

Comparison of respiratory responses to *Metarhizium anisopliae* extract using two different sensitization protocols[☆]

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Abstract

Metarhizium anisopliae, an entomopathogenic fungus, is a prototypic microbial pesticide licensed for indoor control of cockroaches, a major source of allergens. We have previously demonstrated allergy and asthma-like responses in BALB/c mice intraperitoneally (IP) sensitized in the presence of adjuvant and intratracheally (IT) challenged with the soluble factors from *M. anisopliae* crude antigen (MACA) (Ward et al., 1998, 2000). This protocol has been used frequently to establish animal models of allergenicity. However, the sensitization protocol is artificial and not representative of an environmental exposure. Concern has been raised that this protocol might produce allergic responses that would not occur under normal environmental exposure conditions. The objective of this study was to compare responses in mice to MACA by two exposure protocols: (1) exclusive respiratory exposures without adjuvant (representative of environmental exposures) and (2) intraperitoneal sensitization in the presence of adjuvant followed by IT challenge (the traditional approach). The intratracheal protocol consisted of four IT exposures of 10 µg MACA in 50 µl HBSS each over a 4-week period. A vehicle control group of mice was exposed IT to HBSS. The intraperitoneal protocol consisted of IP sensitization with 25 µg MACA in 0.2 ml of 1.3% alhydrogel (aluminum hydroxide) followed 14 days later with an IT challenge (10 µg MACA/50 µl HBSS). Airway reactivity responsiveness to methacholine was assessed, serum and bronchoalveolar lavage fluid (BALF) samples were obtained, and the lungs were fixed for histopathology at 1, 3, and 8 days following the last MACA IT challenge. Both groups exhibited immune and pulmonary responses typical of allergic asthma. In general, local responses in the lung, including inflammatory responses (eosinophils, lymphocytes, and macrophages), BALF IgE, and functional responses to

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; S.E., standard error of the mean.

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methacholine were greater in the IT sensitized group compared to the IP sensitized group, whereas the systemic IgE response was greater in the IP sensitized group. The BALF IL-5 cytokine levels were elevated before and throughout the eosinophil influx. IL-4 was detected in the BALF of IP sensitized, but not IT sensitized mice. Histopathologic changes in the two groups were similar in nature but more severe in the IT mice. The results suggest that the IP sensitization protocol does not induce the level of respiratory responsiveness that results from sensitization by a physiologically relevant route of exposure. Thus total serum IgE levels, which were greater following IP sensitization, may not be the best indicator of allergen potency, at least with respect to respiratory responses. © 2000 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Biopesticide; *Metarhizium anisopliae*; Allergic airways

1. Introduction

A number of microorganisms have been developed for use as biopesticides. These agents, which are non-pathogenic for humans, are desirable alternatives to more toxic chemical pesticides. However, they do contain potential allergens. Test methods are needed to assess this possibility, identify allergenic components, and rank their relative potency against proteins known to produce allergic airway disease in humans. The entomopathogenic fungus *Metarhizium anisopliae* is a prototype organism to study allergic potential and to develop test methods to assess other microbial pesticides or protein contaminants. *M. anisopliae* is marketed for indoor use to control cockroaches in the United States and has been studied as a pest control agent for a variety of insects (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).

We have recently shown that BALB/c mice intraperitoneally (IP) sensitized with *M. anisopliae* crude antigen (MACA) in the presence of adjuvant followed by intratracheal (IT) challenge induces immune responses, pulmonary inflammation (Ward et al., 1998), airway hyperresponsiveness, and lung pathology (Ward et al., 2000) consistent with allergic airways disease. Systemic sensitization with adjuvants, which has been used extensively in the development of animal models for allergenicity (Tamura et al., 1986; Ohmori et al., 1995; Watanabe et al., 1995; Wilder et al., 1999), is logistically simpler and faster than sensitization by the respiratory route. However, this sensitization protocol may not accurately reflect

responses to exposure by the more environmentally relevant respiratory route. In particular systemic sensitization with adjuvant was perceived to be a more aggressive protocol that might result in allergic responses that would not occur following sensitization by the respiratory route. To this end several investigators have developed sensitization protocols that rely strictly on the respiratory route and avoid the use of adjuvant (Ritz et al., 1993; Kawabata et al., 1996; Sarlo et al., 1997; Robinson et al., 1998).

This study was designed to address this concern with respect to MACA sensitization. The objective of this study was to compare responses in mice to MACA by two exposure protocols: (1) exclusive respiratory exposures without adjuvant; and (2) intraperitoneal sensitization in the presence of adjuvant followed by IT challenge.

2. Materials and methods

2.1. Animals

Female BALB/c mice (Charles River, Raleigh, NC), 7 weeks old, 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 1 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). Proteins 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 (Fig. 1)

The three groups of 18 mice each were treated as follows.

1. IP mice were intraperitoneally (IP) sensitized with 25 µg MACA in 0.2 ml of 1.3% alhydrogel (Superfos Biosector: aluminum hydroxide gel adjuvant; Accurate, Westbury, NY) followed 14 days later with an IT challenge (10 µg MACA/50 µl HBSS). Optimal sensitization and challenge doses and exposure protocol were chosen based on dose response information obtained in an earlier study (Ward et al., 1998).

2. IT mice were intratracheally (IT) exposed 4 × over a 4-week period to 10 µg MACA in 50 µl HBSS by a modification of the methods of Hatch et al. (1981) as previously described in Ward et al. (1998).
3. Control mice (vehicle control) were IT exposed 4 × over a 4-week period to HBSS. Several other potential controls IP treated with vehicle, adjuvant alone or chitin media proteins in the presence of adjuvant or no treatment (naïve) were omitted because previous studies (Ward et al., 1998, 2000) have demonstrated that MACA IT challenge of these controls resulted in non-specific responsiveness on day 1 post challenge only. Specific responses were never observed in these controls and non-specific response did not occur on subsequent days post challenge.

Airway reactivity hyperresponsiveness to methacholine was assessed 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. Serum was assayed for total IgE. BALF samples were assayed for total IgE and cytokines.

2.4. Airway responsiveness to methacholine

Animals were anesthetized with 1.5 g urethane/kg body weight, tracheostomized, and intubated with a T shaped cannula adapted to measure airway opening pressure and flow. The rate of

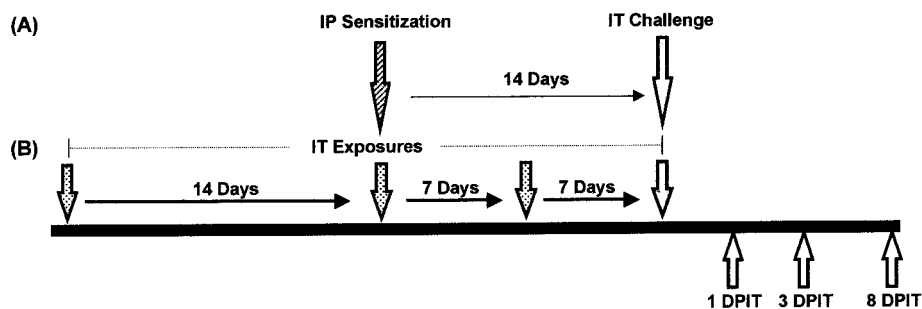


Fig. 1. Time line for experimental design. Mice (A) were administered MACA in aluminum hydroxide adjuvant IP followed by a MACA IT challenge (IP group) or (B) were administered MACA (IT group) or HBSS (control group) by IT. Airway responsiveness, pulmonary inflammation, and IgE levels were determined 1, 3, and 8 days post IT challenge (DPIT).

ventilation, controlled by the speed of the switching solenoids, was determined for each animal based on allometric scaling of body weight versus 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. Airway opening pressure (P_{ao}) was monitored directly and total respiratory system resistance (R_T) and compliance (C_T) were calculated using a custom-written software program as described previously (Gavett et al., 1999). Briefly, R_T and C_T were calculated for each breath using the constant flow inflation single compartment model (Bates et al., 1985) from the y -intercept coefficient of pressure versus volume (PV) array divided by isoflow (R_T), and the inverse of the slope of the PV array (C_T). Prior to the first methacholine (Mch) challenge, a stable baseline was recorded for 2 min. Subsequently, the mice were challenged with four doubling doses of Mch (50 μ g to 400 μ g/kg body weight) delivered as a bolus over 1 s via a jugular cannula connected to an electronically controlled syringe pump containing methacholine (0.1 mg/ml). A 2-min recovery period was allowed between each dose. The maximal change in R_T or P_{ao} following each dose is expressed as percent increase over baseline immediately prior to each dose (e.g. $[(\text{peak } R_T - \text{baseline } R_T)/\text{baseline } R_T] \times 100$), while the maximal change in total respiratory system compliance (C_T) is expressed as percent decrease from baseline prior to each dose $[(\text{baseline } C_T - \text{minimum } C_T)/\text{baseline } C_T] \times 100$.

2.5. Bronchoalveolar lavage (BAL) and blood collection

Following measurement of airway responsiveness, blood and BAL samples were collected as previously described (Ward et al., 1998). Briefly, blood samples were collected by cardiac puncture using a 23-gauge 1-inch needle and stored at room temperature for 1–2 h prior to centrifugation. Serum was stored at -70°C . After severing the diaphragm and rib cage and exposing the trachea,

a blunt 24-gauge needle was inserted into the trachea and tied in place. The lungs were lavaged twice with 1-ml aliquots of HBSS. The BAL fluid (BALF) aliquots were pooled and stored on ice. Total cell counts were performed using a haemocytometer and viability assessed by trypan blue exclusion. Cytospin preparations of 100–150 μ l BALF were made by centrifugation onto glass slides (Shandon Cytospin: 200 rpm, 15 min). Following Wright-Giemsa (Fisher Scientific) staining, cells were differentially counted at 200 cells per slide (one slide per animal). The BALF was centrifuged at 1200 rpm for 15 min at 4°C to pellet the remaining cells. Aliquots of BALF supernatant were assayed for total protein and LDH, and the remainder was stored at -20°C for IgE and cytokine assays.

2.6. Total IgE, IL-4, and IL-5 ELISAs

2.6.1. Total IgE ELISA

Reagents and incubation periods were at room temperature to avoid uneven and non-specific binding. Certified 96-well microtiter plates (Costar, Cambridge, MA) were coated with rat anti-mouse IgE (Pharmingen, San Diego, CA) at 2.5 μ g/ml in phosphate buffered saline (PBS) pH 7.3 (Pharmingen) sealed, and incubated overnight at 4°C . The plates were washed three times between each incubation with PBS and 0.5% polyoxyethylene-sorbitan monolaurate 20 (Tween 20; Sigma, St. Louis, MO). To block non-specific binding, 200 μ l of PBS plus 1% BSA was added to plates. After 30 min, plates were washed as described above and mouse serum or BALF samples diluted in blocking buffer, negative controls (blocking buffer only), and mouse IgE standard (positive control) (monoclonal anti-trinitrophenol (TNP); Sigma), in 2-fold dilutions from 800 to 1.5625 μ g/ml, were applied. Following a 1-h incubation and wash (as described above), biotinylated rat anti-mouse IgE (Pharmingen) was added to all wells at a concentration of 2.5 μ g/ml in blocking buffer. After a 1-h incubation and six washes, 0.25 μ g/ml of streptavidin alkaline phosphatase (Kirkegaard and Perry Laboratories, Gaithersburg, MD) was added and the plates incubated for 30 min. Following six washes, phos-

phatase substrate (p -nitrophenyl phosphate disodium; Sigma 104, Sigma), dissolved in substrate buffer (768.7 mM Tris (hydroxymethyl) aminomethane, 2 mM $MgCl_2 \cdot 6H_2O$, and 0.5 mM $ZnCl_2$, pH 8.2) was added to the plates (incubation for at least 20 min). Optical density was read on a Thermomax[®] Plate Reader (Molecular Devices, Menlo Park, CA) at a wavelength of 405 nm. Softmax Pro[®] version 2.6.1 (Molecular Devices) software was used for data collection and conversion from optical density to protein concentrations.

2.6.2. IL-4 ELISA

Plates were coated overnight with rat anti-mouse IL-4 (R&D Systems, Minneapolis, MN) in PBS. Plates were washed as described above and subsequently blocked with blocking buffer (1% BSA and 5% sucrose in PBS) for 1 h. BALF samples diluted with assay buffer (0.1% BSA, 0.05% Tween 20 in Tris buffered saline, pH 7.3) and IL-4 standards (purified recombinant mouse IL-4; R&D Systems, Minneapolis, MN), in 2-fold dilutions from 2000 to 3.906 pg/ml, were applied. Following a 2-h incubation and washes the biotinylated second antibody (biotin-goat-anti-mouse IL-4; R&D Systems) was added and incubated for 2 h. Streptavidin-horseradish peroxidase (HRP) (Zymed, South San Francisco, CA) was added to the plates and they were incubated for 20 min. Subsequently, the substrate tetramethylbenzidine (TMB, Dako, Carpinteria, CA) was added and incubated for 20–30 min in the dark. The optical density was read at 650 nm. Softmax Pro[®] version 2.6.1 software was used for data collection and conversion from optical density to protein concentrations.

2.6.3. IL-5 ELISA

The IL-5 ELISA was performed as previously described (Ward et al., 2000). Briefly, microtiter plates were coated with anti-mouse IL-5 (PharMingen) in a carbonate-bicarbonate pH 9.4 buffer (BupH buffer pack, Pierce, Rockford, IL). The biotinylated detection antibody (biotin-anti-mouse rIL-5; R&D Systems) was diluted in

Tris buffered saline + 0.1% BSA. The IL-5 standard (purified recombinant mouse IL-5; PharMingen) was in 2-fold dilutions providing a range from 480 to 1.875 pg/ml. Streptavidin-HRP (Zymed) and substrate were applied and optical density determined as described above. Softmax Pro[®] version 2.6.1 software was used for data collection and conversion from optical density to protein concentrations.

2.7. Total protein and lactate dehydrogenase (LDH) assays

BALF samples were assayed for total protein using Pierce Coomassie Plus Protein Assay Reagent (Pierce). Concentrations were determined from a standard curve using BSA standards obtained from Sigma. Additionally, the BALF samples were assayed for LDH using a commercially prepared kit and controls from Sigma. The assay reagent proportions remained the same but volumes were reduced for use on the Cobas Fara II Centrifugal Spectrophotometer (Hoffman-LaRoche, Branchburg, NJ).

2.8. Histopathology

Following BAL, the lungs were fixed in 10% formalin (phosphate buffered), embedded in paraffin, and sectioned. One slide per mouse was prepared by sectioning the lung along the main stem bronchus and staining with hematoxylin-eosin solution prior to pathological analysis. The entire lung was examined at several magnifications. The relative degree of severity of inflammatory, degenerative, and proliferative changes was graded on a scale of 1 to 5: 1 = minimal, 2 = slight/mild, 3 = moderate, 4 = moderately severe, 5 = severe/high. The pathology scores for lung sections were determined as follows: incidence is the percent of mice with the lesion, and severity indices are determined by dividing the sum of the numerical scores (1–5) for a particular lesion by the number of animals examined (Experimental Pathology Laboratories, Research Triangle Park, NC). Diffuse alveolar macrophage accumulation

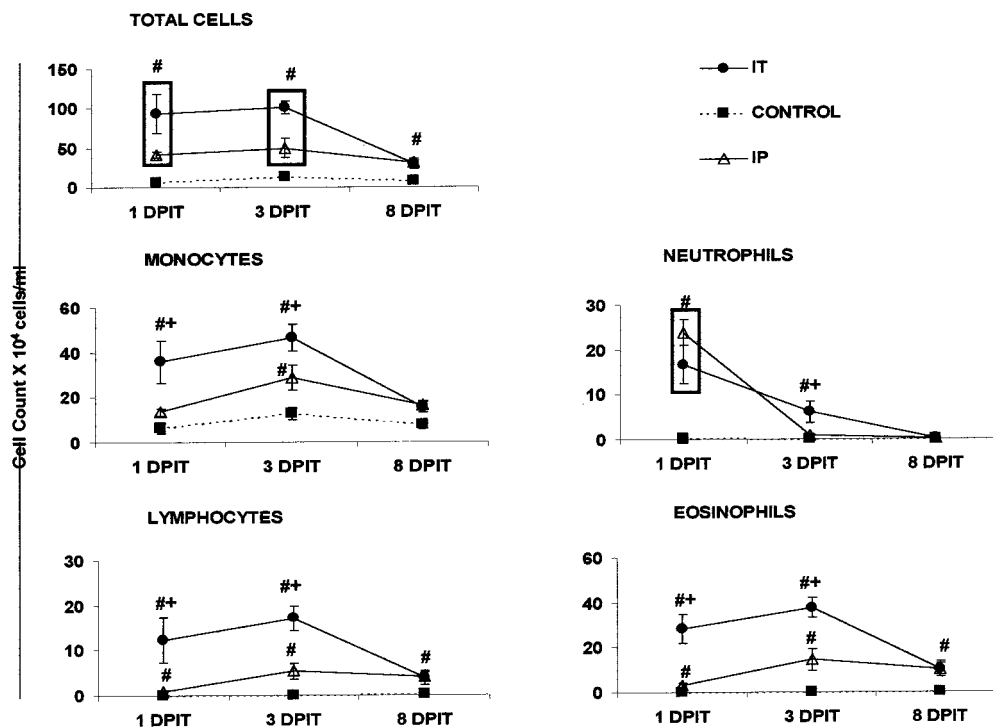


Fig. 2. BALF total and differential cell counts. Data shown represent mean $\pm$ S.E. Significant difference from (+) IP sensitized and (#) control mice at $P < 0.05$. Boxed data points share significant difference. $n = 3-5$.

consisted of activated macrophages present in alveolar air spaces in a large area of the lung parenchyma. Alveolitis consisted of focal thickening of alveolar septae, often with inflammatory cells in the affected alveolar spaces. Edema was diagnosed when there was a distinct distended area surrounding vessels that contained proteinaceous fluid.

2.9. 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 less than 0.05. Adjustments in significance levels, for multiple comparisons, were made using a modified Bonferroni correction.

3. Results

3.1. Total and differential cell counts

Although both MACA sensitized groups exhibited an influx of inflammatory cells (monocytes, neutrophils, eosinophils) following challenge, in general immune mediated cellular influx (lymphocytes, eosinophils) in the IT sensitized group was significantly higher than in the IP/adjuvant sensitized group (Fig. 2). Neutrophil responses were roughly equal at 1 DPIT and were reduced in both groups by 3 DPIT. However, the response in the IT sensitized group resolved more slowly and counts were significantly different from IP and control mice at 3 DPIT. The IT neutrophil count was 6-fold higher than IP and control neutrophil counts. Both treatment groups exhibited significantly increased lymphocyte, monocyte, and eosinophil numbers compared to controls at both 1 and 3 DPIT, but these re-

sponses were all significantly greater in the IT sensitized group. The comparison of IT to IP lymphocyte, monocyte, and eosinophil counts represented 12-, 2.6-, and 9-fold increases at 1 DPIT and 3-, 1.6-, and 2.6-fold increases at 3 DPIT, respectively. While there was considerable resolution of these responses by 8 DPIT, lymphocyte and eosinophil counts were still significantly higher than controls and there was no difference in the cell numbers of the two MACA treated groups at this time point.

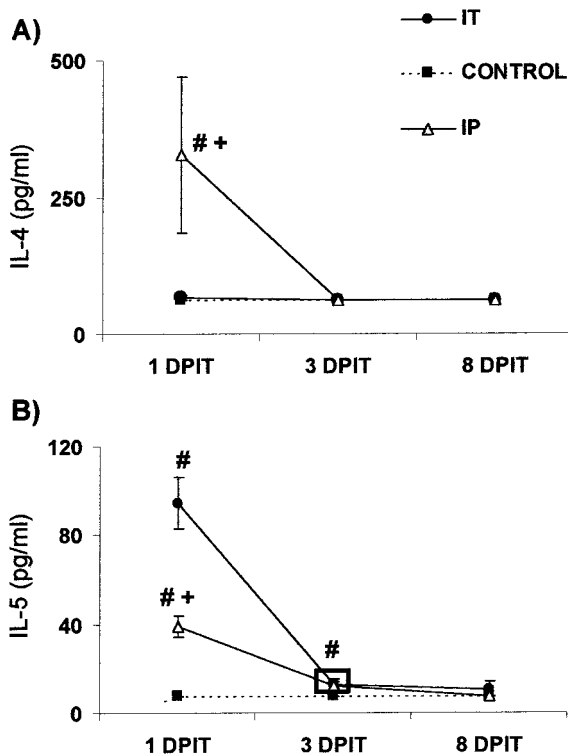


Fig. 3. BALF cytokines measured by ELISA. (A) IL-4: samples were assayed at 1:2 dilution with the assay level of detection at 31.25 pg/ml. (B) IL-5: samples were assayed undiluted with the assay level of detection at 7.81 pg/ml. Data shown represent mean $\pm$ S.E. (#) Indicates significant difference from control mice and (+) from exclusively IT exposed mice at $P < 0.05$. Boxed data points share significant difference. $n = 3-6$.

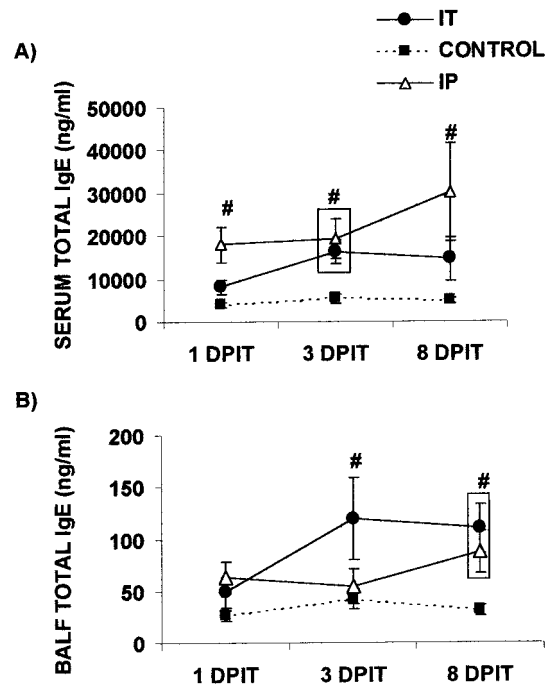


Fig. 4. Serum (A) and BALF (B) total IgE. Data shown represent mean $\pm$ S.E. Significant difference from (#) control mice at $P < 0.05$. Boxed data points share significant difference. $n = 3-6$. The effective level of detection was 500 ng/ml for serum samples and 50 ng/ml for BALF samples.

3.2. Cytokines

IL-4 levels for the IP mice were dramatically elevated at 1 DPIT (Fig. 3A) and then fell to background levels by 3 DPIT. In contrast, IL-4 levels in the IT and control were at or near the level of detection at all time points. IL-5 (Fig. 3B) levels were significantly increased in both IT and IP mice compared to controls at 1 DPIT. IT sensitized mice secreted significantly more IL-5 than IP mice (2.4-fold higher). By day 3 IL-5 levels were reduced but significantly higher than control levels.

3.3. Serum and BALF total IgE

IP mice had significantly elevated levels of serum total IgE at all three time points when compared to the HBSS controls (Fig. 4A). For the IT mice serum total IgE reached the level of

significance only on day 3. BALF total IgE was significant at 8 DPIT for IP sensitized mice (Fig. 4B). In contrast, BALF total IgE in IT mice was significant at both 3 and 8 DPIT.

3.4. Total protein and LDH levels

Both sensitization protocols caused significantly elevated BALF total protein and LDH levels compared to HBSS treated controls at 1 DPIT (Fig. 5). However, only the animals that were exposed exclusively by the IT route maintained significantly elevated levels at 3 DPIT. By 8 DPIT both the total protein and LDH had returned to near normal levels.

3.5. Airway responsiveness

3.5.1. Airway pressure

Both IP and IT mice demonstrated increased

airway opening pressure compared to controls in response to Mch challenge, particularly at the higher challenge doses (Fig. 6A). There was no statistical difference in airway pressure between IT and IP mice.

3.5.2. Compliance

Respiratory system compliance was significantly reduced in both IT and IP mice compared with control mice at all three time points (Fig. 6B). On day 3 the reduction in compliance was significantly greater in IT mice compared with IP mice at all doses.

3.5.3. Airway resistance

There were no significant differences between the treatment groups with the exception of the higher Mch doses on day 3 (Fig. 6C). On day 3 the IT mice were not as responsive to Mch challenge as were the IP mice (200- and 400- μ g Mch doses) or the control mice (400- μ g Mch dose).

3.6. Histopathology

Lung lesions were present in all groups. The mice were lavaged before excision and fixation of the lungs. While even the gentlest lavage could result in some lung damage, the HBSS control mice had limited lesions at any time point; with the exception of perivascular mononuclear infiltrate (Table 1), these lesions were not consistent within the group. This suggests that the lavage had minimal effect on the lung pathology in the MACA treatment groups. Lesions in the IT and IP sensitized mice were similar but more severe in the IT sensitized mice (Table 1, Fig. 7). The severity and incidence of most lesions in each group decreased over time. Table 1 summarizes the incidence and severity of the more significant lesions. Fig. 7 shows a comparison of representative lesions of the IT and IP groups, particularly as it pertains to perivascular and peribronchial cellular infiltrates. At 1 DPIT the IT mice appear to have a more extensive cellular infiltrate than the IP mice. By day 3 the cellular infiltrate has dissipated somewhat in the IT mouse but is more extensive in the IP mouse. The lesions appear similar in both exposure protocols 8 days following challenge.

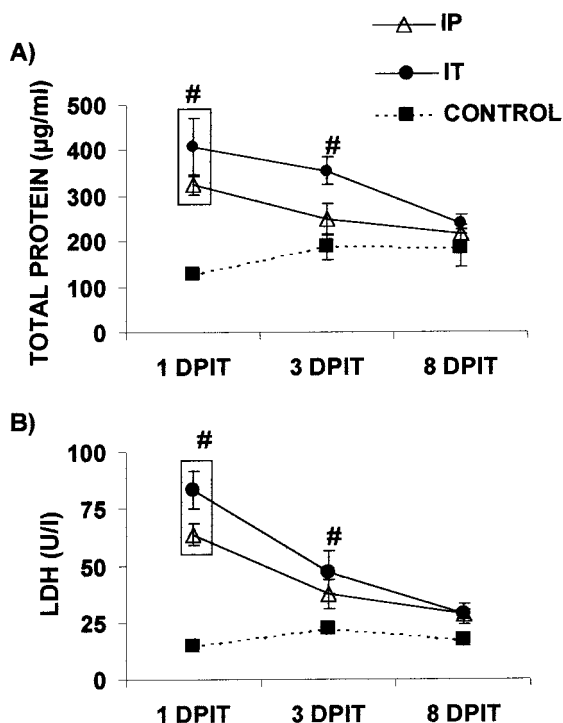


Fig. 5. BALF total protein (A) and LDH (B). Data shown represent mean $\pm$ S.E. (#) Significant difference from control mice at $P < 0.05$. Boxed data points share significant difference. $n = 4-6$.

Table 1
Summary of the incidence and severity of lung pathology^a

	1 DPIT			3 DPIT			8 DPIT		
	Incidence (%) (severity)			Incidence (%) (severity)			Incidence (%) (severity)		
	IP	IT	HBSS	IP	IT	HBSS	IP	IT	HBSS
Alveolar macrophage (diffuse accumulation)	33% (0.3)	100% (1.5)		25% (0.3)	100% (1.0)			25% (0.3)	
Alveolitis	100% (1.0)	25% (0.5)		25% (0.3)				50% (0.5)	25% (0.3)
Edema		25% (0.3)						25% (0.3)	
<i>Peribronchial</i>									
Eosinophil	67% (0.7)	100% (1.0)		50% (0.5)	100% (1.0)			50% (0.5)	
Neutrophil	67% (0.7)	100% (1.0)		100% (1.0)	67% (0.7)		75% (0.8)	25% (0.3)	
Mononuclear	100% (1.0)	100% (1.3)		100% (1.0)	67% (0.7)		100% (1.0)	50% (0.5)	
<i>Perivascular</i>									
Eosinophil	100% (1.0)	75% (1.0)		75% (0.8)	100% (1.0)			75% (0.8)	
Neutrophil	100% (1.0)	100% (1.0)		100% (1.0)	100% (1.0)		100% (1.0)	75% (0.8)	
Mononuclear	100% (1.3)	100% (1.8)	75% (0.8)	100% (1.0)	100% (2.0)	25% (0.3)	100% (1.0)	100% (1.3)	50% (0.5)

^a Mononuclear infiltrates were described as mainly lymphocytes with some macrophages. $n = 3-4$.

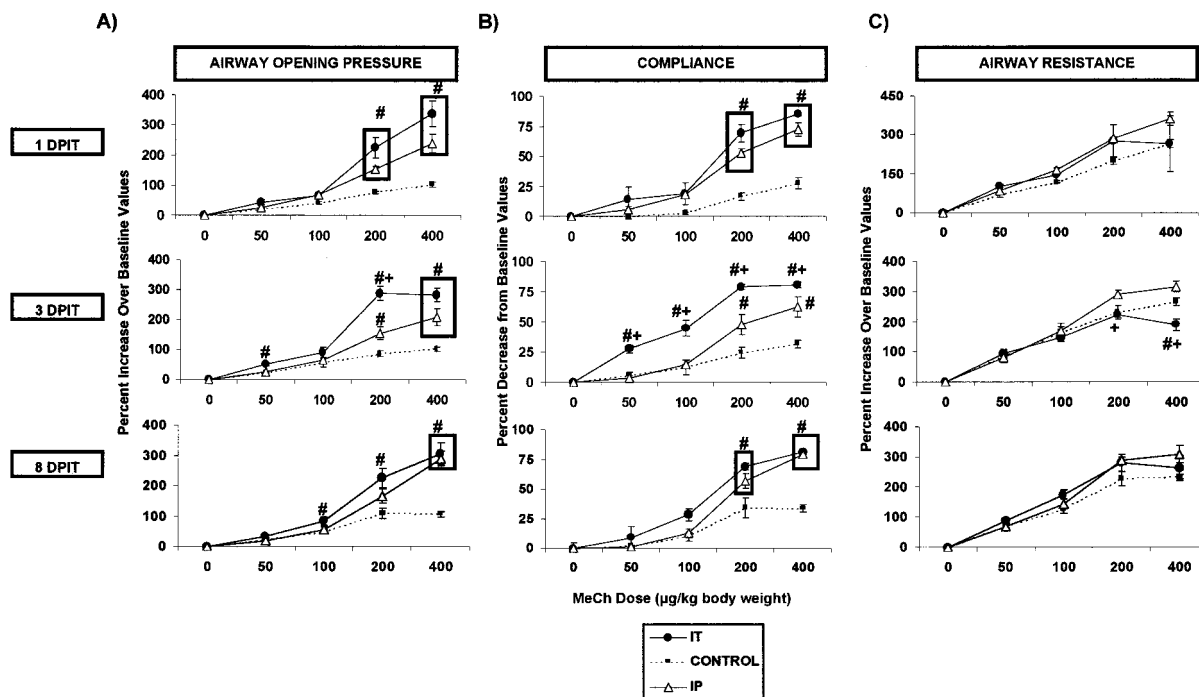


Fig. 6. Increases in airway opening pressure (A) and airway resistance (C) and decreases in compliance (B) relative to baseline response values. Data shown represent mean $\pm$ S.E. Significant difference (#) from control mice and (+) from IP sensitized mice at $P < 0.05$. Boxed data points share significant difference. $n = 3-5$.

4. Discussion

We have previously demonstrated that *M. anisopliae* crude antigen (MACA) contains components that induce immunologic, inflammatory (Ward et al., 1998), physiologic, and histopathologic (Ward et al., 2000) responses characteristic of allergy. A clear potential for allergenicity and induction of airway hyperresponsiveness by *M. anisopliae* was demonstrated. However, the IP/adjuvant sensitization protocol is not environmentally relevant. The current study extends these observations and demonstrates that mice sensitized by the more relevant respiratory route also demonstrate allergic responses to MACA. While the 10-µg IT dose is higher than what would be expected in a naturally occurring exposure, this dose is composed of a mixture of fungal proteins. Only a small fraction of these fungal proteins is detectable by IgE from MACA exposed mice.

Some interesting differences in responses of mice sensitized by the different protocols were noted.

When previously sensitized allergic asthmatics are subsequently exposed to the specific antigen, an immediate bronchoconstriction occurs (Orange et al., 1971). The cytotoxic antibody IgE mediates this immediate (Type I) hypersensitivity to allergens and is a major determinant in occupational and environmental respiratory diseases (Chan-Yeung and Lam, 1986; Pollart et al., 1988). We detected elevated levels of BALF IL-4 and serum and BALF total IgE in IP sensitized mice. Elevated IgE was also detected in BALF and serum of IT sensitized mice, but the serum response was less than in IP sensitized mice. However, the BALF IgE response was detected earlier (3 DPIT) in the IT sensitized mice. Interestingly, the BALF IL-4 response seen in IP mice is apparently absent in IT mice at the time points examined. There may be differences in the kinetics

and/or the magnitude of the IL-4 response with repeated IT exposures. If BALF IL-4 was produced following the earlier IT exposures of this protocol it would have been missed in the current sampling schedule. If the magnitude of the IL-4 response is greatly reduced with repeated IT exposures, the IL-4 secreted may all be bound or below the level of detection in the current assay. In addition to the importance of IL-4 in Th2 cell differentiation (Swain et al., 1990; Kopf et al., 1993), IL-4 initiates B-cell immunoglobulin class switching to IgE (Bergstedt-Lindqvist et al., 1988; Finkelman et al., 1988). While these events are important early in the disease process, IL-4 may no longer be required following repeated exposures.

Between 2 and 8 h after antigen exposure a more prolonged late phase reaction occurs, characterized by increased respiratory mucus hypersecretion, increased influx of inflammatory cells (eosinophils), and non-specific (pharmacological) airway hyperresponsiveness (Larson, 1985). Using both sensitization protocols, we observed elevated levels of IL-5, which is known 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), eosinophil viability (Kita et al., 1992; Ohnishi et al., 1993), and granule protein release (Kita et al., 1992). The IL-5 response was consistent with the eosinophil influx. Airway hyperresponsiveness characteristic of the late phase response was also observed in MACA sensitized mice. Recently, adoptive transfer experiments by Lambert et al. (1998) have shown that activated lymphoid cells rather than IgE play a significant role in this late phase response. Our data indicate that these late phase responses were greater following repeated respiratory exposure to MACA than in IP sensitized mice.

It has been suggested that the responses observed following IP sensitization with adjuvant might be exaggerated by what was perceived as a more rigorous sensitization protocol. Additionally, the IP route of sensitization is often criticized as not physiologically relevant. Several studies have proposed repeated six to eight IT or intranasal (IN) exposures as a more relevant means

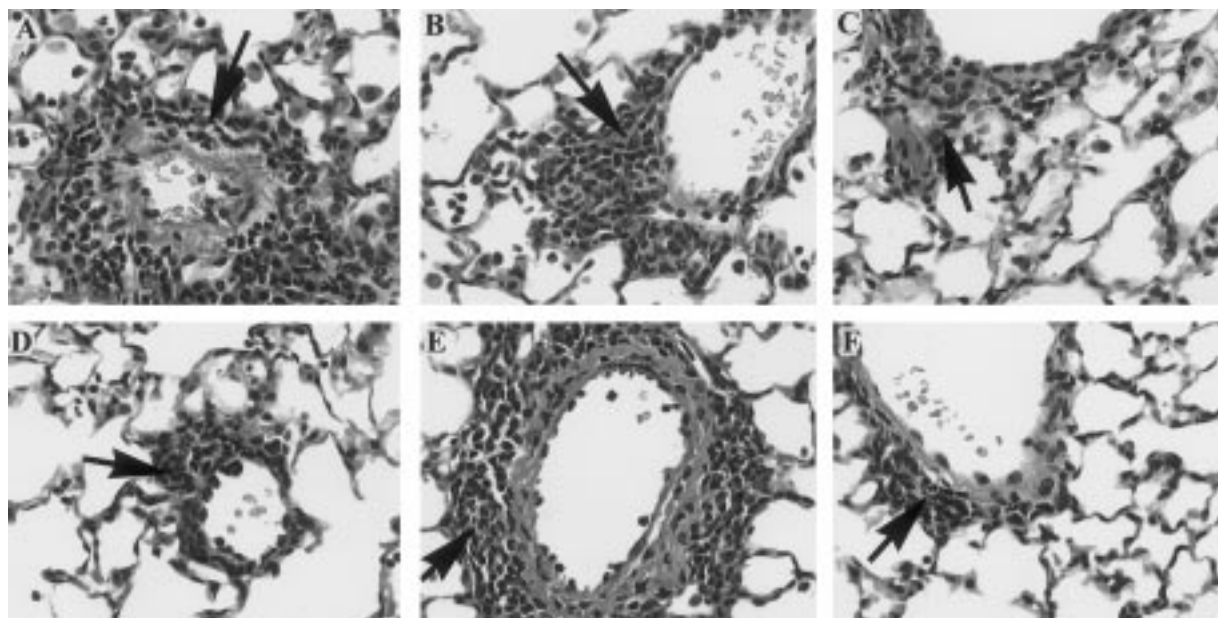


Fig. 7. Representative histopathology micrographs show perivascular and/or peribronchial infiltrates (arrows) in IT MACA sensitized and challenged mice (A–C) and IP/adjuvant MACA sensitized and IT challenged mice (D–F) at different time points. A and D at 1 day post challenge, B and E at 3 days post challenge, and C and F at 8 days post challenge. (400 × magnification)

of assessing the potential allergenicity of proteins (Ritz et al., 1993; Kawabata et al., 1996; Sarlo et al., 1997; Robinson et al., 1998). In this study we have demonstrated that four repeated IT exposures produced enhanced allergic and inflammatory responses in the lung compared to the responses from the IP protocol, whereas the serum total IgE response was greater in mice sensitized IP. The data suggests that indicators known to be associated with the immediate response were greater following IP/adjuvant sensitization, but late phase responses were greater following IT sensitization. Because we were unable to assess immediate airway responses in this study, no firm conclusion can be drawn.

The development of biological agents for pest control has been driven by the toxicity associated with many chemical pesticides. Therefore, test methods are needed to assess the allergic potential of microbial pesticides. In this study we have shown that IT exposure to *M. anisopliae* antigen extract induces immune-mediated inflammatory responses, airway hyperresponsiveness, and lung pathology typical of allergic disease. Therefore, an aqueous crude extract appears to be very useful for initial screening of biologics for an allergic response. The identification and subsequent purification of the immunodominant allergens will allow assessment of relative potency. It may also be informative to assess the relative potency of this extract compared with a benchmark allergen such as alcalase (Ritz et al., 1993; Sarlo et al., 1997). Our data suggest that the assessment of relative potency should not be limited to a comparison of serum IgE responses, but should also include an indicator of late phase responses. In the present study the magnitude of the serum total IgE response was not necessarily predictive of the magnitude of effects on airway hyperresponsiveness. This is not surprising since the latter appears to be cell-mediated rather than IgE-mediated (Lambert et al., 1998). In conclusion, with the exception of serum total IgE, responses to antigen challenge were greater in IT sensitized mice as compared to IP/adjuvant sensitized mice. This may reflect some compartmentalization of immune responses such that systemic sensitization results in better systemic responses and local sen-

sitization results in better local responses. Alternatively, repeated local exposures may be more effective in stimulating late phase responses.

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