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A draining lymph node assay (DLNA) for assessing the sensitizing potential of proteins

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ABSTRACT

There is a need for a simple and predictive model to identify the respiratory sensitization potential of (novel) proteins. The present study examined the use of a mouse draining lymph node assay (DLNA) approach, employing several routes of exposure, as a possible starting point for assessing protein sensitization potential. Consistent with the experimental procedure for the standard local lymph node assay (LLNA), female BALB/c mice were dosed dermally (topical), intranasally (IN) or by oropharyngeal aspiration (OP) on days 1, 2 and 3, and proliferation in the relevant draining lymph nodes was measured on day 6. For each route, the auricular, superficial cervical and tracheobronchial lymph nodes (TBLN) were evaluated following treatment with Subtilisin Carlsberg (SUB; a potent sensitizer/allergen), ovalbumin (OVA; a potent food allergen), β-lactoglobulin (BLG; a moderate food allergen), and keyhole limpet hemocyanin (KLH; a strong immunogen with no reports of respiratory sensitization). Initial studies with OVA indicated that dermal administration did not stimulate lymph node proliferation. Responses in the tracheobronchial lymph node were most dramatic (stimulation indices up to 100) and reproducible for both the IN and OP routes. In a comparative experiment, all proteins induced lymph node proliferation with a rank order potency of SUB > KLH > OVA > BLG. The influence of the endotoxin content on lymph node proliferation was determined to be minimal, and did not impact the rank order potency. Molecular characterization of the TBLN at an equipotent proliferative dose was conducted for select gene transcripts based on research examining chemical sensitizers. Expression profiles differed among the four proteins, but the relevance of these responses was not clear and they did not further discriminate their allergic potential. These data illustrate both the opportunities and challenges associated with the examination of the draining lymph node proliferative response to assess the allergenic potential of proteins.
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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; Boverhof et al., 2008a). Many studies on proteins have focused on the elicitation stage of allergy with little examination of sensitization. The guinea pig has been a prominent animal species for researching respiratory allergy potential of proteins with intratracheal and inhalation models serving as tools for ranking the proteins (Robinson et al., 1998; Blaikie and Basketter, 1999a). The use of the guinea pig model, however, has certain drawbacks including long experimentation 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, 1999a). Due to these limitations, researchers have explored the mouse as an experimental model which led to the development of an intratracheal dosing approach and the mouse intranasal test (MINT) which have been applied for the assessment of the relative allergic potential of detergent enzymes (Kawabata et al., 1996; Robinson et al., 1998). However, these assays were reported to have several deficiencies in predictive accuracy when compared to results produced with guinea pig models, which has limited their broader use and acceptance (Blaikie and Basketter, 1999a,b).

The mouse local lymph node assay (LLNA) is an accepted/guideline approach for detection of contact sensitizers (Hattan and Sailstad, 2001; Cockshott et al., 2006). The LLNA assesses the sensitizing potential of a test material through topical application on the dorsal surface of the ear on three consecutive days and subsequent examination of the proliferative response in the draining auricular lymph nodes (Kimber et al., 2002). In addition to identifying contact sensitizers, it is generally accepted that respiratory

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sensitizers will also test positive in the LLNA, despite the fact that these compounds are rarely associated with clinical allergic contact dermatitis (Vanoirbeek et al., 2003; Holsapple et al., 2006; van Loveren et al., 2008). This is because the initial sensitization or induction phase of allergy is considered to be similar, but the subsequent downstream molecular and cellular events dictate the ultimate allergic state that is developed. As an extension of this, the current view is that a chemical that tests negative in the LLNA can be regarded as lacking the potential for both contact and respiratory sensitization (Roggen et al., 2008; Vanoirbeek et al., 2003; Holsapple et al., 2006; van Loveren et al., 2008). However, the important consideration to this interpretation is that not all materials that test positive in the LLNA are respiratory sensitizers.

As a widely accepted approach for the assessment of contact sensitization potential, the LLNA may represent an important starting point for the assessment of the respiratory sensitization potential of proteins. The dermal route of exposure becomes a major consideration for the wider use of the LLNA as it is not a relevant route for high molecular weight protein allergens. Exploring alternate routes of exposure, including intranasal (IN) and oropharyngeal administration (OP), has the potential to circumvent this limitation. While these routes have previously been used to research aspects of respiratory allergy for both low and high molecular weight compounds (Keane-Myers et al., 1998; Woolhiser et al., 2000b; Sailstad et al., 2003), they have not been extensively applied for the characterization of respiratory sensitization potential through the examination of lymph node responses. Modification of the current LLNA through the use of these alternate routes of exposure and analyzing proliferative responses in the relevant draining lymph nodes represents a novel approach to extend the use of this assay for detection of the respiratory sensitization potential of both high and low molecular weight compounds. Such an approach has been recently applied for the examination of low molecular weight chemicals following head/nose-only exposures (Arts et al., 2008). The present study was conducted to assess the use of dermal (topical), intranasal and oropharyngeal administration in the LLNA for the evaluation of the respiratory sensitization potential of proteins. It was hypothesized that the use of IN and OP administration, and monitoring the response in the relevant draining lymph node, may alleviate the limitations associated with dermal applications when examining the respiratory sensitization potential of proteins while leveraging a well-accepted assay and endpoint for the assessment of low molecular weight contact sensitization potential.

2. Materials and methods

2.1. Animals

Female BALB/c mice, 8–12 weeks of age, were used in all studies (Charles River Laboratories, Inc., Kingston, NY). 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 procedures involving the use of animals were reviewed and approved by a veterinarian and the Institutional Animal Care and Use Committee (IACUC) of The Dow Chemical Company and were conducted in facilities fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC International). BALB/c mice were selected because of their general acceptance and suitability for toxicity testing, and availability of historical background data in this laboratory. BALB/c mice have been successfully used by other laboratories in LLNA protocols (van't Erve et al., 1998; Hariya et al., 1999; Woolhiser et al., 2000a; Ehling et al., 2005). This strain has also been used extensively in the scientific community for researching various aspects of immunology and respiratory allergy due in part to its predominant Th2 and high-IgE response (Gajewska et al., 2001; Dearman et al., 2003b; Vanoirbeek et al., 2003, 2006; Duez et al., 2004).

2.2. Test materials

Test proteins were chosen to cover a range of apparent respiratory sensitization potencies. Subtilisin (SUB; a potent respiratory sensitizer/allergen), ovalbumin (OVA; a strong food and respiratory allergen), and β-lactoglobulin (BLG; moderate–weak allergen), were all obtained from Sigma–Aldrich (St. Louis, MO). Keyhole Limpet Hemocyanin (KLH; a strong immunogen with no reports of respiratory sensitization and a potential negative control protein) was obtained from Calbiochem (San Diego, CA). All dosing solutions were prepared in sterile pyrogen-free saline. Since many protein test materials may contain appreciable amounts of endotoxin/lipopolysaccharide (LPS), all proteins were analyzed for LPS content. The level of bacterial endotoxin was determined at SGS Northview Laboratories, Inc. (Northbrook, Illinois) using a chromogenic LAL (Limulus Amoebocyte Lysate) Bacterial Endotoxin test and expressed as endotoxin units (EU)/μg protein.

2.3. Intranasal instillation and oropharyngeal aspirations

Intranasal instillations were accomplished by anesthetizing the mice (3% isoflurane in oxygen) and gently holding in a supine position with the nose pointed slightly up and allowing the mice to aspirate the instillate into the nasal cavity 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 (Farraj et al., 2003, 2004, 2006).

Oropharyngeal aspirations were accomplished by anesthetizing the mice with 3% isoflurane in oxygen. Anesthetized mice were removed from the induction box and placed in a supine position on an inclined polycarbonate sheet (approx. 30° from vertical). To perform the instillations, the mouth was opened and the tongue was then gently withdrawn using padded forceps to expose the oropharyngeal/tracheal junction and block the esophagus. While holding the tongue, a 50 μl sample was pipetted into the back of the oral cavity. The tongue was held to prevent swallowing until the dose was aspirated from the oropharyngeal cavity. This approach has been published by a number of researchers and has been shown to result in effective delivery of test material to the right and left lungs (Keane-Myers et al., 1998; Woolhiser et al., 2000b; Sailstad et al., 2003).

2.4. Conduct of draining lymph node assay

The DLNA was conducted using the same general approach as that specified for the LLNA (Hattan and Sailstad, 2001; OECD, 2002). IN and OP administration of the test material (50 μl/mouse) was performed as described above. Dermal (topical) administration was performed as per the standard LLNA where the test material was applied to the dorsal surface of each ear. To prevent test material loss, a Pluronic® L92 block copolymer surfactant (L92) was added to the dermal dosing solutions at a concentration of 1% to promote test material retention on the ear. Addition of 1% L92 creates a vehicle that facilitates evaluation in an aqueous solvent while providing prolonged dermal contact through its wetting properties (Ryan et al., 2002; Boverhof et al., 2008b). L92 was purchased from BASF (Mount Olive, NJ). A minimum of four female mice/group received the indicated concentration of each protein or vehicle (physiological saline) once daily for three consecutive days. Non-instilled control mice were also included in select studies as an additional control. All mice were weighed on days 1, 2, 3, and 6. On day 6, mice received a 250 μl intravenous (i.v.) injection (via the lateral tail vein) of phosphate-buffered saline (PBS) containing 20 μCi of ^{3}H-thymidine (specific activity 2 Ci/mmol; Amersham code TRA310, Buckinghamshire, United Kingdom). Approximately 5 h post-administration, the mice were euthanized via CO_2 asphyxiation and the relevant draining lymph nodes were removed. Lymph nodes that were examined varied by study and were selected as relevant draining lymph nodes for the examined routes of exposure. These included the auricular lymph nodes (located at the bifurcation of the jugular veins; drain the ear), tracheobronchial lymph node (located along the trachea superior to the tracheal bifurcation; drain the lung), superficial cervical lymph nodes (located anterior to the salivary gland; drain the nasal region) and popliteal lymph nodes (located posterior to the knee; examined as a distal negative control). All processing of lymph nodes, measurement of tritiated thymidine (^{3}HTdR) incorporation and calculation of stimulation index (SI) and EC_3 values (concentration resulting in a 3-fold SI, and the threshold for a positive response) were conducted according to guidelines and as previously published (OECD, 2002; Boverhof et al., 2008b, 2009). Although calculation of an EC_3 for categorization purposes may not be appropriate for intranasal and oropharyngeal routes of exposure, these values were calculated to facilitate comparisons of relative sensitization potency between the different routes of exposure and across different lymph nodes (Basketter et al., 1999).

2.5. Molecular characterization of lymph node responses

Select gene expression responses were monitored in the draining lymph node using quantitative real-time PCR (QRTPCR) as described previously (Boverhof et al., 2009). Briefly, lymph nodes from individual mice were excised and placed in RNAlater (Applied Biosystems, Foster City, CA), and total RNA was extracted using the RNeasy Mini Kit (Qiagen, Valencia, CA). RNA was quantified using the ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE) and the quality assessed using a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). Total RNA (500 ng) was reverse transcribed using a high capacity cDNA reverse transcription kit (Applied Biosystems). cDNA was used as template in subsequent QRTPCR assays

which were conducted using pre-designed TaqMan Gene Expression assay reagents on an ABI 7500 real-time PCR sequence detection system (Applied Biosystems). Relative expression between treated and vehicle control samples were determined using the comparative Ct method ($\Delta\Delta C_T$ Method) with beta actin used as the endogenous control. A full listing of all pre-designed TaqMan assays used in these analyses can be found in Supplementary Table 1. Examined genes included Ki67 (antigen identified by monoclonal antibody Ki67), IL4 (interleukin 4), IL21 (interleukin 21) and Ifng (interferon gamma). Statistical analysis was performed on the ΔC_T values for each animal as these values represent the normalized dependent variables from the experimental analysis.

2.6. Histological analysis

For the study that examined gene expression markers in the tracheobronchial lymph node, histological responses in the lung were also examined to determine any significant differences in lung histology at an equipotent dose based on the tracheobronchial lymph node proliferative response. Lungs were inflated to an approximately normal inspiratory volume with neutral, phosphate-buffered 10% formalin using a hand-held syringe and blunt needle and transferred to a container with the same fixative for a minimum of 48 h. The fixed left lung lobe was trimmed free of the trachea and extrapulmonary bronchus. Two transverse tissue blocks were taken at approximately the level of the 5th (G5) and 11th (G11) airway generations to evaluate tissue responses in large (G5) and small (G11) diameter conducting airways. The tissue blocks were embedded in paraffin, sectioned at approximately 6 μm, and stained with hematoxylin and eosin for histopathologic evaluation.

2.7. Statistical analysis

Data were analyzed by a one-way analysis of variance. In cases where data did not pass Bartlett's test for homogeneity of variance, data were log transformed prior to statistical analysis. When differences were indicated by the ANOVA, a comparison of treated versus control groups was done using a Dunnett's *t*-test.

3. Results

3.1. Comparison of dermal, IN, and OP routes of administration using OVA

Mice received one of three concentrations of OVA at 0.005%, 0.05%, or 0.5% (corresponding to 2.5, 25 and 250 μg/mouse) or vehicle (physiological saline) once daily for three consecutive days and were sacrificed on day 6. Administration of OVA did not result in any effects on body weight for any route of exposure. Proliferative responses were monitored in the auricular and tracheobronchial lymph nodes which drain the ear and lung, respectively. Popliteal lymph nodes were also monitored as distal negative controls. Results for each route of exposure and lymph node are presented in Fig. 1. Dermal administration of OVA did not induce a proliferative response in any of the lymph nodes. IN and OP administration of OVA did not induce a significant proliferative response in the auricular or popliteal lymph nodes. However, a significant proliferative response was noted in the tracheobronchial lymph node at the 0.5% dose level for both routes of exposure. Stimulation indices at this dose, relative to vehicle controls, were 7.8 and 14.1 for IN and OP routes, respectively. The greater response after OP administration may be due to more effective deposition of the test material in the lung when compared to IN administration. The simulation indices for OVA were used to calculate EC_3 values of 0.18% and 0.09% for IN and OP routes of exposure, respectively.

3.2. Comparison of protein responses after IN and OP administration

Mice received three concentrations of each protein or vehicle (physiological saline) once daily for three consecutive days and were sacrificed on day 6. In the case of OVA, BLG and KLH the low, mid and high doses were 0.03%, 0.3% and 3%, corresponding to doses of 15, 150 and 1500 μg/mouse. Due to irritant properties and previously reported toxicity, the low, mid and high doses for SUB were 0.001%, 0.003% and 0.01% corresponding to doses of 0.5, 1.5 and 5 μg/mouse.

IN and OP administration of SUB, OVA, KLH or BLG did not result in statistically significant effects on body weight on any measurement day. To assess the sensitization potential of the different proteins, proliferative responses were monitored in the superficial cervical and tracheobronchial lymph nodes, which drain the nasal cavity and lung, respectively. Auricular lymph nodes, which drain the ear, were also included as negative controls. As expected, IN and OP administration did not elicit a proliferative response in the auricular lymph nodes for any of the proteins, at any dose level (data not shown). OP administration did not result in a significant proliferative response in the superficial cervical nodes for any protein, at any dose. A similar lack of response in the superficial cervical nodes was observed for the IN route of exposure with the exception of KLH, which induced a statistically significant increase in disintegrations per minute (dpm) at the 0.3% and 3% dose levels with stimulation indices of 3.5 and 5.4, respectively (data not shown). In contrast to the auricular and superficial cervical nodes, the tracheobronchial node exhibited a proliferative response to each of the proteins by both routes of exposure (Fig. 2). In general, as with the response to OVA in the initial study, proliferative responses in the tracheobronchial node were greater in magnitude and exhibited statistical significance at lower doses when administered by the OP route versus the IN route. Stimulation indices were used to calculate EC_3 values for each protein by each route of exposure. Although calculation of an EC_3 (estimated concentration resulting in a 3-fold SI) for categorization purposes may not be appropriate for IN and OP routes of exposure, these values were calculated to facilitate assessment and comparison of relative sensitization potency

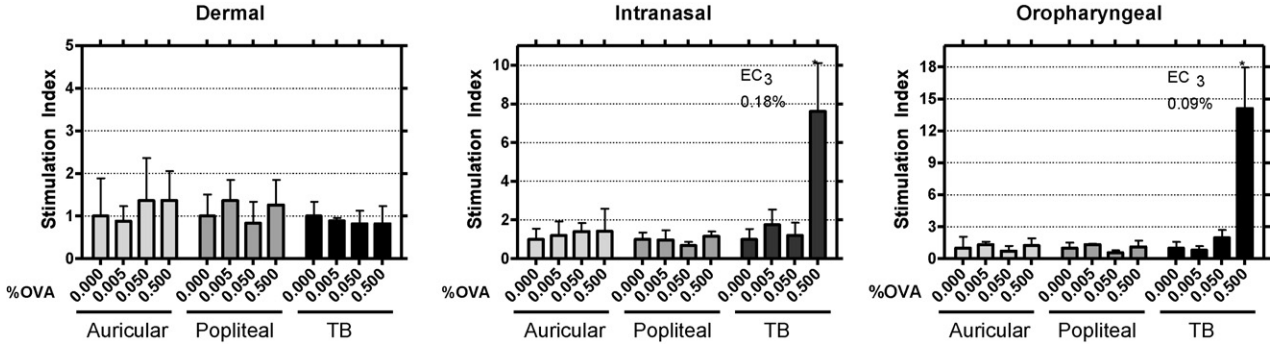

Fig. 1. Stimulation index (SI) values for each route of exposure and each monitored draining lymph node after treatment with the indicated concentrations of OVA. Mice were dosed on days 1, 2 and 3 and the proliferative responses in the lymph nodes were determined by ^{3}HTdR incorporation as per the standard LLNA. Values are expressed relative to the vehicle control and represent the mean ± standard deviation. Average dpm values for vehicle control auricular, popliteal and tracheobronchial nodes were approximately 600, 250 and 120 dpm, respectively. EC_3 values, the concentration required to induce a stimulation index of 3, are presented when applicable. Doses of 50 μl of 0.005%, 0.05%, or 0.5% OVA correspond to 2.5, 25 and 250 μg/mouse.
$p < 0.05$ when compared to vehicle control (TB = tracheobronchial lymph node).

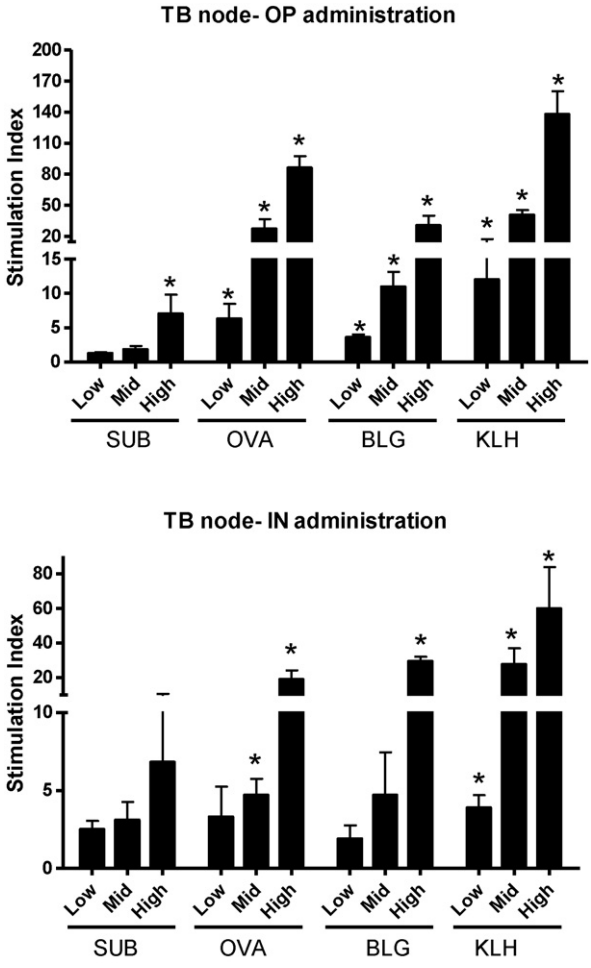

Fig. 2. Stimulation index (SI) values from the tracheobronchial lymph node for each protein after OP or IN administration. Mice were dosed on days 1, 2 and 3 and the proliferative responses in the tracheobronchial lymph node determined by ^{3}HTdR incorporation as per the standard LLNA. Values are expressed relative to the vehicle control and represent the mean ± standard deviation. Average dpm values for vehicle control auricular, superficial cervical and tracheobronchial nodes were approximately 650, 600 and 100 dpm, respectively. EC_3 values, the concentration required to induce a stimulate index of 3, are presented in Table 1. For OVA, BLG and KLH the low, mid and high doses were 0.03%, 0.3% and 3%, corresponding to doses of 15, 150 and 1500 μg/mouse. Due to irritant properties, the low, mid and high doses for SUB were 0.001%, 0.003% and 0.01% corresponding to doses of 0.5, 1.5 and 5 μg/mouse. *$p < 0.05$ when compared to vehicle control.

(Basketter et al., 1999). Potency rankings in the tracheobronchial lymph node after OP administration, as determined through comparison of EC_3 values, were: SUB (0.0046%) > KLH (0.014%) > OVA (0.021%) > BLG (0.025%) (Table 1). Potency in the tracheobronchial lymph node after IN administration followed the same rank order of: SUB (0.0034%) > KLH (0.028%) > OVA (0.038%) > BLG (0.164%).

Table 1
EC_3 values calculated using stimulation indices from TB lymph nodes for each route of exposure.

Protein	EC_3 (% protein)	
	IN	OP
SUB	0.0034	0.0046
OVA	0.0379	0.021[a]
BLG	0.164	0.025[a]
KLH	0.028[a]	0.014[a]

IN = intranasal instillation; OP = oropharyngeal aspiration; TB = tracheobronchial lymph node.

[a] EC_3 values calculated using log-linear equation.

Table 2
Endotoxin levels in 1 mg/ml samples of each protein and the calculated endotoxin unit (EU) level in each dose group.

Sample	Endotoxin level (EU/ml)	EU/μg protein	EU/dose		
			Low	Mid	High
Saline	Less than 0.005	NA	NA	NA	NA
SUB[a]	Less than 5	0.005	0.0025	0.0075	0.025
OVA[b]	572	0.572	8.58	85.8	858
BLG[b]	1920	1.92	28.8	288	2880
KLH[b]	66.3	0.0663	0.9945	9.945	99.45

[a] Low, mid and high doses of 0.001%, 0.003% and 0.01%, respectively (50 μl/mouse) corresponding to doses of 0.5, 1.5 and 5 μg/mouse.

[b] Low, mid and high doses of 0.03%, 0.3% and 3%, respectively (50 μl/mouse) corresponding to doses of 15, 150 and 1500 μg/mouse. NA = not applicable.

These rankings do not correlate with the hypothesized sensitization potential of these proteins. These data suggest that although the lymph node proliferative response may be an indicator of the immunogenic potential, additional characterization of the response may be required to assess the sensitization/allergic potential.

Due to the potential for varying amounts of endotoxin to be present in different protein samples and its potential influence on allergic responses, endotoxin levels were analyzed in each of the protein samples. Results of these analyses are displayed in Table 2 and are provided as endotoxin units (EU)/μg of protein and as the endotoxin levels in each dose group. These results indicate the rank order of endotoxin levels for the proteins was BLG > OVA > KLH > SUB which varies inversely with the EC_3 potency rankings. This prompted an additional study to determine the effect of endotoxin on the lymph node response.

3.3. Effect of endotoxin on the proliferating lymph node response

A dose level of 0.3% OVA was used in the evaluation as this protein/dose level induced a robust yet moderate proliferative response in the tracheobronchial lymph node such that the enhancement or suppression of lymph node proliferation could be detected. Two dose levels of LPS, 100 and 1000 EU, were tested alone and in combination with 0.3% OVA by the OP route and the proliferative response in the tracheobronchial lymph node was monitored. The levels of LPS were selected to represent the range of endotoxin levels observed in protein samples above. The results of this study are presented in Fig. 3. The data indicate the addition of endotoxin resulted in the enhancement of OVA-induced lymph node proliferation. Therefore, the varying endotoxin levels between the different proteins tested above would not be expected to change the rank order potency for the proliferative response in the tracheobronchial lymph node.

3.4. Examination of gene expression markers

Gene expression responses were examined in the draining lymph node to assess the potential of this approach to provide additional discriminating information on the sensitization/allergy potential of the administered proteins. For this evaluation, equipotent doses of SUB, OVA, KLH and BLG were administered via the OP route and gene expression responses were monitored in the tracheobronchial lymph node. The EC_{10} dose (estimated concentration resulting in a 10-fold SI) was chosen as the equipotent dose as it represents a dose that would yield a significant proliferative response and facilitate comparisons across all proteins. The EC_{10} was calculated using SI values generated in the previous experiment. These calculations yielded doses of 0.014% (7 μg/mouse), 0.078% (39 μg/mouse), 0.026% (13 μg/mouse) and 0.266% (133 μg/mouse) for SUB, OVA, KLH and BLG, respectively. For this examination, mice dosed with SUB displayed toxicity (body weight loss) which may

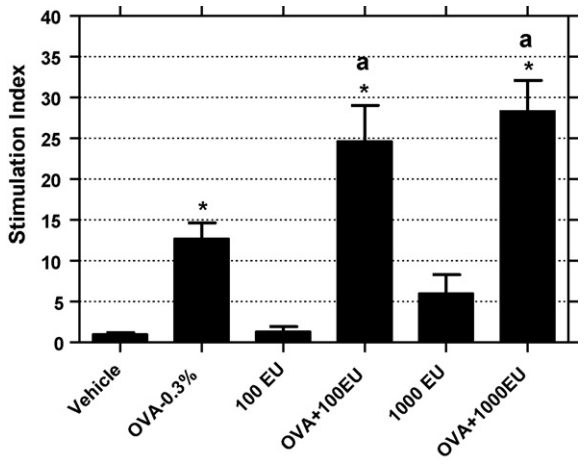

Fig. 3. Examination of the influence of endotoxin on the proliferative response in the draining lymph node. Mice were dosed on days 1, 2 and 3 and the proliferative response in the tracheobronchial lymph node was determined by ^{3}HTdR incorporation as per the standard LLNA. All mice dosed with OVA were dosed at a level of 0.3% which corresponds to a dose of 150 μg/mouse. Stimulation index values are expressed relative to the vehicle control and represent the mean ± standard deviation. *$p < 0.05$ when compared to vehicle control; a indicates $p < 0.05$ when compared to OVA alone; EU = endotoxin units.

confound the interpretation of the data for this protein. All other proteins had no effect on body weight. Gene expression markers were chosen to provide an indication of the proliferative response (Ki67) and the predominant T-helper cell response (IL4 and IL21 have been associated with a Th2 response while Ifng has been associated with a Th1 response). All proteins resulted in induction of the transcript for the proliferative marker Ki67, although the magnitude of the response was different (Fig. 4). Each protein also induced IL4 in a pattern similar to that observed for Ki67, suggesting this transcript may correlate with the proliferation response. Induction of IL21 did not follow the same pattern as the highest induction was observed for OVA followed by BLG, SUB and KLH. In contrast, Ifng displayed slight suppression for all proteins relative to the vehicle control which was not statistically significant.

Lung histology was also examined as part of this experimental evaluation. Relative to vehicle, all protein treated groups displayed slight, multifocal, bronchial goblet cell hyperplasia. Inflammation of the alveolar, peribronchial and/or perivascular regions was also observed for all proteins and was most prominent in mice exposed to SUB followed by OVA, BLG and KLH. Overall, these histological observations were considered to be changes which are characteristic of acute pulmonary exposure to foreign proteins and did not offer further insight into the allergenic potential of the proteins. Additional insight offered by histological endpoints would likely require the examination of later time points which coincide with the developed allergic state. However, these data do confirm test material delivery to the lung after OP administration.

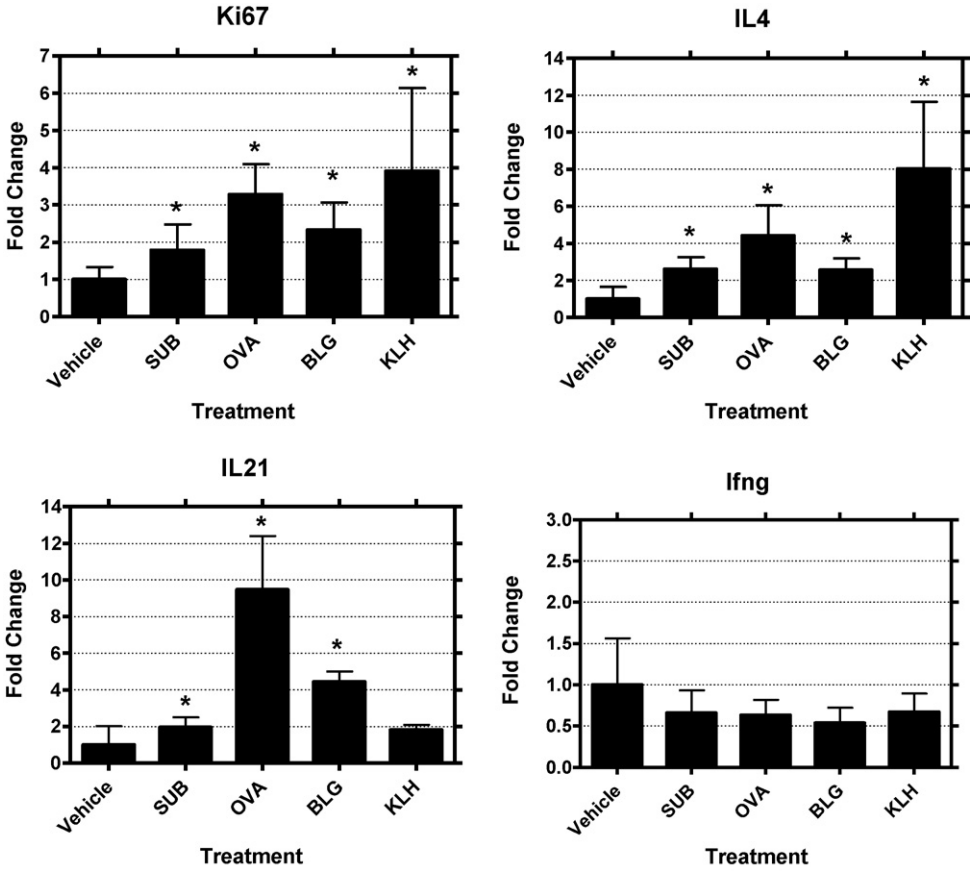

Fig. 4. Molecular characterization of the tracheobronchial lymph node. Groups of mice received equipotent doses of each protein and the expression level of select transcripts were monitored by quantitative real-time PCR. Equipotent dose was selected as the calculated EC_{10} value, the concentration required to induce a stimulate index of 10, corresponding to doses of 0.014% (7 μg/mouse), 0.078% (39 μg/mouse), 0.026% (13 μg/mouse) and 0.266% (133 μg/mouse) for SUB, OVA, KLH and BLG, respectively. Data points represent the fold change relative to vehicle ± standard deviation of at least 5 independent samples. Gene identities are denoted by their gene symbols: Ki67 (antigen identified by monoclonal antibody Ki67), IL4 (interleukin 4), IL21 (interleukin 21) and Ifng (interferon gamma). *$p < 0.05$ when compared to vehicle control.

4. Discussion

The present study examined the use of a LLNA approach which employed alternate routes of exposure to determine if the proliferative response in the draining lymph node correlates with the known allergenic potential/potency of various proteins. The results indicate, as expected, dermal exposure to proteins is not effective for inducing lymph node responses. However, IN and OP administration of proteins was effective in inducing lymph node proliferative responses, most notably in the tracheobronchial lymph node, which drains the lung. These results indicate the general responsiveness of the draining lymph node to administered proteins which may suggest utility for this endpoint in evaluating the sensitization potential of proteins. However, comparison of proteins revealed the lymph node response may be an indicator of their immunogenic potential and additional characterization may be necessary to assess the sensitization/allergic potential. The data also highlight a number of additional considerations beyond lymph node proliferation which need to be taken into account when assessing the allergenicity of proteins. These include issues such as immunogenicity versus allergenicity, identification of an appropriate negative control protein, and assessment of endotoxin levels.

Although the goal of this study was to evaluate the utility of a draining lymph node assessment as a potential endpoint for the assessment of protein allergenicity, using an approach anchored to the design of the LLNA, interpretation of the data suggests that the lymph node proliferative response is more likely an indicator of their immunogenic potential. The proteins evaluated in this study included SUB, a potent respiratory sensitizer/allergen, OVA, a potent food allergen commonly used in respiratory allergy research, BLG, a moderate food allergen, and KLH a strong immunogen with no reports of respiratory sensitization. Therefore, the expected rank order potency for allergic potential was SUB > OVA > BLG > KLH, however, the observed rank order for the lymph node proliferative responses was SUB > KLH > OVA > BLG. The higher than expected ranking for KLH is likely due to its strong immunogenicity. An alternative explanation could be that KLH is an allergen in this mouse model. However, additional studies in this lab that have monitored the IgE, IgG1, IgG2a and respiratory responses to these proteins in a sensitization/elicitation model do not support this as the data indicated that OVA was clearly more allergenic than KLH (Krieger et al., in preparation). Therefore, the proliferative response observed in the draining lymph node in the present study is likely attributable to immunogenicity of the proteins and should not be assigned to allergenicity. Such an interpretation is consistent with the notion that most proteins are inherently immunogenic, however, only few are able to cause stimulation of the quality required for allergic sensitization and production of IgE (Matsuda et al., 2006; Dearman and Kimber, 2009). Therefore, a key research area has been to develop an understanding of the properties of proteins that confer allergenicity rather than only immunogenicity. To date, no key characteristics to discriminate these classes have been identified (Holsapple et al., 2006). For this reason, we engaged in efforts to further characterize the draining lymph node response through examination of gene expression responses.

Much of the research aimed at discriminating immunogenic and allergenic proteins has been in the area of understanding the balance between Th1 and Th2 cytokines (Holsapple et al., 2006). The current understanding is that proteins that are allergenic have an immune response that is skewed toward Th2 cell activation which can lead to IgE production. Furthermore, Th1 and Th2 cytokines are known to have suppressive effects on one another, while regulatory T-cells are also thought to play a role in modulating these responses. Therefore, the development of an allergic state is a multifaceted response in which differentiated T-cells and their secreted cytokines appear to play an integral role. For this reason, the transcript response in the proliferating lymph nodes was examined to determine if transcripts encoding key cytokines may offer additional insight into the allergenic potential of the proteins. Ki67, a commonly used marker of proliferation, was examined as a surrogate marker of the proliferative response. Despite the selection of equipotent doses, the responses for this gene may indicate differing proliferative responses between the proteins. Transcript levels for IL4, a classic Th2 cytokine, correlate strongly with the Ki67 proliferative response suggesting the IL4 transcript response may not have distinguishing potential, consistent with previous reports (Selgrade et al., 2006). Ifng, a classic Th1 cytokine, was unchanged by all proteins and therefore did not provide any further discrimination. The lack of response for this gene is consistent with recent reports indicating that the regulation of this cytokine in response to sensitizers is at the level of the protein as opposed to the transcript (Dearman et al., 2008). Interestingly, IL21 displayed a pattern of induction wherein the potency ranking differed from that of Ki67 and IL4 with OVA > BLG > SUB > KLH. IL21 is a cytokine that has been reported to be preferentially expressed in Th2 cells and inhibit interferon-gamma production in developing Th1 cells, thereby supporting development of a Th2 response (Wurster et al., 2002). The response for this transcript suggests that a more specific profiling of the lymph node, beyond simple proliferation, may offer additional clues as to the allergenic potential of proteins. Such profiling would involve the examination of a wide range of transcripts, cytokines or other responses to fully assess the discriminating potential of alternative endpoints.

There are few studies in the literature that have examined negative control proteins as part of the evaluation of the allergenicity model or endpoint. Inclusion of a negative control protein is important in demonstrating the specificity of the response. However, interpretation of a negative response should be made with sufficient knowledge of not only the allergenicity of the protein but also the immunogenicity. This is because the major histocompatibility complex (MHC) class II haplotype can vary among strains of mice and can play an important role in immune recognition. For this reason, researchers have recommended evaluating both IgG and IgE responses to proteins and only when a negative IgE response is observed in the presence of a vigorous IgG response can conclusions be made with regards to lack of allergenic potential (Dearman and Kimber, 2009). Evaluation of allergic and non-allergenic foods has shown that protein extracts from these foods vary in their relative immunogenicity in an allergy sensitive mouse model but this immunogenicity alone does not distinguish allergenic from non-allergenic foods (Birmingham et al., 2002). Although immunogenicity may be an important component toward the development of allergy, it alone does not predict allergenic potential, therefore, the difficulty that remains is the availability of proteins which are immunogenic and do not induce endpoints or responses associated with the development of allergy in the various research models. One approach is to use purified proteins from foodstuffs that are considered to lack significant allergenic activity, such as proteins from spinach, lettuce or potato. The potato agglutinin seems to be a good candidate for a negative control protein. Potato agglutinin induced vigorous specific IgG antibody production in BALB/c mice after intraperitoneal injection confirming its immunogenic potential, but it failed to stimulate marked IgE antibody production (Dearman et al., 2003a). Another potential option would be the use of mouse serum albumin (MSA). Intranasal administration with MSA did not result in the production of specific-IgE as indicated by negative responses in the PCA assay (Krieger et al., in preparation). However, this work did not evaluate the immunogenic potential of the MSA in terms of the IgG response. 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 both

its immunogenic and allergenic potential. It is clear that development and evaluation of models for allergenicity should include both positive and negative control proteins; however, we should not lose sight of the fact that the ultimate goal is prediction of allergic potential in humans and not rodents. Therefore, development of these models should be closely tied to case history reports such that the developing models are predictive of the allergic potential in humans.

Assessment of endotoxin levels is also an important consideration when conducting research to characterize the allergic potential of proteins. Many protein test materials are purified from Gram-negative bacterial hosts and therefore have the potential to contain appreciable amounts of LPS (endotoxin). Data derived from the literature indicate conflicting results as to the enhancing or suppressing influence of LPS on the subsequent induction of allergic responses (Mizoguchi et al., 1986; Slater et al., 1998; Eisenbarth et al., 2002; Ormstad et al., 2003; Watanabe et al., 2003). Evaluation of the LPS content of the proteins examined in this study revealed the proteins differed dramatically in their LPS content and in a manner that varied inversely to the observed proliferative responses. To help facilitate a meaningful interpretation of the results, the influence of LPS on the proliferative response was examined using OVA as the test protein. The results of this examination indicated that the addition of endotoxin resulted in the enhancement of OVA-induced lymph node proliferation. Therefore, the varying endotoxin levels between the different proteins would not have changed their rank order potency for the lymph node proliferation response. Although it is unknown how the proliferative response that was monitored correlates with true allergenic potential of the proteins, these data reiterate the need to properly characterize the LPS content of examined proteins to help ensure complete and proper data interpretation.

In summary, the present study evaluated the utility of a draining lymph node approach for the assessment of the respiratory sensitizing potential of proteins after IN or OP administration. The results indicate that both administration approaches resulted in robust proliferative responses in the draining lymph node, however, the rank order response seemed to correlate with immunogenicity as opposed to allergenicity. Select transcript responses were examined in the draining lymph node to further characterize the response. Overall, the data illustrate both the opportunities and challenges associated with the examination of the draining lymph node response to proteins. Future research that further explores this response should consider additional endpoints and/or optimized dosing and temporal examinations to more fully assess the allergenic potential of proteins.

Conflict of interest statement

None.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.toxlet.2009.12.020.

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