

Allergen-triggered airway hyperresponsiveness and lung pathology in mice sensitized with the biopesticide *Metarhizium anisopliae*[☆]

Marsha D.W. Ward ^{a,*}, Sharon L. Madison ^b, Denise M. Sailstad ^b,
Stephen H. Gavett ^b, MaryJane K. Selgrade ^b

^a Curriculum in Toxicology, University of North Carolina, Chapel Hill, NC 27599, USA

^b National Health and Environmental Effects Research Laboratory, US Environmental Protection Agency, Research Triangle Park, NC 27711, USA

Received 4 April 1999; accepted 3 October 1999

Abstract

Metarhizium anisopliae is an entomopathogenic fungus recently licensed for indoor control of cockroaches, a major source of allergens. While *M. anisopliae* has been shown to be non-infectious and non-toxic to mammals there has been only limited research on potential allergenicity. Using a mouse model, we previously demonstrated allergic immune and inflammatory responses to this agent. The present study was designed to determine whether these responses were associated with changes in pulmonary responses, lung pathology, and the cytokine profile in bronchoalveolar lavage fluid (BALF). Soluble factors from fungal components were combined in equal protein amounts to form *M. anisopliae* crude antigen (MACA). BALB/C mice were intratracheally (IT) challenged with 10 µg MACA 14 days post intraperitoneal sensitization with 25 µg fungal antigen in aluminum hydroxide adjuvant. Physiological and cellular changes were examined. The mice were tested for airway hyperresponsiveness before (No Chal) and after (1, 3, and 8 days post challenge (DPIT)) MACA IT challenge. Subsequently, serum, BALF and the lungs were harvested. All treatment groups concurrently demonstrated significant non-specific pulmonary inflammation (neutrophil influx) and increased pulmonary sensitivity to methacholine (Mch) at 1 DPIT MACA challenge. Where as both adjuvant treated and naïve mice airway responses had returned to near normal levels by 3 DPIT, mice

Abbreviations: BAL, bronchoalveolar lavage; BALF, bronchoalveolar lavage fluid; DPIT, days post MACA IT challenge; HBSS, Hank's balanced salt solution; IP, intraperitoneal; IT, intratracheal; MACA, *Metarhizium anisopliae* crude antigen; Mch, methacholine; PMN, polymorphonuclear leukocytes; SEM, standard error of the mean.

[☆] Disclaimer: This research paper has been reviewed by the National Health and Environmental Effects Research Laboratory, US Environmental Protection Agency and approved for publication. Approval does not signify that the contents necessarily reflects the views and policies of the agency, nor does mention of trade names or commercial products constitute endorsement or recommendation for use.

* Corresponding author. Present address: United States Environmental Protection Agency, MD 92, 86 TW Alexander Drive, Research Triangle Park, NC 27711, USA. Tel.: +1-919-5411193.

E-mail address: ward-marsha@epamail.epa.gov (M.D.W. Ward)

which were previously sensitized with MACA were still hyperresponsive to Mch challenge at 3 and 8 DPIT. This hyperresponsiveness correlates with eosinophil and lymphocyte influx, which is maximal at 3 DPIT and still elevated at 8 DPIT. Interleukin (IL) 5 was elevated for all treatment groups at 1 DPIT but only the MACA sensitized mice maintained elevated levels for both 3 and 8 DPIT. Furthermore, MACA sensitized mice had a more extensive inflammatory histopathology at all examined time points with peribronchial and perivascular infiltrates, like those associated with allergic responsiveness, peaking at 3 DPIT. These pulmonary pathologic changes appeared to be consistent with elevated levels of serum and BALF total IgE, BALF IL-4, eosinophils, and lymphocytes following MACA IT challenge in MACA sensitized mice. There were no significant differences among the three treatment groups with regard to BALF interferon (IFN) γ . The cytokines profiled indicate a Th2-type response, which is reflected in the cellular influx and total IgE induction. These data further indicate that immune inflammatory responses, observed in mice following MACA sensitization and challenge, are associated with physiologic changes and histopathology characteristic of allergic disease. © 2000 Published by Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Fugus; Mouse; Antigen

1. Introduction

Atopy, the presence of immediate (Type I) bronchial hyperreactivity to specific allergens, and other Th2 responses are major determinants in occupationally and environmentally induced respiratory diseases. These immune responses are of particular concern because, in addition to the medical and economic impact on the general population, about 75% of asthma cases are triggered by allergens (US DHHS, 1991; Platts-Mills and Carter, 1997). Morbidity and mortality due to asthma have increased considerably over the past 20 years. The reasons are not entirely clear, but have potential linkage to the environment (US DHHS, 1991; Vogel, 1997).

Respiratory hypersensitivity is an atopic response requiring previous exposure to allergens for elicitation of specific IgE (IgG1 in guinea pigs). Upon subsequent exposure of allergic asthmatics an immediate bronchoconstriction occurs as a result of IgE cross-linking of mast cells and release of mediators. Between 2 and 8 h after exposure a more severe and prolonged (late phase) reaction occurs. Respiratory hyper-mucus secretion, increased influx of inflammatory cells (eosinophils), and nonspecific (pharmacological) airway hyperresponsiveness (Larson, 1985) characterize this late phase reaction. The recruited inflammatory cells, particularly eosinophils, release mediators important in late phase responses (DeMonchy et al., 1985). Additionally, these hypersensitivity responses that have been associated

with increased airway hyperresponsiveness to nonspecific bronchoconstrictors may last several days following allergen challenge (Marsh et al., 1985; Murphy et al., 1986). Recently, Lambert et al. (1998), in adaptive transfer experiments, have shown that activated lymphoid cells rather than IgE play a significant role in this late phase response.

The toxicity associated with many chemical pesticides has provided the impetus to develop biological agents for pest control including the entomopathogenic fungus, *Metarhizium anisopliae*. *M. anisopliae*, which has been used to control a variety of pests (Young, 1974; Latch and Falloon, 1976; Al-Aidroos and Roberts, 1978; Ferron, 1981; Agudelo-Silva and Wassink, 1984; Riba et al., 1986; Gillespie, 1988; Samuels et al., 1989, 1990; Barson et al., 1994), is marketed for indoor cockroach control in the United States. Cockroaches are infected when they pass through a chamber. The effectiveness of the pesticide depends on the infection subsequently spreading through the entire colony. Therefore, the potential concentration of *M. anisopliae* in indoor air depends on the size of the cockroach infestation and the degree to which other conditions support replication and dissemination of the organism. While these biological agents, used in pest control, have been assessed for pathogenicity and toxicity, there has been limited research with regard to allergenicity. Fungi and molds, particularly their spores, are thought to provoke allergic responses and contribute to indoor air health

problems. *M. anisopliae* var. *anisopliae* spores are cylindrical to oval shaped with a length of 3.5–9.0 μm (Tulloch, 1976). However, in indoor situations, individuals will likely be exposed not only to spores but also to mycelium (hyphae) and the inducible proteases and chitinases. Although, exposure assessment studies have not been done, it is probable that MACA antigens will become airborne along with cockroach antigens. There is at least one report of immune responses to both vegetative components and spores of a microbial pesticide (Bernstein et al., 1999).

We have used a mouse model to assess the potential allergenicity of *M. anisopliae*, which we selected as a prototype organism to study allergic potential and to develop methods to screen organisms used for bio-remediation. Previously we demonstrated immune and inflammatory responses in this model that are characteristic of immediate-type hypersensitivity and late phase responses (Ward et al., 1998). Specifically, mice sensitized with MACA in the presence of adjuvant were found to have significantly elevated levels of serum and bronchoalveolar lavage fluid (BALF) total IgE, BALF Interleukin (IL-) 4, eosinophils, and lymphocytes following MACA intratracheal (IT) challenge. This study was designed to determine whether immune and inflammatory endpoints reflected changes in respiratory function and pathology characteristic of allergic asthma and to further characterize cytokine responses.

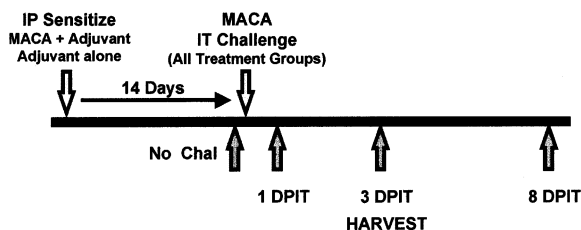


Fig. 1. Time line for experimental design. All treatment groups (MACA + adjuvant, Adjuvant alone and sensitization naïve mice) were intratracheally (IT) challenged with MACA 14 days after intraperitoneal (IP) sensitization. The mouse airway reactivity test was performed and blood, BALF, and lungs were harvested before and at 1, 3, and 8 days after MACA challenge.

2. Methods

2.1. Animals

Fifty-day old, female Balb/c mice (Charles River, Raleigh, NC) were group-housed in polycarbonate cages with bedding made of pine shavings in an environmentally controlled, American Association for Accreditation of Laboratory Animal Care (AAALAC)-accredited vivarium. Sentinel mice were monitored serologically and were found to be free of Sendai, mouse pneumonia, mouse hepatitis, other murine viruses, and mycoplasma. Mice also were monitored for, and found to be free of, ectoparasites and endoparasites. Mice were maintained on a standard diet (Purina rodent lab chow, St. Louis, MO) and water ad libitum. Mice were acclimated one week at this facility prior to the start of the experiment.

2.2. Fungal antigen preparation

M. anisopliae strain 1080 was obtained from USDA-ARS Entomopathogenic Fungus Collection in Ithaca, NY. The MACA, composed of Hank's balanced salt solution (HBSS) extracts from mycelium and spores as well as inducible enzymes, was produced as previously described (Ward et al., 1998). The proteins with greater than 3000 molecular weight were concentrated using Centrprep-3 (MWCO 3000) (Amicon, Beverly, MA) and assayed for total protein concentration (as described below). Equal protein concentrations of each component were combined to form the *M. anisopliae* crude antigen (MACA).

2.3. Experimental design

To evaluate the potential of MACA to elicit airway hyperresponsiveness, 8 week old, female Balb/c mice were sensitized intraperitoneally (IP) with 25 μg of MACA in a total volume of 0.2 ml of 1.3% aluminum (alum) hydroxide adjuvant [Accurate, Westbury, NY; Alhydrogel (Superfos Biosector)] (MACA treatment group) (Fig. 1). The two control groups were: (1) an alum adjuvant only (0.2 ml alhydrogel) inoculated group (ADJUV) and (2) a naive group (no sensitization)

(NAÏVE). Additionally, the NAÏVE/No Chal mice are designated NORMAL for the airway reactivity test. Fourteen days after sensitization all groups were IT challenged with 10 µg MACA in 50 µl HBSS (divalent cation-free) by a modification of the methods of Hatch et al. (1981) as previously described in Ward et al. (1998). The mouse airway reactivity test was performed prior to challenge (No Chal) and at 1, 3, and 8 days post MACA IT challenge (DPIT). Subsequent to the mouse airway reactivity test, serum and bronchoalveolar lavage fluid (BALF) samples were obtained and the lungs removed and fixed for histopathology. Only BALF samples were used for cytokine assays.

2.4. Mouse airway reactivity test

Animals were anesthetized with 1.5 g/kg body weight urethane, tracheostomized and intubated with a T-shaped cannula adapted to measure airway opening pressure and flow. The rate of ventilation, controlled by the speed of the switching solenoids, was determined for each animal based on allometric scales of body weight vs. lung volumes/ventilation rates (Costa et al. 1992). Heart rate and waveform were monitored throughout the experiment (Sinus Rhythm Analyzer, Micro-Med, Louisville, KY). Spontaneous breathing during ventilation was blocked by IP injection of succinylcholine chloride at 15 mg/kg body weight. Flow and pressure measurements for each breath were averaged over 6 s to calculate the compliance and airway resistance according to the constant flow inflation method (Bates et al., 1985). Prior to the first methacholine (Mch) challenge a stable baseline was recorded for 2 min. Subsequently, the mice were challenged with four doubling doses (50 µg to 400 µg/kg body weight) of Mch delivered as a bolus over one second via a jugular cannula connected to an electronically controlled syringe pump containing 0.1 mg/ml methacholine. A 2 min recovery period was allowed between each dose. Results collected throughout the challenge and recovery periods, were expressed as the effective dose (ED) of Mch that caused a 100% increase in airway pressure (ED100) and airway resistance (ED100) over baseline values and a

20% decrease in compliance (ED20). These ED values were compared to normal mice (MACA and adjuvant naïve, prior to MACA IT challenge) at the various time points.

2.5. Bronchoalveolar lavage (BAL) and cardiac bleeds

Following the mouse airway reactivity test, blood and BAL samples were collected as previously described (Ward et al., 1998), with the exception that HBSS was used as the lavage medium. Total cell counts, performed by trypan blue exclusion on a haemocytometer, were followed by centrifugation at $100 \times g$ for 15 min at 4°C. The BALF was stored at -70°C for IgE and cytokine assays. A total of 100–150 µl of the BALF cells, resuspended in 0.5 ml HBSS, were centrifuged onto glass slides (Shandon Cytospin, $60 \times g$ for 5 min). Following Wright–Giemsa (Fisher Scientific) staining, differential cell counts were performed by counting 200 cells per slide.

2.6. Histopathology

Following the mouse airway reactivity test and BAL, the lungs were fixed in 10% formalin (phosphate buffered), embedded in paraffin, and sectioned. The lung sections were stained with hematoxylin–eosin solution prior to pathological analysis. The relative degree of severity of inflammatory, degenerative, and proliferative changes was graded on a scale of 1–5 by a pathologist (Peter Mann of Experimental Pathology Laboratories, Research Triangle Park, NC). Group severity indices were determined by dividing the sum of the numerical scores for a particular lesion by the number of animals examined in a group.

2.7. TOTAL IgE and cytokine ELISA

The total IgE ELISA performed as previously described (Ward et al., 1998) used rat α -mouse IgE (Southern Biotechnology, Birmingham, AL) at 2.5–5 µg/ml in a carbonate-bicarbonate pH 9.4 buffer as the capture antibody and biotinylated sheep anti-mouse IgE (The Binding Site, Birmingham, England) as the detection antibody. Addi-

tionally, a mouse monoclonal IgE anti-dinitrophenyl (DNP) (Sigma Chemical, St. Louis, MO) was used as the positive control.

The IL-4 ELISA performed as previously described (Ward et al., 1998) used anti-mouse IL-4 (Endogen, Woburn, MA) in PBS (Dulbecco's phosphate buffered saline, pH 7.4, GIBCO, Grand Island, NY) as the capture antibody and Biotin-anti-Mouse IL-4 (Endogen, Woburn, MA) as the detection antibody. Additionally, purified recombinant mouse IL-4 (Endogen, Woburn, MA), applied in two-fold dilutions from 640 to 5 pg/ml, was the positive control.

2.8. IL-5 ELISA

Reagents and incubation periods were kept at room temperature (20–25°). Certified 96-well microtiter plates were washed between each incubation step. Following coating with anti-mouse IL-5 (PharMingen, San Diego, CA) in a carbonate-bicarbonate pH 9.4 buffer (BupH buffer pack, Pierce, Rockford, IL), the plates were incubated overnight at 4°C. Subsequently, the plates were washed twice with PBS (1.19 mM sodium phosphate, 8.1 mM sodium phosphate dibasic, 154 mM sodium chloride in distilled H₂O, pH 7.4) containing 0.05% Tween-20, pH 7.3–7.5 (PBS/Tween) and blocked with PBS containing 10% newborn calf serum (PBS/NCS) for 2 h at room temperature. The plates were washed twice with PBS/Tween prior to the addition of samples and IL-5 standards. BALF samples, undiluted or at a 1:2 dilution in PBS/NCS, were added to the plates and then incubated overnight at 4°C. The IL-5 standard (purified recombinant mouse IL-5, PharMingen, San Diego, CA), in two-fold dilutions from 480 to 1.875 pg/ml, also was applied. Following six washes with PBS/Tween, the biotinylated secondary antibody (Biotin-anti-Mouse rIL-5, R&D Systems, Minneapolis, MN) in Tris buffered saline + 0.1% BSA was added and incubated 2 h at room temperature. Following six washes with PBS/Tween, streptavidin horseradish peroxidase (Zymed, South San Francisco, CA) diluted in PBS/NCS was added and incubated 30 min. Subsequently, the substrate tetramethylbenzidine (TMB, Dako, Carpinteria, CA) was added

and incubated a minimum of 30 min in the dark. The optical density was read at 650 nm using a Thermomax^R Plate Reader (Molecular Devices, Menlo Park, CA).

2.9. IFN- γ ELISA

Reagents and incubation periods were kept at room temperature (20–25°). Certified 96-well microtiter plates were washed between each incubation step with PBS (1.19 mM sodium phosphate, 8.1 mM sodium phosphate dibasic, 154 mM sodium chloride in distilled H₂O, pH 7.4) containing 0.05% Tween-20, pH 7.3–7.5. The plates were coated overnight with anti-mouse IFN- γ (R&D Systems, Minneapolis, MN) in PBS. Subsequently, the plates were blocked for 1 h with blocking buffer (1% BSA, 5% sucrose, 0.05% sodium azide, in PBS, pH 7.4). BALF samples, diluted 1:2 in dilution buffer (20 mM Tris base, 150 mM sodium chloride, 0.1% BSA, pH 7.3), were added to the plates and incubated 2 h. The IFN- γ standard (purified recombinant mouse IFN- γ , R&D Systems, Minneapolis, MN), in two-fold dilutions from 2000 to at least 7.8 pg/ml also was applied. Following washing, the biotinylated second antibody (Biotin-anti-Mouse IFN- γ , R&D Systems, Minneapolis, MN) was added and incubated 2 h. The streptavidin horseradish peroxidase diluted in dilution buffer and the substrate tetramethylbenzidine were sequentially added and incubated 20 min with the substrate incubation in the dark. The reaction was stopped with the addition of 100 μ l/well 0.18 M sulfuric acid. The optical density was read at 450 nm using a Thermomax^R Plate Reader.

2.10. Statistics

The data were analyzed using analysis of variance. Pairwise comparisons were performed as subtests of the overall model. In cases of comparing a control group to other groups, Dunnett's test was used. Significance was attributed to an effect or difference if the probability was < 0.05. Adjustments in significance levels, for multiple comparisons, were made using a modified Bonferroni Correction (Thomas, 1973).

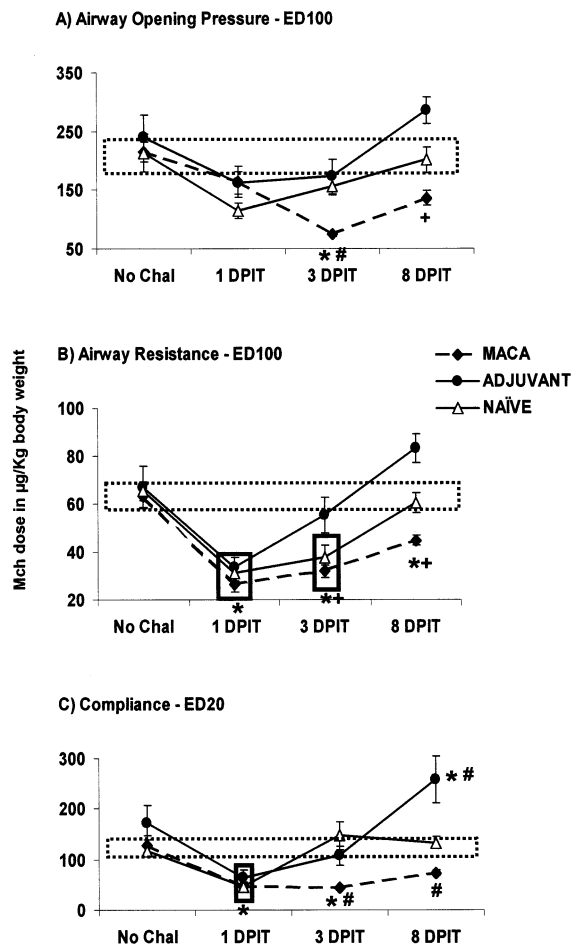


Fig. 2. Airway reactivity during bronchial provocation with methacholine (Mch) was assessed before and at 1, 3, and 8 days post MACA IT challenge. (A) airway pressure and (B) airway resistance expressed as Effective Dose 100 (the dose of Mch required to produce a 100% increase over baseline). (C) compliance expressed as Effective Dose 20 (the dose of Mch required to produce a 20% decrease from baseline). The dashed box represents the mean $\pm$ SEM of the normal (MACA naïve, no challenge) mice. Data shown represents the mean $\pm$ SEM. $n = 4-5$ per group. Significant difference from (*) normal mice; (+) adjuvant group; (#) both controls within time point ($P < 0.05$). Boxed data points share significance. (DPIT, days post MACA IT challenge).

3. Results

3.1. Airway responsiveness

Prior to MACA IT challenge, all treatment

groups were essentially equivalent in their sensitivity to Mch for each of the parameters examined (Fig. 2). Increased sensitivity to Mch (lower dose) is indicative of increased hyperreactivity in the airways. The calculated Mch dose to cause a 100% increase in airway pressure for untreated mice was 213 μ g Mch/kg body weight. The only significant increase in sensitivity to Mch compared to the untreated controls occurred at 3 DPIT by the MACA sensitized and challenged mice (Fig. 2A). The calculated Mch dose to cause a 100% increase in airway resistance for untreated mice was 66 μ g Mch/kg body weight. At 1 DPIT all treatment groups were equivalent and were significantly more sensitive to Mch challenge than untreated controls. By 3 DPIT MACA and IT challenged Naïve mice were still significantly more sensitive than untreated controls while adjuvant sensitized mice were returning to untreated mice-Mch sensitivity levels. At 8 DPIT, MACA sensitized and challenged mice were still significantly more sensitive to Mch challenge than untreated controls. While Naïve, MACA challenged mice had returned to near normal sensitivity, adjuvant sensitized mice were shown to be significantly less sensitive to Mch challenge than were the untreated controls (Fig. 2B). The calculated Mch dose to cause a 20% decrease in lung compliance for untreated mice was 118 μ g Mch/kg body weight. Again at 1 DPIT all treatment groups were equivalent and were significantly more sensitive to Mch challenge than untreated controls. However, by 3 DPIT only the MACA sensitized and challenged mice were significantly more sensitive to Mch challenge than were the untreated mice. By 8 DPIT MACA mice hyperreactivity were significantly different from both the adjuvant and naïve groups. However, the adjuvant treated mice were less sensitive to Mch challenge than were the untreated mice (Fig. 2C).

3.2. Histopathology

Prior to MACA IT challenge there was no evidence of allergic disease in the lungs of any of the treatment groups. While all treatment groups

had alveolar lesions at 1 DPIT, lesions in the lungs of the MACA sensitized mice were more extensive (Table 1). Only in the MACA-sensitized group was bronchial epithelial hypertrophy evident (1/3) and the character of the perivascular cellular infiltrate was more acute [polymorphonuclear leukocytes (PMN)]. By 3 DPIT, there was both perivascular and peribronchial cellular infiltration in all treatment groups but the incidence and severity of the acute infiltrates was increased in the MACA sensitized mice. Bronchial epithelial hypertrophy was present in the adjuvant and MACA sensitized mice. Again the severity was increased for the MACA sensitized animals. 8 DPIT the peribronchial and perivascular infiltrates in the naïve mice were mononuclear, while the adjuvant sensitized mice had only perivascular mononuclear infiltration. The MACA-sensitized mice had greater severity of both the peribronchial and perivascular mononuclear infiltration than either the naïve or adjuvant mice or the MACA-sensitized mice sacrificed at the 3 DPIT. However, the severity of the inflammatory infiltrate in MACA sensitized mice had decreased from the 3 DPIT levels. Additionally, only the MACA sensitized mice exhibited bronchial epithelial hypertrophy. Examples of the lung sections are shown in Fig. 3.

3.3. Cytokines

As had previously been seen there was a dramatic increase in BALF IL-4 only in MACA sensitized and challenged mice and only at 1 DPIT (Fig. 4A). While BALF IL-5 (Fig. 4B) was significantly elevated for all treatment groups at 1 DPIT, levels were significantly higher and persisted through 8 DPIT in the MACA sensitized and challenged mice. However, there were no significant differences in BALF IFN- γ among the treatment groups (Fig. 4C).

3.4. Total and differential cell counts

To verify that mice used in this study were eliciting basic allergic responses comparable to those previously described (Ward et al., 1998), total and differential cell counts from the BALF

were examined. Prior to challenge, total cell counts (Fig. 5) from the BALF of both MACA sensitized and control (adjuvant and naïve) mice were similar. At 1 DPIT there was a doubling in total cell counts for all treatment groups. By 3 DPIT there was a further and significant increase in the total cell counts of MACA sensitized mice when compared to No Chal and both naïve and adjuvant control mice. At 8 DPIT the MACA treatment group cell counts were reduced but still significantly elevated compared to control groups. Differential cell counts (Fig. 5) indicated that prior to challenge the predominant cell type was monocytic (97–98%) ranging from $\approx 17\text{--}19 \times 10^4$ cells/ml. MACA sensitized and challenged mice demonstrated significant increases in lymphocyte and eosinophil counts at both 3 and 8 DPIT. However, BALF lymphocytes were only slightly increased and eosinophils were barely detectable in controls. There was a substantial neutrophilic influx at 1 DPIT for all treatment groups that had returned to pre-challenge levels by 3 DPIT.

3.5. Serum and balf total IgE

Prior to IT challenge (≈ 2 weeks following sensitization) and throughout the experiment only the MACA sensitized mice showed significant increases in serum total IgE when compared to control groups ($P < 0.05$) (Fig. 6). None of the treatment groups had elevated levels of BALF total IgE prior to MACA IT challenge but by 8 DPIT MACA sensitized mice had significantly increased levels of BALF total IgE.

4. Discussion

This study demonstrated that MACA sensitized mice display not only immune inflammatory responses characteristic of allergy but also the physiological and the histopathological responses that are consistent with allergic airway disease. The temporal relationship of the significant findings in this study is summarized in Fig. 7. BALF cytokines are maximal prior to the maximal influx of BALF eosinophils and lymphocytes, which coincides with maximal lung pathology and respiratory physiology abnormalities.

Table 1
Histopathology scores for lung sections^a

	1 DPIT			3 DPIT			8 DPIT		
	Incidence (severity)			Incidence (severity)			Incidence (severity)		
	NAÏVE	ADJUV	MACA	NAÏVE	ADJUV	MACA	NAÏVE	ADJUV	MACA
Bronchial epithelial hypertrophy	0	0	1/3 (0.3)	0	1/3 (0.3)	1/3 (0.7)	0	0	2/3 (1.3)
Peribronchial mononuclear cell infiltration	0	0	0	1/3 (0.3)	1/3 (0.3)	1/3 (0.3)	2/3 (0.7)	0	2/3 (0.7)
Peribronchial polymorphonuclear (PMN) cell infiltration	0	0	0	1/3 (0.3)	0	2/3 (1.0)	0	0	2/3 (0.7)
Perivascular mononuclear cell infiltration	3/3 (1.0)	3/3 (1.0)	1/3 (0.3)	3/3 (1.0)	2/3 (0.7)	2/3 (1.0)	1/3 (0.3)	2/3 (0.7)	3/3 (1.7)
Perivascular PMN cell infiltration	1/3 (0.3)	1/3 (0.3)	3/3 (2.0)	2/3 (0.7)	0	3/3 (2.7)	0	0	3/3 (2.0)

^a Incidence indices were determined by the number of mice with the pathological feature over the number of mice examined. Severity indices were determined by dividing the sum of the numerical score for a particular lesion by the number of animals examined.

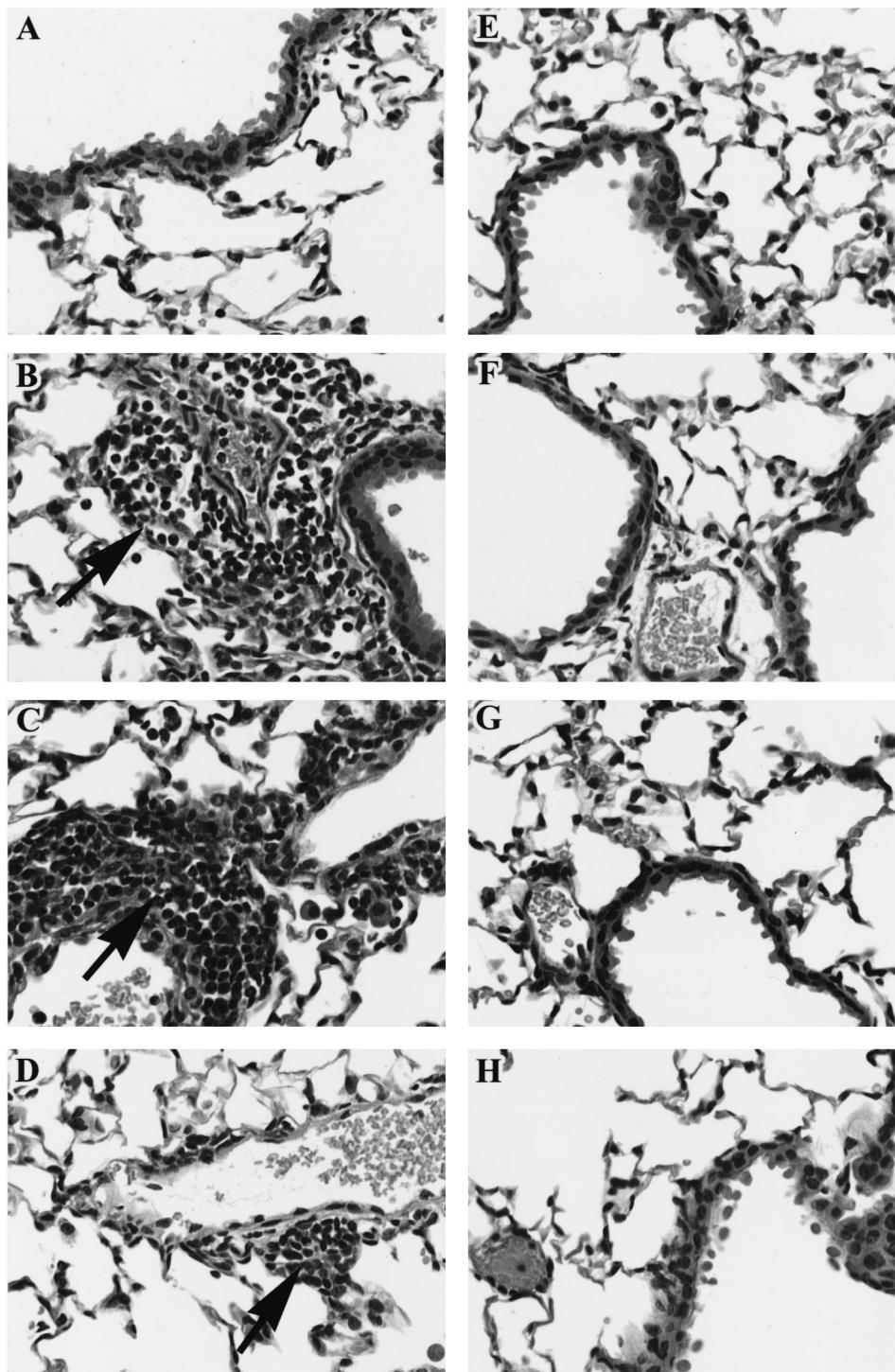
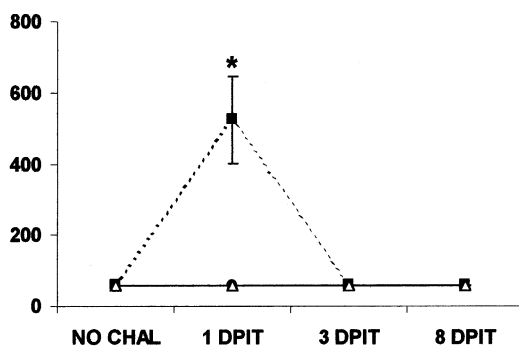


Fig. 3. Histopathology micrographs of mouse lungs showing peribronchial and/or perivascular infiltrates (arrows) in MACA sensitized (A–D) and adjuvant treated (E–H) mice at different time points. A and E before IT challenge, B and F at 1 day post challenge, C and G at 3 days post challenge, and D and H at 8 days post challenge. Slides were stained with hematoxylin and eosin and magnification $\times 400$.

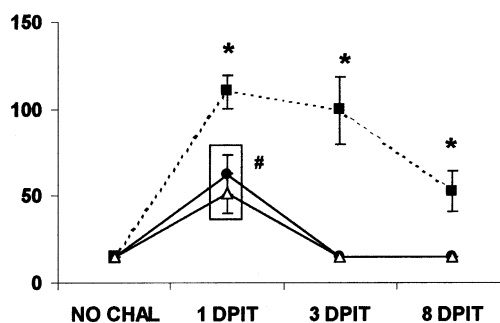
The T helper 2 cell (Th2)-like responses have been associated with allergy and asthma. Specifically, the Th2 cytokine IL-4 has shown autocrine activity in Th2 cell differentiation (Swain et al., 1990; Kopf et al., 1993), Ig class switching to IgE (Bergstedt-Lindqvist et al., 1988; Finkelman, et al., 1988), and mast cell development (Madden et al., 1991). In our study elevated IL-4 in BALF and increased total IgE occurred only in MACA sensitized mice indicating that this microorganism induces a Th2 response. Furthermore, IL-5, another Th2 cytokine shown to be essential in proliferation and maturation of eosinophil precursors (Yamaguchi et al., 1988) as well as eosinophil recruitment to the lung (Van Oosterhaut et al., 1996), viability (Kita et al., 1992; Ohnishi et al.,

1993), and eosinophil granule protein release (Kita et al., 1992) was also elevated in MACA sensitized mice. CD4 + Th2 lymphocytes are not the only source Th2-associated cytokines. Mast cells have also been shown to secrete IL-4. Additionally, mast cells (Kung et al., 1995) and activated eosinophils (Broide et al., 1992) have been shown to secrete IL-5. In our studies elevated levels of both IL-5 and IL-4 appear in BALF by 1 day following MACA IT challenge. While IL-4 was at background levels by 3 DPIT, IL-5 remained elevated through 8 DPIT paralleling the increase in eosinophil and lymphocyte numbers (3 and 8 DPIT). Adjuvant alone did not drive these cytokine responses as seen in the mice sensitized with adjuvant only (ADJUV). Furthermore, it

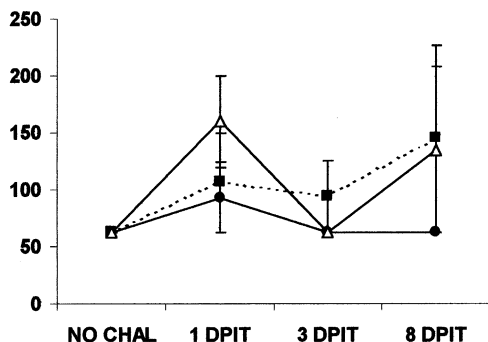
A) IL-4 (pg/ml)



B) IL-5 (pg/ml)



C) IFN- γ (pg/ml)



---■--- MACA
 —●— ADJUV
 —△— NAIVE

Fig. 4. BALF cytokines measured by ELISA. (A) IL-4: the level of detection was 60 pg/ml; (B) IL-5: the level of detection was 15 pg/ml; (C) IFN- γ : the level of detection was 62.50 pg/ml. Data shown represents the mean $\pm$ SEM. $n = 3-5$ mice per group. Significant difference from both controls and No Chal mice (*), from No Chal mice (#) ($P < 0.05$). (DPIT-days post MACA IT challenge).

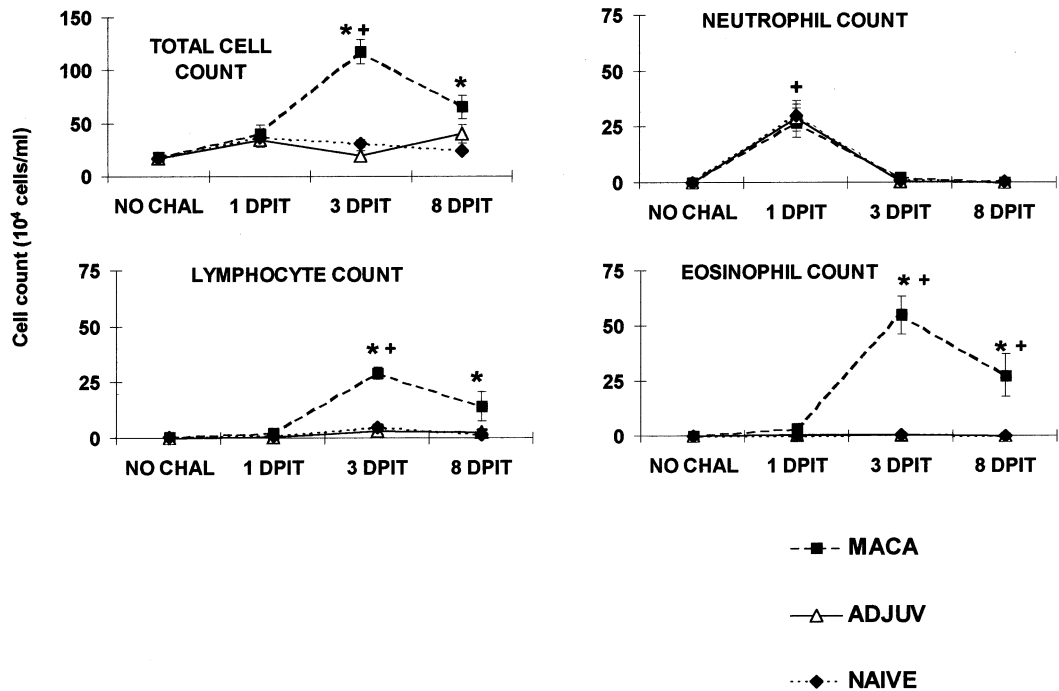


Fig. 5. BALF total and differential cell counts. Data shown represents the mean $\pm$ SEM. $n = 4-6$ per group. (*) Significant difference from both controls; (+) significant difference from within treatment group ($P < 0.05$). (DPIT, days post MACA IT challenge).

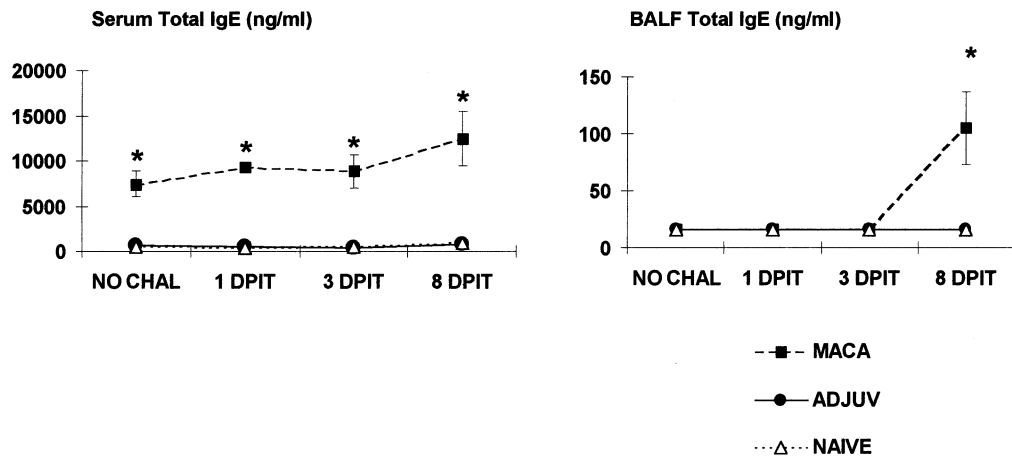


Fig. 6. Serum and BALF Total IgE. Data shown represents the mean $\pm$ SEM. The level of detection was 12.5 ng/ml. $n = 4-5$ per group. (*) Significant difference from both controls ($P < 0.05$). (DPIT-days post MACA IT challenge).

was previously (Ward et al., 1998) shown that the chitin media (with adjuvant) sensitized controls did not develop increased serum total IgE. Since media proteins in the presence of adjuvant did not

result in IgE increases it can be concluded that not all proteins even in the presence of alum induce allergic responses. IFN- γ , on the other hand, has been shown to inhibit development of

Th2 cells (Gajewski and Fitch, 1988) and IL-4-induced immune responses (Coffman and Carty, 1986; Li et al., 1996). Furthermore, IFN- γ receptor deficient mice displayed a persistent eosinophilic inflammation compare to wild-type controls as a result of a sustained Th2 cytokine profile (Coyle et al., 1996). While this evidence strongly suggests a role for IFN- γ in modulation of the allergic responses, we did not observe differences in the IFN- γ response prior to or following MACA challenge.

MACA challenge resulted in significantly increased sensitivity to Mch for all treatment groups at 1 DPIT coinciding to the time when non-specific, neutrophilic inflammation was evident. Both adjuvant treated and naïve mice airway responses had returned to near normal levels by 3 DPIT again corresponding to the decline in non-specific inflammation. However, those mice sensitized with MACA prior to MACA respiratory challenge were still hyperresponsive at 8 DPIT. Interestingly, by 8 DPIT adjuvant treated/MACA challenged mice were actually less sensitive to Mch challenge than were untreated controls, although the reasons for this hyporesponsiveness are not apparent. This data indicates that both non-specific inflammatory effects induced by

MACA respiratory challenge in previously unexposed mice and the more persistent specific inflammation observed with allergic sensitization are associated with increased hyperreactivity. Furthermore, histopathological analysis found that in general the MACA sensitized mice appeared to have more extensive lung pathology at all time points. Peribronchial and perivascular infiltrates associated with allergic responsiveness peak at 3 DPIT and become more mononuclear by 8 DPIT. Additionally, diffuse bronchial epithelial hyperplasia was evident in MACA sensitized mice at all time points. Pulmonary hyperresponsiveness and pathology persisted for at least 8 days in sensitized mice.

Work to move away from the use of adjuvants and towards a respiratory route of sensitization is currently underway. Furthermore, we have recently identified up to 4 proteins that induce an IgE response. These allergens represent $\approx 7.12\%$ of the MACA proteins (unpublished data). Purification of these proteins would provide the means to compare the potency of MACA to other well studied indoor air allergens such as dust mite.

We have extended the allergy/mouse model for the fungal allergen(s) of *M. anisopliae* by characterizing the respiratory hyperresponsiveness and

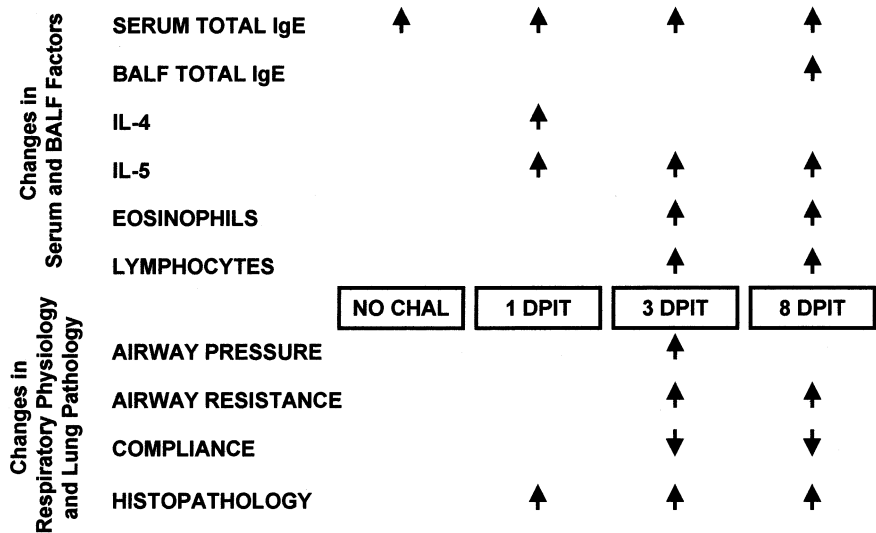


Fig. 7. Summary of study results. Arrows represent significant differences found in MACA sensitized and challenged mice compared to either or both control groups (adjuvant sensitized or sensitization naïve mice followed by one MACA IT challenge).

lung pathology in MACA sensitized and challenged mice. Furthermore, the airway hyperresponsiveness, lung pathology, and cytokine responses are features consistent with those observed in allergic asthmatics. Therefore, this model may be useful, not only to assess the allergic potential of *M. anisopliae* but also more generally to assess the potential allergic responses of other microbes used in the indoor environment.

Acknowledgements

The authors thank Debora Andrews, Elizabeth Boykin and Judy Richards of the US EPA for technical assistance, Don Doerfler of the US EPA for statistical analysis and Dr. Peter Mann of Experimental Pathology Laboratories, Research Triangle Park, NC for histopathological analysis.

References

- Agudelo-Silva, F., Wassink, H., 1984. Infectivity of a Venezuelan strain of *Metarhizium anisopliae* to *Aedes aegypti* larvae. *J. Invertebr. Pathol.* 43 (3), 435–436.
- Al-Aidroos, K., Roberts, D.W., 1978. Mutants of *Metarhizium anisopliae* with increase virulence toward mosquito larvae. *Can. J. Genet. Cytol.* 20, 211–219.
- Barson, G., Renn, N., Bywater, A.F., 1994. Laboratory evaluation of six species of entomopathogenic fungi for the control of the house fly (*Musca domestica* L.), a pest of intensive animal units. *J. Invertebr. Pathol.* 64 (2), 107–113.
- Bates, J.H., Rossi, A., Milic-Emili, J., 1985. Analysis of the behavior of the respiratory system with constant inspiratory flow. *J. Appl. Physiol.* 58, 1840–1848.
- Bergstedt-Lindqvist, S., Moon, H.-B., Persson, U., Moller, G., Heusser, C., Severinson, E., 1988. Interleukin 4 instructs uncommitted B lymphocytes to switch to IgG1 and IgE. *Eur. J. Immunol.* 18, 1073–1077.
- Bernstein, I.L., Bernstein, J.A., Miller, M., et al., 1999. Immune responses in farm workers after exposure to *Bacillus thuringiensis* pesticides. *Environ. Health Perspect.* 107, 575–582.
- Broide, D.H., Paine, M.M., Firestein, G.S., 1992. Eosinophils express interleukin 5 and granulocyte macrophage-colony-stimulating factor mRNA at sites of allergic inflammation in asthmatics. *J. Clin. Invest.* 90, 1414–1424.
- Coffman, R.L., Carty, J., 1986. A T cell activity that enhances polyclonal IgE production and its inhibition by interferon- γ . *J. Immunol.* 136 (3), 949–954.
- Costa, D.L., Tepper, J.S., Raub, J.A., 1992. Interpretations and limitations of pulmonary function testing in small laboratory animals. In: Parent, R.A. (Ed.), *Comparative Biology of the Normal Lung*. CRC Press, Boca Raton, FL, pp. 367–399.
- Coyle, A.J., Tsuyuki, S., Bertrand, C., Huang, S., Aguet, M., Alkan, S.S., Anderson, G.P., 1996. Mice lacking the IFN- γ receptor have an impaired ability to resolve a lung eosinophilic inflammatory response associated with a prolonged capacity of T cells to exhibit a Th2 cytokine profile. *J. Immunol.* 156, 2680–2685.
- DeMonchy, J.G.R., Kauffman, H.F., Venge, P., Koeter, G.H., Jansen, H.M., Sluiter, H.J., De Vries, K., 1985. Bronchoalveolar eosinophilia during allergen-induced late asthmatic reactions. *Am. Rev. Respir. Dis.* 131, 373–376.
- Ferron, P., 1981. Pest control by the fungi *beauveria* and *metarhizium*. In: Burgess (Ed.), *Microbial Control of Pests and Plant Diseases*. Academic Press, London, New York, pp. 465–482.
- Finkelman, F.D., Katona, I.M., Urban, J.F. Jr., Holmes, J., Ohara, J., Tung, A.S., Sample, J.V., Paul, W.E., 1988. IL-4 is required to generate and sustain in vivo IgE responses. *J. Immunol.* 141 (7), 2335–2341.
- Gajewski, T.F., Fitch, F.W., 1988. Anti-proliferative effect of IFN- γ in immune regulation I. IFN- γ inhibits the proliferation of Th2 but not Th1 murine helper T lymphocyte clones. *J. Immunol.* 140 (12), 4245–4252.
- Gillespie, A.T., 1988. Use of fungi to control pests of agricultural importance. In: Burge, M.N. (Ed.), *Fungi in Biological Control Systems*. University Press, Manchester, pp. 37–60.
- Hatch, G.E., Slade, R., Boykin, E., Hu, P.C., Miller, F.J., Gardner, D.E., 1981. Correlation of effects of inhaled versus intratracheally injected metals on susceptibility to respiratory infection in mice. *Am. Rev. Respir. Dis.* 124, 167–173.
- Kita, H., Weiler, D.A., Abu-gazaleh, R., Sanderson, C.J., Gleich, G.J., 1992. Release of granule proteins from eosinophils cultured with IL-5. *J. Immunol.* 149, 629–635.
- Kopf, M., Le Gros, G., Bachmann, M., Lamers, M.C., Bluethmann, H., Kohler, G., 1993. Disruption of the murine IL-4 gene blocks Th2 cytokine responses. *Nature* 362, 245–248.
- Kung, T.T., Stelts, D., Zurcher, J.A., Jones, H., Umland, S.P., Kreutner, W., Egan, R.W., Chapman, R.W., 1995. Mast cells modulate allergic pulmonary eosinophilia in mice. *Am. J. Respir. Cell Mol. Biol.* 12, 404–409.
- Lambert, A.L., Winsett, D.W., Costa, D.L., Selgrade, M.J.K., Gilmour, M.I., 1998. Transfer of allergic airway responses with serum and lymphocytes from rats sensitized to dust mite. *Am. J. Respir. Crit. Care Med.* 157, 1991–1999.
- Larson, G.L., 1985. Hypersensitivity lung disease. *Ann. Rev. Immunol.* 3, 59–85.
- Latch, G.C.M., Falloon, R.E., 1976. Studies on the use of *Metarhizium anisopliae* to control *Oryctes rhinoceros*. *Entomophaga* 21, 39–48.

- Li, X.-M., Chopra, R.K., Chou, T.-Y., Schofield, B.H., Wills-Karp, M., Huang, S.-K., 1996. Mucosal IFN- γ gene transfer inhibits pulmonary allergic responses in mice. *J. Immunol.* 157, 3216–3219.
- Madden, K.B., Urban, J.F. Jr., Ziltener, H.J., Schrader, J.W., Finkelman, F.D., Katona, I.M., 1991. Antibodies to IL-3 and IL-4 suppress helminth-induced intestinal mastocytosis. *J. Immunol.* 147 (4), 1387–1391.
- Marsh, W.R., Irvin, G.G., Murphy, K.R., Behrens, B.L., Larsen, G.L., 1985. Increases in airway reactivity to histamine and inflammatory cells in bronchoalveolar lavage after the late asthmatic response in an animal model. *Am. Rev. Respir. Dis.* 131, 875–879.
- Murphy, K.R., Wilson, M.C., Irvin, C.G., Glezen, L.S., Marsh, W.R., Haslett, C., Henson, P.M., Larsen, G.L., 1986. The requirement for polymorphonuclear leukocytes in the late asthmatic response and heightened airways reactivity in an animal model. *Am. Rev. Respir. Dis.* 134, 62–68.
- Ohnishi, T., Kita, H., Weiler, D., et al., 1993. IL-5 is the predominant eosinophil-active cytokine in the antigen-induced pulmonary late-phase reaction. *Am. Rev. Respir. Dis.* 147, 901–907.
- Platts-Mills, T.A.E., Carter, M.C., 1997. Asthma and indoor exposure to allergens. *N. Eng. J. Med.* 336 (19), 1382–1384.
- Riba, G., Keita, A., Soares, G.G. Jr., Ferron, P., 1986. Comparative studies of *Metarhizium anisopliae* and *Tolypocladium cylindrosporum* as pathogens of mosquito larvae. *J. Am. Mosq. Control Assoc.* 2 (4), 469–473.
- Samuels, K.D.Z., Pinnock, D.E., Allsopp, P.G., 1989. The potential of *Metarhizium anisopliae* (Metschnikoff) Sorokin (Deuteromycotina: *Hyphomycetes*) as a biological control agent of *Inopus rubriceps* (Macquart) (Diptera: *Stratiomyidae*). *J. Aust. Ent. Soc.* 28, 69–74.
- Samuels, K.D.Z., Pinnock, D.E., Bull, R.M., 1990. Scarabeid larvae control in sugarcane using *Metarhizium anisopliae*. *J. Invertebr. Pathol.* 55, 135–137.
- Swain, S.L., Weinberg, A.D., English, M., Huston, G., 1990. IL-4 directs the development of Th2-like helper effectors. *J. Immunol.* 145 (11), 3796–3806.
- Thomas, D.A.H., 1973. Multiple comparisons among means, a review. *The Statistician* 22, 16–42.
- Tulloch, M., 1976. The genus *Metarhizium*. *Trans. Br. mycol. Soc.* 66, 407–411.
- US Department of Health and Human Services, 1991. Guidelines for the diagnosis and management of asthma. Publication No 91-3042. National Institutes of Health, Bethesda, MD.
- Van Oosterhout, A.J.M., van Ark, I., Hofman, G., Van Der Linde, H.J., Fattah, D., Nijkamp, F.P., 1996. Role of interleukin-5 and substance P in development of airway hyperreactivity to histamine in guinea-pigs. *Eur. Respir. J.* 9, 493–499.
- Vogel, G., 1997. New clues to asthma therapies. *Science* 276 (13), 1643–1646.
- Ward, M.D.W., Sailstad, D.M., Selgrade, M.J.K., 1998. Allergic responses to the biopesticide *Metarhizium anisopliae* in Balb/c mice. *Toxicol. Sci.* 45, 195–203.
- Yamaguchi, Y., Suda, T., Suds, J., Eguchi, M., Miura, Y., Harada, N., Tominaga, A., Takatsu, K., 1988. Purified interleukin 5 supports the terminal differentiation and proliferation of murine eosinophilic precursors. *J. Exp. Med.* 167, 43–56.
- Young, E.C., 1974. The epizootiology of two pathogens of the coconut palm rhinoceros beetle. *J. Invertebr. Pathol.* 24, 82–92.