



Assessment of the respiratory sensitization potential of proteins using an enhanced mouse intranasal test (MINT)



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ABSTRACT

There remains a need for a simple and predictive animal model to identify potential respiratory sensitizers. The mouse intranasal test (MINT) was developed to assess the relative allergic potential of detergent enzymes, however, the experimental endpoints were limited to evaluation of antibody levels. The present study was designed to evaluate additional endpoints (serum and allergic antibody levels, pulmonary inflammation and airway hyperresponsiveness (AHR)) to determine their value in improving the predictive accuracy of the MINT. BDF1 mice were intranasally instilled on days 1, 3, 10, 17 and 24 with subtilisin, ovalbumin, betalactoglobulin, mouse serum albumin or keyhole limpet hemocyanin; challenged with aerosolized methacholine or the sensitizing protein on day 29 to assess AHR, and sacrificed on day 29 or 30. Under the conditions of this study, evaluation of AHR did not improve the predictive power of this experimental model. Allergic antibody responses and IgG isotype characterization proved to be the most sensitive and reliable indicators of the protein allergenic potential with BAL responses providing additional insight. These data highlight that the evaluation of the respiratory sensitization potential of proteins can be best informed when multiple parameters are evaluated and that further improvements and refinements of the assay are necessary.

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1. Introduction

There remains a need for a simple and predictive animal model to identify compounds with the potential to induce respiratory sensitization and allergy (Blaikie and Basketter, 1999b). Respiratory sensitization is defined as an immune state which results in symptomatic respiratory disease or distress upon inhalation of an allergen to which an individual has been previously sensitized (Briatico-Vangosa et al., 1994). Clinically, respiratory sensitization is described as respiratory allergy, with allergic asthma and/or rhinitis the most common resulting manifestations (Briatico-Vangosa et al., 1994). In humans, the allergic response of the respiratory tract to inhaled protein or peptide antigens is associated with spe-

cific antibody responses, those of the immunoglobulin E (IgE) class being the most common, and it has been shown that IgE antibody-dependent mechanisms mediate the allergic respiratory hypersensitivity response to proteins (Briatico-Vangosa et al., 1994; Kimber et al., 1996). In addition to elevated serum IgE, respiratory sensitization is characterized by allergen-induced airway inflammation and airway hyperresponsiveness (AHR) (Taube et al., 2004). Allergen-induced airway inflammation is marked by an influx of leukocytes (especially lymphocytes, monocytes, neutrophils and eosinophils) into the airways (Meade and Woolhiser, 2002; Kraneveld et al., 2002). AHR is defined as an extreme bronchoconstrictor response elicited by exposure to a stimulus that would not affect a normal individual (Briatico-Vangosa et al., 1994; De Sanctis and Drazen, 1997).

The guinea pig has been a prominent model for researching respiratory allergy potential of proteins with intratracheal and inhalation models serving as tools for ranking the respiratory allergy potential of proteins (Robinson et al., 1998; Blaikie and Basketter, 1999b). The use of the guinea pig as a model has several advantages including the ability to display respiratory reactions similar to those observed in human allergic diseases (Briatico-Vangosa et al., 1994) and a well developed bronchial smooth muscle compartment which can respond vigorously to antigen and facilitates the detection of airway constriction in both early and late pulmonary hypersensitivity responses (Pauluhn and

Abbreviations: AB/PAS, Alcian Blue/Periodic Acid Schiff sequence; AHR, airway hyperresponsiveness; ANOVA, analysis of variance; BAL, bronchoalveolar lavage; BLG, beta-lactoglobulin; ELISAA, enzyme-linked immunosorbent assay; H&E, hematoxylin and eosin; HRP, horseradish peroxidase; IACUC, Institutional Animal Care and Use Committee; KLH, keyhole limpet hemocyanin; MCh, methacholine; MHC, major histocompatibility complex; MINT, mouse intranasal test; MSA, mouse serum albumin; MV, minute volume; OVA, ovalbumin; PCA, passive cutaneous anaphylaxis; Penh, enhanced pause; SUB, subtilisin; TV, tidal volume; WBP, whole-body plethysmography.

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Mohr, 2005). Furthermore, the pulmonary inflammatory response is comprised of both eosinophilic and neutrophilic infiltrates, similar to that developed by humans. The use of the guinea pig model, however, has certain drawbacks including long experimental timelines, the lack of species-specific reagents and differing allergic antibody isotypes when compared to humans (Briatico-Vangosa et al., 1994; Robinson et al., 1998; Blaikie and Basketter, 1999b). Due to these limitations, researchers have explored the use of mice as an experimental model, which led to the development of an intratracheal dosing approach and the mouse intranasal test (MINT). Both have been applied for the assessment of the relative allergic potential of detergent enzymes (Kawabata et al., 1996; Robinson et al., 1996, 1998). However, these assays were reported to have several deficiencies in predictive accuracy, when compared to results produced with guinea pig models, which have limited their broader use and acceptance (Blaikie and Basketter, 1999b).

The MINT developed by Robinson et al. (1996, 1998) included an assessment of allergenicity (although limited) as it evaluated total and enzyme-specific antibody levels as endpoints. As stated previously, the components of the immune-mediated airway sensitization response include elevated IgE antibody levels, as well as inflammation of the airways and AHR. The goal of the present study was to evaluate a mouse model which examined each of these endpoints in an effort to determine their value as enhancements to the MINT assay to help improve the specificity and predictive accuracy of this model for the identification of proteins with the potential to induce respiratory allergy. The investigation involved sensitization and challenge with several proteins of varying allergic potential and evaluation of the subsequent airway inflammation (measured via total and differential leukocyte counts of bronchoalveolar lavage (BAL) fluid and pulmonary histopathology), airway hyperreactivity (measured via whole-body plethysmography), and serum immunoglobulin response.

2. Materials and methods

2.1. Animals

Female BDF1 mice, 9–10 weeks of age, were used in this study (Charles River Laboratories, Inc., Portage, Michigan). This strain was selected as it has shown to respond to allergenic stimuli with a strong TH₂-type (IgE/IgG1-mediated) immune response and to accurately predict human responses to detergent enzymes (Robinson et al., 1996). To minimize the number of animals used, only female mice were evaluated as no gender differences in respiratory sensitization have been observed. Mice were housed one per cage in stainless steel wire bottom cages with LabDiet Certified Rodent Diet (PMI Nutrition International, St. Louis, Missouri) and water provided *ad libitum*. All mice were fed and handled in compliance with standards set forth by the U.S. Animal Welfare Act and recommendations in the National Institutes of Health "Guide for the Care and Use of Laboratory Animals". The procedures performed on the mice were reviewed and approved by a veterinarian and the Institutional Animal Care and Use Committee (IACUC).

2.2. Test substances

The selected proteins were chosen to cover a range of respiratory sensitization potencies. Subtilisin (SUB), a serine protease and known human respiratory sensitizer, ovalbumin (OVA), a strong food and respiratory allergen, beta-lactoglobulin (BLG), a moderate-weak allergen, and mouse serum albumin (MSA), a putative negative control, were all obtained from Sigma–Aldrich (St. Louis, Missouri). Keyhole Limpet Hemocyanin (KLH), a strong immunogen previously shown to elicit pulmonary immune responses, was obtained from Calbiochem (San Diego, California). Based on existing data regarding allergenicity, the expected rank order potency for the proteins was SUB > OVA ≥ KLH > BLG > MSA for each of the experimental endpoints (Boverhof et al., 2010). All test material dosing solutions were prepared in sterile pyrogen-free saline (Butler Animal Health Supply, Dublin, Ohio). Acetyl-β-methylcholine chloride (methacholine hydrochloride; MCh), a well-characterized bronchoconstrictor, was obtained from Sigma–Aldrich (St. Louis, Missouri).

2.3. Intranasal instillation

Intranasal instillations were accomplished by anesthetizing the mice with 3% Isoflurane (Butler Animal Health Supply, Dublin, Ohio) in oxygen and gently holding each animal in a supine position with the nose pointed slightly up and allowing the

mice to aspirate the instillate into the nasal airways as it was deposited as a series of fluid beads at the external nares. For intranasal instillations the dosing volume was 50 µl (25 µl/naris). This procedure is simple, reproducible, and results in exposure of both the upper and lower airway epithelium to the instillate (Hotchkiss et al., 1998; Massaro et al., 2004; Yang et al., 2004). This route of exposure has previously been used to research aspects of respiratory allergy for both low and high molecular weight compounds (Robinson et al., 1996; Farraj et al., 2003, 2004, 2006).

2.4. Experiment 1

A dosing regimen based on the work of Robinson et al. (1996) was followed. Female BDF1 mice ($n = 6$ /group) were intranasally instilled with saline (0 µg) or 0.05, 0.5, or 5.0 µg of SUB, OVA, BLG, or MSA in a total of 50 µl of pyrogen-free saline (25 µl/naris), on days 1, 3, 10, 17, and 24 of the study (Fig. 1). An additional group of six non-instilled mice were also included in this study to serve as naive controls. On test day 29, all animals received an aerosol challenge of MCh while being monitored for respiratory parameters. After measurement of a baseline exposure to air, mice received increasing concentrations of MCh (0%, 0.3%, 0.6%, 1.3%, 2.5%, 5% in saline). Each exposure step included a 3 min exposure to aerosolized MCh followed by a 5 min recovery period. After completion of the pulmonary function testing, animals were sacrificed and lung lavage samples were taken for BAL analysis and serum was taken for Passive Cutaneous Anaphylaxis (PCA) analysis.

2.5. Experiment 2

The dosing regimen from experiment 1 was followed. Female BDF1 mice ($n = 6$ /group) were intranasally instilled with saline (0 µg) or 0.5, 5, or 25 µg of OVA, BLG, or KLH in a total of 50 µl of pyrogen-free saline (25 µl/naris), on days 1, 3, 10, 17, and 24 of the study (Fig. 1). The top dose level was increased to 25 µg in an attempt to stimulate a more pronounced pulmonary response. For SUB, only the 0, 0.5 and 5 µg dose groups were evaluated as a 25 µg dose would produce excessive irritation. An additional group of six non-instilled mice were also included in this study to serve as naive controls. On test day 29, all animals within an exposure group (SUB, OVA, KLH or BLG) and their respective saline-instilled controls were exposed as a group for 10 min to an aerosolized 1% solution of their respective protein and then immediately transferred to individual whole-body plethysmography (WBP) chambers to monitor respiratory parameters for 120 min post-exposure. Groups of saline-instilled (0 µg protein/instillation) mice with no previous exposure to the test protein were similarly exposed to each protein aerosol. Mice were sacrificed on day 30 at which time lung lavage samples were taken for BAL analysis and blood from the abdominal aorta was collected to obtain serum for PCA, IgG1 and IgG2a analysis.

2.6. Respiratory parameters

Tidal volume (TV), minute volume (MV), and enhanced pause (Penh) were measured by whole-body plethysmography for each animal and these data were examined for their predictive value. Penh has been suggested as a predictive indicator of AHR following allergic sensitization in some strains of mice (Hamelmann et al., 1997). All animals were acclimated to the WBP chambers for 30 min and 60 min, respectively, on the 2 days prior to initiation of pulmonary function testing. On test day 29 all animals received an aerosol challenge and were monitored with a Buxco® WBP system (Sharon, Connecticut) for treatment-related changes in respiratory parameters. Penh was calculated as described by Hamelmann et al. (1997).

For experiment 1, following a 10 min baseline exposure to air, animals received increasing concentrations of MCh (0%, 0.3%, 0.6%, 1.3%, 2.5%, 5% in isotonic saline) for 3 min followed by a 5 min recovery period. For each MCh exposure concentration data were collected during both the 3 min exposure period and the 5 min recovery period. Qualitative assessment indicated that the maximal Penh, MV and TV response occurred during the 5 min recovery period following each MCh exposure, therefore, statistical analyses were performed on mean Penh, MV, and TV values for each experimental group during the recovery periods. For experiment 2, animals received a 10 min aerosol challenge to a 1% solution of the respective protein, followed by a 120 min post-challenge monitoring period. For analysis, the 120 min post-exposure response period was divided into 5 min intervals and the mean response for each animal within each 5 min interval was determined. Separate statistical analyses were performed on early phase (0–30 min) and late phase (90–120 min) responses.

2.7. BAL

Mice were anesthetized with isoflurane/O₂ and euthanized via exsanguination. The lungs of the euthanized mice were then lavaged with three 1 ml volumes of saline. The recovered bronchoalveolar lavage (BAL) fluid was immediately placed on ice until processed for analysis of total and differential cell counts. The total number of nucleated cells recovered in the BAL fluid was determined using a hemacytometer. Cell smears were made with a cytocentrifuge (Shandon Cytospin III, Thermo Electron Corporation, Pittsburgh, Pennsylvania) and stained with Diff-Quick (Sigma Chemical Co., St. Louis, Missouri). Differential cell counts for BAL fluid was

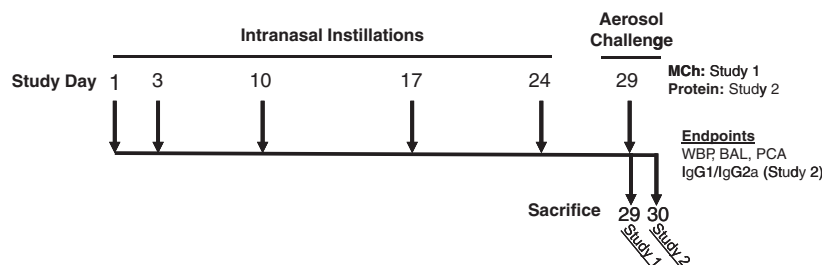


Fig. 1. Experimental design for sensitization and challenge studies. For both experiments, a dosing regimen based on the work of Robinson et al. (1996) was followed. In experiment 1, mice were intranasally instilled with saline or protein on days 1, 3, 10, 17, and 24. On test day 29 mice received an aerosol challenge of MCh while being monitored for respiratory parameters. After completion of the pulmonary function testing, animals were sacrificed and lung lavage samples were taken for BAL analysis and serum was taken for PCA analysis. In experiment 2, mice were intranasally instilled with saline or protein on days 1, 3, 10, 17, and 24. On test day 29, mice received an aerosol challenge of their respective induction protein while being monitored for respiratory parameters. Animals were sacrificed on day 30 and lung lavage samples were taken for BAL analysis and serum was taken for PCA analysis and determination of serum IgG1 and IgG2a.

performed by counting 200 cells per animal. The total number of each cell type recovered by lavage (e.g., neutrophil, macrophage, lymphocyte, eosinophil, or epithelial cell) was estimated by multiplying the total recovered cells per animal by the percentage occurrence of that cell type in the corresponding differential count. After lavage, the lung was inflated to approximately the normal inspiratory volume with 10% neutral buffered formalin, the trachea was ligated and the entire heart/lung block was transferred to a container with the same fixative until processed for histopathologic analyses.

2.8. Histopathology

Following fixation, the lungs of selected animals were processed for light microscopy by standard histologic techniques. Paraffin-embedded tissues were sectioned approximately six μm thick, stained with hematoxylin and eosin (H&E) or Alcian Blue (pH 2.5)/Periodic Acid Schiff sequence (AB/PAS) and examined via light microscopy. AB/PAS was used to identify both neutral and acidic mucosubstances stored within the cytoplasm of mucous cells in the nasal respiratory epithelium.

2.9. Passive cutaneous anaphylaxis assay

The passive cutaneous anaphylaxis (PCA) assay is recognized as a direct *in vivo* approach to characterize the specific allergic antibody response associated with systemic exposure to protein allergens (Dearman et al., 2003; Hilton et al., 1994). In the present experiments, serum samples from intranasally sensitized mice (neat or diluted in sterile saline) were pooled for each dose group and injected intradermally into the ears of naïve BALB/c mice (20 μl per site,) using 100 μl Hamilton Gastight[®] syringes (Hamilton Company, Reno, Nevada) fitted with a 0.5 in., 28 G, 10–12° beveled needle. Four to six naïve mice were evaluated per protein dose group. Twenty-four hours later, intradermally sensitized mice were injected intravenously (i.v.) via the tail vein with a saline solution containing 0.5% Evans Blue and 1 mg/ml of the protein of interest (SUB, OVA, BLG, MSA, or KLH). Thirty minutes following the i.v. injections, the development of blue cutaneous anaphylactic reactions at the sites of intradermal sensitization were evaluated and reactions that had dimensions of at least 3 mm in diameter were scored as positive reactions. Serum samples were tested at increasing dilutions for each protein and dose group to characterize the serum antibody titer with a sample size of 4–6 mice per group. In the case of positive PCA responses data were reported as the highest dilution that resulted in a positive response.

2.10. Determination of serum IgG1 and IgG2a

Specific IgG1 and IgG2a levels were measured using an enzyme-linked immunosorbent assay (ELISA) method similar to that described by Dearman et al. (2000). Briefly, Nunc microtiter plates were coated overnight with the protein of interest (5 $\mu\text{g}/\text{ml}$ or 50 $\mu\text{g}/\text{ml}$ for IgG1 and IgG2a determinations, respectively), in PBS (pH 7.4). The plates were blocked with 5% non-fat milk. An initial 10-fold dilution of each serum sample plus serial 2-fold dilutions were added and bound antigen-specific antibody was detected using diluted horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG1 (Southern Biotech 1070-05) or goat anti-mouse IgG2a (Southern Biotech 1080-05). For detection, tetramethylbenzidine substrate was added and plate absorbency determined at 450 nm (VersaMax[™] microplate reader, Molecular Devices, Sunnyvale, California). Regression curves were obtained and analyzed using SoftMax[®] Pro 4.0 software, Molecular Devices (Sunnyvale, California). The serum titer was defined as the reciprocal of the dilution that demonstrated an optical density value of 0.5. Values that did not reach an optical density of 0.5 were reported as the minimum dilution (10).

3. Statistical analyses

For experiment 1, saline-instilled and non-instilled controls for each BAL and pulmonary function parameter were compared using a *t*-test ($p \leq 0.05$). If no difference was observed, the two control groups were pooled. If a difference was detected, only saline-instilled control data were used for comparisons.

BAL, pulmonary function data and protein-specific antibody titers were first tested for equality of variance using Bartlett's test. If the results from Bartlett's test were significant at $\alpha = 0.01$, then the data for the parameter was subjected to a transformation to obtain equality of the variances. The transformations that were examined were the common log, the inverse, and the square root, in that order. The data were reviewed and an appropriate form of the data was selected. These data were then subjected to analysis of variance (ANOVA) for factors of protein, dose and time (pulmonary function data only). Significant differences were evaluated using a post hoc comparison procedure to identify the source of the variance. The level of significance was set at $p \leq 0.05$. Only descriptive data (means and standard deviations) were reported for PCA results (incidence of response and titers).

4. Results

4.1. Experiment 1

4.1.1. Respiratory parameters

Whole-body plethysmography was used to identify exposure-related effects of the instilled proteins on pulmonary function. On test day 29, pulmonary function testing was performed on all groups of mice. MCh, a well-characterized bronchoconstrictor, was used to assess protein-induced alterations in pulmonary function and general airway reactivity. Mice received increasing concentrations of aerosolized MCh (0%, 0.3%, 0.6%, 1.3%, 2.5%, 5% in saline) in a WBP system and Penh, TV and MV values were determined. MCh challenge resulted in a dose-dependent increase in Penh, however, when compared to saline-instilled control mice, there were no significant treatment related alterations in Penh for any of the proteins (data not shown). Examination of TV and MV responses revealed dose-related increases for SUB and MSA while OVA and BLG did not elicit any treatment-related effects on these parameters (data not shown). Saline-instilled controls exhibited increased Penh, TV and MV responses when compared to non-instilled controls which may have influenced the ability to detect protein-specific responses. Overall, under the conditions of this study, assessment of pulmonary function did not yield data that could differentiate between proteins.

4.1.2. BAL

Total and differential cell counts were performed on all BAL samples to assess the effect of repeated intranasal instillation of the test proteins on the types and numbers of pulmonary inflammatory cells. There were no significant differences in the total number or types of BAL cells recovered from saline-instilled and non-instilled controls, therefore, the data from these two groups were combined as a single control group ($n = 10$) for subsequent statistical analyses. Only mice instilled with 5.0 μg /instillation SUB had significantly elevated numbers of total BAL cells compared to controls (Fig. 2). The increase in total cells reflected statistically-significant increases in the number of macrophages, neutrophils and lymphocytes (Fig. 2). Repeated intranasal instillation of OVA, BLG or MSA had no effect on the types or numbers of pulmonary inflammatory cells.

4.1.3. Histopathology

Eosinophils were not observed in BAL from mice in any experimental group. This was unexpected given that eosinophilic inflammation is a hallmark of allergic airway disease and asthma. BAL provides data regarding the numbers and types of cells present within the airway lumen and alveolar air space of the lung, but may not accurately reflect the population of inflammatory cells present within the lung tissue. To assess treatment-related intramural inflammation and airway remodeling, lungs from animals instilled with the highest concentration (5 μg /instillation) of each of the proteins as well as saline- and non-instilled controls were fixed and processed for histopathologic examination (Table 1; Fig. 3).

Treatment-related morphologic alterations were observed in mice instilled with SUB, OVA or BLG (Table 1). The microscopic lesions were characterized by mucous cell hyperplasia/metaplasia within the airway epithelium with peribronchiolar and perivascular accumulations of inflammatory cells including eosinophils, mononuclear cells, and neutrophils. Intracellular eosinophilic inclusions, ranging from very slight to moderate in severity, were observed in surface epithelial cells located in regions of mucous cell hyperplasia/metaplasia in SUB- and OVA-instilled mice. Images highlighting these lesions in SUB animals in comparison to control animals are provided in Fig. 3. Based on the frequency and severity of the microscopic lesions the relative potency of the test proteins to induce eosinophilic inflammation and airway remodeling was SUB $\gg$ OVA $>$ BLG $\gg$ MSA.

4.1.4. PCA

The PCA assay was used to characterize the specific allergic antibody response after sensitization to the test proteins. The assay was conducted in naïve mice using pooled sera obtained from sensitized mice upon sacrifice on test day 29 and the results are listed in Table 2. Sera from SUB sensitized mice displayed modest positive responses at 0.05 and 0.5 μg dose levels, while sera from the 5 μg dose group yielded a robust response as indicated by a 100% incidence at a 1:512 dilution. Sera from OVA sensitized mice did not elicit a positive response at the 0.05 μg dose level, however, positive responses were observed for the 0.5 and 5 μg dose levels at dilutions of 1:11 and 1:27, respectively. Serum samples from BLG and MSA sensitized mice yielded negative PCA responses for all dose groups. Saline instilled and uninstilled control serum samples were tested concurrently and were also negative. Based on the results of this assay, the rank order potency for induction of specific allergic antibody is SUB $>$ OVA $>$ BLG = MSA.

4.2. Experiment 2

To address concerns identified in experiment 1 with respect to lack of differentiating responses for BAL and respiratory parameters,

experiment 2 explored the use of protein-specific challenge as a potential means to increase the specificity and magnitude of the response and enhance the predictive power of the assay (see materials and methods). The immunogenic neoantigen KLH was included in experiment 2. Nasal instillation dose levels were also increased to 0.5, 5 and 25 μg for each of the proteins in an effort to stimulate a more robust response. The exception was SUB for which only 0.5 and 5 μg were evaluated as a 25 μg dose would produce excessive irritation. Serum levels of IgG1 and IgG2a were also examined in this experiment to provide potential additional insights into the protein-induced immunological state.

4.2.1. Respiratory parameters

As in experiment 1, WBP was used to identify exposure-related effects on AHR and pulmonary functional parameters, however rather than using MCh challenge, specific protein challenge was used (Fig. 1). On test day 29, pulmonary function testing was performed on all groups of mice. Animals received a 10 min whole-body aerosol challenge to a 1% solution of the respective sensitizing protein, followed by a 120 min post-challenge monitoring period in WBP chambers where Penh, TV and MV values were determined. For purposes of data analysis and interpretation, the time-dependent changes in respiratory parameters were divided into early- and late-phase responses (early phase: 0–30 min following protein aerosol challenge; late phase: 90–120 min following protein aerosol challenge). Fig. 4 summarizes the time-dependent changes in Penh following a single aerosol challenge to the sensitizing proteins SUB, OVA, KLH and BLG. Compared to non-sensitized, saline-instilled, control mice exposed to the same 1% sensitizing protein aerosols, mice sensitized and challenged with SUB and OVA had both early- and late-phase increases in Penh ($\sim$ 1.7–/2.8-fold and 1.3–/1.4-fold increase in early/late phase responses, respectively; Fig. 5) but only in groups repeatedly instilled with the highest concentration of protein (5 μg and 25 μg per instillation, respectively). For BLG, mice instilled and challenged with 5 μg BLG/installation had a small but significant late-phase increase in Penh compared to late-phase Penh levels in non-sensitized, saline instilled mice challenged with 1% BLG aerosol. Aerosol exposure to 1% KLH had no effect on Penh values in any KLH sensitized group, compared to non-sensitized saline-instilled control mice exposed to the same KLH aerosol.

Examination of TV and MV responses revealed no dose-related changes for any of the proteins (data not shown). Under the conditions of this study, pulmonary function testing did not provide additional predictive strength to identify the sensitization potential of proteins.

4.2.2. BAL

Total and differential cell counts were performed on all BAL samples to assess the effect of an aerosolized protein challenge on the types and numbers of inflammatory cells in the airway and alveolar lumen of protein-sensitized/protein-challenged compared to non-sensitized/protein-challenged controls. Overall, BAL responses for experiment 2 were more pronounced than for experiment 1.

Sensitization to SUB, OVA or KLH by repeated intranasal instillations followed by protein aerosol challenge resulted in a statistically-significant alteration in the total numbers or types of cells recovered by BAL, when compared to saline-instilled mice exposed once to the same protein aerosol (Fig. 6). Differences in the magnitude and character of the inflammatory response between test proteins, as well as a dose-dependency (instilled dose) within each test protein group, were also observed.

Compared to saline-instilled mice exposed to aerosolized SUB, mice instilled with 5.0 μg SUB/installation had a statistically-identified increase (3.4-fold) in total BAL cells which was primarily due

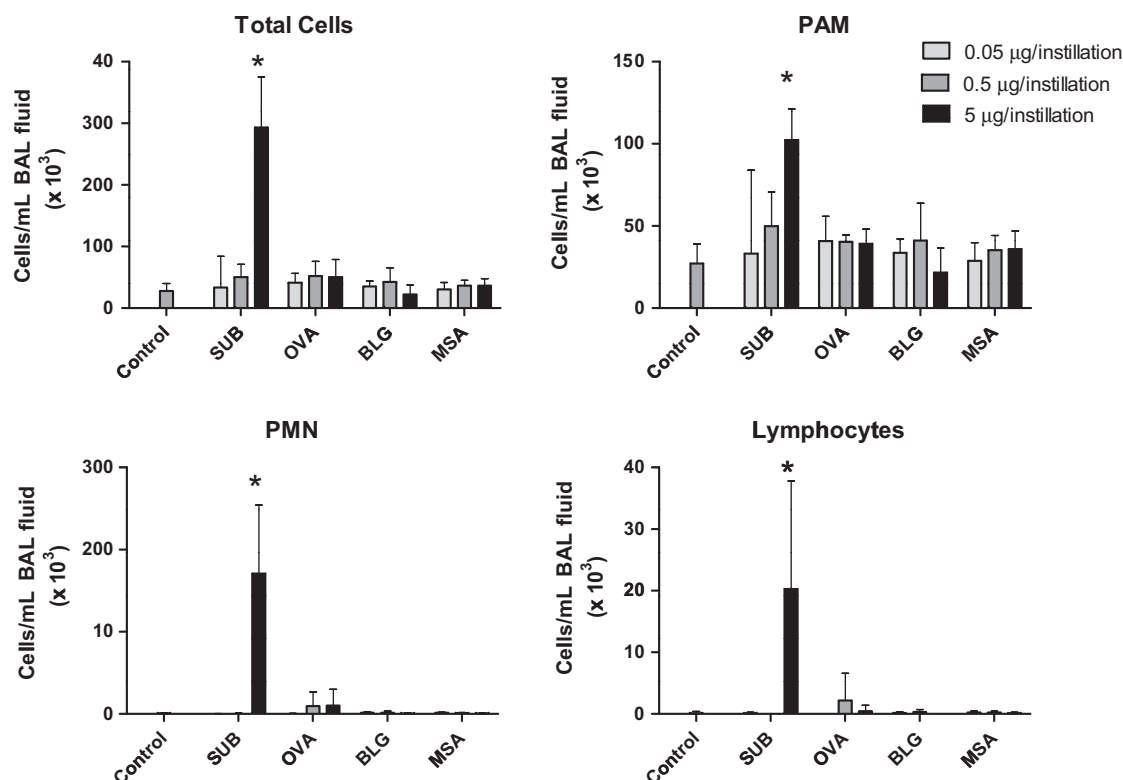


Fig. 2. Experiment 1 – total and differential cell counts in BAL fluid. Cell numbers in the BAL fluid were determined for each mouse in each protein dose group ($n = 4$ –6/dose group). Mice were intranasally instilled with SUB, OVA, BLG or MSA on test days 1, 3, 10, 17 and 24 and BAL fluid was obtained on test day 29 after MCh challenge. Control values represent the combined response of the saline-instilled and non-instilled controls ($n = 10$). Statistical significance relative to controls ($p \leq 0.05$) is indicated by *. PAM = pulmonary alveolar macrophage; PMN = polymorphonuclear neutrophil.

Table 1
Experiment 1 – histopathologic observations.

Treatment	Non-instilled controls	Saline-instilled controls	SUB	OVA	BLG	MSA
LUNG (number examined)	(6)	(6)	(6)	(4)	(5)	(6)
Infiltration, eosinophils and monocytes, increased, perivascular/peribronchial, multifocal						
– Very slight	0	0	0	2	2	0
– Slight	0	0	0	0	0	0
– Moderate	0	0	6	1	0	0
Infiltration, neutrophils, increased, perivascular/peribronchial, multifocal						
– Very slight	0	0	0	1	0	0
– Slight	0	0	4	0	0	0
– Moderate	0	0	2	0	0	0
Mucous cell hyperplasia/metaplasia, increased, airway epithelium, multifocal						
– Very slight	0	0	0	1	0	0
– Slight	0	0	0	0	1	0
– Moderate	0	0	6	1	0	0
Eosinophilic inclusions, increased, airway epithelium, multifocal						
– Very slight	0	0	1	0	0	0
– Slight	0	0	1	0	0	0
– Moderate	0	0	4	1	0	0

Bold values interpreted to be treatment related.

$n = 4$ –6/dose group based on sample suitability.

to a large, statistically-identified increase in BAL eosinophils (Fig. 6). Mice instilled with 0.5 µg SUB/institution had a statistically-significant decrease in total BAL cells as well as BAL neutrophils and eosinophils. This was explained by the fact that there was a statistically-identified increase in the total number and type of BAL cells recovered from saline-instilled (naïve) mice challenged with aerosolized SUB, when compared to unexposed control mice. Compared to unexposed control mice, saline instillation followed by challenge to aerosolized SUB resulted in an 11.5-fold increase

in the mean number of BAL cells. This reflected an increase in all cell types, but primarily neutrophils and eosinophils. These data highlight that a single challenge to a test protein can alter the number and types of inflammatory cells in BAL, which needs to be considered in the assignment of experimental control groups and the final interpretation of the data.

There was a dose-dependent increase in the total number of BAL cells recovered from OVA-instilled mice relative to saline-instilled OVA-challenged controls, but the increase in cell numbers

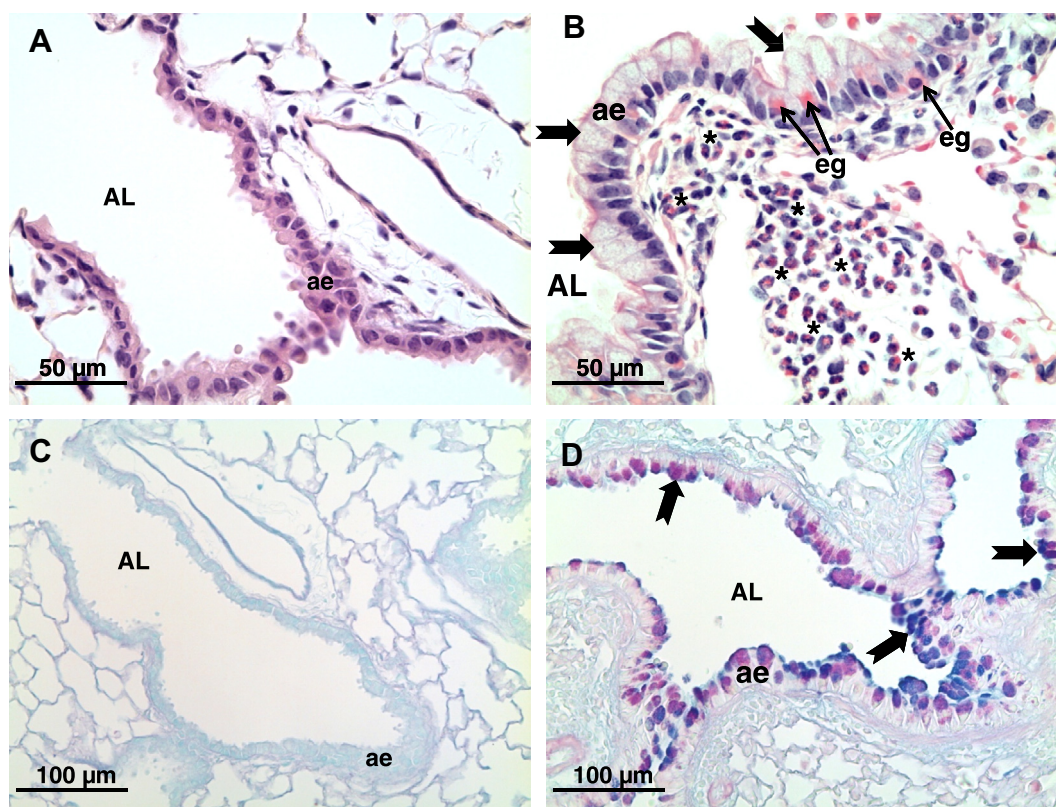


Fig. 3. Experiment 1 – comparison of pulmonary histopathological responses to saline and SUB. Images depict light photomicrographs of terminal bronchiolar airways in lungs of mice repeatedly instilled with saline (control; A and C) or SUB (5 μ g/instillation; B and D). Lung sections were stained with hematoxylin & eosin (A and B) for evaluation of tissue morphology or Alcian Blue (pH 2.5)/Periodic Acid Schiff's sequence (C and D) to detect acidic and neutral mucosubstances. AL = airway lumen; ae = airway epithelium; eg = eosinophilic globules in airway epithelial cells (hyalinosis). Thick arrows point to stored intraepithelial mucosubstances in mucous goblet cells. Thin arrows point to eosinophilic globules (eg) present in some airway epithelial cells (hyalinosis). Peribronchiolar/perivascular accumulations of eosinophils are marked by an asterisk (*). Images for SUB are provided as a representative example of the identified lesions relative to saline controls. Similar but less pronounced effects were noted for OVA and BLG instilled animals.

Table 2

Experiment 1 – specific reagenic antibody responses determined using passive cutaneous anaphylaxis assay^a.

Dose group	SUB		OVA		BLG		MSA	
	Dilution ^b	Incidence (%) ^c	Dilution	Incidence (%)	Dilution	Incidence (%)	Dilution	Incidence (%)
Uninstilled	Neat	0	Neat	0	Neat	0	Neat	0
Saline	Neat	0	Neat	0	Neat	0	Neat	0
0.05 μ g	1:4	33	Neat	0	Neat	0	Neat	0
0.5 μ g	Neat	50	1:11	50	Neat	0	Neat	0
5 μ g	1:512	100	1:27	100	Neat	0	Neat	0

^a Sera were obtained on test day 29 following intranasal instillation of proteins on test days 1, 3, 10, 17 and 24 and MCh exposure on day 29.

^b Data expressed are the maximum dilution of sera tested; neat indicates undiluted serum was tested.

^c Incidence represents the percentage of mice responding at the corresponding dilution with 4–6 naïve mice per dose group.

only reached the level of statistical significance in mice instilled with 25 μ g/instillation. The dose-dependency of prior OVA instillations was reflected in significant increases in neutrophils, lymphocytes and eosinophils (Fig. 6). Mice repeatedly exposed to 5.0 or 25.0 μ g KLH/instillation had significant increases in total BAL cells that were primarily the result of large, statistically-significant increases in the number of eosinophils in the BAL fluid. Only 5.0 μ g KLH-instilled mice had a significant increase in the number of pulmonary alveolar macrophages, when compared to saline-instilled KLH-exposed control mice. No increase in total BAL cells was observed in mice repeatedly instilled with 0.5, 5.0, or 25.0 μ g BLG, compared to saline-instilled BLG-exposed controls.

Although differences were evident in the magnitude and types of BAL inflammatory cell responses resulting from repeated intranasal instillation of the test proteins, all of the test proteins

induced a dose-dependent increase in BAL eosinophils, a key effector cell associated with allergic airway response. Based on the difference in the magnitude of this response, these data suggest a relative potency of the immune-mediated response to the test proteins of SUB $\geq$ KLH > OVA > BLG.

4.2.3. PCA

The PCA assay was again used to characterize the specific allergic antibody response after sensitization to the test proteins. The assay was conducted in naïve mice using pooled sera obtained from sensitized mice upon sacrifice on test day 30 and the results are listed in Table 3. Sera from SUB sensitized mice displayed a modest positive response at the 0.5 μ g dose level, while sera from the 5 μ g dose group yielded a robust response as indicated by a 100% incidence at a 1:320 dilution. Sera from OVA-sensitized mice

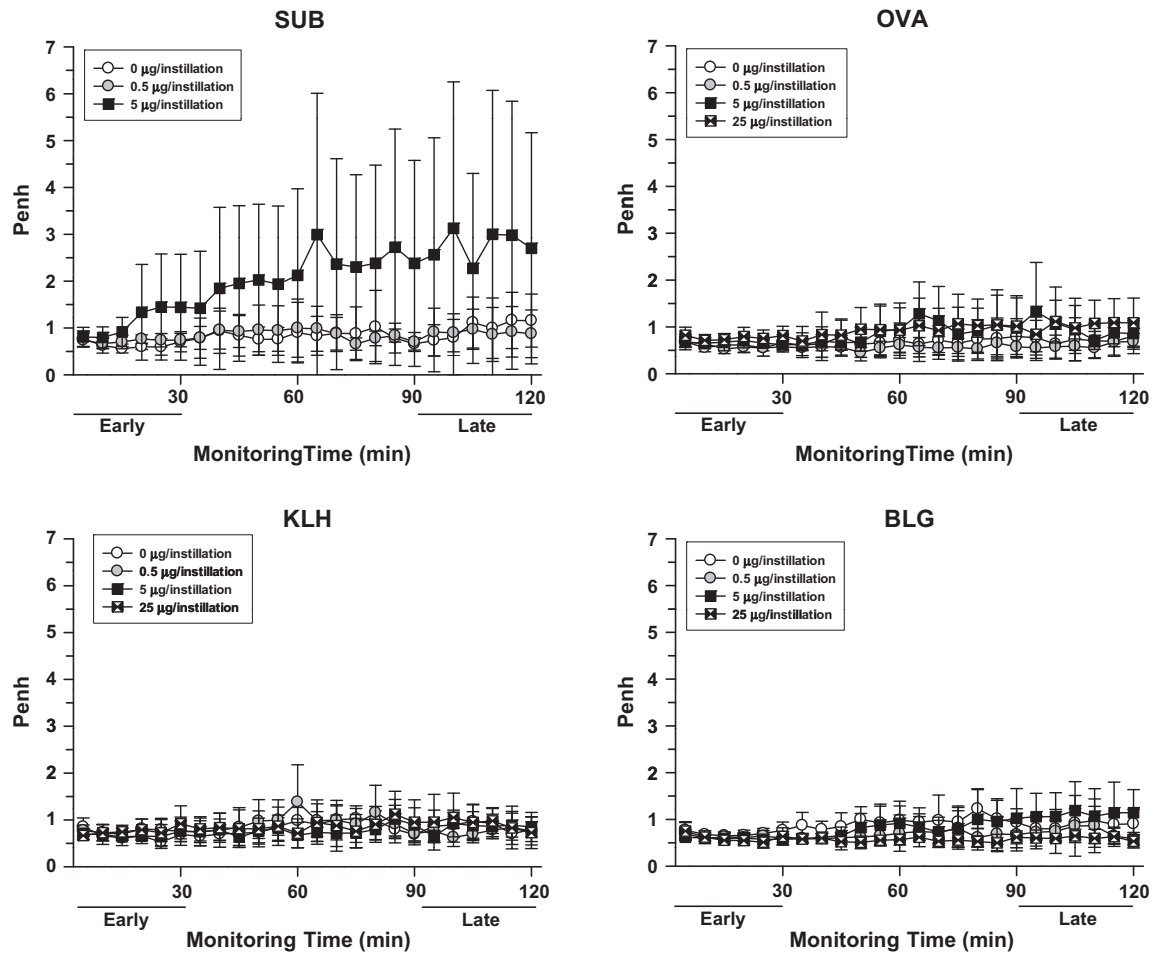


Fig. 4. Experiment 2 – time dependent Penh responses to 1% protein aerosol challenge. Mice were intranasally instilled with saline (non-sensitized control), SUB, OVA, BLG or KLH on test days 1, 3, 10, 17 and 24. On test day 29, saline- and protein-instilled mice received a 10 min challenge to an aerosol of a 1% solution of the sensitizing protein. Immediately after exposure mice were transferred to barometric whole-body plethysmographs to measure Penh for 120 min after exposure. The data were analyzed for treatment-related early- (0–30 min) and late-phase (90–120 min) effects on Penh, relative to the response of protein-challenged, saline-instilled, control mice in each experimental group. Symbols represent the mean Penh value ($\pm$ standard deviation) at 5-min intervals for each experimental group ($n = 5$ –6/group).

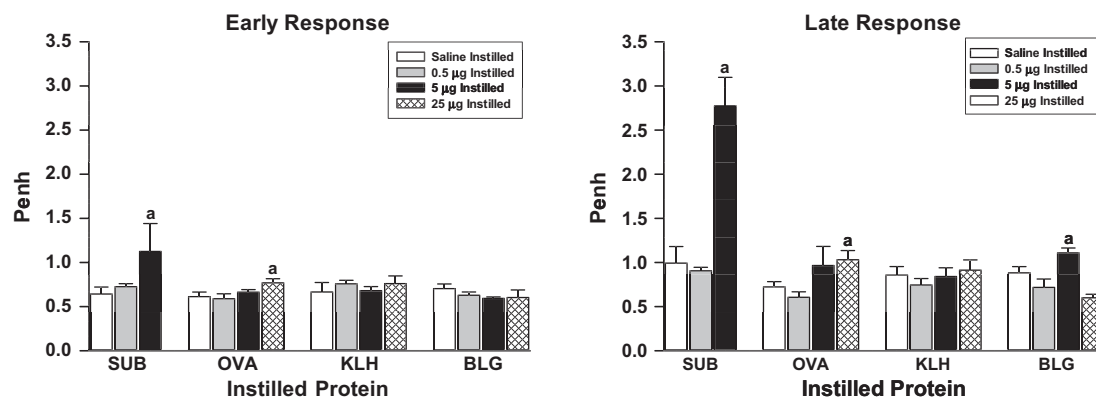


Fig. 5. Experiment 2 – early- and late-phase Penh responses to 1% protein aerosol challenge. Summary of early- and late-phase effects of a 1% protein aerosol challenge on Penh levels in mice intranasally instilled with saline or the aerosol challenge protein. Bars represent the mean Penh values of mice in each experimental group averaged over the time intervals of 0–30 min following challenge (early-phase) and 90–120 min following challenge (late-phase) $\pm$ standard deviation. a = statistically increased relative to saline-instilled control mice exposed to the sensitizing protein aerosol. ($p < 0.05$; $n = 5$ –6/group).

elicited a modest positive response at the 0.5 μg dose level, however, more robust positive responses were observed for the 5 and 25 μg dose levels with an incidence of 100% at dilutions of 1:64 and 1:256, respectively. Serum samples from all BLG sensitized

dose groups yielded weak PCA responses with little to no serum dilution. Sera from KLH sensitized mice elicited robust positive responses for the 5 and 25 μg dose levels with an incidence of 100% at dilutions of 1:32 and 1:53, respectively. For each of the proteins

tested, serum from non-sensitized, protein challenged control mice did not yield a positive response when tested undiluted. Based on the results of this assay, the rank order potency for induction of specific allergic antibody was SUB > OVA > KLH > BLG.

4.2.4. Determination of serum IgG1 and IgG2a

Serum prepared from blood samples collected during necropsy was also assayed for IgG1 and IgG2a, which, along with PCA results, may provide an indication of T-cell helper phenotype as previously proposed (Dearman et al., 2000). Each test protein elicited antibody production following repeated intranasal administration to mice (Fig. 7). SUB and OVA both induced a strong dose-dependent increase in IgG1 protein. KLH also induced a strong IgG1 response, however the highest induction was approximately an order of magnitude lower than that of SUB and OVA. The induction of IgG1 by BLG was modest and was at least three orders of magnitude lower than that of SUB and OVA. IgG2a production was robust and comparable for SUB, OVA, and KLH treated mice, but was below the detection limit (0.5 optical density) of the analysis

approach in mice administered BLG. Ratios of IgG1 to IgG2a were calculated for each protein to provide an indication of the predominant T-cell phenotype. The relative differences in the induction of IgG1 and IgG2a are evident in Fig. 8 which depicts the IgG1:IgG2a ratios for each of the proteins. Both SUB and OVA display IgG1:IgG2a ratios of approximately 1000 while KLH ratios were approximately 10. These data suggest that SUB and OVA generated a more pronounced Th2 response than KLH. BLG demonstrated small increases in IgG1 without any IgG2a production which led to a dose-dependent increase in ratios which were slightly greater than that of KLH at the high dose, despite much lower production of antibody. This suggests BLG may be more prone to induce a Th2 phenotype, but without robust immunogenicity.

5. Discussion

The mouse intranasal test (MINT) was developed to assess the relative allergic potential of detergent enzymes through the

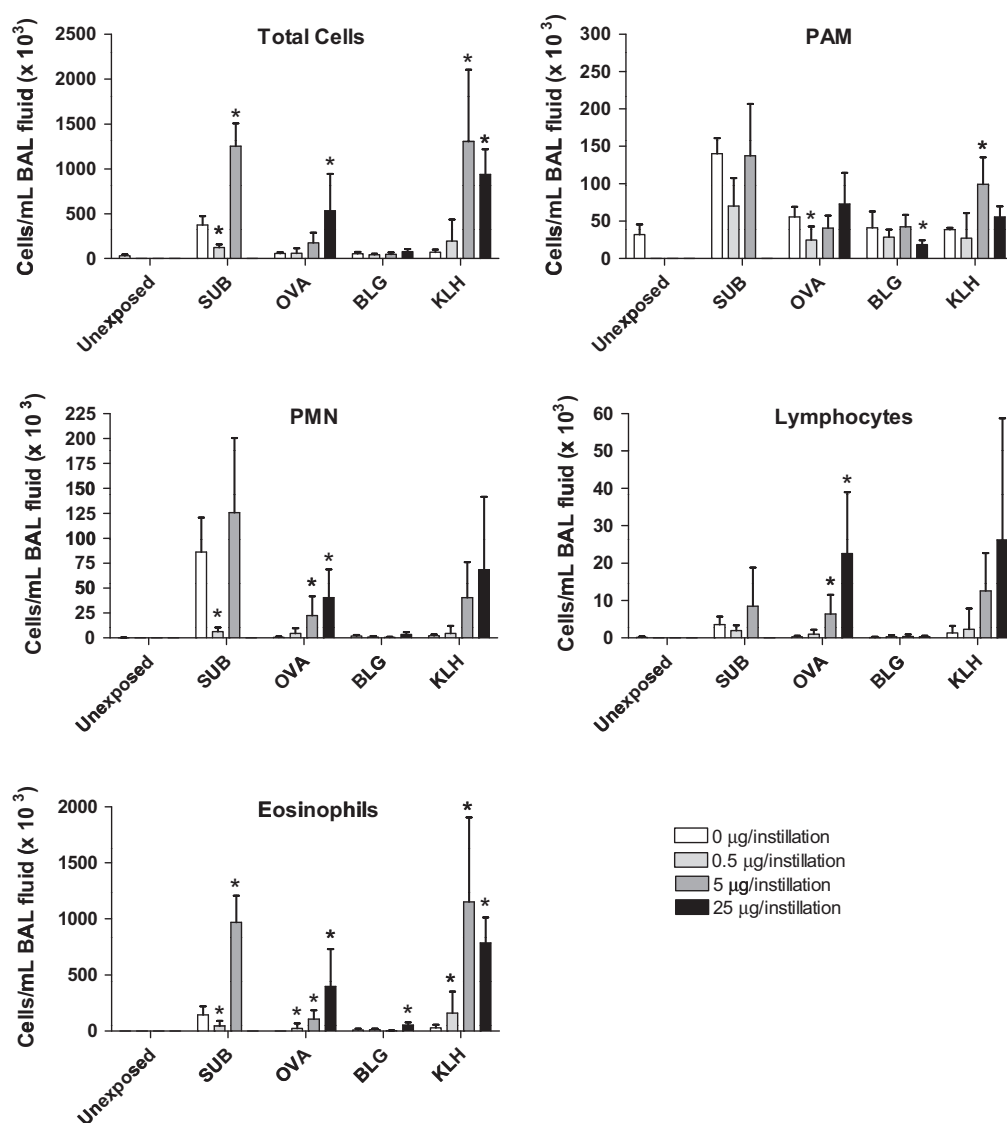


Fig. 6. Experiment 2 – total and differential cell counts in BAL fluid. Cell numbers in the BAL fluid were determined for each mouse in each protein dose group ($n = 4$ –6/dose group). Mice were intranasally instilled with SUB, OVA, BLG or KLH on test days 1, 3, 10, 17 and 24 and BAL fluid was obtained on test day 30, after protein-specific challenge. Unexposed controls represent the response in non-sensitized/non-challenged mice, while the 0 μ g/instillation group represents the non-sensitized/protein challenged control mice. Statistical significance relative to the respective non-sensitized/protein challenged controls ($p \leq 0.05$) is indicated by *. PAM = pulmonary alveolar macrophage; PMN = polymorphonuclear neutrophil.

evaluation of enzyme-specific antibody (IgG1) levels. However, additional components of an immune-mediated allergic airway response such as inflammation of the airways, airway epithelial remodeling (mucous cell metaplasia) and airway hyperresponsiveness (AHR) are not routinely evaluated in the MINT. The present study sought to further expand the MINT approach through inclusion of these additional endpoints in an effort to develop a predictive animal model to identify proteins with the potential to induce respiratory sensitization and allergy. The investigation involved sensitization and challenge with several proteins of varying allergic potential and evaluated the subsequent airway inflammation (measured via total and differential leukocyte counts of BAL fluid and pulmonary histopathology), airway hyperreactivity (measured as an increase in Penh in response to aerosol challenge with MCh or the sensitizing protein in WBP chambers), and serum immunoglobulin response. Based on existing data regarding allergenicity, the expected rank order potency for the proteins was predicted to be SUB > OVA $\geq$ KLH > BLG $\geq$ MSA for each of the experimental endpoints.

Allergen-induced airway inflammation is characterized by an influx of leukocytes (especially lymphocytes, monocytes, neutrophils and eosinophils) into the airways (Meade and Woolhiser, 2002; Kraneveld et al., 2002; Koya et al., 2006). Cellular analysis of BAL fluid provides a rapid and sensitive indicator of inflammation and can also be used as an assessment of pulmonary allergic responses (Farraj et al., 2007; Pauluhn, 2008). For experiment 1, in which animals were repeatedly exposed to the sensitizing proteins by intranasal instillation and AHR was assessed by exposure to aerosolized MCh, BAL responses were minimal and only animals intranasally sensitized with 5.0 μ g SUB showed a significant increase in leukocyte numbers. Although differentiation of cell subpopulations of animals in the 5.0 μ g SUB group showed a pattern of airway inflammation characterized by increased numbers of neutrophils, pulmonary macrophages and lymphocytes in the lung, eosinophils were not observed in BAL from mice in any experimental group. This was unexpected given that eosinophilic inflammation is a hallmark of allergic airway disease and asthma (Whitehead et al., 2003; Ichinose et al., 2004), however, the time selected for BAL analysis may have been too long after the last intranasal protein challenge (5 days) to identify a transient protein-induced influx of eosinophils. Morokata et al. (2005) reported that the time of peak pulmonary eosinophil influx in BDF1 mice sensitized by intraperitoneal administration of ovalbumin/alum and challenged by inhalation of aerosolized ovalbumin was 72 h. These data may highlight the importance identifying the time of peak inflammation when selecting an appropriate sampling interval to assess pulmonary inflammation by BAL. While analysis of BAL provides a sensitive measure of inflammation at the time of lavage, it provides only a snapshot of a dynamic cellular process and does not provide information on inflammatory cell populations within the lung tissue compartment. Histopathologic evaluation of lung tissue from animals in experiment 1 revealed the presence of eosinophils in the lung tissue compartment of animals instilled

with 5.0 μ g SUB, and to a lesser extent those instilled with 5.0 μ g OVA.

In experiment 2 the dose levels of sensitizing protein were increased and a specific protein aerosol challenge replaced the non-specific MCh challenge from experiment 1, resulting in an increase in the magnitude of the inflammatory response as well as the detection of eosinophils in BAL. The relative potency of the immune-mediated response to the test proteins determined in experiment 2 (SUB > OVA > KLH > BLG), correlated well with the documented allergic immune response of these proteins (Robinson et al., 1996, 1998; Hamelmann et al., 1997; Blaikie and Basketter, 1999a; Heederik et al., 2002). KLH, included because it is a strong immunogen, produced a robust BAL response. The observed flattening of the KLH dose–response may be due to attenuation of the immune response due to the relatively high sensitizing dose (25 μ g) of this immunogenic protein (Riedl et al., 2005). Another interesting observation was that non-sensitized (saline instilled) mice challenged with aerosolized SUB displayed robust BAL responses, likely due to the irritating properties of SUB at the aerosol challenge concentration of 1%. This highlights the importance of including appropriate non-sensitized challenge control groups as part of these sensitization/challenge assays to facilitate complete interpretation of the response.

In addition to monitoring pulmonary BAL responses, WBP was used to measure Penh as a potential endpoint for assessing allergenic potential. Penh, which in some studies correlates with AHR, has been used as a non-invasive technique to investigate allergic sensitization in mice (Taube et al., 2004; Vaickus et al., 2010; Hamelmann et al., 1997). Under the conditions of this study, AHR measured as an increase in Penh following MCh or specific protein aerosol challenge was not a reliable endpoint to discriminate the respiratory sensitization potential of one agent from another. This may be due in part to the experimental design of this study. In this study, mice were sensitized to specific proteins by repeated intranasal instillation, without prior systemic sensitization through another route of exposure. Sensitization via dermal and intraperitoneal (i.p.) routes has been used extensively to predict respiratory sensitization potential of low molecular weight chemicals and proteins and has been shown to successfully elicit an altered AHR response (Vanoirbeek et al., 2004; Baqueiro et al., 2010; De Vooght et al., 2010). In each case, the initial systemic sensitization was followed by respiratory tract exposure with the sensitizing material. The intranasal route used in the current studies, although a more physiologically relevant route of exposure for proteins (i.e., respiratory tract exposure to assess respiratory sensitization potential) may not have been robust enough to result in the cellular and structural airway changes associated with AHR in this short-term assay.

Another contributing factor in the relative lack of protein-induced alterations in AHR, measured as a change in Penh, may be the strain of mouse used in this study. BDF1 mice were chosen based on their ability to accurately predict human responses to detergent enzyme using the MINT based on induction of antibody responses as originally developed by Robinson et al. (1996);

Table 3

Experiment 2 – specific reagenic antibody responses determined using passive cutaneous anaphylaxis assay^a.

Dose group (μ g)	SUB		OVA		BLG		KLH	
	Dilution ^b	Incidence (%) ^c	Dilution	Incidence (%)	Dilution	Incidence (%)	Dilution	Incidence (%)
0	Neat	0	Neat	0	Neat	0	Neat	0
0.5	1:2	67	1:2	60	Neat	17	Neat	17
5	1:320	100	1:64	100	Neat	50	1:32	100
25	–	–	1:256	100	1:1	50	1:53	100

^a Sera were obtained on test day 30 following intranasal instillation of proteins on test days 1, 3, 10, 17 and 24 and protein challenge on day 29.

^b Data expressed are the maximum dilution of sera tested; neat indicates undiluted serum was tested.

^c Incidence represents the percentage of mice responding at the corresponding dilution with 4–6 naive mice per dose group.

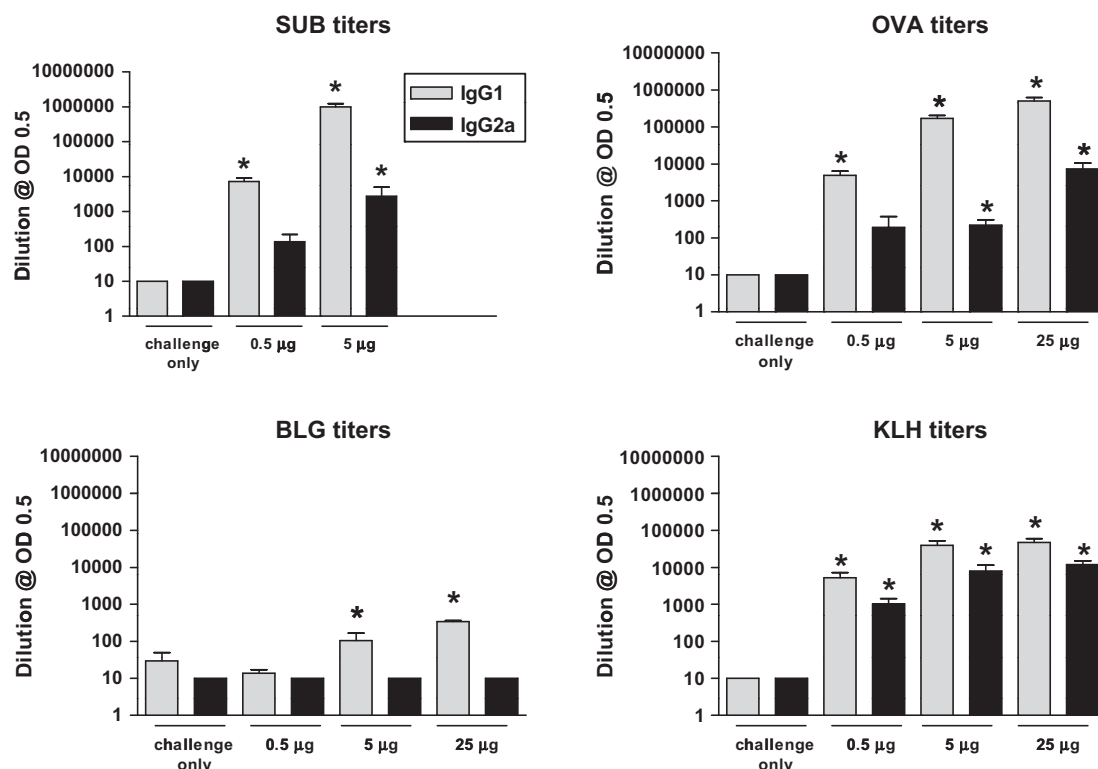


Fig. 7. Experiment 2 – serum levels of IgG1 and IgG2a after protein sensitization and challenge. Specific IgG1 and IgG2a levels were measured using an ELISA method. The serum titer was defined as the reciprocal of the dilution that demonstrated an optical density value of 0.5. Challenge only mice represent the response in the non-sensitized/protein challenged controls. Data represent the mean and standard deviation of $n = 4$ –6 mice/dose group. Statistical significance relative to controls ($p \leq 0.05$) is indicated by *.

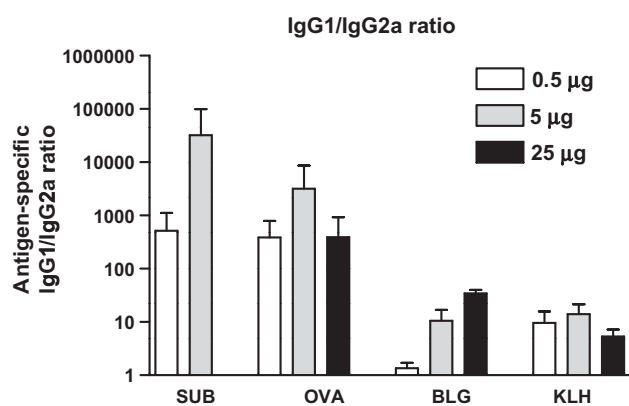


Fig. 8. Experiment 2 – ratio of IgG1 to IgG2a after protein sensitization and challenge. Ratios of IgG1 to IgG2a were calculated for each protein to provide an indication of the predominant Th-cell phenotype.

however they have not been widely used in other models of protein- or chemical-induced AHR and their effectiveness and appropriateness for monitoring this endpoint is largely unknown. Several studies have shown that mouse strains differ in their AHR responses to allergens (De Vooght et al., 2010; Swedin et al., 2010; Van Hove et al., 2009; Atochina et al., 2003). BDF1 (i.e., B6D2F1) mice are a cross between female C57BL/6 and male DBA/2 mice and both strains have been shown to be poor responders in terms of allergen-induced AHR which may be contributing to the lack of responsiveness in the current study.

The present study also examined antibody responses to the proteins through the use of the PCA assay to assess allergic antibody production and through ELISA evaluation of IgG1 and IgG2a. The

PCA assay has been used to characterize the specific IgE antibody response associated with systemic exposure to protein allergens and it is recognized by the scientific community as an approach to generate data on protein allergenicity (Hilton et al., 1994; Atherton et al., 2002; Dearman et al., 2003). The advantage of the PCA assay is its functional response which involves monitoring a cutaneous allergic endpoint as a consequence of allergen/IgE interaction. However, the assay is very labor-intensive and not entirely specific to IgE (Birmingham et al., 2003). In the second experiment, the PCA data was complemented by the assessment of serum IgG1 and IgG2a levels. Increases in serum IgG1 have been used as a marker of overall immunogenicity and in mice IgG1 is also considered as a potential surrogate for IgE induction and as an indicator of a Th2 cellular response that is associated with the etiology of respiratory allergy (Blaikie and Basketter, 1999b; Dearman et al., 2000). In contrast, IgG2a production has been associated with interferon gamma production and a predominant Th1 cellular response. Therefore the relative induction of IgG1 and IgG2a has been proposed as an indicator of the Th2/Th1 response and the subsequent allergenic potential of the protein (Stevens et al., 1988; Dearman et al., 2000). The data generated as part of this study revealed significant differences between the examined proteins with respect to these endpoints. Serum from mice intranasally instilled with SUB and OVA showed a robust specific-IgE response consistent with previously generated PCA data (Hilton et al., 1994, 1997) and the known allergenic potential of these proteins (Robinson et al., 1996, 1998; Hamelmann et al., 1997; Blaikie and Basketter, 1999a; Heederik et al., 2002). Consistent with the strong IgE response, SUB and OVA also induced strong IgG1 responses with titers for this isotype up to 1000-fold greater than that for IgG2a. These data suggest the development of a strong Th2 cellular response that would be expected to yield a robust specific-IgE re-

sponse. In contrast, intranasal administration of BLG failed to induce a consistent and biologically relevant response in the PCA assay. This was somewhat unexpected given the known allergenic potential of this protein and previous reports of IgE induction (Kitagawa et al., 1995; Adel-Patient et al., 2003; Hazebrouck et al., 2009). Potential reasons for this discrepancy could include the source of the protein or a reduced potential for sensitization by the intranasal route. The lack of specific IgE was consistent with the weak induction of serum IgG1. Intranasal administration with MSA did not result in the production of specific-IgE as indicated by negative responses in the PCA assay. Although serum IgG1 and IgG2a were not determined for this protein, they would be expected to show little to no induction. These data suggest that MSA may be a useful as a negative control protein; however, its utility in this role will require additional characterization of its immunogenic and allergenic potential. KLH induced an increase in specific-IgE in the PCA assay, although the response was less robust when compared to that of SUB and OVA. Further examination of the literature revealed previous reports indicating positive PCA responses for this protein (Brzezinska-Blaszczek et al., 1980). Interestingly, KLH resulted in a robust IgG1 induction but this was also accompanied by a strong induction of serum IgG2a that differed by only 10-fold. This response is consistent with the robust immunogenicity of KLH and suggests a more mixed Th response when compared to SUB and OVA which may account for the lower specific IgE response. While the PCA titers are less than those of SUB and OVA, the induction of specific IgE observed following KLH administration confirms its allergenicity in this assay. The strong immunogenicity of KLH may be a confounder to predicting allergenicity potency via PCA due to the role that IgG1 is thought to play in the sensitization and activation of murine mast cells (Miyajima et al., 1997; Birmingham et al., 2003). Such results may limit broad use and interpretation of PCA data as a true indicator of specific-IgE and highlight the potential utility of serum IgG1 and IgG2a as part of the overall assessment of the Th cellular response and the potential allergenicity. These endpoints would require a more comprehensive analysis with a wider range of proteins before their true utility could be assessed. Overall, the specific IgE production, as indicated by the PCA assay, provided a sensitive and reproducible indication of the allergenic potential of the proteins. Furthermore, comparison of the serum IgG1 and IgG2a responses to that of the PCA assay indicate that characterization of these IgG isotypes may serve as a sensitive and more easily attainable indicator of the allergenic potential.

In the present study, several endpoints previously used in murine models of immune-mediated allergic airway disease were evaluated as potential additions to the MINT assay. The data indicate that allergic antibody responses and IgG isotype characterization proved to be sensitive and reliable indicators of the protein allergenic potential. This is likely due to the fact that the production of antibodies may occur more rapidly and at exposure concentrations far lower than those needed to produce significant airway remodeling or changes in pulmonary function or cellular inflammation (Briatico-Vangosa et al., 1994). Differences in allergic antibody responses between test proteins were demonstrated, with SUB, OVA and KLH producing the highest titers. BAL responses after protein-specific challenge were also sensitive indicators of allergic potential with SUB, OVA and KLH showing roughly dose-dependent induction of eosinophils in the BAL. The results are consistent with published results showing SUB as a known human respiratory sensitizer, OVA as a known food-borne antigen, and KLH as a potent immunogenic neoantigen which has been shown to induce airway responses after sensitization and challenge (Riedl et al., 2005; Schatz et al., 1977; Weissman et al., 1990). In addition, both SUB and OVA have been shown to induce structural alterations in the upper and lower airways, including mucous cell hyperplasia

and metaplasia and accumulation of inflammatory leukocytes (neutrophils, eosinophils, and lymphocytes) within airway mucosa (Farraj et al., 2003). As expected, both BLG and MSA resulted in minimal to no responses for all endpoints. However, assessment of airway hyperreactivity did not provide additional data that improved the ability to predict the sensitization potential of proteins. Although these data highlight that further improvements and refinements of the assay may be necessary, it is important to recognize that each protein is unique in the biological response it elicits, and that the components of the immune-mediated airway sensitization response include elevated serum antibody levels, inflammation of the airways and AHR. Therefore, no matter how sensitive one specific endpoint may appear, the respiratory sensitization potential of proteins may be best predicted when multiple parameters are evaluated.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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