 10 mM HEPES (pH 7.4). Following red blood cell lysis, cells were re-homogenized in HBSS containing 0.5% hexadecyltrimethylammonium bromide (HTAB; pH 7.4) and freeze thawed in liquid nitrogen. Levels of eosinophil peroxidase in lung homogenates were determined using the O-phenylenediamine substrate (Sigma; OPD 1.5 mM, H_2O_2 6.6 mM; pH 8). The reaction was stopped using H_2SO_4 (4 M) and plates read in a MRX microplate reader at 490. Data was analyzed using Revelation software.

2.7. Determination of Der p1 specific antibody levels in sera

Antibody levels in sera, were determined by ELISA. Unless otherwise indicated serum samples and antibodies were diluted in PBS/1% BSA and wells washed between each incubation step with PBS/0.05% Tween-20. Round

bottomed 96-well microtitre plates (Costar) were coated overnight at 4 °C with either rat anti-mouse IgE (R35-72; Pharmingen), or with Der p1 (5 µg/ml), diluted in carbonate buffer (0.1 mM NaHCO₃, pH 8.2). After washing, non-specific binding was blocked by incubating wells PBS/3% BSA for 2 h at 37 °C. Plates were washed and serial dilutions of serum samples added in duplicates (IgE 1:5, 1:10, 1:20; IgG1 1:50, 1:100, 1:500; IgG2a 1:10, 1:20, 1:50). The plates were incubated overnight at 4 °C. After washing, biotinylated rat anti-mouse IgG1 (LO-MG1-2; Serotec) or rat anti-mouse IgG2a (Lo-MG2a-7; Serotec) was added at 1000 ng/ml and plates incubated for 2 h at room temperature. To determine Der p1 specific IgE levels in sera, Der p1 (10 µg/ml) was added and plates incubated at 37 °C for 60 min. After washing, plates were incubated for a further 45 min with biotinylated 4C1 (1:1000 dilution). Bound biotinylated antibodies were detected by incubating with streptavidin-peroxidase for 45 min at room temperature and visualized using the TMB substrate. Plates were read in a microtitre reader at 450 nm using the MRX microplate reader and Revelation software.

2.8. Statistics

Data represents mean values ± S.E.M., with five mice per group. Differences between mean values were analyzed by one way analysis of variance (ANOVA) using the Dunnett Multiple comparison parametric test and Instat software. $P < 0.05$ were considered statistically significant.

3. Results

3.1. Prophylactic effects of intranasal administration of an immunodominant peptide on Der p1 allergen induced airway eosinophilia

Eosinophil infiltration of the airways is a predominant feature of allergic asthma. To investigate whether the intranasal administration of a peptide covering an immunodominant T cell epitope of Der p1; p111–139, could protect against the induction of Th2 mediated airway inflammatory responses to Der p1, eosinophil numbers in the BAL and lung tissue were determined. Groups of mice were administered intranasally either Der p1; p111–139 peptide (5 × 100 µg) alone or encapsulated in MEA particles on five consecutive days. Mice were then immunized systemically and challenged by instillation of Der p1 into the airways (Fig. 1). Eosinophil numbers in the lung tissue and BAL were determined 24 h after allergen challenge. A significant reduction ($P < 0.05$) of 66.3% in eosinophil numbers in the BALF was observed in mice treated intranasally with MEA encapsulated Der p1; p111–139 peptide, as compared with the control group receiving PBS (Fig. 2A). This correlated with a significant ($P < 0.01$) reduction of 54.4% in eosinophil numbers in the lung tissue (Fig. 2B). No

reduction in eosinophil levels in the BAL or lung tissue were observed in mice administered Der p1; p111–139 peptide alone (Fig. 2). The suppression of eosinophilia was antigen dependent since empty MEA particles had no effect. Preferential uptake of MEA-peptide by immunosuppressive APC such as pulmonary dendritic cells (DC) or alveolar macrophages may account for reductions in Th2 mediated inflammation. Alternatively, the kinetics of peptide released systemically from MEA particles may, by altering the frequency and duration of TCR ligation, reduce the activation threshold required for T cell tolerance induction.

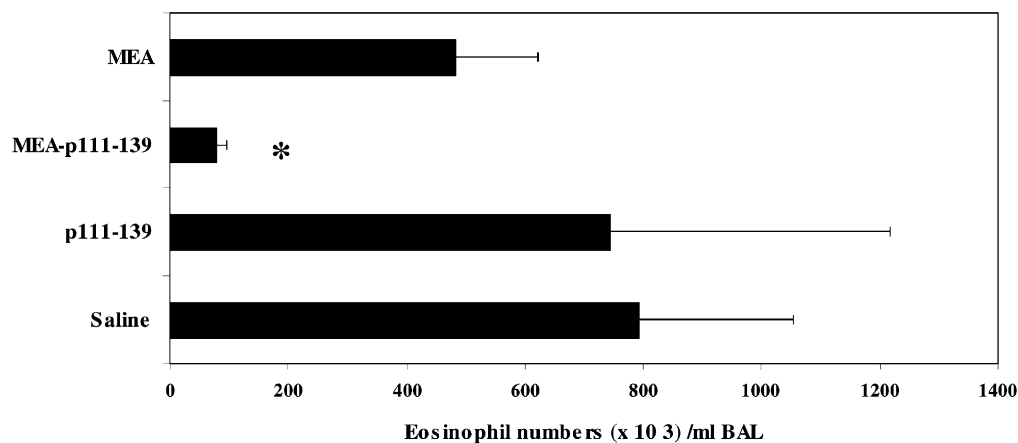
3.2. Cytokine levels in the BALF

Allergic asthmatics have elevated levels of the cytokines IL-4 and IL-5 present in the BALF [1]. These cytokines play a critical role in initiating and sustaining airway inflammation and eosinophilia. To determine whether reductions in eosinophil numbers in the lung and BALF of MEA-peptide intranasally treated mice, were due to a loss of allergen reactive CD4⁺ T helper activity, levels of these cytokines were measured in BALF of mice 24 h after challenge with Der p1. Marked reductions in levels of IL-4 (35%) and IL-5 (62%) were observed in the BALF of mice treated i.n. with MEA encapsulated Der p1; p111–139, as compared with control PBS treated mice. Reductions in IL-5 levels (48%), were also observed in mice treated intranasally with Der p1; p111–139 peptide alone. No parallel reduction was observed in IL-4 levels, suggesting that other factors in addition to IL-5 influenced eosinophil levels (Fig. 3).

3.3. Mucosal tolerance leads to reductions in antigen specific production of both Th1 and Th2 cytokines, produced systemically

To investigate whether reductions in eosinophilia was due to a Th2 to Th1 cytokine shift, splenocytes obtained from individual mice were cultured in the presence of Der p1 or the immunodominant peptide Der p1; p111–139. Cytokine levels in the supernatants were assayed after 72 h (Fig. 4). In comparison with the control saline treated group, Der p1 reactive T cells obtained from mice administered MEA-Der p1; p111–139 had reduced levels of both the Th2 (IL-5 (69%), IL-13 (67%)) and Th1 (IFN-γ (82%)) cytokines. Reductions in airway eosinophilia were, therefore, due to a loss of antigen specific T cell responsiveness and eosinophil recruitment, rather than a Th1 shift in cytokine production. The loss of T cell responsiveness to intact Der p1, following mucosal tolerance induction with an immunodominant T cell epitope (Der p1; p111–139), may be due to linked suppression resulting in a loss of responsiveness to minor T cell epitopes within Der p1. Peptide alone, failed to tolerize Der p1 reactive T cells, suggesting that MEA particles enhanced the tolerogenicity of the peptide. Whereas, the inhibitory effect of MEA-peptide on Der p1 specific responses is antigen dependent, the significant reduction in IFN-γ levels

(A) Eosinophils in BAL



(B) Lung tissue

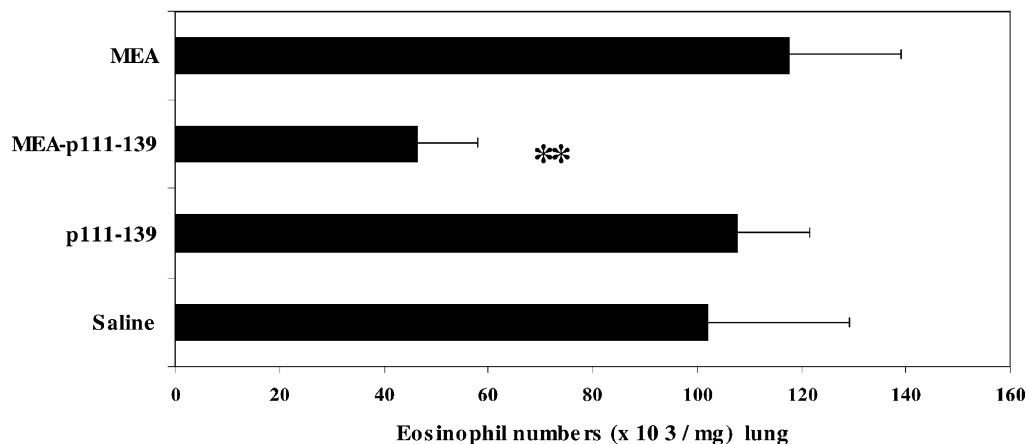


Fig. 2. The effect of i.n. treatment with Der p1; p111–139 on levels of eosinophils in BALF and lung tissue of mice sensitised and challenged with Der p1. (A) Eosinophil numbers in BALF were determined by Diff/Quick staining of cytopins. For each treatment group, cell number were determined as a percentage of total cell numbers per ml BALF and expressed as mean $\pm$ S.E.M. Differences in means were significant (* $P < 0.05$). (B) Eosinophil numbers in lung tissue were determined by measuring eosinophil peroxidase levels in lung homogenates. The data is presented as mean eosinophil numbers $\pm$ S.E.M. for each group of mice. Differences in means were significant (** $P < 0.01$).

($P < 0.001$) may be largely due to a non-specific effect of MEA particles. Background cytokine levels were negligible (Fig. 4).

3.4. Antibody levels and isotype are not significantly suppressed by intranasal peptide treatment

To determine whether the i.n. administration of a single T cell epitope peptide could influence B cell responses, Der p1 specific antibody levels in sera were measured by ELISA. Der p1 specific IgE, IgG1 and IgG2a levels were not reduced in mice receiving MEA-peptide as compared with control mice, although there was a 42% reduction in Der p1 specific IgG1 levels, in sera obtained from Der p1; p111–139 treated mice. Mice treated with MEA particles alone, had increased levels of specific IgE in sera, which may be linked to the non-specific reduction in IFN- γ production (Fig. 5).

3.5. Pulmonary eosinophilia is reduced following local allergen challenge and correlates with an increase in the ratio of antigen induced IL-10:IL-5

We investigated whether or not IL-10 plays a role in the intranasal peptide induced Th2 tolerance to Der p1 allergen. Supernatants obtained from spleen or pooled bronchial lymph node cells, were cultured with Der p1 or Der p1; p111–139 and assayed for IL-10 production by ELISA. There was an increase in levels of IL-10 production by antigen specific T cells obtained from the draining lymph nodes of mice treated i.n. with MEA-Der p1; p111–139 or peptide Der p1; p111–139 alone, as compared with cells obtained from control mice. IL-10 levels produced by splenocytes obtained from intranasally tolerized mice were not significantly greater than in the control group. Comparisons of the ratio of IL-10 to IL-5, revealed a significant increase in the

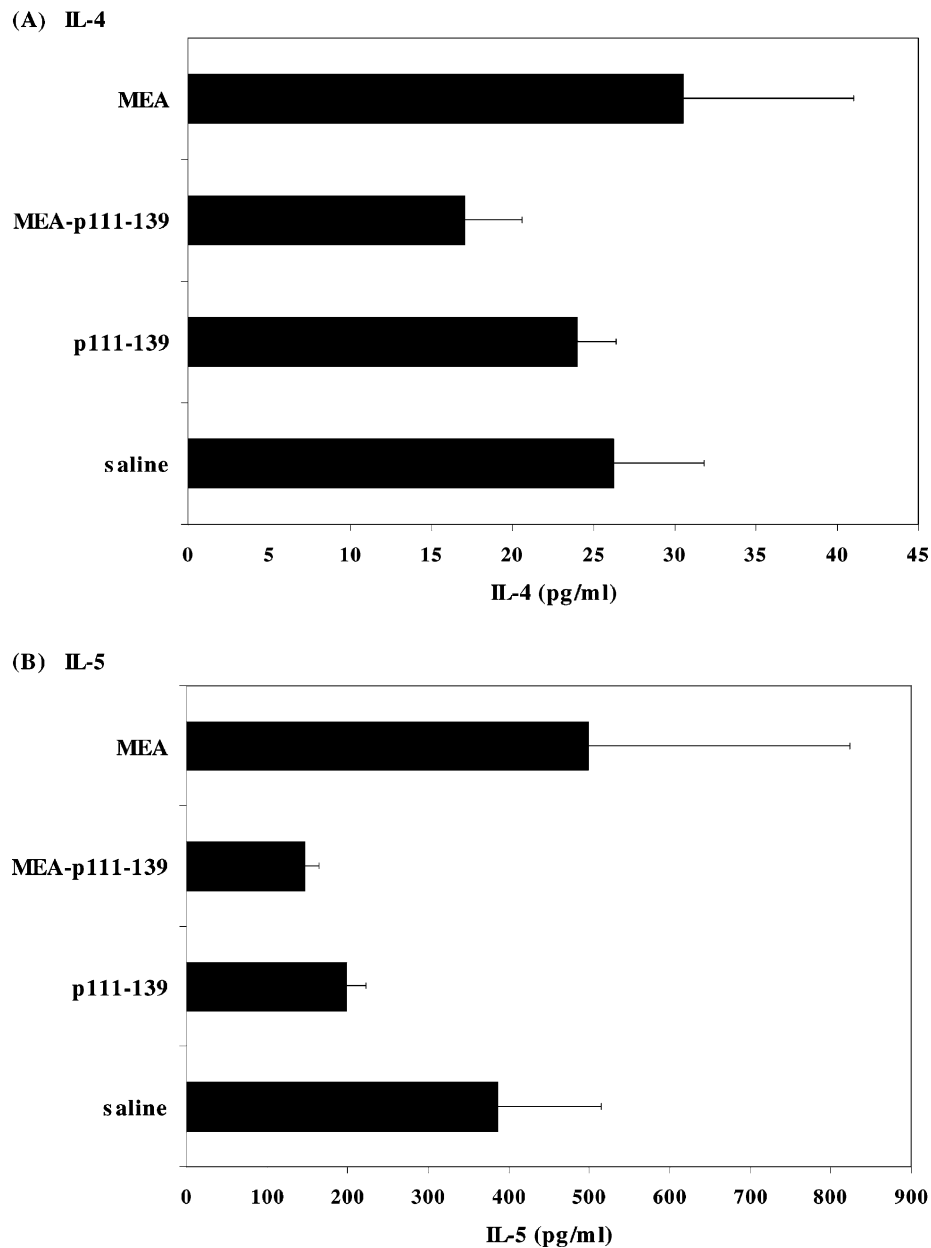


Fig. 3. Reduction in levels of the Th2 cytokines IL-4 and IL-5 in BALF of mice treated with MEA-peptide (Der p1; p111–139), correlated with reduced numbers of eosinophils in BALF and lung tissue. IL-4 (A) and IL-5 (B) in BALF obtained from mice 24h after challenge of the airways with Der p1 were determined by ELISA. The data is presented as mean $\pm$ S.E.M. for each treatment group.

ratio of IL-10, which correlated with a reductions in lung eosinophilia. Increases were observed in cultures of spleen and draining lymph node cells obtained from MEA-peptide treated mice (Fig. 6).

3.6. Effect of the therapeutic intranasal administration of Der p1; p111–139 peptide to Der p1 sensitized mice

Studies have demonstrated that the intranasal administration of allergen to mice sensitized systemically, can lead to airway inflammation. We investigated whether the intranasal administration of a Der 1 peptide, using the therapeutic regimen (Fig. 1), leads to reductions or exacerbations

in established Th2 mediated response to Der p1. For this purpose, groups of mice primed by systemic immunization with Der p1, were treated intranasally with peptide (Der p1; p110–131) alone or encapsulated into MEA particles. Therapeutic administration of MEA-peptide or peptide alone did not exacerbate airway eosinophilia in sensitised mice. In contrast, the intranasal administration of Der p1 allergen to sensitised mice, lead to increases in airway eosinophilia of 70% and an increase of 55% in Der p1 specific IgG1 levels in sera ($P < 0.01$). Increases in eosinophilia and specific IgG1 in pre-sensitised mice were not elevated on intranasal administration of Der p1 adsorbed to MEA. Fig. 7A). In contrast, administration of Der p1; p110–131 peptide or MEA-peptide

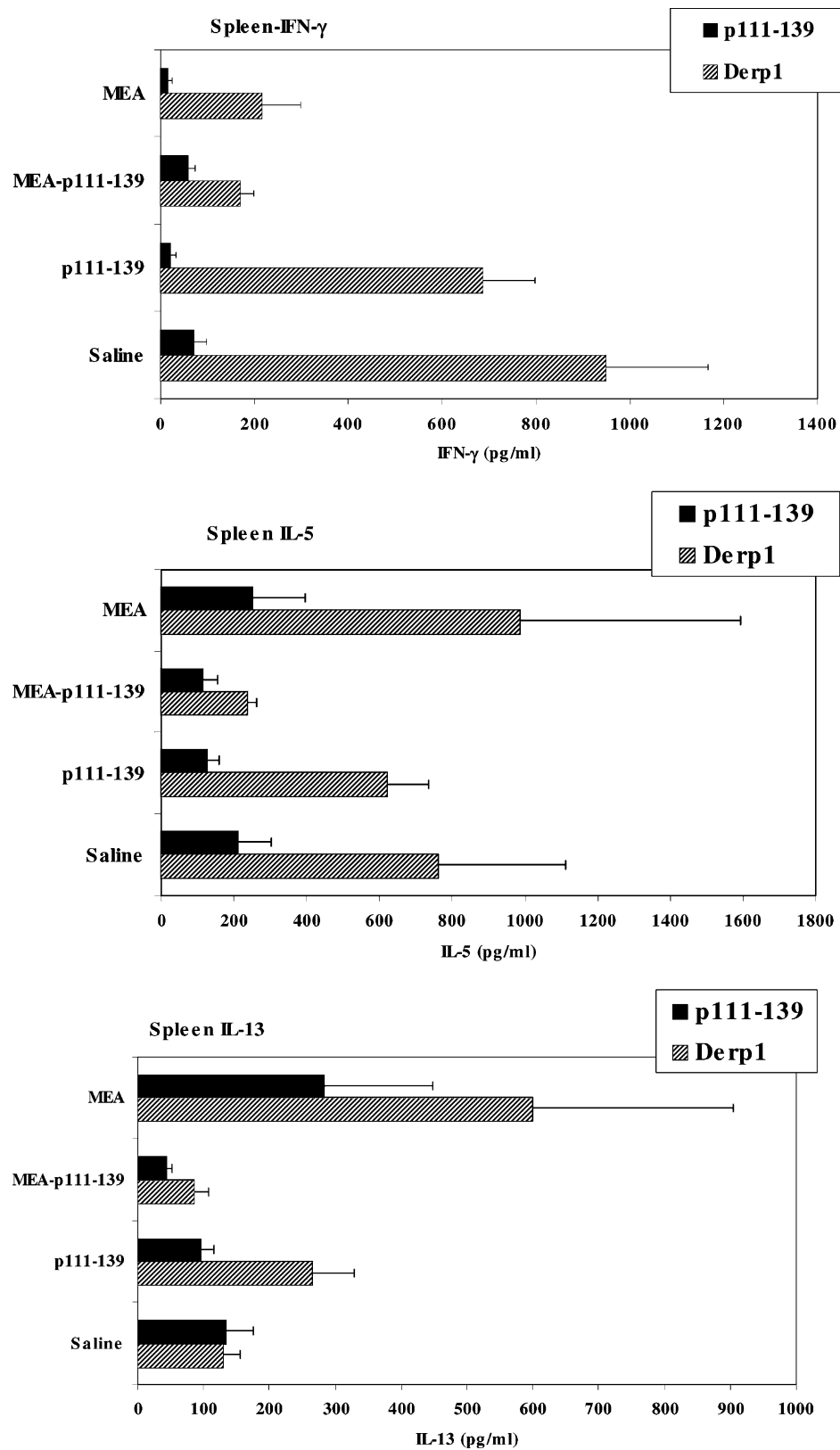


Fig. 4. Reduction in both Th2 (IL-5) and Th1 (IFN- γ) cytokine levels. Splenocytes obtained from individual mice were cultured (5×10^6 per ml) in the presence of either Der p1 or the peptide Der p1; p111–139 ($10 \mu\text{g/ml}$). Supernatants were collected after 72 h and cytokine levels assayed by ELISA. Cytokines in the supernatants of splenocytes cultured in media alone or an irrelevant antigen were at baseline levels. The data is presented as mean $\pm$ S.E.M. for each group of mice. Differences in means were significant (* $P < 0.001$).

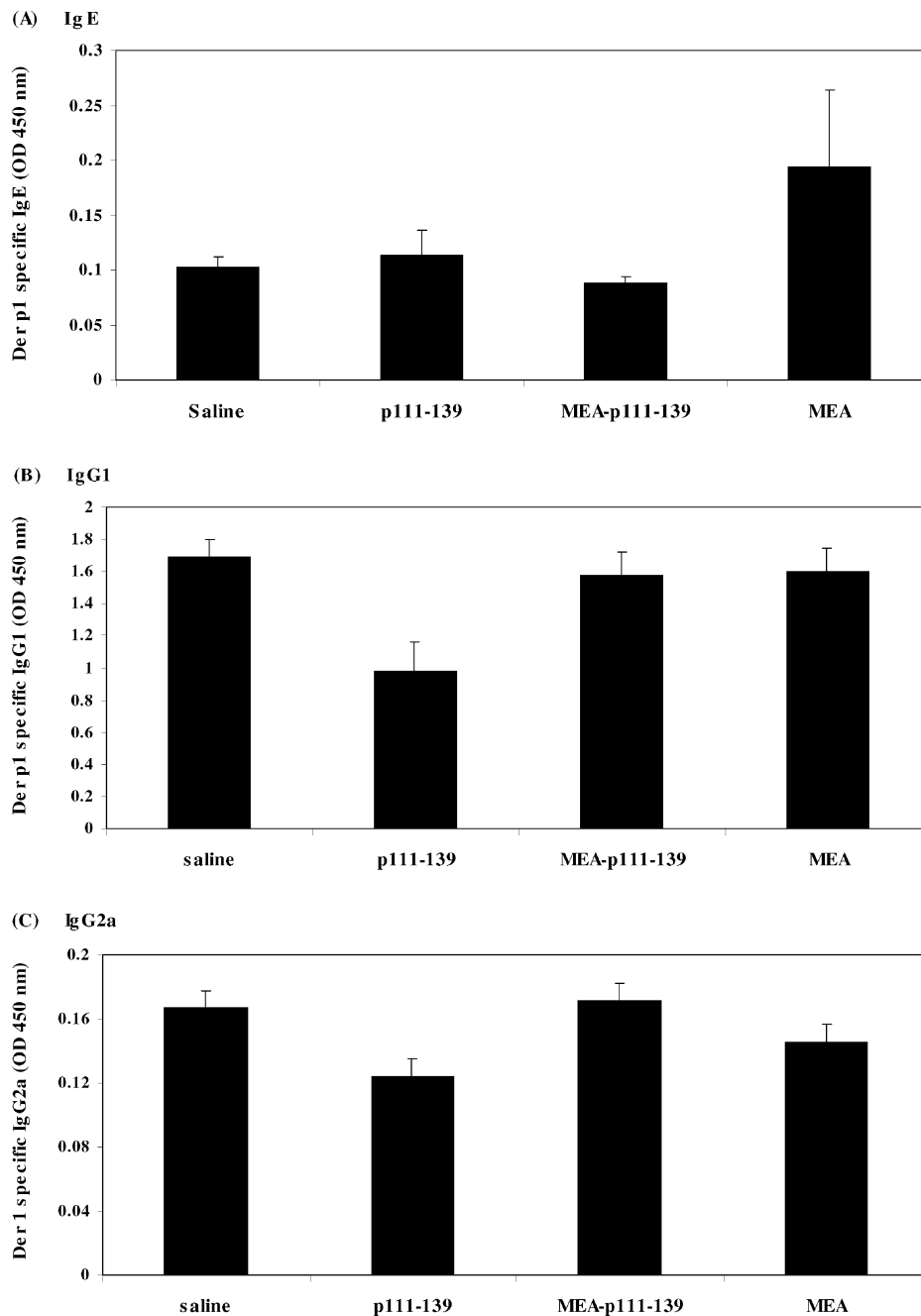


Fig. 5. Der p1 specific IgE and IgG levels in sera. Der p1 specific antibodies in sera were determined on day 45. Antibody levels, measured by ELISA are presented for the following dilutions; specific IgE (1/20 dilution), IgG1 (1/100 dilution) and IgG2a (1/10 dilution). The data is presented as OD 405 nm (mean $\pm$ S.E.M.) for each group.

to systemically sensitised mice, lead to non-specific reductions in Der p1 specific IgG1 (60%, 52%) and IgG2 (47%, 59%) levels in sera, as compared to control mice (Fig. 7).

4. Discussion

Allergic asthma is thought to arise following a loss of tolerance to inhaled allergen. The exposure of naive mice to aerosolized allergen has been shown to protect against the

induction of allergic responses [11]. A number of groups have demonstrated that exposure of the airway mucosa to soluble allergen by intranasal administration can lead to the induction of tolerance. However, concerns about the potential for triggering IgE mediated inflammatory responses, have prompted investigations on the use of peptides, which contain T cell epitopes in therapy.

Previous studies demonstrated that residues p111–139 of Der p1 contained an immunodominant T cell epitope. Stimulation with a peptide covering this region induced

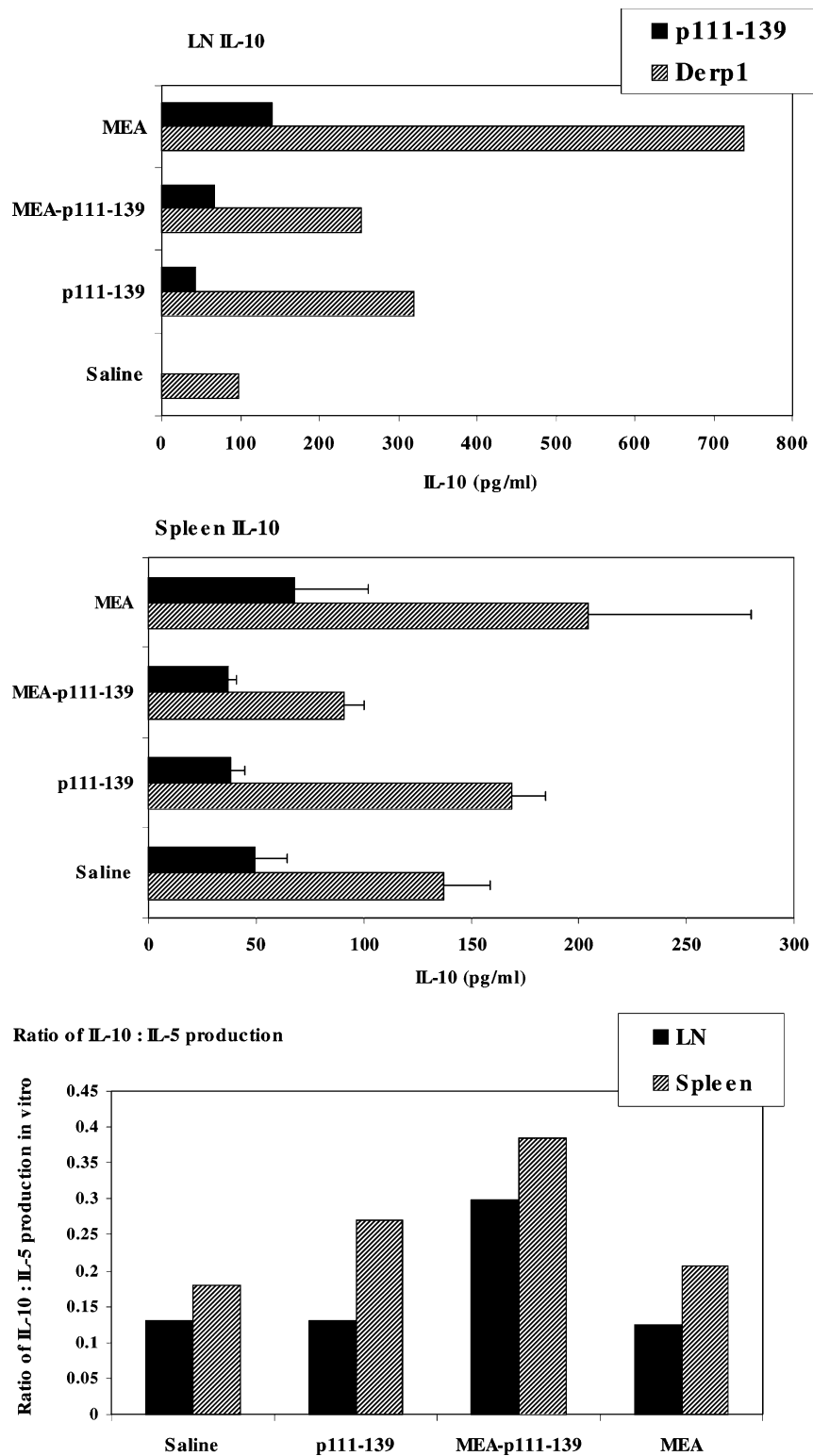


Fig. 6. Intranasal MEA-peptide treatment results in an increase in the ratio of IL-10 to IL-5 produced by Der p1 reactive T cells in vitro. IL-10 and IL-5 levels in supernatants obtained from Der p1 (10 μ g/ml) stimulated cells obtained from the spleen or bronchial lymph nodes, were assayed by ELISA. Mean values from individual spleen and pooled lymph node cells were used for each group. The data is presented as the ratio of IL-10 to IL-5 produced by each treatment group.

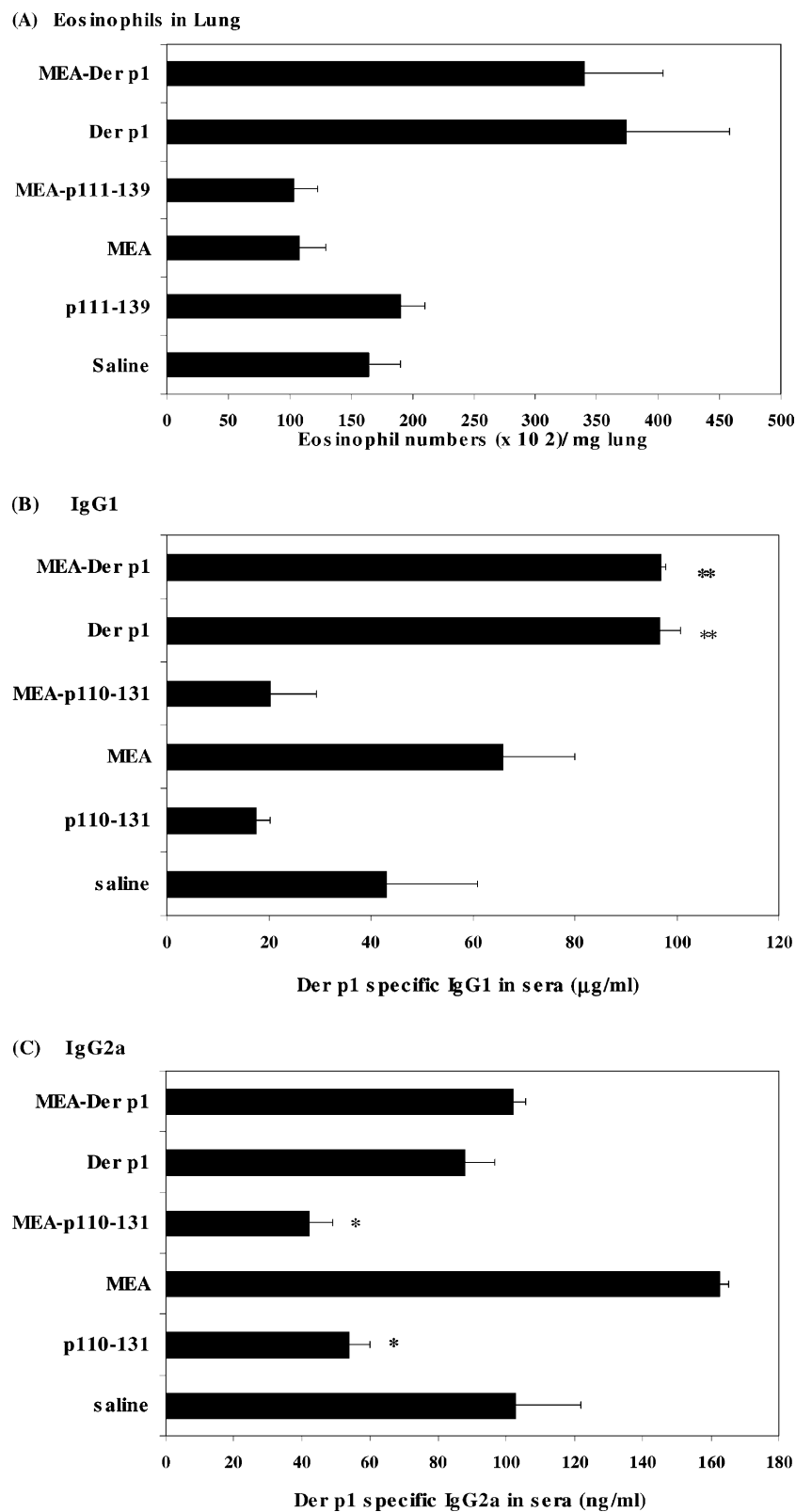


Fig. 7. Therapeutic intranasal administration of Der p1 peptide to sensitised mice causes in reduction in systemic humoral responses but not in airway eosinophilia, following challenge with Der p1. Groups of mice sensitised systemically to Der p1, received three consecutive intranasal administrations of either peptide or Der p1, alone or encapsulated in MEA particles. Control mice received PBS or MEA alone. Eosinophil numbers in the lung were determined using the quantitative eosinophil peroxidase assay (A), Der p1 specific IgG1 (B) and IgG2a (C) levels in sera were determined by ELISA. The data is presented here as mean $\pm$ S.E.M. for each treatment group. Differences in mean between the treatment and control saline group are significant (* $P < 0.05$, ** $P < 0.01$) using Dunnett multiple comparison test.

lympho-proliferative responses in human T cell clones, and murine T cell lines obtained from Der p1 immunized mice expressing the H-2^b haplotype [17,18]. The intranasal administration of peptide Der p1; p111–139 to naïve mice protected against the induction of Th1 mediated DTH responses, following immunization with Der p1 in CFA [19]. In this study, we demonstrate, using a murine model of pulmonary inflammation that the intranasal delivery of an immunodominant T cell epitope peptide can also protect against the induction of Th2 mediated eosinophilia, which is a principal feature of allergic asthma. Mice administered MEA encapsulated peptide intranasally had significantly reduced numbers of eosinophils in the BAL and lung tissues after inhalation of the Der p1 allergen. Mucosal tolerance is linked to the degree to which the CD4⁺ T cell population is initially activated, since the intranasal administration of peptide alone, failed to protect against the induction of eosinophilia. Antigen concentration and TCR affinity are critical in determining the strength of initial activation signals that precede T cell tolerance [20,21]. Our data suggest that by using MEA encapsulated peptide, we were able to increase the concentration of peptide released systemically thus strengthening the initial TCR mediated activation signal.

Eosinophil infiltration of the airways is a characteristic feature of allergic asthma. Eosinophils contribute towards airway inflammation not only by the release of inflammatory mediators, but also as antigen presenting cells, capable of amplifying localized Th2 mediated responses [22]. The recruitment and differentiation of eosinophil progenitor cells in the bone marrow as well as the infiltration and survival of eosinophils at sites of inflammation are all regulated by IL-5. Signaling by IL-4 or IL-13 through the IL-4R α chain is also critical for development of an allergic asthma phenotype [23]. Reductions in levels of the cytokines IL-4 and IL-5 in the BAL fluid of mice challenged with Der p1, may account for reduced eosinophil numbers in the airways of mice administered MEA-peptide intranasally. However, a decrease in levels of IL-5 in the BALF did not result in a reduction in airway eosinophilia in peptide treated mice, suggesting that other factors contributed to the reduction in eosinophil numbers. A separate study, investigating systemic responses generated by the intranasal administration of overlapping peptides from PLA2, demonstrated a shift in cytokine profile, with an increase in the ratio of IFN- γ to IL-4 [13]. Here we fail to demonstrate a Th2 to Th1 shift in cytokine profile by antigen specific cells following successful intranasal therapy. Comparison of levels of cytokines produced by spleen and draining lymph node (data not shown) cells obtained from control or MEA-peptide treated mice, revealed a reduction in levels of the Th2 (IL-5, IL-13) as well as the Th1 (IFN- γ) cytokines. Reductions in allergen specific production of both Th1 and Th2 cytokines were also observed in a separate study using a recombinant allergen of Bet v1 [12]. The general view that Th1 cytokines, by suppressing Th2 responses may protect against

progression of allergic disease, is not supported by data, which indicates that elevated IFN- γ levels can exacerbate airway inflammatory responses to allergen [24,25].

Studies involving the intranasal administration of whole allergen, demonstrated that the induction of peripheral T cell tolerance was accompanied by modified B cell responses, with reductions in specific IgE and changes in the ratio of IgG1 to IgG2a levels in sera [12,13]. Data obtained from this study, demonstrate that the prophylactic use of a short linear T cell epitope peptide, while suppressing local Th2 dependent inflammatory responses in the airways, did not greatly influence B cell dependent responses such as antibody levels in sera or isotype. In contrast, the intranasal administration of a T cell epitope peptide p110–131, to mice systemically sensitised with Der p1, lead to a reduction in B cell dependent antibody responses, but failed to suppress airway eosinophilia. There is considerable data from animal models of asthma, which demonstrate that many of the processes of asthma, including eosinophil infiltration of the bronchial mucosa and bronchial hyper-responsiveness are elicited independently of the presence of B cells and IgE, but are dependent on the presence of T cells [26,27]. The induction of tolerance in the allergen specific T cell population by mucosal delivery of peptide containing a T cell epitope is sufficient to cause a reduction in eosinophil levels in the airways, independently of any effect on B cell responses. Failure to significantly downregulate allergen induced eosinophilia following mucosal delivery of peptide to sensitised mice, suggests that it may be more difficult to suppress established Th2 mediated responses, by tolerance induction. This view is supported by a recent publication demonstrating that ongoing Th2 responses in the lungs of mice prevented induction of tolerance to aerosolized OVA [28].

We have attempted to address the mechanism underlying the induction of Th2 tolerance. Linked suppression is thought to account for the loss of responsiveness to all epitopes within a protein, following the induction of T cell tolerance using a single immunodominant epitope. This form of suppression may be mediated via an antigen non-specific cognate interaction with APC [29], possibly involving signaling through Notch ligands and their receptors [30] and/or through the production of immunosuppressive cytokines such as IL-10 and TGF- β . There has been considerable evidence demonstrating that TGF- β plays a role in the loss of responsiveness arising due to the induction of mucosal tolerance [12,31]. Animal studies have indicated that the immunoregulatory cytokine IL-10 is a critical factor in the induction of mucosal tolerance to a Th1 response to autoantigen [32]. Clinical studies have demonstrated an increase in IL-10 production by allergen reactive CD4⁺ T cells in the periphery following SIT [33]. However, until now, a role for IL-10 in the induction of tolerance to allergen and consequently a reduction in Th2 mediated airway inflammation has not been demonstrated using *in vivo* models. In this study, we have shown that after prophylactic ther-

apy with peptide alone or encapsulated in MEA particles, there is an increase in levels of IL-10 production following antigen stimulation of cells obtained from lymph nodes draining the airways. Reductions in eosinophil numbers correlated with a parallel reduction in IL-5 production. IL-5 is a critical differentiation and survival factor for eosinophils as well as a major chemoattractant. It is therefore, likely that IL-10 induced tolerance in the allergen reactive CD4⁺ Th2 population, possibly during antigen presentation in the draining lymph nodes, would result in a loss of localized IL-5 production and consequently in eosinophil levels in the airways. Recent evidence suggests that the production of IL-10 by pulmonary DC may be critical for the induction of mucosal tolerance [34]. IL-10 is a critical growth factor for a population of antigen specific CD4⁺ CD45RB low CD25⁺ T regulatory type 1 (Tr1) cells, which are thought to be central to the induction and maintenance of peripheral tolerance [35,36]. The transfer of Tr1 T cell clones, but not Th1 or Th2 clones, at the time of OVA immunization inhibited antigen specific serum IgE responses [37]. Although originally described as a murine Th2 factor, it is now recognized that IL-10 inhibits Th2 mediated responses, including airway eosinophilia [38]. The ability of IL-10 to suppress DC maturation may be central to the induction of T cell tolerance and immunoregulation by Tr1 cells [39,40].

Acknowledgements

This work was supported by ALK-Abello A/S, the European Commission, The British Lung Foundation, Wellcome Trust and Medical Research Council.

References

- [1] Robinson DS, Hamid Q, Ying S, et al. Predominant TH2 like bronchoalveolar T-lymphocyte population in atopic asthma. *New Engl J Med* 1992;326:298.
- [2] Hamid QA, Minshall EM. Molecular pathology of allergic disease. Part I: Lower airway disease. *J Allergy Clin Immunol* 2000;105:20–36.
- [3] van Neerven RJJ, Wikbor T, Lund G, et al. Blocking antibodies induced by specific allergy vaccination prevent the activation of CD4⁺ T cells by inhibiting serum-IgE-facilitated allergen presentation. *J Immunol* 1999;163:2944–52.
- [4] Furin MJ, Norman PS, Creticos PS, et al. Immunotherapy decreases antigen-induced eosinophil migration into the nasal cavity. *J Allergy Clin Immunol* 1999;88:27–32.
- [5] Varney VA, Hamid QA, Gaga M, et al. Influence of grass pollen immunotherapy on cellular infiltration and cytokine mRNA expression during allergen-induced late-phase cutaneous response. *J Clin Invest* 1993;92:644–51.
- [6] Secrist H, Chelen CJ, Wen Y, Marshall JD, Umetsu DT. Allergen immunotherapy decreases interleukin 4 production in CD4 T cells from allergic individuals. *J Exp Med* 1993;178:2123–30.
- [7] Akdis CA, Akdis M, Blesken T, et al. Epitope-specific T cell tolerance to phospholipase A2 in bee venom immunotherapy and recovery by IL-2 and IL-15 in vitro. *J Clin Invest* 1996;98:1676–83.
- [8] Weiner HL. Oral tolerance for the treatment of autoimmune diseases. *Annu Rev Med* 1997;48:341–51.
- [9] Metzler B, Wraith DC. Mucosal tolerance in a murine model of experimental autoimmune encephalomyelitis. *Annu New York Acad Sci* 1996;778:228–42.
- [10] Frew AJ, Smith HE. Sublingual immunotherapy. *J Allergy Clin Immunol* 2001;107:441–4.
- [11] McMenamin C, Holt PG. The natural immune response to inhaled soluble protein antigens involves major histocompatibility complex (MHC) class I-restricted CD8⁺ T cell-mediated but MHC class II-restricted CD4⁺ T cell-dependent immune deviation resulting in selective immunosuppression of immunoglobulin E production. *J Exp Med* 1993;178:889–99.
- [12] Wiedermann U, Jahn-Schmid B, Bohle B, et al. Suppression of antigen specific T and B cell responses by intranasal or oral administration of recombinant Bet v 1, the major birch pollen allergen, in a murine model of type I allergy. *J Allergy Clin Immunol* 1999;103:102–10.
- [13] Astori M, von Garnier C, Kettner A, Dufour N, Corradin G, Spertini F. Inducing tolerance by intranasal administration of long peptides in naive and primed CBA/J mice. *J Immunol* 2000;165:3497–505.
- [14] Muller U, Akdis CA, Fricker M, et al. Successful immunotherapy with T-cell epitope peptides of bee venom phospholipase A2 induces specific T-cell anergy in patients allergic to bee venom. 1998;101:747–54.
- [15] Pene J, Desroches A, Paradis L, et al. Immunotherapy with Fel d 1 peptides decreases IL-4 release by peripheral blood T cells of patients allergic to cats. *J Allergy Clin Immunol* 1998;102:571–8.
- [16] Haselden BM, Kay AB, Larche M. Immunoglobulin E-independent major histocompatibility complex-restricted T cell peptide epitope-induced late asthmatic reactions. *J Exp Med* 1999;189:1885–94.
- [17] Hoyne GF, Callow MG, Kuo M-C, Thomas WR. Inhibition of T cell responses by feeding peptides containing major and cryptic epitopes. Studies with the Der p 1 allergen. *Immunology* 1994;83:190–5.
- [18] Higgins JA, Thorpe CJ, Hayball JD, O'Hehir RE, Lamb JR. Overlapping T-cell epitopes in the group I allergen of *Dermatophagoides* species restricted by HLA-DP and HLA-DR class II molecules. *J Allergy Clin Immunol* 1994;93:891–9.
- [19] Hoyne GF, O'Hehir RE, Wraith DC, Thomas WR, Lamb JR. Inhibition of T cell and antibody responses to house dust mite allergen by inhalation of the dominant T cell epitope in naive and sensitised mice. *J Exp Med* 1993;178:1783–8.
- [20] Janssen EM, van Oosterhout AJ, Nijkamp FP, van Eden W, Wauben MHM. The efficacy of immunotherapy in an experimental murine model of allergic asthma is related to the strength and site of T cell activation during therapy. *J Immunol* 2000;165:7207–14.
- [21] Benson JM, Campbell KA, Guan Z, et al. T-cell activation and receptor downmodulation precede deletion induced by mucosally administered antigen. *J Clin Invest* 2000;106:1031–8.
- [22] MacKenzie JR, Mattes J, Dent LA, Foster PS. Eosinophils promote allergic disease of the lung by regulating CD4⁺ Th2 lymphocyte function. *J Immunol* 2001;167:3146–55.
- [23] Grunig G, Warnock M, Eakil AE, et al. Requirement for IL-13 independently of IL-4 in experimental asthma. *Science* 1998;282:2261–2.
- [24] Tsioura DC, Blumenthal RL, Berry G, DeKruyff RH, Umetsu DT. Mechanisms preventing allergen-induced airway hyperreactivity: role of tolerance and immune deviation. *J Allergy Clin Immunol* 2000;106:239–46.
- [25] Hansen G, Berry G, DeKruyff RH, Umetsu DT. Allergen-specific Th1 cells fail to counterbalance Th2 cell-induced airway hyperreactivity but cause severe airway inflammation. *J Clin Invest* 1999;103:175–83.
- [26] Korsgren M, Erjefalt JS, Korsgren O, Sunder F, Persson CG. Allergic eosinophil-rich inflammation develops in lungs and airways of B cell-deficient mice. *J Exp Med* 1997;185:885–92.
- [27] Hamelmann E, Takeda K, Schwartz J, Vella AT, Irvin CG, Gelfand EW. Development of eosinophilic airway inflammation and airway

- hyperresponsiveness requires interleukin-5 but not immunoglobulin E or B lymphocytes. *Am J Respir Cell Mol Biol* 1999;21:480–9.
- [28] Hurst SD, Seymour BWP, Muchamuel T, Kurup VP, Coffman RL. Modulation of inhaled antigen-induced IgE tolerance by ongoing Th2 responses in the lung. *J Immunol* 2001;166:4922–30.
- [29] Thornton AM, Shevach EM. Suppressor effector function of CD4⁺ CD25⁺ immunoregulatory T cells is antigen non-specific. *J Immunol* 2000;164:183–90.
- [30] Hoyne GF, Le Roux I, Corsin-Jimenez M, et al. Serrate 1-induced Notch signalling regulates the decision between immunity and tolerance made by peripheral CD4⁺ T cells. *Int Immunol* 2000;12:177–85.
- [31] Haneda K, Sano K, Tamura G, et al. Transforming growth factor- β -secreted from CD4⁺ T cells ameliorates antigen-induced eosinophilic inflammation. *Am J Respir Cell Mol Biol* 1999;21:268–74.
- [32] Burkhart C, Liu GY, Anderton SM, Netzler B, Wraith DC. Peptide-induced T cell regulation of experimental autoimmune encephalomyelitis: a role for IL-10. *Int Immunol* 1999;11:1625–34.
- [33] Akdis CA, Blesken T, Akdis M, Wuthrich B, Blaser K. Role of interleukin 10 in specific immunotherapy. *J Clin Invest* 1998;102:98–106.
- [34] Akbari O, DeKruyff RH, Umetsu DT. Pulmonary dendritic cells producing IL-10 mediate tolerance induced by respiratory exposure to antigen. *Nature Immunol* 2001;2:725–31.
- [35] Asseman C, Mauze S, Leach MW, Coffman RL, Powrie F. An essential role for interleukin 10 in the function of regulatory T cells that inhibit intestinal inflammation. *J Exp Med* 1999;190:995–1004.
- [36] Groux H, Bigler M, O'Garra A, et al. A CD4⁺ T-cell subset inhibits antigen-specific T-cell responses and prevents colitis. *Nature* 1997;389:737–42.
- [37] Cottrez F, Hurst SD, Coffman RL, Groux H. T regulatory cells 1 inhibit a Th2-specific response in vivo. *J Immunol* 2000;165:4848–53.
- [38] Zuany-Amorim C, Haile S, Leduc D, et al. Interleukin-10 inhibits antigen-induced cellular recruitment into the airways of sensitised mice. *J Clin Invest* 1995;95:2644–51.
- [39] Steinbrink K, Wolf M, Jonuleit H, Knop J, Enk AH. Induction of Tolerance by IL-10 treated dendritic cells. *1997;159:4772–80.*
- [40] Jonuleit J, Schmitt E, Schuler G, Knop J, Enk AH. Induction of interleukin 10-producing, non-proliferating CD4 (+) T cells with regulatory properties by repetitive stimulation with allogeneic immature human dendritic cells. *J Exp Med* 2000;192:1213–22.