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THE USE OF Th2 CYTOKINE GENE EXPRESSION IN THE NASAL AND PULMONARY AIRWAYS OF MICE TO IDENTIFY HUMAN CHEMICAL RESPIRATORY ALLERGENS

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By

Aimen Khader Farraj

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ABSTRACT

THE USE OF Th2 CYTOKINE GENE EXPRESSION IN THE NASAL AND PULMONARY AIRWAYS OF MICE TO IDENTIFY HUMAN CHEMICAL RESPIRATORY ALLERGENS

By

Aimen Khader Farraj

Exposure to low molecular weight (LMW) chemical allergens is the leading cause of occupational respiratory diseases such as asthma and allergic rhinitis. There are currently no well-validated and widely accepted methods for identifying chemical respiratory allergens due in part to the uncertainty in mechanism(s) and the variability across animal models in the route of exposure, the endpoints assessed and the tissues from which the endpoints are measured. Nonetheless, LMW chemical-induced allergic airway diseases share many pathologic and immunologic features with allergic airway diseases induced by larger proteins, including enhanced Th2 cytokines within the airways. An *in vivo* murine model of allergic airway disease was used to study the pathologic and immunologic responses to known chemical respiratory sensitizers and non-sensitizers after intra-airway instillation. I hypothesized that Th2 cytokine gene expression in the nasal and/or pulmonary airways of mice may be used to identify chemicals with the potential to elicit allergic responses in the respiratory tract.

Intranasal (IN) sensitization and challenge with adjuvant-free ovalbumin in A/J mice elicited mucous cell metaplasia and lymphocytic/eosinophilic infiltration in pulmonary airways that correlated with increased lung-derived Th2 cytokine

mRNA and serum IgE, characteristic of allergic airway disease. Cannabinol, (CBN), an inhibitor of Th2 cytokine expression, administered via intraperitoneal (IP) injection, suppressed the ovalbumin-induced increase in intraepithelial mucus (IM) in the pulmonary airways, serum IgE, and lung-derived IL-4 mRNA. IN delivery of CBN inhibited the ovalbumin-induced increase in serum IgE, the inflammatory cells in the lung, and lung-derived IL-13 mRNA levels, but not the IM in the pulmonary airways or the lung-derived increase in other Th2 cytokines.

IN sensitization and challenge with the chemical respiratory allergens toluene diisocyanate (TDI) and trimellitic anhydride (TMA) elicited some of the characteristic pathologic features of LMW chemical-induced allergic rhinitis and TMA caused increased nasal airway-derived Th2 cytokine gene expression. Neither TDI nor TMA exposed mice had pulmonary lesions, but had increased lung-derived Th2 cytokine mRNA and serum IgE. The contact allergens dinitrochlorobenzene (DNCB) and oxazolone (OXA) did not induce any nasal or pulmonary airway lesions or increases in Th2 cytokine mRNA in the lung, but DNCB did elicit an increase in serum IgE.

The results suggest that IN sensitization and challenge may be effective in eliciting the characteristic features of protein- or chemical-induced allergic airway disease. CBN inhibited some features of the ovalbumin-induced allergic airway response highlighting the importance of Th2 cytokines in the pathogenesis of the allergic airway response in this murine model and the potential therapeutic utility of CBN. Airway Th2 cytokine mRNA expression and/or serum IgE levels may be used to identify protein or chemical respiratory allergens.

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In the name of Allah (Arabic word for God), the Most Beneficent, the Most Merciful. All praise and thanks are due to Allah, the sole Creator and Sustainer of this universe and who alone we worship, for facilitating the completion of this work. Our Lord, may the mention of Muhammad (last of a series of Messengers including Adam, Abraham, Noah, Moses and Jesus sent by Allah to guide humankind) and all the other true Prophets and Messengers be exalted in the heavens. I ask Allah to make this humble effort beneficial to all humankind and to forgive my shortcomings.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Images in this dissertation are presented in color

LIST OF ABBREVIATIONS

AB/PAS	alcian blue/Periodic acid Schiff's reagent
APC	antigen presenting cell
BALF	bronchoalveolar lavage fliud
BSA	bovine serum albumin
CBN	cannabinol
CD4	cluster of differentiation 4
C_T	cycle threshold
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNCB	dinitrochlorobenzene
Δ^9-THC	Δ^9-tetrahydrocannabinol
EA/OO	ethyl acetate/olive oil
ECP	eosinophilic cationic protein
GM-CSF	granulocyte macrophage-colony stimulating factor
GSH	glutathione
HDI	hexamethylene diisocyanate
IFN	interferon
Ig	immunoglobulin
IL	interleukin
IM	intraepithelial mucosubstances
IN	intranasal
IP	intraperitoneal
IS	internal standard
kDa	kiloDalton
LMW	low molecular weight
MBP	major basic protein
MCM	mucous cell metaplasia
MINT	mouse intranasal test
mRNA	messenger ribonucleic acid
MTB	mycobacterium tuberculosis
OXA	oxazolone
PAF	platelet activating factor
PEL	permissible exposure limits
RADS	reactive airway dysfunction syndrome
rcRNA	recombinant RNA
RNA	ribonucleic acid
RPA	RNase Protection Assay
RT	reverse transcriptase
RT-PCR	reverse transcriptase-polymerase chain reaction
SEM	standard error of the mean
TB	tracheobronchial
TDI	toluene diisocyanate

Th	T-helper
TMA	trimellitic anhydride
TMB	tetramethyl benzidine
VCAM-1	vascular cell adhesion molecule 1
Vs	Volume Density

CHAPTER 1

INTRODUCTION

Occupational Respiratory Diseases

There are a variety of respiratory illnesses that result from occupational exposure. Some of the most frequent diagnoses include acute pulmonary edema, mesothelioma, and diseases that result from allergic sensitization of the respiratory tract such as allergic alveolitis, allergic rhinitis, and asthma (Kimber et al, 1997). Occupational asthma and allergic rhinitis are the most frequently diagnosed occupational respiratory illnesses (Beach et al, 2000). Epidemiologic studies suggest that 10 to 25 % of adult asthma is linked to workplace exposure (Petsonk, 2002). The Surveillance of Work and Occupational Respiratory Disease (SWORD) program in the United Kingdom found that 26 % of all work-related respiratory illness was due to occupational asthma (Lombardo and Balmes, 2000). The incidence of occupational asthma amongst workers in the United States ranges from 29 to 710 cases per million workers (Petsonk, 2002). There was a 70 % increase in cases of occupational asthma in Finland from 1986 to 1993 (Petsonk, 2002). Allergic rhinitis resulting from occupational exposure is less well documented than occupational asthma partly because of inadequate diagnostic methods (WHO, 1999). Hypersensitivity of the upper respiratory tract, however, often coexists with occupational asthma. Several studies suggest that allergic rhinitis due to workplace exposure to chemicals precedes and may be more common than occupational asthma (Bernstein, 1993; Grammer et al, 2002).

Hallmark features of Allergic Airway Disease of the Lung

Allergic airway diseases such as asthma result from inappropriate immune responses to commonly inhaled foreign agents such as those found in the environment (e.g., pollen or cockroach antigen) or in occupational settings (e.g., dander from laboratory animals or isocyanates). The offending agent is usually termed an antigen or allergen in reference to the allergic reaction it provokes. Allergy consists of an initial generally symptom-free sensitization phase followed by an effector phase that produces the symptoms characteristic of allergy. Asthma is a chronic inflammatory disease of the lung that has increased in prevalence, morbidity, and mortality in recent years. The main physiological abnormalities of asthma are airflow obstruction and airway hyper-responsiveness (Wills-Karp, 1999). The airways of asthmatics are characterized by the presence of chronic inflammation with infiltration of the bronchial mucosa by lymphocytes, eosinophils, neutrophils, and mast cells, along with increased mucus production and epithelial desquamation, and airway remodeling such as hyperplasia of the airway epithelium, smooth muscle hypertrophy and fibrosis (Wills-Karp, 1999; Holgate, 2000).

Many factors contribute to the development of asthma. Nonetheless, the single-most identifiable predisposing factor for the development of asthma is atopy (Kay, 2001). Individuals with atopy have a hereditary predisposition to produce an IgE-mediated response against common allergens and have one or more atopic diseases such as allergic rhinitis, asthma, and eczema (Kay, 2001). In general, adults and children without atopy mount a low-grade immunologic

response to allergen exposure characterized by IgG1 and IgG4 antibodies (Kay, 2001). After repeated low-dose exposure to allergens, atopic individuals develop specific IgE antibodies to the allergens. Subsequent exposure to the allergen(s) initiates a secondary humoral response. Immediately after re-exposure, the response is characterized by rapid onset of mucosal edema, increases in airway smooth muscle tone and airway narrowing associated with mast cell degranulation. A few hours later and persisting for several days, a late-phase response takes place that is identified by airway narrowing associated with the migration of eosinophils, lymphocytes, and neutrophils from the blood into the lung parenchyma and airway (Wills-Karp, 1999). This type of response characterizes Type I hypersensitivity responses that take place rapidly (within hours) after re-exposure to a particular allergen.

Mechanisms of Allergic Airway Diseases of the Lung

The mechanism(s) underlying the pathogenesis of allergic airway disease are being elucidated. There are many lines of evidence that implicate the T lymphocyte and specifically the CD4+ T cell in the orchestration of this aberrant response. Evidence for the participation of T lymphocytes includes increased numbers of CD4+ T cells in bronchoalveolar lavage fluid (BALF) and a concurrent decrease in the number of CD4+ T cells in peripheral blood following allergen challenge (Wills-Karp, 1999). The Th2 subset of CD4+ T cells and the cytokine profile associated with it have been consistently found in both BALF and biopsies of allergic asthmatics (Wills-Karp, 1999). CD4+ T cells are classified

into functionally distinct Th1 or Th2 subsets based on the restricted cytokine profiles expressed by *in vitro* murine T cell clones (Bruijnzeel-Koomen et al, 2000). Th1 cells produce IFN-γ and IL-2 promoting the development of a cell-mediated immune response involving cytotoxic T cells and macrophages. Th2 cells produced IL-4, IL-5, IL-9, IL-10, and IL-13 involved in promoting humoral immune responses, typical of atopy and parasitic infections. A similar functional dichotomy has been found in human T cells (Andersson et al, 2001). In allergic airway disease, Th2 cell activity predominates, while Th1 activity is nominal. Several factors are involved in the development of CD4+ Th2 cells in allergic processes including the local cytokine environment, the level and type of antigen, the antigen presenting cell, and the delivery of co-stimulatory signals form the antigen presenting cell. The paracrine release of IL-12 derived from antigen presenting cells promotes the development of Th1 responses, while the autocrine production of IL-4 by Th2 cells promotes the development of Th2-type responses (Wills-Karp, 2001). Several lines of evidence support the role of Th2 cytokines in the pathophysiologic features of allergic airway diseases such as asthma. The differentiation of uncommitted T cell precursors into Th2 cells is largely driven by IL-4 as demonstrated by the abrogation of airway hyperreactivity after administration of a monoclonal antibody against IL-4 during the period of immunization in a murine model of asthma (Corry et al, 1996). IL-4 is also a growth factor for mast cells and up-regulates vascular cell adhesion molecule-1 (VCAM-1) expression which leads to preferential migration of eosinophils into tissues (Wills-Karp, 1999). IL-4 and IL-13 are required for B cells to undergo

class switching to go from producing IgM to producing IgE (Frew, 1996). IL-5 regulates the maturation and activation of eosinophils. IL-5-deficient mice have markedly fewer eosinophils in the lamina propria of airways than normal mice after provocation and antibody blockade inhibits antigen-induced eosinophilia and late-phase hyperresponsiveness (Foster et al, 2000 and Karras et al, 2000).

Hypersecretion of mucus and mucous cell metaplasia (MCM) are important features of allergic airway disease. IL-9 plays a large role in the stimulation of mucin (a mucus protein found within a mucous cell) production in airways as demonstrated by the inhibition of mucin production in respiratory epithelial cells via treatment with antibodies against IL-9 in dogs (Longphre et al, 1999). IL-4 and IL-13 also induce mucin gene expression in airway epithelium (Shim et al, 2001). IL-13 has also been proven to be necessary for the expression of allergic airway hyperresponsiveness and sufficient to induce it. Administration of recombinant IL-13 conferred an asthma-like phenotype to non-immunized mice (Grunig et al, 1998).

Pathogenesis of Allergic Airway Disease of the lung

The pathogenesis of allergic airway disease may be summed up by a proposed series of events beginning with the inhalation of the antigen. After inhalation, the antigen is believed to interact with the follicular dendritic cell found above the basement membrane of the airway epithelium throughout most of the respiratory tract (Wills-Karp, 1999). Dendritic cells are antigen-presenting cells that recognize and process the antigen and migrate to the local draining lymph

nodes of the lung, up-regulate expression of co-stimulatory ligands on their surfaces (i.e., B7), and interact with resting T lymphocytes, initiating a primary immune response (Figure 1.1). Uncommitted T cell precursors (Th0) differentiate into one of two subtypes depending on the nature and dose of the antigen and the local cytokine microenvironment. In the presence of IL-4, Th0 cells differentiate into Th2 cells (Wills-Karp, 1999). IL-12 primes CD4+ T cells for Th1 differentiation, which later secretes IFN-γ, critical for cell-mediated immunity and an inhibitor of Th2 differentiation (Warbrick et al, 1999). Newly differentiated armed Th2 cells secrete a unique pattern of cytokines that includes IL-4, IL-5, IL-6, IL-9, IL-10 and IL-13. Initial contact with the antigen causes B cells to differentiate into memory B cells. The Th2 cell then interacts with the B cell that has already made initial contact with the antigen; Th2 cells secrete IL-4 and IL-13 that drive 'class switching' in B cells that convert from IgM-producing naïve B cells to IgE-producing memory B cells (Bruijnzeel-Koomen et al, 2000). IgE molecules secreted by the B cell bind FcεRI receptors on the surface of mast cells in the bronchial mucosa. Subsequent exposure to the allergen causes the IgE producing memory B cells to proliferate and differentiate into IgE-secreting plasma cells (Figure 1.2). The allergen then 'cross-links' two IgE molecules on the surface of the mast cell causing degranulation of mast cell mediators. The mast cell contains several potent mediators of inflammation including histamine, platelet activating factor (PAF) and leukotrienes which contribute to the vasodilation, increased vascular permeability, and airway narrowing associated

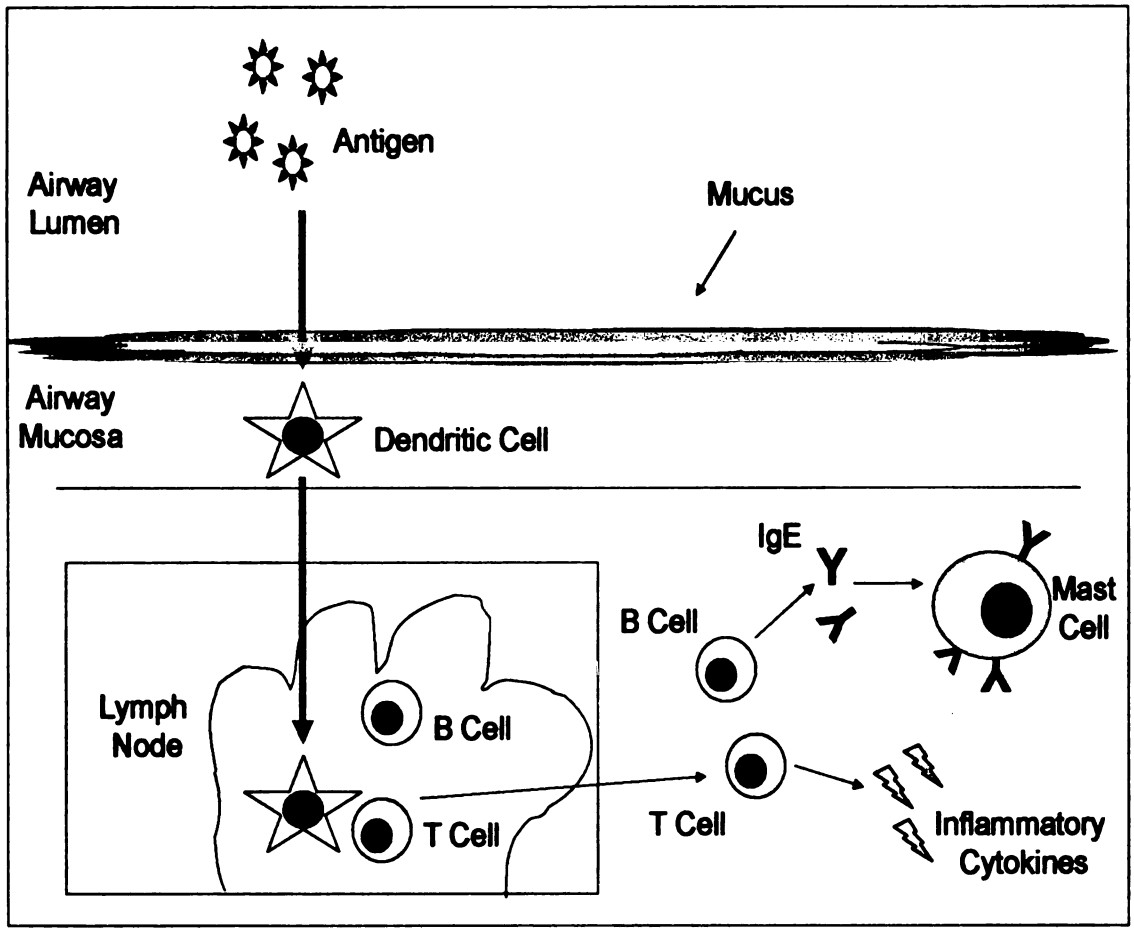


Figure 1.1: Primary exposure to a high molecular weight protein allergen (>1 kDa).

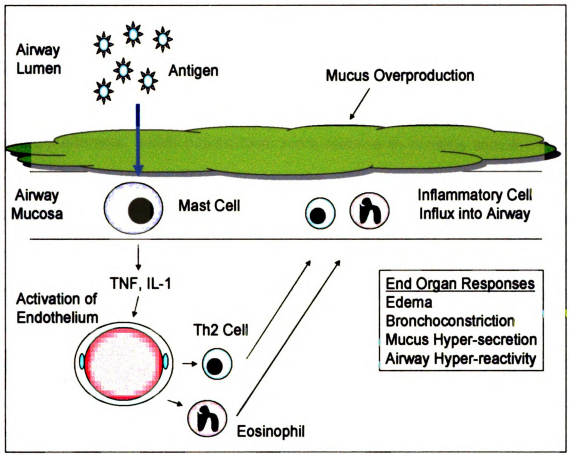


Figure 1.2: Secondary Exposure to a high molecular weight protein allergen (>1kDa).

with asthma (Frew, 1996). Re-exposure to the allergen also causes the infiltration of plasma cells and Th2 cells into the bronchial mucosa. Th2 cells also release IL-5, which promotes the growth and recruitment of eosinophils. Eosinophils release leukotrienes, eosinophil cationic protein (ECP) and major basic protein (MBP) that cause epithelial cytotoxicity, airway hyperreactivity and narrowing, and increased mucus production. Bare epithelium exposes the sensory afferents, which makes them more reactive to noxious stimuli. Major basic protein binds and inhibits M_2 receptors located on the nerve terminals of airway parasympathetic nerves that limit acetylcholine release; this contributes to the airway hyperreactivity (Adamko et al, 1999). IL-4, IL-9, and IL-13, also released by the Th2 cell contribute to airway hyperreactivity and mucus overproduction.

Allergic Airway Disease of the Nose

Allergic rhinitis is an inflammatory disease of the nasal mucosa affecting 10 to 20 % of the world's population (Andersson et al, 2000). It is frequently caused by exposure to perennial or seasonal allergens present indoors and outdoors (Andersson et al, 2000). Pollens, house dust mites and animal dander are major causes of allergic rhinitis (Andersson et al, 2000). Itching, followed by sneezing occurs very rapidly after allergen deposition in the nasal mucosa. These symptoms then abate followed by an onset of nasal watery secretion a few minutes after nasal provocation. This may last for a few hours if the provocative dose is sufficiently strong. Nasal obstruction, due in part to

hypersecretion of mucus is also generated within minutes and may continue for hours (Andersson et al, 2000). Sensory nerve stimulation is also a feature of allergic rhinitis (WHO, 1999). The mucosal exudation of plasma to the airway lumen is also a fast process. Like asthma, the main predisposing factor for allergic rhinitis is atopy. IgE antibodies are known to play a key role by triggering the release of mediators that are responsible for the allergic symptoms (Andersson et al, 2000). The histologic features are characteristic of Type I immediate hypersensitivity. The nasal mucosa shows large numbers of T- and B-lymphocytes, mast cells, neutrophils, plasma cells and dendritic cells and mucus overproduction (Andersson et al, 2000). The nasal mucosa in allergic rhinitis is also characterized by the infiltration of large amounts of eosinophils (van de Rijn et al, 1999). Th2 cytokines, similar to allergic asthma, have been shown to play a role in allergic rhinitis. Nasal biopsies taken 24 hours after nasal allergen challenge demonstrated an increased number of cells expressing mRNA hybridization signals for IL-3, IL-4, IL-5 and GM-CSF in patients with allergic rhinitis (Andersson et al, 2000). Nasal allergen provocation, followed by nasal mucosa biopsy 24 hours after challenge in patients with allergic rhinitis is associated with a cellular infiltrate where CD4+ T cells and eosinophils are prominent (Andersson et al, 2000).

Occupational Asthma Definition and Pathophysiology

Occupational asthma may be defined as a condition that is characterized by variable airflow limitation and/or airway hyperresponsiveness due to causes

and conditions attributable to a particular occupational environment and not to stimuli encountered outside the workplace (Bernstein et al, 1993). There are two types of occupational asthma that are distinguished by whether they appear after a latency period. One is the immunologic type where there is a latency period for most high and some low molecular weight agents. It is characterized by an asymptomatic period during which sensitization to a substance in the work environment occurs. The clinical expression of asthma symptoms is then elicited by low concentrations of the causative agent. Asthma may persist for years even after removal from further exposure. The reason for this is unclear, but may perhaps be due to the immunologic response, which is initially very specific, but gradually becomes more nonspecific as the inflammatory response in the airway becomes more established (Beach et al, 2000). The prolonged morbidity correlates directly with the length of time the worker is exposed to the causative agent prior to diagnosis and removal from further exposure (Kimber et al, 1997). The nonimmunologic type, also known as 'irritant asthma' or reactive airways dysfunction syndrome (RADS), may occur after a single or multiple exposures to nonspecific chemicals at high concentrations (Malo and Chan-Yeung, 2001). The term 'irritant' is commonly used by investigators studying the mechanisms of occupational allergic airway diseases to refer to chemicals that cause a nonspecific immune response at the site of contact, either the airway mucosa or the skin, without the requirement for prior exposure or sensitization to the chemical (Ban and Hettich, 2001).

Pathologic features in the airways of patients with occupational asthma are similar to those found in individuals with non-occupational asthma. They include airway smooth muscle hypertrophy, mucosal edema, increased mucus production, deposition of collagen beneath the basement membrane, and epithelial desquamation (Beach et al, 2000). A cellular infiltrate of inflammatory cells consisting of lymphocytes, eosinophils, and neutrophils is present in the airway and bronchial mucosa. The impact on pulmonary function includes non-specific airway hyperreactivity and bronchoconstriction (Beach et al, 2000).

Occupational Allergic Rhinitis Pathophysiology

Upper respiratory tract hypersensitivity involving the nose often coexists with asthma in the occupational setting (WHO, 1999). Similar to occupational asthma, the causes and conditions of occupational allergic rhinitis are attributable to a particular occupational environment and not to stimuli encountered outside the workplace. The pathophysiology of LMW chemical-induced allergic rhinitis is similar to that induced by proteins. With occupational agents, rhinitis is a more common manifestation than asthma because the nasal mucosa is more accessible to deposition of dusts and vapors (e.g., baker's flour, isocyanates, wood dusts, and animal dander) (Holgate et al, 2000). Some studies suggest that allergic rhinitis due to workplace exposure to chemicals precedes occupational asthma (Bernstein, 1993; Leynaert et al; 2000, Grammer et al, 2002). The symptoms include itching, sneezing, nasal congestion. They are accompanied by afferent nerve stimulation and the release of neuropeptides

such as substance P and tachykinins (WHO, 1999). There is a local accumulation of CD4+ T-lymphocytes, eosinophils, neutrophils and mast cells. Allergies, however, have to be differentiated from toxic and irritative mechanisms. Allergenic chemicals are often also irritants. Strongly toxic chemicals may elicit the characteristic symptoms of allergic rhinitis by directly damaging the mucosa after single contact (WHO, 1999). Milder irritants such as solvents may cause hyperreactivity after repeated contact (WHO, 1999).

Agents Linked to Occupational Asthma and Allergic Rhinitis

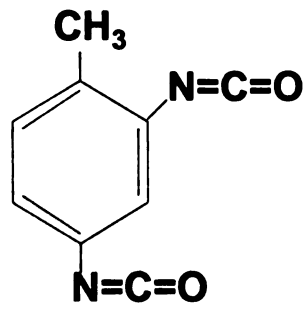
There are many occupations associated with asthma and allergic rhinitis including health care, animal handling, farms, bakeries, laboratory work, detergent manufacture, work with paints, plastics, and adhesives, work with jewelry making, work with metal salts, nickel plating, and the tanning industry (Petsonk, 2002). There are over 200 documented organic and inorganic agents that have been linked to the development of occupational asthma (Petsonk, 2002). These include high molecular weight agents including organic dusts, plant and animal proteins and proteins existing in natural rubber latex (Petsonk, 2002). The Occupational Safety and Health Administration (OSHA) has set Permissible Exposure Limits (PEL) for some of the agents associated with occupational asthma such as platinum salts, and certain isoycanates, but many agents that cause asthma in the workplace still lack PELs.. Among the most important classes of agents are low molecular weight (LMW) chemicals that have a molecular weight less than $1X10^3$ kDa (Kimber et al, 1997). Several

epidemiologic studies have found that highly reactive LMW chemicals are the leading cause of occupational asthma and allergic rhinitis. These include the Sentinel Event Notification System for Occupational Risks (SENSOR) surveillance system sponsored by the National Institute for Occupational Safety and Health (NIOSH) of the United States (Jajosky et al, 1999) and the SWORD surveillance program of the United Kingdom (Meyer et al, 1999). LMW chemicals linked to occupational asthma include flour and grain dusts, proteins from laboratory animals, glutaraldehyde, wood dusts, isocyanates and acid anhydrides (Kimber et al, 1997). Toluene diisocyanate (TDI) and trimellitic anhydride (TMA) are leading causes of occupational asthma and allergic rhinitis (Figure 1.3). TDI is used in polyurethane, plastics, and varnish industries, while TMA is a main component of epoxy resins.

Mechanisms of LMWC-induced Occupational Asthma and Allergic Rhinitis

The underlying mechanisms associated with the induction of asthma and allergic rhinitis by LMW chemicals have not been full elucidated. The pathophysiologic changes in the airways of patients with LMW chemical- induced asthma and rhinitis resemble those of allergy to larger agents such as pollens and animal dander. LMW chemical respiratory allergens, unlike most of the larger allergenic proteins, are either inherently chemically reactive or are metabolized in vivo into chemically reactive species (Karol et al, 2001). The physicochemical characteristics of LMW chemicals that are most closely associated with their allergenicity are transport and reactivity (Karol, 2001). In

Toluene Diisocyanate



Trimellitic Anhydride

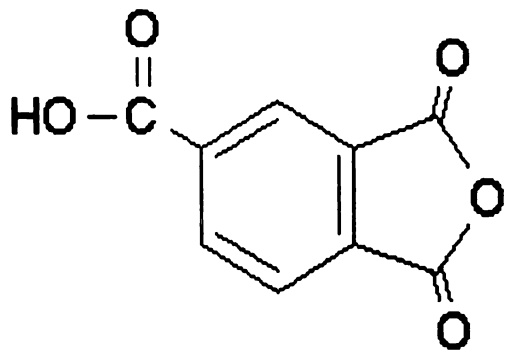


Figure 1.3: Chemical structures of Low Molecular Weight Chemicals Linked to Allergic Airway Disease

order to elicit their biologic or toxic activity, the LMW chemical must reach its appropriate target. The internal regions of the nasal airways and lung are separated from the external environment by the airway epithelium. There is data that suggests that the airway epithelium is the critical target of inhaled LMW chemical allergens. LMW chemical allergens are typically electrophiles or proelectrophiles capable of reacting with hydroxyl, amino, and thiol groups on endogenous proteins (Figure 1.4) (Karol et al, 2001). LMW chemicals conjugate with endogenous proteins including human serum albumin, tubulin, keratin, glucose-regulated protein, trans-1,2 dihydrobenzene-1,2 diol dehydrogenase, actin, and glutathione (GSH) in the respiratory tract (Beach et al, 2000; Lange et al, 1999; Day et al, 1997; Wisnewski et al, 2000). TDI colocalizes with tubulin on the cilia of differentiated human epithelial cells in vitro (Lange et al, 1999). Following inhalation of hexamethylene diisocyanate, keratin 18 was identified as the predominant diisocyanate-conjugated protein in human endobronchial biopsy samples, whereas albumin was the predominant diisocyanate-conjugated protein in BALF (Wisnewski et al, 2000). Low molecular weight agents are unique in that they only acquire immunogenicity after linkage with an endogenous protein carrier. The new multi-valent hapten-protein conjugates are then capable of inducing and eliciting an immune response, the specificity of which may be directed against the hapten carrier protein or new antigenic determinants in the conjugate, either singly or in combination (Kimber et al, 1997). Another mechanism by which LMW chemicals can lead to the production of new antigenic sites is by inducing cross-linking of endogenous proteins such as albumin. A

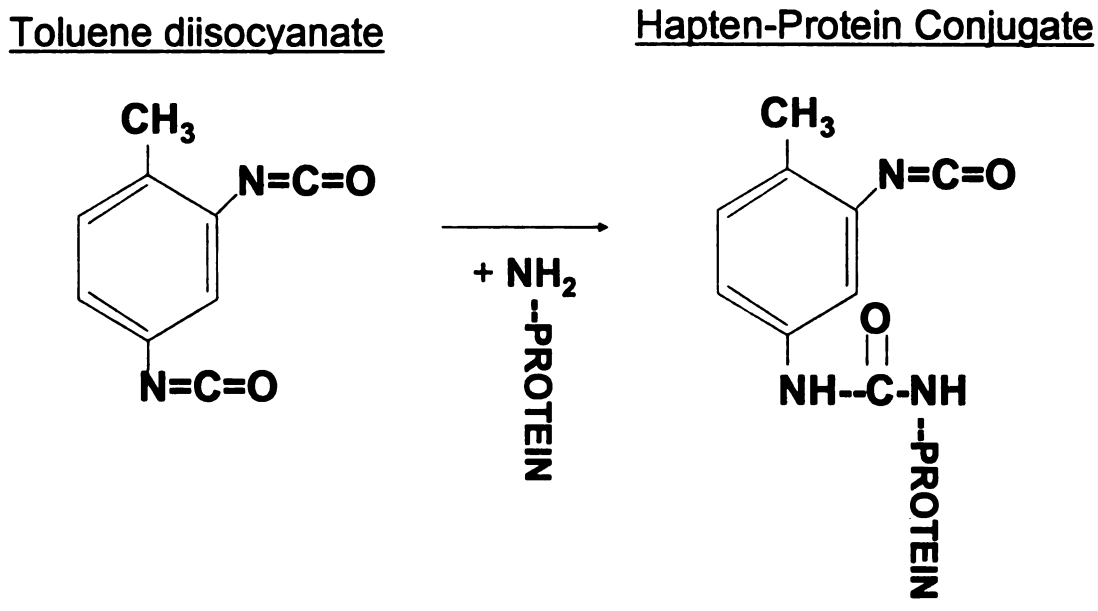


Figure 1.4: Toluene diisocyanate reacts with the amino terminus of a protein.

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number of patients suffering from glutaraldehyde-induced occupational asthma have exhibited antibodies against antigenic sites resulting from the glutaraldehyde-induced cross-linking of endogenous proteins (Beach et al, 2000).

Serum IgE in LMW Chemical-induced Occupational Allergic Airway Disease

The development of new antigenic sites as a result of conjugation of a chemical with human protein or endogenous protein cross-linkage may, in susceptible individuals, result in the production of IgE antibodies to the site in the same way as to large molecular weight substances, thus resulting in asthma and/or allergic rhinitis. In general, however, only 20 % of the subjects with TDI-induced occupational asthma demonstrate an increased level of serum TDI-specific IgE antibodies (Tee et al, 1998). The classic atopic response to allergen mediated by IgE antibodies (Type I hypersensitivity) accounts for only a small proportion of the total cases of occupational asthma induced by isocyanates. Similarly, IgE antibodies to acid anhydrides have been detected in individuals with occupational asthma from these substances, and the presence of these antibodies has been associated with the presence of disease (Beach et al, 2000). But like TDI, the development of specific IgE antibodies to TMA is inconsistent. In LMW chemical-induced allergic rhinitis, eliciting agents such as anhydrides, metallic salts, and diisocyanates only occasionally have been shown to induce IgE production (WHO, 1999). Thus, the available data suggest that an IgE-mediated mechanism is not the predominant manner by which individuals become sensitized to LMW chemicals. There is some controversy, however, in

the value of IgE levels due, as some investigators suggest, to the methods used to detect the antibodies. Some groups have suggested that the methods used to measure serum IgE were inadequate and that the use of more optimal detection methods to measure IgE levels will reveal a stronger association between IgE and LMW chemical-induced allergic airway disease (Kimber and Dearman, 2002). In most of the assays used to detect serum IgE against specific chemical allergens in humans or in animal models, the antigen substrate used is in the form of a hapten-protein conjugate. The most prevalent protein used to prepare such conjugates has been albumin (Kimber and Dearman, 2002). Tests for specific IgE that make use of hapten-protein conjugates are likely to identify only those patients in which binding to albumin has stimulated IgE production, not those in which IgE may have been stimulated by other conjugates (Griffin et al, 2001). This may have led to the underestimation of IgE levels and possibly false diagnosis. The recent development of an antibody specific for TMA and not a particular conjugate by one group of investigators (Griffin et al, 2001) may help clarify the role of IgE in LMW chemical-induced allergic airway disease.

Some individuals exposed to TDI or TMA develop specific IgG antibodies (Beach et al, 2000). Less is known, however, about how the presence of IgG antibodies is translated into symptomatic disease than is known about IgE-mediated asthma and allergic rhinitis. It is also unclear whether a specific subclass of IgG antibody is particularly relevant in the causation of asthma or allergic rhinitis (Beach et al, 2000).

Irritant Asthma as an Alternative Mechanism

A toxicologic mechanism exists apart from immune-mediated effects in LMW chemical-induced asthma-like symptoms. Individuals exposed to high concentrations of irritants may develop a syndrome known as Reactive Airways Dysfunction Syndrome (RADS) (Beach et al, 2000). RADS or irritant asthma was described in a number of reports following exposure to agents as diverse as chlorine, glacial acetic acid, sulfuric acid, hydrochloric acid, perchloroethylene, TDI, ethylene oxide and smoke (Beach et al, 2000). It is characterized by the apparent lack of a period of significant symptom-free exposure during which sensitization might occur, a relatively high level of exposure to the agent in question, onset of symptoms within a few minutes or hours of exposure, and ongoing symptoms and airway hyperresponsiveness persisting for months or years afterwards (Beach et al, 2000). Some of the pathologic features in the airways of patients with irritant-induced asthma include inflammation consisting of lymphocytes, epithelial shedding, and subepithelial fibrosis, similar to immune-mediated asthma (Beach et al, 2000). There is little evidence, however, of any eosinophils in the airways of patients with RADS (Beach et al, 2000). Although there is considerable overlap in the clinical features of RADS and allergic asthma, the mechanisms underlying RADS are less clearly understood than asthma arising from immunologic mechanisms. The changes in cytokines that may underlie this type of inflammatory response have not been fully characterized (Beach et al, 2000). Data from the SWORD study in the United Kingdom showed that less than 10 % of inhalation injuries that resulted from high

exposure to a chemical were followed by persistent asthma (Lombardo and Balmes, 2000). The predominant mechanism of LMW chemical-induced allergic airway disease thus appears to be immunologic.

Th2 Cytokines in LMW Chemical-Induced Allergic Airway Disease

Despite the apparent irregularity in mechanism and presence of specific antibodies, many lines of evidence implicate the T lymphocyte and specifically the preferential expression of the Th2 phenotype in LMW chemical-induced occupational asthma. For example, the production of IL-4 and IL-5 proteins was increased in bronchial biopsies of patients with TDI-induced asthma (Maestrelli et al, 1997). In addition, the selective enhancement of Th2 cytokines was detected in several murine models of TMA-induced asthma (Betts et al, 2002; Dearman et al, 2002; Vandebriel et al, 2000). One group reported that not only does treatment with a LMW chemical allergen, i.e., hexamethylene diisocyanate (HDI), lead to an enhancement of Th2 cytokines but that the HDI-induced airway inflammation in the lung was dependent on the presence of Th2 cytokines in a murine model (Herrick et al, 2002). Stimulation of isolated lymph node cells in culture in mice was used to demonstrate that the respiratory sensitizers TMA, TDI and phthalic anhydride led to preferential Th2 induction indicated by increased IL-4 expression, while the contact allergen dinitrochlorobenzene, induced the expression of the Th1 cytokine, IFN-γ (Vanderbriel et al, 2000). Dearman and colleagues demonstrated that TDI exposure induced high levels of IL-4 and IL-10, but low levels of IFN-γ in local draining lymph nodes (Dearman et

al, 1996). In another report, investigators distinguished five acid anhydrides as respiratory sensitizers based on their capacity to induce Th2 cytokine secretion (Dearman et al, 2000). Glutaraldehyde-stimulated lymph node cells also displayed the Th2 pattern of cytokine secretion (Dearman et al, 1999).

There has been no report in the literature that has linked local Th2 cytokine gene expression in the nose with the induction of LMW chemical-induced allergic rhinitis.

While in asthma caused by diisocyanates specific antibodies are unusual, an increase in activated T lymphocytes, mast cells, and eosinophils has been observed, similar to the appearance in allergic asthma. An alternative mechanism by which preferential Th2 expression may take place may involve the interaction of the chemical with glutathione (GSH). In vitro studies have shown that TDI avidly forms bis adducts with GSH and these adducts transfer monoisocyanato-monoglutathionyl-TDI to a sulfhydryl-containing peptide (Lange et al, 1999). This may lead to the depletion of GSH; recent evidence has implicated a lowered cellular GSH content in promoting the Th2 phenotype that is characteristic of allergy. Antigen presenting cells (APC) help regulate the balance between Th1 and Th2 response patterns, e.g., APCs secrete IL-12, which drives IFN-γ production, and the Th2-associated cytokine IL-10 inhibits APC IL-12 production and thereby drives IL-4 production (Peterson et al, 1998). GSH-depletion is accompanied by a loss of IL-12 production, which leads to loss of IFN-γ production (Peterson et al, 1998). APC depleted of GSH, however, still produce IL-4 similar to the levels of normal APC (Peterson et al, 1998). GSH-

depletion may thus be an additional mechanism by which LMW chemicals preferentially induce the Th2 pattern of cytokine expression.

Need for an Animal Model of LMW Chemical-induced Allergic Airway Disease

Occupational asthma and allergic rhinitis due to exposure to LMW chemicals are increasing in morbidity and associated with decreased worker productivity and the loss of billions of dollars annually (Lombardo and Balmes, 2000). Knowledge of the potential of a chemical to sensitize the respiratory tract will help to establish thresholds of exposure and/or permissible exposure limits. There are currently fewer than 400 chemicals with known PELs for inhalation exposure of the many thousands of chemicals used in industry (OSHA, 1993). The identification of potential chemical allergens used in industry will also serve to restrict their use. It may also enhance precautionary measures to limit exposure to chemical allergens such as improved ventilation, the placement of monitors to gauge the level of the chemical concentration in the air, and the use of personal respirators. The need to further elucidate the mechanism(s) of LMW chemical-induced allergic airway disease and to develop a reliable method of identifying chemicals that have the potential to elicit allergic airway disease in humans, especially in the workplace, has led to the development of animal models.

Many groups have attempted to model LMW chemical-induced allergic airway disease in animals using guinea pigs, rats, or mice to further understand

the mechanism(s) of LMW chemical-induced allergic airway disease and to identify potential human allergens. These models have been used to demonstrate the irritant/toxic and/or immunologic effects of LMW chemicals. Murine models are increasing in popularity because of the advantages they offer relative to other species including the availability of genetically manipulated strains and a vast array of diagnostic reagents. Also, the allergen-induced changes in murine airways are similar to the pathologic sequelae that take place in the airways of sensitized individuals after allergen challenge (Gelfand, 2002). Some murine models have induced several of the hallmark pathologic features of LMW chemical-induced allergic airway disease in the pulmonary airways with HDI (Herrick et al, 2002), TDI (Lee et al, 2002) and TMA (Regal et al, 2001). Increased Th2 cytokines has also been demonstrated in murine models of allergic airway disease induced by TMA (Betts et al, 2002), HDI (Herrick et al, 2002), and TDI (Matheson et al, 2001). Still other murine models have demonstrated elevated serum IgE levels with LMW chemical exposure in mice (Ban and Hettich, 2001; Matheson et al, 2001; Scheerens et al, 1999)

Despite the multitude of murine models, there remains a lack of a well validated and widely accepted animal model of LMW chemical-induced allergic airway disease. The failure to establish such an animal model is due in part to the variability across existing animal models in a number of respects including the route of exposure, the endpoints that are analyzed, the tissues from which the endpoints are measured, and whether it models irritant-induced asthma or immune-mediated asthma.

A Comparison of the Routes of Exposure in Animal Models

Animal models differ, for example, in the route of exposure used to sensitize the animals. Although inhalation is the most common and relevant route of exposure to LMW chemicals, there is a growing belief that the dermal route of exposure is sufficient to induce sensitization of the respiratory tract to LMW chemicals. In fact, some animal models have been able to elicit some of the characteristic features of LMW chemical-induced allergic airway disease including airway inflammation using dermal sensitization followed by intra-airway challenge (Herrick et al, 2002, Regal et al, 2002). However, a recent study showed that dermal sensitization followed by intra-airway challenge w/ TMA in rats resulted in a qualitatively different complement of immunocompetent cells in BALF than intra-airway sensitization and challenge (Vohr et al, 2002). That is, intra-airway induction followed by airway challenge with TMA caused an influx of macrophages, granulocytes and dendritic cells into the lung that dermal sensitization and intra-airway challenge with TMA failed to do. This suggests that the route of exposure influences the response within the respiratory tract and raises the possibility that dermal exposure to LMW chemicals in animal models may not adequately model human allergic airway disease that results exclusively from inhalation exposure to LMW chemicals.

The animal model described in this thesis project used a method of intra-airway administration, i.e. intranasal instillation, to both sensitize and challenge mice to LMW chemicals. Intranasal instillation more closely resembles the natural route of exposure to airborne allergens than dermal application. It also

offers a number of advantages compared to other methods of intra-airway administration, including aerosol exposure. Aerosolized LMW chemical exposure in animal models requires aerosol generation devices and exposure chambers, which are costly, complicated, and may pose a health risk to the person conducting these exposures because of chemical leak (Ebino et al, 1999). Intranasal instillation, in contrast, is a simple, inexpensive, safe and noninvasive method of exposing both the upper and lower respiratory tract of laboratory rodents to potential allergens. In addition, the urgent need to assess the respiratory allergenicity of numerous chemicals necessitates that the animal model rapidly and efficiently screen LMW chemicals. The ease and practicality of intranasal instillation may facilitate such screening.

A Comparison of Endpoints Measured in Animal Models

Related to the route of exposure is the type of endpoint measured and the tissue in which the endpoint is measured. Some animal models have successfully distinguished known sensitizers of the respiratory tract from non-sensitizers based upon the ability of the chemical to elicit a Th2 cytokine response in lymph nodes draining the site of dermal application (Betts et al, 2002; Dearman et al, 2002). These studies usually only consist of an induction phase and do not examine the respiratory tract to limit the time it takes to screen a chemical. The aforementioned evidence that dermal sensitization elicits a different pulmonary airway response than intra-airway sensitization suggests that changes in lymph nodes draining the site of dermal application may not fully

represent changes that take place in the human respiratory tract after inhalation exposure. In addition, although Th2 cytokine expression has been a valuable endpoint in discriminating between sensitizers and non-sensitizers of the respiratory tract, the site where these measurements are made may influence the value and reliability of Th2 cytokine expression as a discriminator. In some studies, Th2 cytokine expression in skin draining lymph nodes was used to distinguish chemical respiratory allergens such as TMA from non-sensitizers of the respiratory tract such as the contact sensitizer DNCB (Betts et al, 2002; Dearman et al, 2002). This was based on the belief that contact sensitizers that don't sensitize the respiratory tract elicit an exclusive Th1 cytokine response in the skin. However, recent studies have demonstrated that contact sensitizers such as DNCB and OXAZ elicit Th2 cytokine increases in the skin and that this increase may play a role in the hypersensitivity responses in the skin that they induce (Urlich et al, 2001; Traidl et al, 1999). This suggests that Th2 cytokine induction in the skin is not unique to respiratory sensitizers and diminishes its value as a potential discriminator when such measurements are made in lymph nodes draining the site of dermal application. Also, non-respiratory sensitizing chemical contact allergens have never been shown to elicit a Th2 response in the respiratory tract, unlike respiratory sensitizers. In addition, exposure to contact allergens has never been linked to hypersensitivity-type responses in the respiratory tract of humans and current murine models of airway exposure to contact allergens have elicited only hypersensitivity reactions in pulmonary airways that are not mediated by Th2 cytokines (van Houweligan et al, 2002;

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Kraneveld et al, 2002). It appears, therefore, that the tissue in which the cytokine measurements are made is as relevant as the endpoint being measured when attempting to distinguish sensitizers from non-sensitizers of the respiratory tract. Measurements within the respiratory tract in an animal model may thus be a more reliable indicator of human disease resulting from inhalation exposure than similar measurements made in the skin. In the murine model of intranasal sensitization and challenge to LMW chemicals described in this thesis project, assessments were made of local Th2 and Th1 cytokine gene expression within the nasal airway and lungs.

A Comparison of Toxic and Immune Effects of Chemicals in Animal Models

Another concern of current murine models is that many do not distinguish irritant effects of a LMW chemical from its immune effects. The dose of the LMW chemical required to elicit allergic airway disease is known to be less than that required to induce sensitization and is likely less than that required to elicit irritation/toxicity of the respiratory tract (Beach et al, 2000). Only very low doses of a chemical respiratory allergen are required for elicitation of allergic symptoms in a sensitized individual. Many epidemiologic studies have found that most individuals who developed asthma-like symptoms were exposed to low concentrations of the chemical respiratory allergen and that irritant-induced asthma due to exposure to high concentrations of the chemical represent only a small minority of cases of LMW chemical-induced asthma (Beach et al, 2000). Despite such data, most experimental models of LMW chemical-induced allergic

airway challenge sensitized animals with high concentrations of the chemical leading to the induction of both a toxic and an immune response within the respiratory tract. They are thus modeling irritant-induced and immune-mediated mechanisms of asthma. The activation of both mechanisms simultaneously may lead to the under or over-valuing of certain pathways when extrapolating to human disease. Given that irritant-induced asthma and rhinitis account for only a small percentage of all cases of occupational asthma and rhinitis, it seems prudent that immune-mediated asthma and allergic rhinitis are more selectively modeled in experimental conditions. In the murine model of LMW chemical-induced allergic airway disease described in this thesis project, pilot studies were performed to select doses of the chemicals that did not cause irritation/toxicity within the respiratory tract before sensitizing and challenging mice with a particular chemical. This was done for the purpose of focusing on immune-mediated mechanisms of LMW chemicals exclusively.

A/J Mouse Used to Model Human Allergic Airway Disease

Many different strains of mice have been used in murine models of LMW chemical-induced allergic airway disease. The most commonly used strain in murine models is the BALB/c strain. The BALB/c strain is a high Th2-responding strain making it very sensitive to aeroallergens and thus ideal for assessing the allergenic effects of chemicals or proteins. A/J mice are similar to BALB/c mice in that they are naturally high Th2 responders and weak Th1 responders (Wills-Karp and Ewart, 1997). Some have postulated that the high Th2 response and

weak Th1 response in A/J mice is due to a deficiency in complement component 5 (C5) (Jagganath et al, 2000). C5 is required for macrophage-mediated killing (Jagganath et al, 2000). A/J mice exposed to mycobacterium tuberculosis (MTB), for example, demonstrate an enhanced growth of MTB accompanied by reduced secretion of several cytokines including IL-12 in comparison to C5 competent strains (Jagganth et al, 2000). IL-12, secreted by antigen presenting cells is required for differentiation of Th1 cells that in turn help orchestrate a cell-mediated immune response to combat such infections (Peterson et al, 1998). C5 may be an important factor in the secretion of IL-12 from APCs and thus the Th1 response. This may partially explain why A/J mice have increased susceptibility to MTB and Plasmodium infections and preferentially express the Th2 phenotype. In addition, Wills-Karp and Ewart, 1997, determined that the A/J strain is genetically predisposed to airway hyperresponsiveness. They also used genetic linkage studies to map airway hyperresponsiveness genes to murine chromosome 6 in the A/J strain. Th2 cytokines are linked to the pathogenesis of airway hyperresponsiveness. The A/J strain may thus react strongly to respiratory allergens and serve as a suitable model for determining whether or not an agent elicits a Th2-mediated response. In a study by Tony Jan and colleagues, 2003, in A/J mice, intraperitoneal sensitization followed by aerosol challenge with ovalbumin elicited many of the characteristic pathologic and immunologic features of allergic airway disease in humans. In the murine model of LMW chemical-induced allergic airway disease described in this thesis project,

A/J mice were selected to model human allergic airway disease due to LMW chemical exposure.

Goals of the Dissertation

This dissertation project tests the overall hypothesis that local Th2 cytokine gene expression in the nasal and pulmonary airways of the A/J mouse is an accurate predictor of the potential of chemicals to elicit allergic airway diseases such as occupational asthma and allergic rhinitis. In order to test this hypothesis, my first objective, discussed in chapter 2, was to develop an animal model of allergic airway disease using A/J mice that are sensitized and challenged via intranasal instillation with the protein allergen ovalbumin in a saline vehicle. Ovalbumin, known to elicit the hallmark features of allergic airway disease in many experimental models, served as the model allergen. The characteristic pathologic and immunologic profile of allergic airway disease elicited by ovalbumin was used as a 'positive control' of allergic responses with which to compare the effects of chemical respiratory sensitizers and non-sensitizers in later studies. The primary endpoints included pathologic responses within the respiratory tract and the assessment of serum IgE. In addition, an assessment of lung-derived Th2 and Th1 cytokine mRNA expression was made.

Before assessing the effects of LMW chemicals, this animal model of ovalbumin-induced allergic airway disease was used to study the mechanisms of allergic airway disease by testing potential therapeutic agents for the purpose of combating allergic airway disease. Cannabinol is a known inhibitor of Th2

cytokine production and may thus provide additional evidence in this model for the link between Th2 cytokines and protein or chemical-induced allergic airway disease. The second objective, discussed in chapter 3 tested the hypothesis that cannabinol, a natural agent known for its immunosuppressive actions, will suppress the pathologic, cytokine and serum IgE responses within the respiratory tract that take place after intranasal sensitization and challenge with ovalbumin. Cannabinol was administered by one of two routes, systemically or via the airways before and during intranasal sensitization and challenge with ovalbumin. The primary endpoints included pathologic responses within the respiratory tract and the assessment of serum IgE. In addition, an assessment of lung-derived Th2 and Th1 cytokine mRNA expression was made. This was done to examine the utility of cannabinol as a potential therapeutic in allergic airway disease.

The third objective, discussed in chapter 4, tested the hypothesis that intranasal sensitization and challenge with the known low molecular weight chemical allergen, toluene diisocyanate (TDI), in an ethyl acetate/olive oil vehicle will elicit the characteristic pathologic and cytokine responses within the respiratory tract of LMW chemical-induced allergic airway disease. TDI is a LMW chemical known to elicit allergic responses of the respiratory tract. The goal was to determine if TDI caused similar pathologic changes characteristic of allergic airway disease to ovalbumin and whether or not the pathologic changes were accompanied by a local up-regulation of Th2 cytokines within the respiratory tract. The primary endpoints included pathologic responses within the respiratory tract and the assessment of serum IgE. In addition, an assessment of lung-

derived Th2 and Th1 cytokine mRNA expression was made. This study would help determine if TDI sensitization and challenge in this model was associated with the production of the Th2 pattern of cytokines specifically.

The fourth and final objective, discussed in chapter 5, tested the hypothesis that intranasal sensitization and challenge with the known chemical respiratory allergen, trimellitic anhydride (TMA), but not the non-respiratory sensitizers, dinitrochlorobenzene (DNCB) and oxazolone (OXAZ), will induce the characteristic features of LMWC-induced allergic airway disease in the nasal and pulmonary airways. The comparison with known non-sensitizers of the respiratory tract was done to determine if such agents will elicit similar responses to the known respiratory allergens. The primary endpoints included pathologic responses within the nasal and pulmonary airways and the assessment of serum IgE. In addition to the assessment of lung-derived Th2 and Th1 cytokine mRNA expression, a similar assessment was made of tissue derived from the nasal airway in this study in mice treated with TMA. The absence of pathologic and cytokine changes in the respiratory tract after intranasal sensitization and challenge with known non-sensitizers will serve to validate this method as one with the capacity to distinguish chemical respiratory sensitizers from non-sensitizers based on their ability to elicit allergic airway pathology and enhanced Th2 cytokine mRNA expression within the respiratory tract.

CHAPTER 2

IMMUNE RESPONSES IN THE LUNG AND LOCAL LYMPH NODE OF A/J MICE TO INTRANASAL SENSITIZATION AND CHALLENGE WITH ADJUVANT-FREE OVALBUMIN

ABSTRACT

Pathologic features of IgE-mediated allergic airway diseases include airway infiltration of inflammatory cells (e.g., lymphocytes, plasma cells, and eosinophils) and mucous cell metaplasia (MCM) in airway epithelium. $CD4^+$ T lymphocytes, specifically those producing a type 2 (Th2) cytokine profile, are necessary for the induction of IgE-mediated allergic airway responses. Most experimental models of IgE-mediated allergic airway disease use systemic (e.g., intraperitoneal) administration of an allergen coupled with an adjuvant to sensitize animals. Cytokine changes are measured in a number of ways including in bronchoalveolar lavage fluid (BALF) or lymph node cells stimulated *ex vivo*. The primary objective of this study was to test the hypothesis that intranasal sensitization and challenge of mice with ovalbumin in the absence of an adjuvant will induce the pathologic features that are characteristic of IgE-mediated allergic airway disease. Another objective was to determine if intranasal delivery of this allergen will result in the induction of a profile of cytokine gene expression in the lung and tracheobronchial (TB) lymph node that is typical of immunologic changes associated with IgE-mediated allergic airway disease. Only mice that were intranasally sensitized and challenged with ovalbumin had pulmonary lesions that included marked MCM in the respiratory epithelium lining the nasal and pulmonary airways, and an associated mixed inflammatory cell influx consisting of lymphocytes, plasma cells and eosinophils. Ovalbumin-treated mice also had enhanced expression of the Th2 cytokine mRNAs IL-4, IL-5, IL-10, and IL-13 in the lung and IL-4 in the TB lymph node,

and concurrent increases in ovalbumin-specific IgE in the serum. The results of this study indicate that A/J mice intranasally instilled with ovalbumin without adjuvant have the hallmark histopathologic and immunologic features of IgE-mediated allergic airway disease of humans.

INTRODUCTION

Ovalbumin is a protein allergen widely used in experimental models of IgE-mediated allergic airway disease that use systemic administration to sensitize animals. Though it is well known that systemic ovalbumin sensitization followed by intranasal challenge will induce features characteristic of IgE-mediated allergic airway disease, it is not clear whether a similar response can be induced by intranasal sensitization and challenge to ovalbumin. The impetus for intranasal administration is that it more closely resembles the natural route of exposure to airborne allergens, i.e., inhalation. It is also a simple, inexpensive, and noninvasive method of exposing both the upper and lower respiratory tract of laboratory rodents to allergens. Most experimental models also include an adjuvant (e.g., alum) along with the allergen. Adjuvants, like alum, are not part of the natural airborne exposure of allergens and may artificially influence Th1 or Th2 responses in the animal. This is evidenced by the production of various immunoglobulin subclasses that may skew the immune response (Rajananthanan et al, 1999).

A large body of evidence supports the critical role of Th2 cytokines in the development and maintenance of IgE-mediated allergic airway disease. For example, interleukin-4 (IL-4) and IL-13 are required for B cells to undergo "class-switching" (i.e., from IgM-producing to IgE-producing B cells) (Frew, 1996) and the inhibition of IL-5 prevents antigen-induced airway eosinophilia and late-phase hyperresponsiveness (Foster et al, 2000; Karras et al., 2000). Most experimental models of IgE-mediated allergic airway disease assess changes in cytokine

expression by measuring the secreted product of stimulated local draining lymph node cells or by analyzing bronchoalveolar lavage fluid (BALF) for protein or cell associated mRNA expression. An alternative method, described in this report, is the measurement of cytokine gene expression in RNA isolated from the lung tissue or from a local draining lymph node.

The primary objective of this study was to determine if intranasal sensitization and challenge of mice with ovalbumin without an adjuvant would effectively sensitize the animals by inducing features characteristic of IgE-mediated allergic airway disease. The second objective of this study was to assess the temporal changes in the expression of multiple cytokine genes in RNA derived from lung tissue and its local draining lymph node using such an exposure regimen. Allergen-induced changes in cytokine gene expression, along with pulmonary and nasal histopathology, and serum IgE levels were assessed in each mouse. This was done to examine the utility of measuring local cytokine gene expression as a biomarker for screening potential respiratory allergens.

MATERIALS AND METHODS

Animals and Allergen Sensitization and Challenge

Ninety male A/J mice (Jackson Laboratories, Bar Harbor, Maine), 6 weeks of age, were randomly assigned to one of eighteen experimental groups (n=5). Mice were free of pathogens and respiratory disease, and used in accordance with guidelines set forth by the All University Committee on Animal Use and Care at Michigan State University. Animals were housed five per cage in polycarbonate boxes, on Cell-Sorb Plus bedding (A&W Products, Cincinnati, OH) covered with filtered lids, and had free access to water and food. Room lights were set on a 12 hr light/dark cycle beginning at 6:00 am, and temperature and relative humidity were maintained between 21-24°C and 40-55 % humidity, respectively. Figure 2.1 depicts the exposure regimen utilized for intranasal sensitization and challenge of mice. The timeline of the exposure regimen used is a modification of that used by Wills-Karp et al. (Wills-Karp et al, 1998) who used systemic sensitization and intratracheal challenge.

Mice were anesthetized with 4% halothane and 96% oxygen. Anesthetized mice were treated by intranasal instillation with 30 µl of 0.5% ovalbumin (Sigma Chemical, St. Louis, MO) in sterile, pyrogen-free saline or 30 µl of saline alone divided evenly between each naris. The sensitization phase consisted of a single instillation once per day for 5 consecutive days. The animals were challenged once, 14 days after the fifth instillation of the sensitization phase via a single instillation of the same volume and

Exposure Regimen

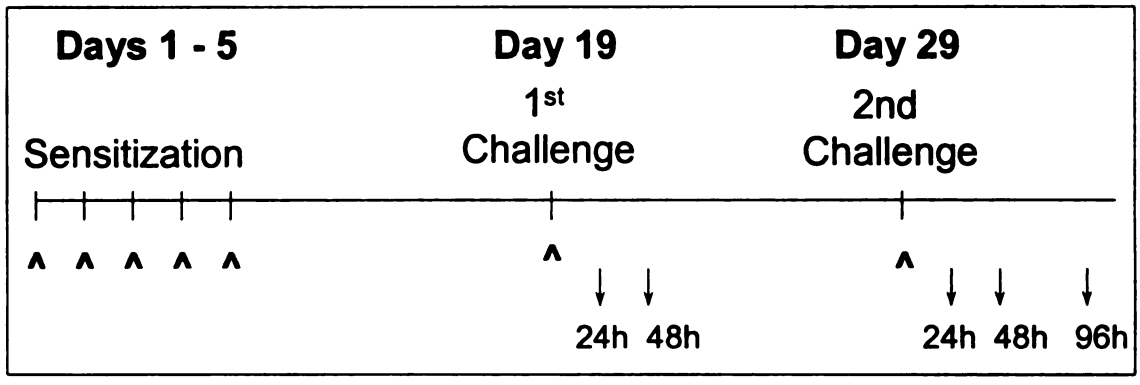

Figure 2.1: Timeline of the exposure regimen used to sensitize and challenge A/J mice with ovalbumin or saline. Mice were sensitized with five consecutive daily intranasal instillations of 30 µl of 0.5% ovalbumin in saline or saline alone. Mice were then challenged two weeks later with a similar volume and sacrificed 24 or 48 hr after challenge or challenged a second time 10 days later followed by sacrifice 24, 48, or 96 hr after double challenge. ^ = intranasal instillation of 30µl of 0.5% ovalbumin in saline or saline alone. ↓ = time after challenge when mice were sacrificed.

concentration as the instillations during the sensitization phase. Mice were sacrificed 24 or48 hr after challenge. There were three different treatment groups for each sacrifice time. One was sensitized and challenged with saline and a second group was sensitized with ovalbumin and challenged with saline. The last group was sensitized and challenged with ovalbumin. Other mice received a second challenge 10 days after the first challenge again with the same volume and concentration as in the first challenge. These mice were sacrificed 24, 48, or 96 hr after the final challenge. There were four different treatment groups for each sacrifice time: 1) sensitization and challenge with saline, 2) sensitization with ovalbumin and challenge with saline; 3) sensitization with saline and first challenge with saline and second challenge with ovalbumin; and 4) both sensitization and challenge with ovalbumin.

Necropsy, Blood Sample Collection, and Tissue Preparation

Before sacrifice, mice were deeply anesthetized via intraperitoneal injection of 0.1 ml of 12% pentobarbital in saline. Blood samples (0.1 to 0.5 ml) were taken from the brachial artery and collected in Beckton Dickinson vacutainers. Serum samples were collected after the blood samples were centrifuged to remove cells and the supernatants were frozen and stored at -20°C. The abdominal aorta and renal artery were then severed to exsanguinate the animals. Immediately after death, the trachea was cannulated and the heart/lung block removed. The tracheobronchial (TB) lymph node was removed and placed in 1 ml Tri reagent (Molecular Research Center, Cincinnati, OH).

Lymph nodes were pooled 2 or 3 per 1 ml Tri reagent within each group. The lymph nodes were then homogenized and stored at -80°C. The right bronchus was clamped using suture thread. Caudal and medial right lung lobes were placed in 2 ml Tri reagent, homogenized and stored at -80°C.

After removal of the right lung lobes, the left lung lobe was intratracheally perfused with 10% neutral buffered formalin at a constant pressure of 30 cm of fixative. After 1 hr, the trachea was ligated, and the inflated left lung lobe was immersed in a large volume of the same fixative for 24 hr. After fixation, the left lung lobe was microdissected along the main axial airway, and two sections were then excised at the level of the fifth (proximal) and eleventh (distal) airway generation (Figure 2.2), as has been described previously in detail (Steiger et al, 1995). Paraffin sections (5 μm) were stained with Alcian blue (pH 2.5) and periodic acid Schiff's (AB/PAS) reagent, which stain neutral and acidic mucosubstances, respectively. Other paraffin sections were stained separately for eosinophilic major basic protein (MBP, described below).

The head of each mouse was removed from the carcass, and the lower jaw and skin were removed. The heads were then immersed in 10% neutral buffered formalin for 24 hr. After fixation, the heads were decalcified in 13% formic acid for 7 days and then rinsed in tap water for at least 4 hr. The nasal cavity of each mouse was transversely sectioned at three specific anatomic locations according to a modified method of Young, 1981, and processed for light microscopy and image analysis. The most proximal nasal section was taken immediately posterior to the upper incisor teeth (proximal); the middle section

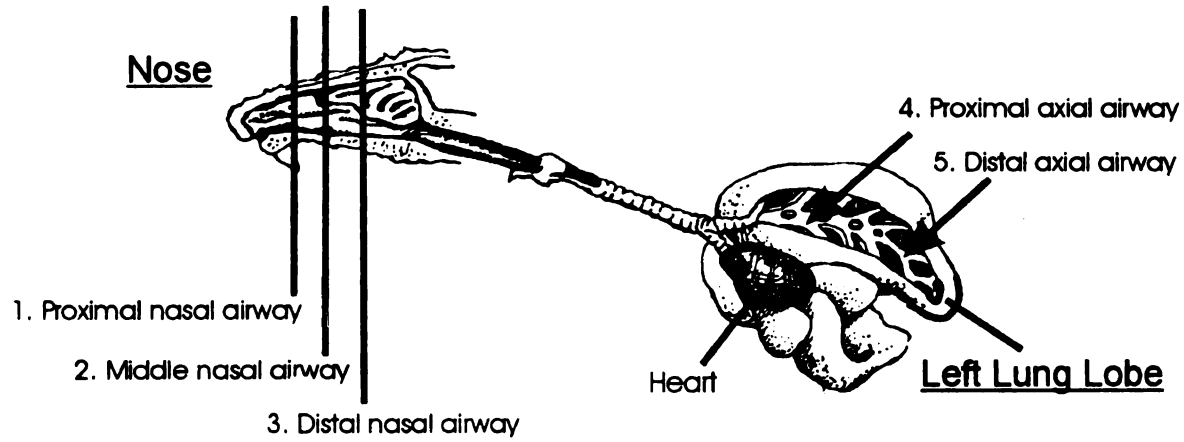

Figure 2.2: Sites of airway tissue selection for morphometric analysis. The cartoon depicts the lateral wall of one nasal passage with the septum removed of the murine respiratory tract and the main axial airway of the left lung lobe. Transverse sections of the nasal cavity were taken 1) immediately posterior to the upper incisor teeth (proximal), 2) at the level of the incisive papilla of the hard palate (middle), and 3) at the level of the second palatal ridge (distal). The left lung lobe was microdissected along the axial airways, and two sections were then excised at the level of the fifth (proximal airway) and eleventh (distal airway) airway generation.

was taken at the level of the incisive papilla of the hard palate (middle); the most distal nasal section was taken at the level of the second palatal ridge (distal) (Figure 2). Tissue sections were embedded in paraffin, at a thickness of 5 microns, and stained with hematoxylin and eosin for histopathologic assessment. Paraffin sections were also stained with AB/PAS to identify intraepithelial mucosubstances (IM).

Immunocytochemistry in the Lung

Unstained hydrated paraffin sections of the left lung lobe were incubated with the blocking solution, Vectastain ABC-AP Kit Normal Goat Serum (Vector, Burlingame, CA). The sections were then incubated with a polyclonal rabbit anti-mouse antibody against eosinophilic MBP generously provided by Drs. Borchers and Lee of the Mayo Clinic of Scottsdale (Scottsdale, Arizona). Treatment with the secondary antibody Vectastain ABC-AP Kit Anti-rabbit IgG followed. Some slides were incubated without the secondary antibody to check for nonspecific binding. Immunoreactive MBP was visualized after treatment with the Vectastain ABC-AP Complex followed by the Vector Red AP Substrate Solution (Denzeler et al, 2000). Nasal tissue sections which contained turbinate bone with bone marrow were also stained for MBP. Eosinophils and their cellular precursors within the bone marrow served as positive controls for the MBP immunohistochemistry.

Morphometry of stored intraepithelial mucosubstances in nasal and pulmonary airways.

The amount of stored mucosubstances in the respiratory epithelium lining the nasopharyngeal meatus in the most distal nasal section and the respiratory epithelium lining the axial pulmonary airway (airway generation 5) in the left lung lobe was estimated by quantifying the volume density of AB/PAS-stained mucosubstances using computerized image analysis and standard morphometric techniques. The area of AB/PAS-stained mucosubstances was calculated by circumscribing the perimeter of the stained material using the Scion Image program (Scion Corporation, Frederick, MD). The length of the basal lamina underlying the surface epithelium was calculated from the contour length of the digitized image of the basal lamina. The volume of stored mucosubstances (volume density, Vs) per unit of surface area was estimated using the method described in detail by Harkema et al, 1987, and was expressed as nl of IM per square mm of basal lamina.

Epithelial and Inflammatory Cell Densities.

Numeric cell density of the surface epithelium lining the proximal axial airway (fifth generation) was determined by counting the total number of epithelial cell nuclei in the surface epithelium and dividing by the length of the underlying basal lamina. The length of the basal lamina was determined as described above. Mucous cell density was also expressed as the number of cells per mm of basal lamina. Eosinophil influx was measured by dividing the

total number of eosinophils in the subepithelial interstitium of the airway wall by the length of the corresponding basal lamina. Eosinophils were identified by their morphologic features (i.e., slightly larger than neutrophils with bilobed nuclei) and by the positive immunohistochemical staining for MBP in the large cytoplasmic granules (dark pink-red granules).

ELISA for Total and Ovalbumin-specific serum IgE

Total serum IgE was measured using a 96-well Immulon ELISA plate (Dynex, Technologies, Chantilly, Virginia) coated with 2 μg/ml anti-mouse IgE (Purified Rat Anti-Mouse IgE Monoclonal Ab, Pharmingen, San Diego, CA) and incubated overnight at 4°C. For measurement of ovalbumin-specific IgE, a second ELISA plate was coated with 0.5% ovalbumin and incubated overnight at 4°C. After washing, the plates were incubated in 3 % Bovine Serum Albumin (3 % BSA) at 37°C for 1 hr. (BSA, CALBIOCHEM, La Jolla, CA). Serum samples at 1:10 dilution were then added followed by incubation at 37°C for 1 hr. After washing, biotinylated anti-mouse IgE (Biotin-conjugated Rat Anti-Mouse IgE Monoclonal Ab, Pharmingen, San Diego, CA) was then added at 2 μg/ml and allowed to incubate at 25°C for 1 hr. After washing, 1.5 μg/ml of streptavidin peroxidase was added followed by incubation at 25°C for 1 hr. After washing, TMB substrate (12.5ml citric-phosphate buffer + 200 μl of TMB stock solution (6mg/ml in DMSO)) was added to produce a color reaction. The reaction was terminated by the addition of 6 N H_2SO_4. Optical density was determined at 450

nm using an EL-808 microplate reader (Bio-Tek instruments, Winooski, Vermont).

The RNase Protection Assay

Total RNA was isolated from lung tissues and the TB lymph node using the TRI REAGENT method (Molecular Research Center, Cincinnati, OH) following the manufacturer's protocol and the integrity of the RNA was checked by performing an ethidium bromide diagnostic gel to check for degradation of RNA. The mCK-1 RiboQuant Mouse Cytokine Multi-Probe Template Set (Pharmingen, San Diego, CA) was used to assay mRNA expressions of IL-4, IL-5, IL-10, IL-13, IL-15, IL-9, IL-2, IL-6, and IFN-γ. The housekeeping genes L32 and GAPDH are included as loading controls. The probes were generated by transcribing with T7 RNA polymerase in the presence of [α-^{32}P] UTP (3000 Ci/mmol). The probe set was hybridized in excess to RNA isolated from the right lung lobe (50 μg) or from the TB lymph node (75 μg) (56°C for 16 hr). Free probe and non-hybridized RNA was then digested with single-stranded RNases using the RiboQuant RNase Protection Assay (RPA) Kit. The remaining RNase-protected probes (only those that have hybridized to target tissue-derived mRNA) were resolved by electrophoresis (3500 V for 1 h) through a 7M urea - 7% acrylamide gel. The gel was dried and specific RNAs were visualized by autoradiography.

Preparation of internal standard for IL-4 quantitative RT-PCR.

A recombinant internal standard (IS) was prepared to quantify IL-4 mRNA expression by quantitative/competitive reverse transcriptase polymerase chain reaction (RT-PCR) as previously described (Condie et al, 1996). Briefly, an artificial/recombinant RNA (rcRNA) was used as an IS containing specific PCR primer sequences for IL-4 that were added to RNA samples in a series of dilutions. A rat β-globin sequence was used as the spacer gene for the IL-4 IS. This method, developed by Vanden Heuvel et al, 1993, avoids sample-to-sample variation of reference gene expression (e.g., β-actin) as well as gene-to-gene differences in amplification efficiency. The IL-4 forward and reverse primer sequences were 5' AAC GAG GTC ACA GGA GAA G 3' and 5' GCT TAT CGA TGA ATC CAG GC 3', respectively. The following was the IS forward primer design from 5' to 3': T7 promoter (TAATACGACTCACTATAGG), IL-4 forward primer (as above), and rat β-globin forward primer (AAGCCTGATGCTGTAGAGCC). The IS reverse primer design from 5' to 3' was: $(dT)_{18}$, IL-4 reverse primer (as above), and rat β-globin reverse primer (AACCTGGATACCAACCTGCC). PCR reaction conditions were performed using 100 ng of rat tailed-genomic DNA. PCR-amplified products were purified using the Wizard PCR Prep DNA Purification System (Promega, Madison, WI) and transcribed into RNA using Promega's Gemini II *In vitro* Transcription System. The rcRNA was subsequently treated with RNase-free DNase to remove the DNA template. After quantifying, calculations were made to determine the number of molecules/μl of IL-4 IS.

Quantitative IL-4 RT-PCR.

Quantitative RT-PCR was performed as described by Gilliland et al, 1990, except that rcRNA was used as an IS instead of genomic DNA with 10 aliquots of rcRNA from 10^4 to 10^8 molecules. Briefly, total RNA (right lung lobe or TB lymph node) and IS rcRNA of known amounts were reverse transcribed into cDNA using oligo$(dT)_{15}$ as primers using reverse transcriptase (RT) (Promega, Madison WI). To ensure that there was no genomic DNA contamination in the RNA isolates, no-RT controls were employed for all samples. A PCR master mixture consisting of PCR buffer, 4 mM $MgCl_2$, 6 pmol each of IL-4 forward and reverse primers, and 2.5 U of Taq DNA polymerase was added to the cDNA samples. Samples were then heated to 94°C for 3 min and cycled 40 times at (1) 95°C (30 sec), (2) 58°C (30 sec), and (3) 72°C (45 sec). After the completion of 40 cycles, a final elongation step at 72°C (5 min) was carried out. PCR products were resolved by electrophoresis in 3% NuSieve 3:1 gel (FMC Bioproducts, Rockland ME) and visualized by ethidium bromide staining. The IL-4 primers produced a 228 bp product from the cellular RNA and a 346 bp product from the IS rcRNA. Quantification was performed using Gel Doc 1000 (BioRad Laboratories, Inc., Hercules, CA) and Multi Analyst Software. The amount of IL-4 mRNA present was determined as described by Gilliland et al, 1990. Briefly, the ratio of the density of the IS rcRNA to IL-4 mRNA was plotted against the amount of IS rcRNA (in molecules) added to each reaction. The point at which the ratio of IS (rcRNA) to IL-4 mRNA was equal to 1 signified the "cross-over point" which represented the amount of IL-4 molecules present in the initial RNA sample.

Statistics

The data obtained from each experimental group were expressed as a mean group value ± the standard error of the mean (SEM). The differences among groups were determined by one way or two-way analysis of variance (ANOVA) and an All Pairwise Comparison Test (Tukey), using SigmaStat software from Jandel Scientific Co. (San Rafael, CA).

RESULTS

Pulmonary Histopathology

The exposure regimen used to sensitize and challenge mice with ovalbumin resulted in distinct pulmonary lesions that were restricted to mice that were intranasally sensitized and challenged with ovalbumin. The principal morphologic alterations in the lungs of these mice were marked MCM in the surface epithelium lining the conducting airways and an associated mixed inflammatory cell influx consisting of eosinophils and mononuclear cells (mainly lymphocytes and plasma cells) in the interstitial tissue surrounding the airways (Figure 2.3). Airway lesions were more severe in the main axial airways and pre-terminal bronchioles, but were also present to a lesser degree in the terminal bronchioles. The interstitial tissue surrounding large blood vessels in the alveolar parenchyma (pulmonary veins) and along the conducting airways (pulmonary arteries) exhibited a similar inflammatory response. Ovalbumin-sensitized mice that received two intranasal challenges of ovalbumin had more severe airway epithelial and inflammatory lesions than ovalbumin-sensitized mice that received only one intranasal challenge of ovalbumin. The inflammatory responses in all of these mice were principally present in the pulmonary airways and large vessels of the lung. No significant ovalbumin-induced inflammatory responses were evident in the alveolar parenchyma.

Treatment-associated changes were absent in the lungs of the control mice that received intranasal sensitization and challenges of saline. The only

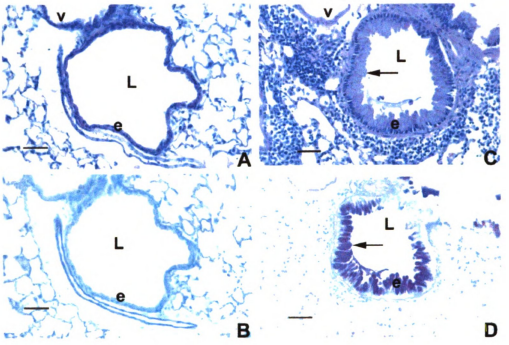

Figure 2.3: Light photomicrographs of conducting airways of the left lung from mice intranasally sensitized and challenged twice with saline (A, B) or 0.5% ovalbumin (C, D) and sacrificed 96 hr after challenge. Tissues were stained with hematoxylin and eosin (A, C) for morphological assessment and Alcian plue (pH = 2.5)/periodic acid Schiff's sequence (AB/PAS) for mucosubstances in the airway epithelium (B, D). Numerous mucous cells (arrow) with AB/PAS-stained mucosubstances are present only in the airway epithelium (e) of the ovalbumin-sensitized and -challenged mouse (C, D). A marked inflammatory cell influx consisting primarily of lymphocytes, plasma cells and eosinophils is also present in the interstitial tissue surrounding the airway of this mouse (C). v = blood vessel; L = airway lumen; Bars = 50 microns.

histologic change in the airways of mice that were sensitized with ovalbumin and received saline during the challenge phase was a minimal MCM limited to the proximal axial airways. This airway epithelial change was more conspicuous in mice that received two saline challenges rather than one saline challenge. No airway inflammation was associated with this minimal epithelial change.

The effect of the sensitization phase was examined by sacrificing mice after the sensitization phase alone. Five consecutive one-day instillations of ovalbumin caused a small increase in IM with no accompanying airway inflammation as assessed on Day 6, indicating that the animals developed the described changes only after challenge.

Stored Intraepithelial Mucosubstances in Pulmonary Airways

The amount of AB/PAS-stained IM in the epithelium lining the main axial airway at the fifth (proximal airway) and eleventh generations (distal airway) increased most in mice that were intranasally sensitized and challenged with ovalbumin (Figure 2.4). Mice that were instilled with saline during the sensitization phase and for the first challenge and with ovalbumin for the second challenge exhibited a 10-fold increase in IM at the fifth generation. This increase, however, was markedly lower than that exhibited by the lungs of mice that were sensitized and challenged with ovalbumin. The mice had a 42-fold increase in the amount of IM compared to the saline-instilled controls. It is notable that although a single ovalbumin challenge was sufficient to induce a significant increase in IM in ovalbumin-sensitized mice, two ovalbumin challenges resulted

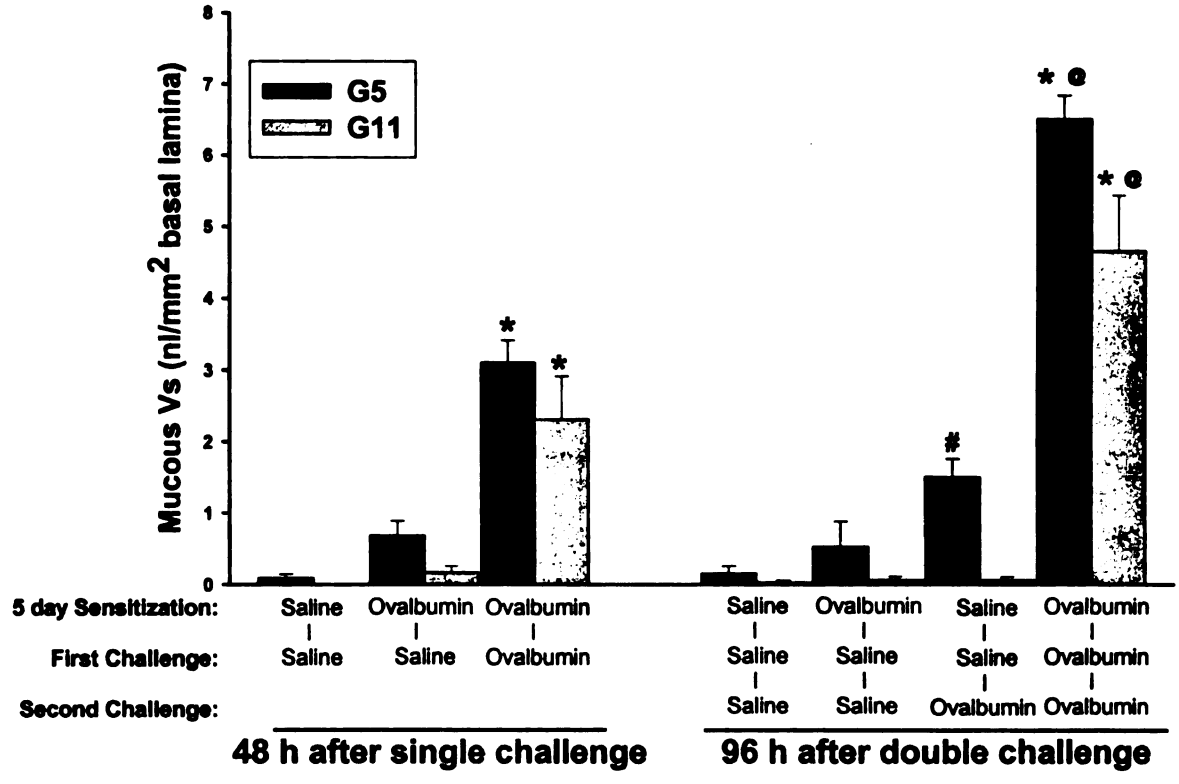

Figure 2.4: Morphometric quantification of AB/PAS-stained material in the surface epithelium lining mouse pulmonary airways at generations 5 (proximal) and 11 (distal)) 48 hr after single challenge or 96 hr after double challenge with ovalbumin. Mice were sensitized with five consecutive daily intranasal instillations of 30μl of 0.5% ovalbumin in saline or saline alone. Mice were then challenged two weeks later with a similar volume and sacrificed 48 hr after challenge or challenged a second time 10 days later followed by sacrifice 96 h after double challenge. Bars represent the Volume Density (Vs) of intraepithelial mucosubstances ± standard error of the mean (n = 5/ group). * = significantly greater than groups challenged similarly ($p<0.05$). # = significantly greater than groups instilled with saline at same airway generation ($p<0.05$). @ = significantly greater than similar group challenged once ($p<0.05$).

in a doubling of the amount of IM as compared to the lungs of mice that received a single challenge (Figure 2.4). Also, the increase in IM was greater in the axial airway at the fifth generation (proximal airway) than at the eleventh generation (distal airway).

Numeric Cell Densities of Epithelial Cells in Pulmonary Airways

A morphometric analysis of the surface epithelium lining the proximal axial airway was performed in mice that were sensitized and challenged twice to estimate the metaplastic transformation of epithelial cells from non-secretory to mucus-secreting goblet cells (Figure 2.5). A 28-fold increase in mucous cell density was observed in mice that received ovalbumin during both sensitization and challenge phases as compared to the saline-only control group. No effect was observed in mice that received ovalbumin only during the sensitization phase or only as the second challenge. It is also notable that there was no evidence of hyperplasia in the main axial airway of the left lung lobe in any of the treatment groups as evidenced by the absence of changes in total epithelial cell density between the various treatment groups.

Pulmonary Airway Eosinophilia

As described above, recruitment of inflammatory cells, specifically eosinophils, into and around subepithelial airway tissues is a hallmark of the allergen-induced airway response. Eosinophils per mm basal lamina were enumerated in the interstitium of the fifth (proximal) and eleventh generation

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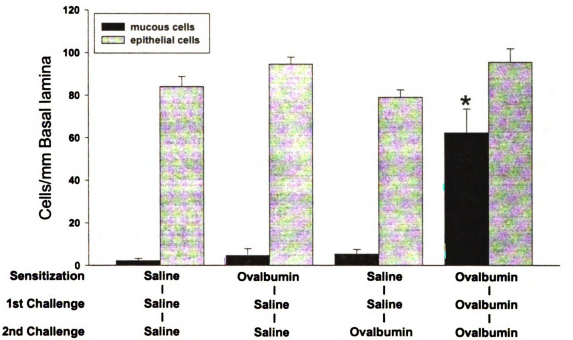

Figure 2.5: Quantification of mucous and epithelial (total) cell density in the surface epithelium lining mouse pulmonary airways (generation 5) 96 hr after double challenge with ovalbumin. Mice were sensitized with five consecutive daily intranasal instillations of 30μl of 0.5% ovalbumin in saline or saline alone. Mice were then challenged two weeks later with a similar volume and challenged a second time 10 days later followed by sacrifice 96 hr after double challenge. Bars represent the number of cells per mm basal lamina ± standard error of the mean (n = 5/group). * = significantly greater than all other groups of this cell type ($p<0.05$).

(distal) axial airway of mice that were sensitized and then challenged twice with either saline or ovalbumin. Mice that were sensitized and challenged with ovalbumin showed a 27-fold and 24-fold increase in the number of eosinophils present in the proximal (generation 5) and distal (generation 11) axial airways of the lung lobe, respectively, as compared to the saline controls (Figure 2.6).

Nasal Histopathology

The exposure regimen used to sensitize and challenge mice with ovalbumin resulted in distinct nasal lesions that were restricted to mice that were intranasally sensitized and challenged with ovalbumin. The principal morphologic alterations in the nasal airways of these mice were MCM in the surface epithelium lining the nasal pharyngeal meatus and an associated mild inflammatory cell influx consisting of eosinophils, lymphocytes and plasma cells in the interstitial tissue surrounding the airways (Figure 2.7).

Stored Intraepithelial Mucosubstances in Nasal Airways

The amount of IM in the airway epithelium lining the nasopharyngeal meatus was also estimated using image analysis and standard morphometric techniques. Ovalbumin sensitization and challenge resulted in an increase in the amount of IM in the respiratory epithelium that lines the nasopharyngeal meatus as compared to the saline controls (Figure 2.8).

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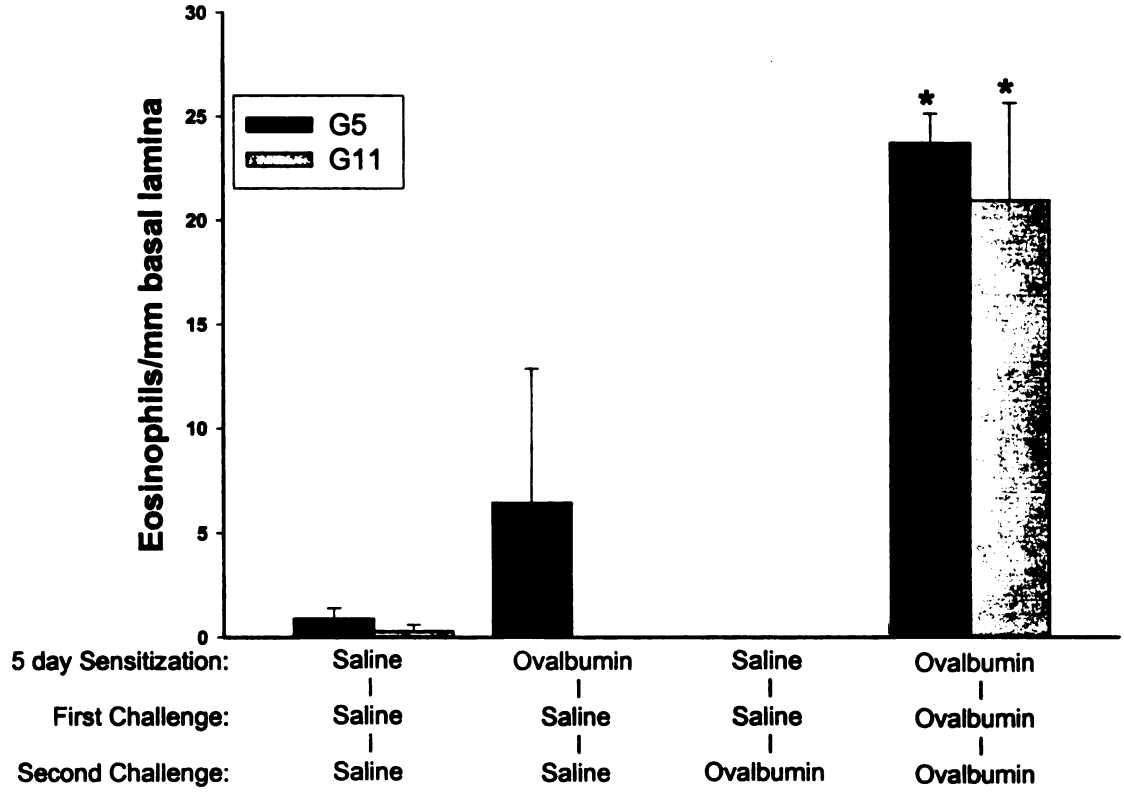

Figure 2.6: Morphometric quantification of eosinophils immunohistochemically stained for major basic protein in mouse airway interstitium (generations 5 and 11) 96 hr after double challenge with ovalbumin. Mice were sensitized with five consecutive daily intranasal instillations of 30μl of 0.5% ovalbumin in saline or saline alone. Mice were then challenged two weeks later with a similar volume and challenged a second time 10 days later followed by sacrifice 96 hr after double challenge. Bars represent the number of eosinophils per mm basal lamina ± standard error of the mean (n = 4/ group). * = significantly greater than all groups at similar airway region ($p<0.05$).

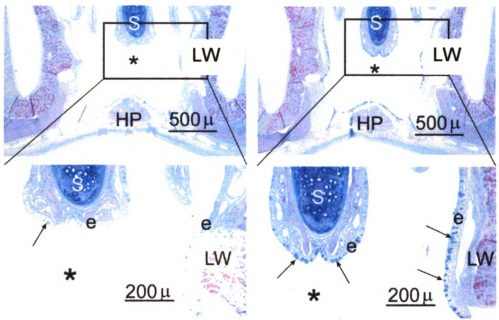

Figure 2.7: Light photomicrographs of the distal region of the nasal cavity at the level of the second palatal ridge of mice 96 hr after sensitization and two challenges with saline (left panels) or ovalbumin (right panels). Tissue sections were histochemically stained with AB/PAS for identification of stored mucosubstances (arrows) in the epithelium lining the nasopharyngeal meatus. The lower panels are enlargements of the boxed regions in the panels shown above. S – nasal septum, HP– hard palate, LW – lateral wall, e – respiratory epithelium.

Serum IgE

None of the treatment groups resulted in a significant increase in serum levels of total IgE (Figure 2.9). Conversely, mice sensitized and challenged with ovalbumin exhibited a marked increase, i.e., a 10-fold increase in ovalbumin-specific serum IgE as compared to saline only controls (Figure 9). Mice that received only ovalbumin sensitizations or only an ovalbumin challenge did not exhibit significant increases in ovalbumin-specific serum IgE.

Cytokine Gene Expression

RNase Protection Assay. Mice that were intranasally sensitized and challenged twice with ovalbumin exhibited an increase in the expression of the Th2 cytokine mRNAs IL-4, IL-5, and IL-13, and the cytokine mRNA of IL-10 in the lung that, with time after challenge, decreased in magnitude as evidenced by less intense bands (Figure 2.10, lanes 6, 10 and 14). The saline controls exhibited no such increase in Th2 cytokine mRNA expression (Figure 2.10, lanes 3-5, 7-9 and 11-13). It is notable that IL-15 and IL-9 mRNA and the Th1 cytokine mRNA IFN-γ were constitutively expressed and that neither ovalbumin nor saline treatments influenced the expression of these cytokine mRNAs in the lung tissue.

There were no differences in cytokine mRNA expression in RNA from the TB lymph node as detected using the RPA (data not shown).

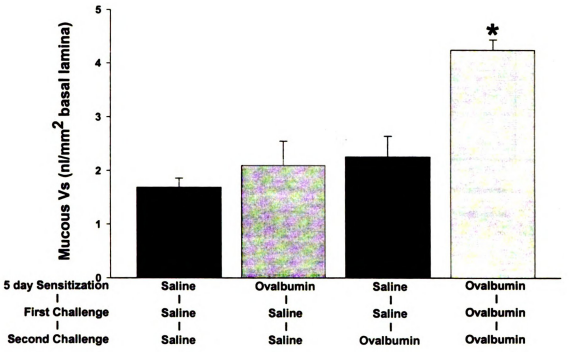

Figure 2.8: Morphometric quantification of AB/PAS-stained material in the surface epithelium lining the nasopharyngeal meatus of mice 96 hr after double challenge with ovalbumin or saline. Mice were sensitized and then challenged twice with ovalbumin or saline. Bars represent the volumetric density (Vs) of intraepithelial mucosubstances +/- the standard error of the mean (n = 5/group). * - significantly greater than all other groups ($p<0.05$).

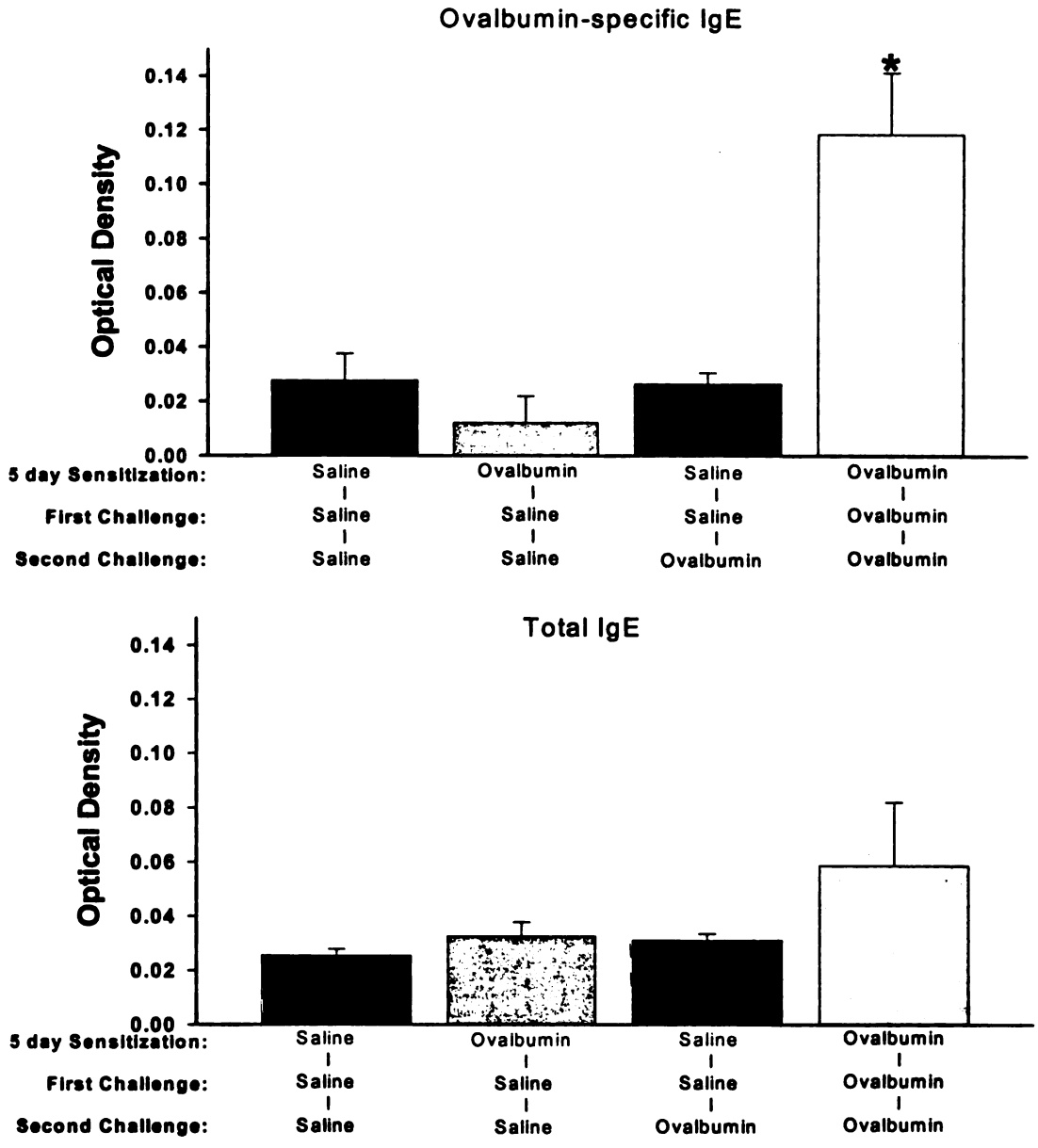

Figure 2.9: Ovalbumin-specific (upper panel) and Total IgE (lower panel) levels in mouse serum isolated 96 h after double challenge with ovalbumin. Mice were sensitized with five consecutive daily intranasal instillations of 30µl of 0.5% ovalbumin in saline or saline alone. Mice were then challenged two weeks later with a similar volume and challenged a second time 10 days later followed by sacrifice 96 hr after double challenge. Bars represent average optical density ± standard error of the mean (n = 5/group). * = significantly greater than all other groups.

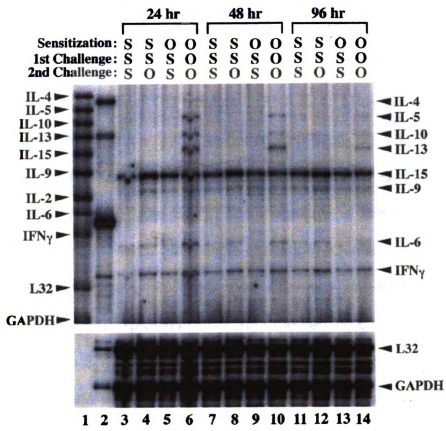

Figure 2.10: Autoradiograph of electrophoretic gel depicting cytokine mRNA expression from RNase Protection Assay performed using RNA isolated from the cranial and medial right lung lobes of mice 24, 48, or 96 hr after double challenge with ovalbumin. Mice were sensitized with five consecutive daily intranasal instillations of 30μl of 0.5% ovalbumin in saline (O) or saline alone (S). Mice were then challenged two weeks later with a similar volume and challenged a second time 10 days later followed by sacrifice 24, 48, or 96 hr after double challenge. Lane 1 = undigested probes for IL-4, IL-5, IL-10, IL-13, IL-15, IL-9, IL-2, IL-6, INF-γ, and the house keeping genes L32 and GAPDH. Lane 2 = mouse control RNA IL-4, IL-10, IL-2, IFN-γ, L32, and GAPDH that each hybridized with their corresponding probe followed by digestion with RNase thus resulting in the small shift in migration. Lanes 3 - 6 = RNA from animals sacrificed 24 hr after double challenge. Lanes 7 - 10 = RNA from animals sacrificed 48 hr after double challenge. Lanes 11 - 14 = RNA from animals sacrificed 96 hr after double challenge. The last lane of each time-point (6, 10, and 14) depicts RNA from animals that were sensitized and challenged with ovalbumin.

RT-PCR. After establishing the profile of Th2 cytokine mRNAs that were expressed using the RPA, RT-PCR was performed on right lung lobe RNA from mice that were sensitized and then challenged once or twice with either ovalbumin or saline. This was done to determine the quantitative differences in the expression of the critical Th2 cytokine mRNA IL-4. Mice that were sensitized and then challenged once with ovalbumin exhibited an approximate 5-fold increase in IL-4 mRNA expression 24 hr after challenge as compared to the saline-instilled controls (Figure 2.11). The magnitude of this increase persisted at that level for at least 48 hr after single challenge. Ovalbumin-sensitized mice that received two ovalbumin challenges exhibited an approximate 40-fold increase in IL-4 mRNA expression 24 hr after the second challenge as compared to controls (Figure 2.12). IL-4 mRNA levels after double challenge were similar 48 hr after exposure but waned in magnitude by 96 hr post exposure, i.e., IL-4 mRNA levels decreased to approximately 16-times those of the saline controls. Mice that received only ovalbumin sensitizations or only an ovalbumin challenge did not exhibit significant increases in IL-4 mRNA expression in the lung.

IL-4 RT-PCR was also performed on RNA isolated from the TB lymph node from mice that were sensitized and then challenged twice with ovalbumin. Ovalbumin-sensitized and challenged mice exhibited a 4-fold increase in IL-4 mRNA expression at 24 hr after challenge compared to saline controls that persisted at that level at least until 96 hr (Figure 2.13).

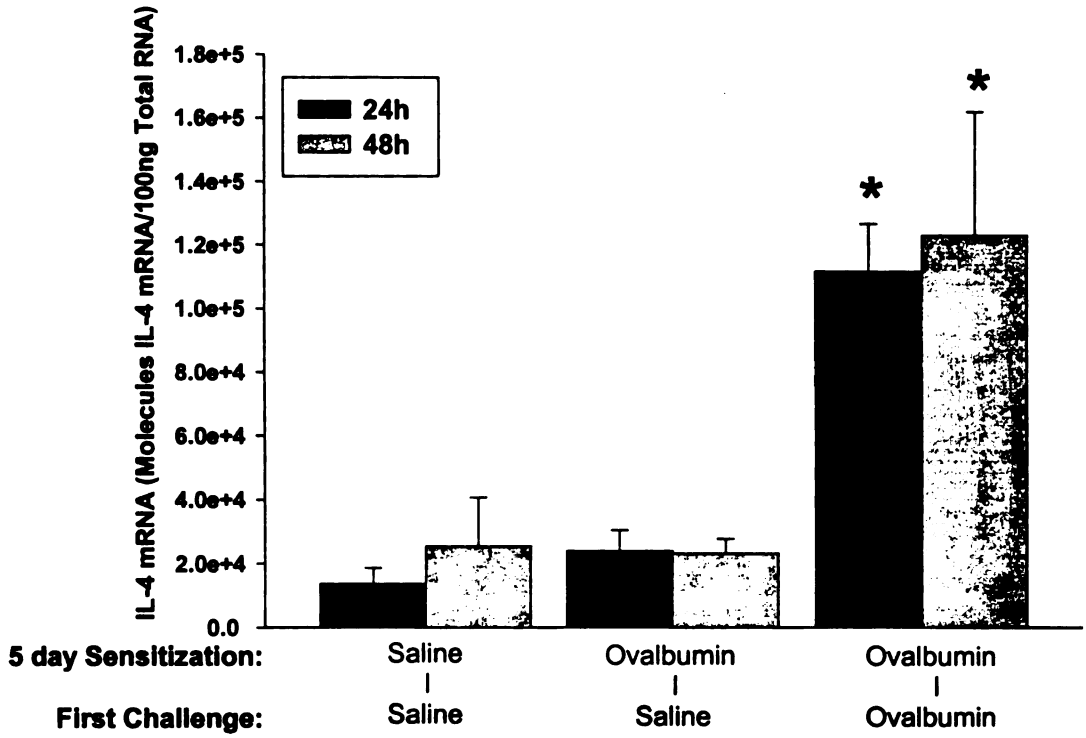

Figure 2.11: Quantification of IL-4 mRNA in cranial and medial right lung lobes 24 or 48 hr after single challenge with ovalbumin using RT-PCR. Mice were sensitized with five consecutive daily intranasal instillations of 30μl of 0.5% ovalbumin in saline or saline alone. Mice were then challenged two weeks later with a similar volume and sacrificed 24 or 48 hr after challenge. Bars represent molecules of IL-4 mRNA per 100 ng of total RNA isolated ± standard error of the mean (n = 5/group). * = significantly greater than all other groups at similar time-point ($p<0.05$).

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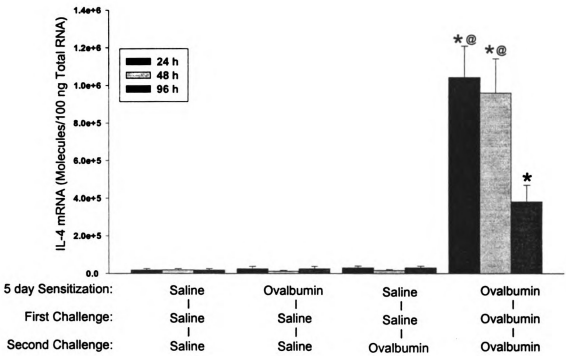

Figure 2.12: Quantification of IL-4 mRNA in cranial and medial right lung lobes 24, 48 or 96 hr after double challenge with ovalbumin using RT-PCR. Mice were sensitized with five consecutive daily intranasal instillations of 30µl of 0.5% ovalbumin in saline or saline alone. Mice were then challenged two weeks later with a similar volume and challenged a second time 10 days later followed by sacrifice 24, 48, or 96 hr after double challenge. Bars represent molecules of IL-4 mRNA per 100 ng of total RNA isolated ± standard error of the mean (n = 5/group). * = significantly greater than all other groups at same time-point ($p<0.05$). @ = significantly greater than similarly-challenged group sacrificed 96 h after challenge ($p<0.05$).

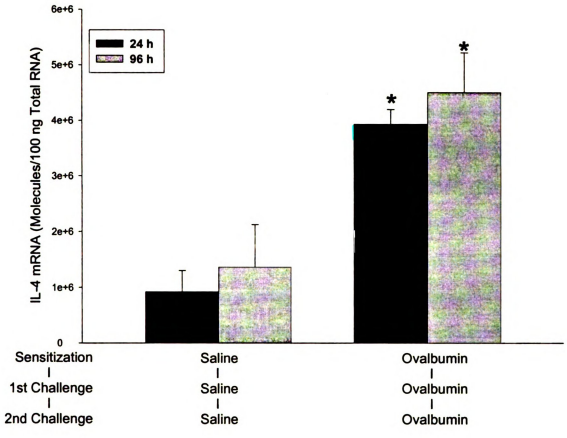

Figure 2.13: Quantification of IL-4 mRNA in tracheobronchial lymph node 24 or 96 hr after double challenge with ovalbumin using RT-PCR. Mice were sensitized with five consecutive daily intranasal instillations of 30μl of 0.5% ovalbumin in saline or saline alone. Mice were then challenged two weeks later with a similar volume and challenged a second time 10 days later followed by sacrifice 24 or 96 hr after double challenge. Bars represent molecules of IL-4 mRNA per 100 ng of total RNA isolated ± standard error of the mean (n = 5/group). * = significantly greater than all other groups at same time-point ($p<0.05$).

DISCUSSION

The primary objective of this study was to determine if the pathologic and immunologic features of IgE-mediated allergic airway disease could be induced in mice by intranasal sensitization and challenge with adjuvant-free ovalbumin. Morphologic changes in the pulmonary airways were correlated with changes in cytokine gene expression in the lung and the TB lymph node after ovalbumin treatment to determine if local cytokine gene expression may be used as a biomarker for the respiratory allergenicity of selected proteins. Intranasal sensitization and challenge with ovalbumin in the absence of an adjuvant elicited the characteristic histopathologic and immunologic features of IgE-mediated allergic airway disease in murine lung. Our results suggest that cytokine gene expression in lung tissue and in a locally draining lymph node of the lung may be a sensitive biomarker for identifying respiratory allergens. In addition, similar histopathologic changes were detected in the nasal airway suggesting that this intranasal exposure method may be used to study allergic rhinitis.

The most common methods typically used to sensitize mice to protein allergens in murine models of IgE-mediated allergic airway disease include intraperitoneal and intra-airway administration. After sensitization, the animals are then challenged via the airways to induce features of IgE-mediated allergic airway disease such as airway hyperresponsiveness and airway inflammation. The intraperitoneal administration of protein allergens is an unnatural route of exposure to aeroallergens. Also, when antigens are intraperitoneally administered in experimental models, they are often coupled with an adjuvant.

An adjuvant, used to potentiate an immune response, can influence the Th1/Th2 cytokine profile and therefore may artificially alter downstream immunologic responses (e.g., immunoglobulin isotypes secreted by B cells) (Rajananthanan et al, 1999).

Mice may also be sensitized to allergens via intra-airway administration. One method of intra-airway administration used to sensitize mice to protein allergens is intratracheal instillation. Intratracheal sensitization of various protein allergens including keyhole limpet hemocyanin (Shen et al, 2002) and latex proteins (Woolhiser et al, 2000) has been shown to successfully induce IgE-mediated allergic airway disease. In addition to intratracheal instillation, some models of IgE-mediated allergic airway disease use aerosolized antigen to sensitize mice to a specific antigen. There are conflicting reports regarding the effectiveness of aerosolized antigen as a method of sensitization. While successful sensitization has been demonstrated with some experimental models (Hamelman et al, 1997), in others, the development of airway inflammation after challenge was not observed (Stampfli et al, 1998).

Very few reported models, however, have used intranasal instillation of antigen as a method of intra-airway administration to sensitize animals. It has been unclear whether a strong immunologic and pathologic response characteristic of IgE-mediated allergic airway disease in the pulmonary airways can be induced by intranasal sensitization (and challenge) to ovalbumin. The intranasal instillation of allergens (i.e., *Schistosoma mansoni* egg antigen, *Aspergillus fumigatus*) has been used to successfully induce features of allergic

rhinitis (Okano et al, 1999; van de Rijn et al, 1998). In addition, the intranasal instillation of allergens has been used to elicit antibody responses for the identification of the respiratory sensitization potential of detergent enzymes in the mouse intranasal test (MINT) (Blakie et al, 1999; Robinson et al, 1996). Our exposure regimen differed from the MINT protocol in the schedule and duration of allergen dosing. The MINT protocol includes instillations on Days 1, 3 and 10 followed by sacrifice on Day 15 (Blakie et al, 1999; Robinson et al, 1996). Our study consisted of five intranasal instillations during a distinct sensitization phase followed by the first challenge (single intranasal instillation) two weeks later and a second challenge 10 days after the first challenge followed by sacrifice from 24 to 96 h after final instillation.

We found that the intranasal instillation of ovalbumin was a simple, inexpensive, and noninvasive method of inducing IgE-mediated allergic airway disease in the upper and lower respiratory tract of the A/J mouse. Adjuvant was not necessary to effectively sensitize the nasal or pulmonary airways to the allergen. Not all of the intranasally inhaled allergen was delivered to the respiratory tract. Some of it was undoubtedly delivered to the esophagus and upper gastrointestinal tract. Our experimental design, however, did not allow us to determine the amount of instillate that was delivered to the gut. In addition, our study was not designed to determine the contribution of the gastrointestinal tract to the overall sensitization of the mouse to ovalbumin.

Many animal models of IgE-mediated allergic airway disease employ continuous administration of the allergen by various routes without any significant

lag period between initial sensitization and subsequent challenge to the allergen. This may increase the risk of making the animal tolerant to the antigen. The exposure regimen used in the present study included a distinct sensitization phase after which no significant immune effect was observed, and a challenge phase that consisted of one or two intranasal instillations. This regimen is somewhat similar to human exposures to many proteins and low molecular weight chemicals in the workplace that ultimately result in occupational asthma. Sensitization to a specific allergen in such cases of occupational asthma usually takes place some time before re-exposure and the subsequent elicitation phase (Beach et al, 2000).

The airways of asthmatics are characterized by the presence of chronic inflammation with infiltration of lymphocytes, plasma cells, eosinophils, and mast cells in the bronchial mucosa and epithelial changes that include MCM (Holgate, 2000; Wills-Karp, 1999). The lung lesions resulting from intranasal sensitization and challenge of mice with ovalbumin included marked MCM in the surface epithelium lining the conducting airways and an associated mixed inflammatory cell influx consisting of eosinophils, lymphocytes, and plasma cells in the interstitial tissue surrounding the airways. Two ovalbumin challenges in sensitized mice induced a more substantial increase in the number of mucus-secreting cells than a single challenge indicating that the two-challenge model elicited a more robust response. There was also a modest increase in the amount of IM in the absence of a cellular or cytokine immune response observed in mice that received ovalbumin only once before sacrifice. These minimal

changes in IM may have been due to some irritating properties of the ovalbumin solution rather than an immune-mediated response. The effect of the sensitization phase was also examined by sacrificing mice after the sensitization phase alone. Five consecutive one-day instillations of ovalbumin caused a similar minimal increase in IM with no accompanying airway inflammation. This minor increase in IM was also likely due to some irritating effect of the inhaled solution on the airway epithelium that resulted in a minimal increase in IM. This response was nowhere near the severity of the MCM induced in the mice that were also challenged with the ovalbumin solution. In addition, only the mice that were both sensitized and challenged with ovalbumin had an inflammatory cell infiltrate accompanying the marked MCM indicating an immune-mediated response. These histologic features were not present in mice that received only ovalbumin sensitization or only ovalbumin challenge.

The single-most predisposing factor for the development of asthma in humans is atopy (Kay, 2001). Individuals with atopy have a hereditary predisposition to produce an IgE-mediated response against common environmental allergens (Kay, 2001). The primary immunoglobulin isotype associated with immediate-type hypersensitivity responses in mice is also IgE (Kimber and Dearman, 1997). Determinations of antigen-specific IgE in the serum of mice that were intranasally sensitized and challenged with ovalbumin showed an increase in ovalbumin-specific IgE. In our study, there appeared to be a requirement for an elicitation phase in order to induce an increase in ovalbumin-specific IgE.

The Th2 subtype of the $CD4^+$ T cell produces a unique profile of cytokines that has been linked to the pathogenesis of IgE-mediated allergic airway disease. Th2 cells produce IL-4, IL-5, and IL-13 (Yssel and Groux, 2000). This Th2 cytokine profile has been consistently found in both bronchoalveolar lavage fluid and biopsies of allergic asthmatics (Wills-Karp, 1999). There are many models of IgE-mediated allergic airway disease that vary in the method used for cytokine analysis with some focusing on changes in BALF-derived cytokine content while others analyze local draining lymph node cells that have been re-stimulated with a mitogen *in vitro*. In the present study, we determined the kinetics of Th2 cytokine gene expression in RNA isolated from the lung and the TB lymph node. Intranasal sensitization and challenge of mice with ovalbumin increased lung-derived mRNA expression of the Th2 cytokines IL-4, IL-5, and IL-13. In addition, there was an increase in IL-10 mRNA. The magnitude of the increases peaked at the earliest measured time, i.e., 24 h after double challenge, and quickly decreased in magnitude by 96 h after double challenge. The kinetics suggests that cytokine gene expression peaks soon after allergen re-exposure and wanes in a short period of time thereafter. The magnitude of IL-4 mRNA expression was several-fold greater after two ovalbumin challenges than after a single challenge. Thus, two ovalbumin challenges produce a more robust cytokine mRNA response than a single challenge. Also, once IL-4 mRNA expression was elevated, it remained elevated for at least 48 h after challenge. Intranasal sensitization and challenge of mice with ovalbumin also increased TB lymph node-derived mRNA expression of IL-4 24 h after challenge and persisted at that

level at least until 96 h. Interestingly, the RNase Protection Assay was not sufficiently sensitive to detect this difference in IL-4 mRNA expression in the TB lymph node. The lack of sensitivity in detecting the increase in IL-4 mRNA is likely due to the high background expression of IL-4 mRNA in the lymph node that may have prevented the detection of an increase in expression in the treated groups. In addition, the fold-increase in IL-4 mRNA expression in the lymph node as determined by PCR was much smaller than that exhibited by the lungs of these animals and suggests that this assay may be unable to detect small increases in expression.

The increase in IL-10 mRNA in this model coincides with the development of the airway pathology suggesting that it may play a role in the induction of the pathologic changes as some murine models suggest (Lee et al, 2002; Makela et al, 2000; Yang et al, 2000). However, the role of IL-10 induction has been somewhat controversial leading some to speculate that its primary role is inhibitory, serving to mitigate the severity of the immune response (Bellinghausen et al, 2001). The exact role of IL-10 in this model is unclear.

Individuals afflicted with asthma often suffer from concurrent IgE-mediated allergic airway diseases of the upper airways such as IgE-mediated allergic rhinitis. The main symptoms of IgE-mediated allergic rhinitis are itching, sneezing, watery discharge and obstructed nose (Andersson et al, 2000). The pathologic features of this nasal airway disease include plasma exudation, hypersecretion of mucus, and cellular infiltrates consisting of T- and B-lymphocytes, mast cells, eosinophils, and plasma cells in the nasal airway

(Andersson et al, 2000). The mechanism of IgE-mediated allergic rhinitis, like asthma, is dependent on Th2 cytokines (Andersson et al, 2000). In the present study, we found that intranasal sensitization and challenge to ovalbumin induced in A/J mice features similar to that of IgE-mediated allergic rhinitis in people. A histopathologic assessment of the nasal cavity of our A/J mice revealed that intranasal sensitization and challenge to ovalbumin caused an increase in the number of mucus-secreting cells in the respiratory epithelium that lines the nasal cavity. This epithelial change was associated with an inflammatory influx consisting of eosinophils and lymphocytes similar to that observed in the lung. Therefore, our exposure regimen elicited some of the characteristic features of IgE-mediated allergic rhinitis and may be used to study allergic rhinitis.

The present study illustrated that intranasal sensitization and challenge of mice to ovalbumin in the absence of an adjuvant successfully induced many of the histopathologic, morphologic and immunologic features associated with IgE-mediated allergic airway disease. In addition, the assessment of local cytokine changes in the lung and its draining lymph node is an effective method for determining changes in cytokine gene expression after intranasal sensitization and challenge to an allergen. The analysis of local cytokine gene expression after intranasal exposure may prove useful in the identification of other allergenic proteins and low molecular weight chemicals.

CHAPTER 3

SUPPRESSION OF THE OVALBUMIN-INDUCED ALLERGIC AIRWAY RESPONSE IN A/J MICE BY INTRANASAL OR INTRAPERITONEAL ADMINISTRATION OF CANNABINOL

ABSTRACT

The pathophysiology of allergic airway diseases such as asthma has been linked to a specific subtype of CD4+ T cells, Th2 cells, and the cytokines they release. A number of studies have shown that cannabinoids inhibit expression of Th2 cytokines and may thus influence Th2 cytokine-dependent allergic airway responses. The present study was designed to test the hypothesis that cannabinol (CBN) administered either via intraperitoneal (IP) injection or intranasal (IN) instillation would suppress the characteristic pathologic and immunologic features of allergic airway disease elicited by intranasal sensitization and challenge with ovalbumin in A/J mice. Morphologic changes in the pulmonary airways were assessed in addition to changes in cytokine gene expression in the lung after ovalbumin and/or CBN treatment. IN sensitization and challenge with ovalbumin elicited marked mucous cell metaplasia (MCM) in the respiratory epithelium lining the pulmonary airways, and an associated mixed inflammatory cell influx consisting of lymphocytes, plasma cells neutrophils, and eosinophils. Ovalbumin-treated mice also had enhanced expression of the Th2 cytokine mRNAs IL-4, IL-5, IL-10, and IL-13 in the lung, and concurrent increases in ovalbumin-specific and total serum IgE. IP administration of CBN attenuated the ovalbumin-induced increase in IM, serum IgE, and lung-derived IL-4 mRNA expression. IN instillation of CBN suppressed the OVA-induced increase in total and antigen-specific serum IgE, the total number of lymphocytes, eosinophils and neutrophils in BALF, and lung-derived IL-13 mRNA expression. Intranasal instillation of CBN had no effect, however, on the increase in IM in the pulmonary

airways and the increase in lung-derived IL-4, IL-5, and IL-10 mRNA expression. The intranasal instillation of CBN, although not as effective as IP administration of CBN, did inhibit some features of the allergic airway response suggesting that CBN may have some utility as a therapeutic/prophylactic when administered via the airways.

INTRODUCTION

In a previous study using a murine model of allergic airway disease, Jan and colleagues, 2003, demonstrated that two separate plant-derived cannabinoids, cannabinol (CBN), and Δ^9-tetrahydrocannabinol (Δ^9-THC), attenuated several of the pathologic and immunologic features of allergic airway disease resulting from IP sensitization and aerosol challenge with ovalbumin (Jan et al, 2003). Subsequently, I developed a murine model of allergic airway disease that more closely resembled the human route of exposure to aeroallergens, i.e., inhalation. This model used intranasal instillations of ovalbumin to both sensitize and challenge mice (refer to Chapter 2 of dissertation, Farraj et al, 2003). Although IP administration of cannabinoids inhibited the allergic airway response resulting from systemic sensitization and airway challenge with ovalbumin, it is not clear whether IP administration of cannabinoids will suppress the allergic response elicited by IN sensitization and challenge with ovalbumin. In addition, the systemic administration of drugs (e.g, oral) in humans often results in a multitude of side effects (Hardman et al, 1995). For this reason, in the development of potential therapeutics/prophylactic to be used by patients for the treatment of allergic airways disease, airway delivery of the drug is preferred to avoid such side effects. One major obstacle in the use of intra-airway administration of cannabinol in experimental models of allergic airway disease has been the poor aqueous solubility of both CBN and Δ^9-THC and the lack of a suitable vehicle for airway delivery. Thus, it is unclear whether

cannabinol administered via the airway would lead to suppression of the OVA-induced allergic airway response in A/J mice.

Asthma is an allergic airway disease of the lung that is characterized by the presence of chronic inflammation of the pulmonary airways with infiltration of the bronchial mucosa by lymphocytes, eosinophils, and neutrophils, along with increased mucus production (Wills-Karp, 1999). Asthma is also associated with increased serum IgE levels and physiological abnormalities including airflow obstruction and airway hyper-responsiveness (Wills-Karp, 1999). The mechanism(s) underlying the pathogenesis of allergic airway disease are currently being elucidated. There are many lines of evidence, however, that implicate the T lymphocyte and specifically the CD4+ T cell. Evidence for the participation of T lymphocytes includes increased numbers of CD4+ T cells in bronchoalveolar lavage fluid (BALF) and a concurrent decrease in the number of CD4+ T cells in peripheral blood following allergen challenge (Wills-Karp, 1999). A specific subtype of the CD4+ T cell, T helper 2 (Th2), produces a unique pattern of cytokine expression. This Th2 pattern has been consistently found in both BALF and biopsies of allergic asthmatics and includes the cytokines IL-4, IL-5, IL-10, and IL-13 (Wills-Karp, 1999). The differentiation of uncommitted T cell precursors into Th2 cells is largely driven by IL-4 as demonstrated by the abrogation of airway hyperreactivity after administration of a monoclonal antibody against IL-4 during the period of immunization in a murine model of asthma (Corry et al, 1996). IL-4 and IL-13 are required for B cells to undergo class switching to go from producing IgM to producing IgE (Frew, 1996). IL-4 and IL-

13 also induce mucin gene expression in airway epithelium (Shim et al, 2001). IL-13 has also been proven to be necessary for the expression of allergic airway hyperresponsiveness and sufficient to induce it. Administration of recombinant IL-13 conferred an asthma-like phenotype to non-immunized mice (Grunig et al, 1998). IL-5 regulates the maturation and activation of eosinophils. IL-5-deficient mice have markedly fewer eosinophils in the lamina propria of their airways than normal mice after provocation and antibody blockade inhibits antigen-induced eosinophilia and late-phase hyperresponsiveness (Foster et al, 2000; Karras et al, 2000).

Cannabinoids are a structurally-related family of compounds produced by the *cannabis sativa* plant. Cannabinoids exert a broad range of physiologic effects; the most extensively studied being those in the CNS and the immune system. Suppression of the immune system by cannabinoids is thought to be mediated, at least in part, through cannabinoid receptors (CB) expressed by leukocytes (Herring et al, 1999). CB1 is the predominant cannabinoid receptor expressed in rat brain (Matsuda et al, 1990), whereas CB2 is expressed by cells of the immune system (Munro et al, 1993; Schatz et al, 1997). Several reported findings suggest that cannabinoids exert inhibitory effects on various aspects of the allergic airway response, thus supporting investigation of the effects of cannabinoids on allergic airway disease. For example, in one study, the smoking of marijuana or ingestion of Δ^9-THC by subjects with chronic asthma of minimal to moderate severity partially reversed asthma-induced bronchoconstriction (Tahskin et al, 1974; Tashkin et al, 1975). Another specific immune effect of

cannabinoids that is relevant to the current study is the effect cannabinoids have on the expression of Th2 cytokines, which mediate the allergic airway response. Cannabinoids inhibit the nuclear factor of activated T cells (NFAT), which is a critical regulator of a number of T cell cytokines including IL-2, IL-4 and IL-5, from binding DNA (Yea et al, 2000; Jan et al, 2002). In addition, in a previous study, IP injections of cannabinoids attenuated the increase in lung-derived Th2 cytokine expression elicited by IP sensitization followed by aerosol challenge with ovablumin (Jan et al, 2003). The effect of cannabinoids on Th2 cytokine expression may underlie the suppression of the asthma pathophysiology. Inhibition of the allergic airway response by CBN would demonstrate the necessity of Th2 cytokines in the pathogenesis of allergic airway disease in this model. This would further support the findings described in Chapter 2 that showed a link between the pathologic changes and the increase in lung-derived Th2 cytokine mRNA expression. CBN-mediated inhibition of the allergic airway response would also suggest that a LMW chemical-induced increase in airway Th2 cytokine expression may be a strong indicator of the allergenic potential of the chemical.

The primary objective of this study was to determine if the cannabinoid, CBN, administered IP or via IN instillation, would attenuate the pathologic and immunologic features of the allergic airway response in A/J mice elicited by IN sensitization and challenge with OVA. In one study, mice sensitized and challenged with ovalbumin were co-treated with IP injections of CBN. In a separate study, mice sensitized and challenged with ovalbumin were co-treated

with IN instillations of CBN. CBN was dissolved in a 1:4 ethyl acetate/olive oil vehicle (EA/OO) when administered via IN instillation. The rationale for using CBN and not Δ^9-THC in the present study is CBN exhibits lower affinity for the CB1 receptor, despite its structural similarity, and thus possesses decreased psychotropic activity, while maintaining its immunomodulatory activity (Jan et al, 2003). Morphologic changes in the pulmonary airways were assessed in addition to changes in cytokine gene expression in the lung after OVA and/or CBN treatment.

MATERIALS AND METHODS

Animals and Allergen Sensitization and Challenge

Forty-four male A/J mice (Jackson Laboratories, Bar Harbor, Maine), 6 weeks of age, were randomly assigned to one of 8 experimental groups in two different studies (n = 5 or 6). Mice were free of pathogens and respiratory disease, and used in accordance with guidelines set forth by the All University Committee on Animal Use and Care at Michigan State University. Animals were housed five per cage in polycarbonate boxes, on Cell-Sorb Plus bedding (A&W Products, Cincinnati, OH) covered with filtered lids, and had free access to water and food. Room lights were set on a 12 hr light/dark cycle beginning at 6:00 am, and temperature and relative humidity were maintained between 21-24°C and 40-55 % humidity, respectively. The exposure regimen used to sensitize and challenge the mice was previously described in detail (refer to Chapter 2 of the dissertation, Farraj et al, 2003). The exposure regimen, outlined in Figure 3.1, is briefly described as follows.

Mice were anesthetized with 4% halothane and 96% oxygen. Anesthetized mice were treated by intranasal instillation with 30 µl of 0.5% ovalbumin (Sigma Chemical, St. Louis, MO) in sterile, pyrogen-free saline or 30 µl of saline alone divided evenly between each naris. The sensitization phase consisted of a single instillation once per day for 5 consecutive days. The mice were challenged once, 14 days after the fifth instillation of the sensitization phase via a single instillation of the same volume and concentration as the instillations during the sensitization phase. The mice were challenged a second time 10

Exposure Regimen for Ovalbumin/Cannabinol Co-Exposure Study

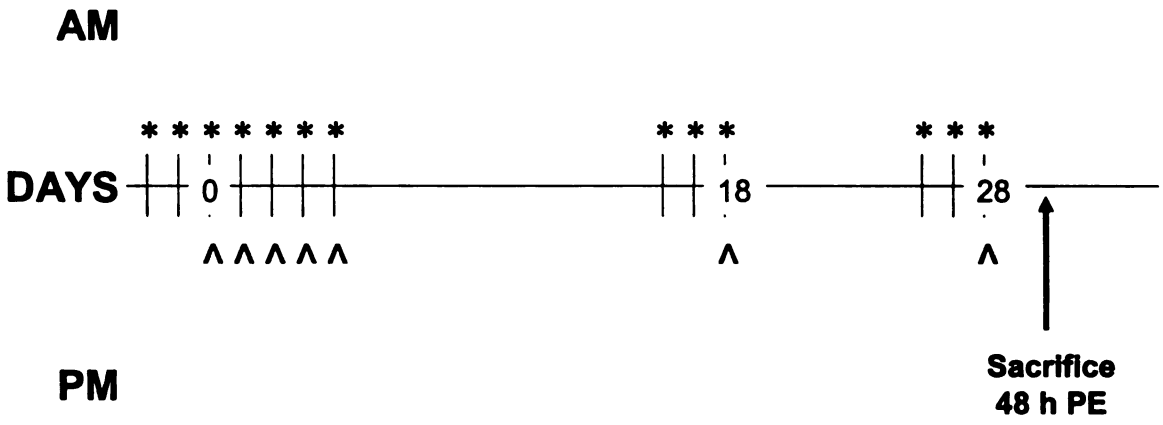

Figure 3.1: Timeline of the exposure regimen used to sensitize and challenge A/J mice with ovalbumin or saline and co-treat with either IP CBN or IN CBN. Mice were co-treated with single IP injections (50 mg/kg) or intranasal instillations (500 μM) beginning 2 days before sensitization and challenge with ovalbumin/saline and the morning of each ovalbumin/saline instillation. Mice were sensitized with five consecutive daily intranasal instillations of 30 μl of 0.5% ovalbumin in saline or saline alone, then challenged once two weeks later with a similar volume, and then challenged again a second time 10 days later followed by sacrifice 48 hr after double challenge. Ovalbumin/saline instillations were administered in the evenings of the CBN administrations. * = IP injection of CBN or IN instillation of CBN. ^ = intranasal instillation of 30μl of 0.5% ovalbumin in saline or saline alone.

days after the first challenge again with the same volume and concentration as in the first challenge. Mice were then sacrificed 48 hr after the second challenge. There were three different treatment groups: 1) sensitization and challenge with saline, 2) sensitization and challenge with ovalbumin; and 3) mice that were co-treated with CBN before, during, and after sensitization and each challenge with ovalbumin. Animals that were exposed to CBN were given IP injections of CBN (50 mg/kg in 200 μl of 5% ethanol and 0.5 % Tween 20 in saline) once per day the two days before ovalbumin/saline sensitization and then once in the mornings before each intranasal instillation of ovalbumin/saline that was administered in the evenings during the sensitization phase. CBN -treated mice also received IP injections once per day of the same concentration the two days before each of the challenges and once the morning of each intranasal challenge with ovalbumin/saline. A group exposed to CBN alone or with saline was excluded from this study because the effect of CBN alone was determined in one of our previous studies (Jan et al, 2003). It was determined from that study that mice treated with CBN alone in did not have any pathologic or immunologic differences within the respiratory tract relative to naïve untreated mice.

A second set of animals was set aside to determine how intra-airway administration of the same formulation of CBN would compare to IP injection in ovalbumin-induced allergic airway disease. The CBN in this set of animals was administered via intranasal instillation. The animals were sensitized and challenged with ovalbumin in the same manner as in the first set of animals. There were four treatments groups (n = 6 or 8): 1) sensitization and challenge

with saline, 2) sensitization and challenge with ovalbumin, 3) mice that were co-treated with CBN before, during, and after sensitization and each challenge with saline and 4) mice that were co-treated with CBN before, during, and after sensitization and each challenge with ovalbumin. Animals that were exposed to CBN were given 60 μl intranasal instillations of 500 μM CBN in 1:4 ethyl acetate/olive oil vehicle per day the two days before ovalbumin/saline sensitization and then once in the mornings before each intranasal instillation of ovalbumin/saline that was administered in the evenings during the sensitization phase. A pilot study was performed that tested for the irritating effects of CBN on the airway epithelium in the nasal airway after intranasal instillation. A dose of 500 μM was selected from a range of doses because it was the highest dose of CBN that was not irritating to the airway epithelium. The 1:4 ethyl acetate/olive oil vehicle has no irritating effects on the respiratory epithelium and allows dissolution of the lipid soluble CBN. CBN-treated mice also received intranasal instillations of CBN once per day of the same concentration the two days before each of the challenges and once the morning of each intranasal challenge with ovalbumin/saline.

Necropsy, Blood Sample Collection, and Tissue Preparation

Before sacrifice, mice were deeply anesthetized via intraperitoneal injection of 0.1 ml of 12% pentobarbital in saline. Blood samples (0.1 to 0.5 ml) were taken from the brachial artery and collected in Beckton Dickinson vacutainers. Serum samples were collected after the blood samples were

centrifuged to remove cells and the supernatants were frozen and stored at -20°C. The abdominal aorta and renal artery were then severed to exsanguinate the mice. Immediately after death, the trachea was cannulated and the heart/lung block removed. Bronchoalveolar lavage fluid (BALF) was collected from the set of animals that received intranasal instillations of CBN. In order to collect BALF, a syringe was then attached to the cannula and BALF was collected from the whole lung via two sequential changes of 1 ml saline. Differential cell counts were determined by cytocentrifuging cells onto glass slides and then staining with Diff-Quik (DADE Behring, Newark, DE). Neutrophils, macrophages, eosinophils and other cell types were microscopically identified and their numbers were determined by multiplying the percentage of each cell type from a total of 200 cells by the total number of cells per ml of lavage fluid. All four right lung lobes were placed in 2 ml Tri reagent, homogenized and stored at -80°C.

After removal of the right lung lobes, the left lung lobe was intratracheally perfused with 10% neutral buffered formalin at a constant pressure of 30 cm of fixative. After 1 hr, the trachea was ligated, and the inflated left lung lobe was immersed in a large volume of the same fixative for 24 hr. After fixation, the left lung lobe was microdissected along the main axial airway, and two sections were then excised at the level of the fifth (proximal) and eleventh (distal) airway generation (Figure 3.2), as has been described previously in detail (Steiger et al, 1995). Paraffin sections (5 μm) were stained

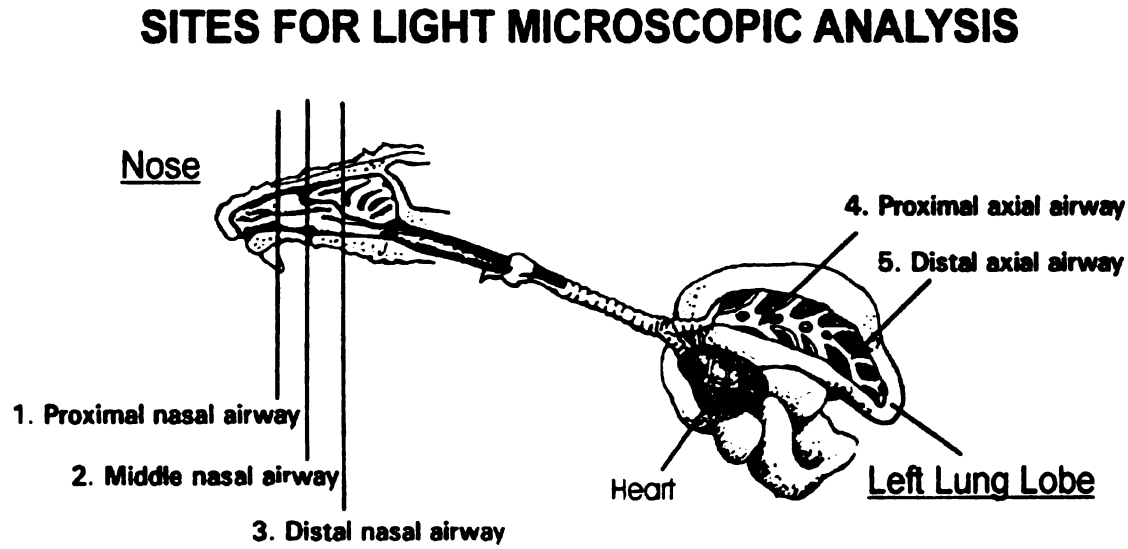

Figure 3.2: Sites of airway tissue selection for morphometric analysis. The cartoon depicts the main axial airway of the left lung lobe. The left lung lobe was microdissected along the axial airways, and two sections were then excised at the level of the fifth (proximal airway) and eleventh (distal airway) airway generation.

with Alcian blue (pH 2.5) and periodic acid Schiff's (AB/PAS) reagent, which stain neutral and acidic mucosubstances, respectively. Other paraffin sections were stained separately for eosinophilic major basic protein (MBP, described below).

Morphometry of stored intraepithelial mucosubstances in pulmonary airways.

The amount of stored mucosubstances in the surface epithelium lining the pulmonary axial airway (airway generation 5, proximal airway) in the left lung lobe was estimated by quantifying the volume density of AB/PAS-stained mucosubstances using computerized image analysis and standard morphometric techniques. The area of AB/PAS-stained mucosubstances was calculated by circumscribing the perimeter of the stained material using the Scion Image program (Scion Corporation, Frederick, MD). The length of the basal lamina underlying the surface epithelium was calculated from the contour length of the digitized image of the basal lamina. The volume of stored mucosubstances (volume density, Vs) per unit of surface area was estimated using the method described in detail by Harkema et al., 1987, and was expressed as nl of IM per square mm of basal lamina.

ELISA for Total and Ovalbumin-specific serum IgE

Total serum IgE was measured using a 96-well Immulon ELISA plate (Dynex, Technologies, Chantilly, Virginia) coated with 2 μg/ml anti-mouse IgE (Purified Rat Anti-Mouse IgE Monoclonal Ab, Pharmingen, San Diego, CA) and incubated overnight at 4°C. For measurement of ovalbumin-specific IgE, a

second ELISA plate was coated with 0.5% ovalbumin and incubated overnight at 4°C. After washing, the plates were incubated in 3 % Bovine Serum Albumin (3 % BSA) at 37°C for 1 hr. (BSA, CALBIOCHEM, La Jolla, CA). Serum samples at 1:10 dilution were then added followed by incubation at 37°C for 1 hr. After washing, biotinylated anti-mouse IgE (Biotin-conjugated Rat Anti-Mouse IgE Monoclonal Ab, Pharmingen, San Diego, CA) was then added at 2 µg/ml and allowed to incubate at 25°C for 1 hr. After washing, 1.5 µg/ml of streptavidin peroxidase was added followed by incubation at 25°C for 1 hr. After washing, TMB substrate (12.5ml citric-phosphate buffer + 200 µl of TMB stock solution (6mg/ml in DMSO)) was added to produce a color reaction. The reaction was terminated by the addition of 6 N H_2SO_4. Optical density was determined at 450 nm using an EL-808 microplate reader (Bio-Tek instruments, Winooski, Vermont).

Quantitative IL-4 Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR).

Total RNA was isolated from lung tissues using the TRI REAGENT method (Molecular Research Center, Cincinnati, OH) following the manufacturer's protocol and the integrity of the RNA was checked by performing an ethidium bromide diagnostic gel to check for degradation of RNA. Quantitative RT-PCR was performed on RNA collected from the first set of animals that received IP injections of CBN. Quantitative RT-PCR was performed as described by Gilliland et al, 1990, except that rcRNA was used as an IS instead of genomic DNA with 10 aliquots of rcRNA from 10^4 to 10^8 molecules.

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Briefly, total RNA (right lung lobe or TB lymph node) and IS rcRNA of known amounts were reverse transcribed into cDNA using oligo$(dT)_{15}$ as primers using reverse transcriptase (RT) (Promega, Madison WI). To ensure that there was no genomic DNA contamination in the RNA isolates, no-RT controls were employed for all samples. A PCR master mixture consisting of PCR buffer, 4 mM $MgCl_2$, 6 pmol each of IL-4 forward and reverse primers, and 2.5 U of Taq DNA polymerase was added to the cDNA samples. Samples were then heated to 94°C for 3 min and cycled 40 times at (1) 95°C (30 sec), (2) 58°C (30 sec), and (3) 72°C (45 sec). After the completion of 40 cycles, a final elongation step at 72°C (5 min) was carried out. PCR products were resolved by electrophoresis in 3% NuSieve 3:1 gel (FMC Bioproducts, Rockland ME) and visualized by ethidium bromide staining. The IL-4 primers produced a 228 bp product from the cellular RNA and a 346 bp product from the IS rcRNA. Quantification was performed using Gel Doc 1000 (BioRad Laboratories, Inc., Hercules, CA) and Multi Analyst Software. The amount of IL-4 mRNA present was determined as described by Gilliland et al, 1990. Briefly, the ratio of the density of the IS rcRNA to IL-4 mRNA was plotted against the amount of IS rcRNA (in molecules) added to each reaction. The point at which the ratio of IS (rcRNA) to IL-4 mRNA was equal to 1 signified the "cross-over point" which represented the amount of IL-4 molecules present in the initial RNA sample.

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Real-time RT-PCR

The evaluation of the relative expression levels of the cytokines IL-4, IL-5, IL-10, IL-13, and IFN-γ mRNA in the lungs of the second set of mice treated with intranasal instillations of CBN was determined using a one-step real-time multiplex RT-PCR using the manufacturer's protocol (Applied Biosystems, Foster City, CA). Briefly, aliquots of isolated tissue RNA (100 ng total RNA) were added to the RT-PCR reaction mixture, which included the target gene (IL-4, IL-5, IL-10, IL-13, or IFN-γ) primers and probe, endogenous reference primers and probe (18S ribosomal RNA), AmpliTaq DNA polymerase and Multiscribe reverse transcriptase (MuLV). The probes are designed to exclude detection of genomic DNA. RNA samples were first reverse transcribed and then immediately amplified by PCR. Following the PCR, amplification plots (change in dye fluorescence versus cycle number) were examined and a dye fluorescence threshold within the exponential phase of the reaction was set separately for the target gene and the endogenous reference (18S). The cycle number at which each amplified product crosses the set threshold represents the C_T value. The amount of target gene normalized to its endogenous reference was calculated by subtracting the endogenous reference C_T from the target gene C_T (ΔC_T). Relative mRNA expression was calculated by subtracting the mean ΔC_T of the control samples from the ΔC_T of the treated samples ($\Delta\Delta C_T$). The amount of target mRNA, normalized to the endogenous reference and relative to the calibrator (i.e., RNA from control) is calculated by using the formula $2^{-\Delta\Delta C}{}_T$.

Statistics

The data obtained from each experimental group were expressed as a mean group value ± the standard error of the mean (SEM). The differences among groups were determined by one way or two-way analysis of variance (ANOVA) and an All Pairwise Comparison Test (Tukey), using SigmaStat software from Jandel Scientific Co. (San Rafael, CA).

RESULTS

Pulmonary Histopathology

Intranasal sensitization and challenge with ovalbumin resulted in distinct pulmonary lesions in the airways of A/J mice. The principal morphologic alterations in the lungs of these mice were marked MCM in the surface epithelium lining the conducting airways and an associated mixed inflammatory cell influx consisting of eosinophils, neutrophils and mononuclear cells (mainly lymphocytes and plasma cells) in the interstitial tissue surrounding the airways (Figure 3.3). Airway lesions were more severe in the main axial airways and pre-terminal bronchioles, but were also present to a lesser degree in the terminal bronchioles. The interstitial tissue surrounding large blood vessels in the alveolar parenchyma (pulmonary veins) and along the conducting airways (pulmonary arteries) exhibited a similar inflammatory response. The inflammatory responses in all of these mice were principally present in the pulmonary airways and large vessels of the lung. No significant ovalbumin-induced inflammatory responses were evident in the alveolar parenchyma. Treatment-associated changes were absent in the lungs of control mice that received intranasal sensitization and challenges of saline.

Mice sensitized and challenged with ovalbumin and co-treated with IP injections of CBN also had undergone MCM in the surface epithelium lining the conducting airways and an associated mixed inflammatory cell influx consisting of eosinophils, neutrophils and mononuclear cells. The lesion, however, was less severe. The MCM in mice co-treated with IP CBN was less severe than in

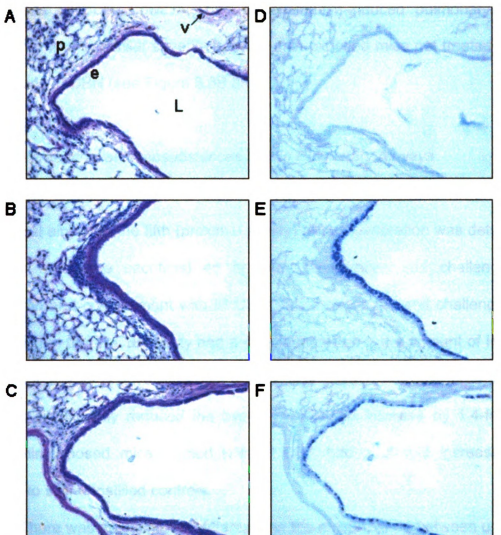

Figure 3.3: Light photomicrographs of the main axial airway of the left lung from mice intranasally sensitized and challenged twice with saline (A, D), 0.5% ovalbumin (B, E), or 0.5 % ovalbumin and co-treated with IP CBN (C, F) and sacrificed 48 hr after challenge. Tissues were stained with hematoxylin and eosin (A, B, C) for morphologic assessment and Alcian blue (pH = 2.5)/periodic acid Schiff's sequence (AB/PAS) for mucosubstances in the airway epithelium (D, E, F). p = alveolar parenchyma, e = airway epithelium, v = blood vessel; L = airway lumen.

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ovalbumin-exposed mice that were not treated with IP CBN (Figure 3.3).

Mice sensitized and challenged with ovalbumin and co-treated with IN instillations of CBN, however, had an ovalbumin-induced pulmonary airway lesion that was of similar severity to ovalbumin-exposed mice not treated with IN instillations of CBN (see Figure 3.3B and E).

Stored Intraepithelial Mucosubstances (IM) in Pulmonary Airways

The amount of AB/PAS-stained IM in the surface epithelium lining the main axial airway at the fifth (proximal airway) airway generation was determined in mice that were sacrificed 48 hr after sensitization and challenge with ovalbumin and co-treatment with IP CBN. Mice sensitized and challenged with ovalbumin in the IP CBN study had a 42-fold increase in the amount of IM in the main axial airway relative to saline-instilled mice (Figure 3.4). Co-treatment with IP CBN significantly reduced the ovalbumin-induced increase by 1.4-fold; i.e., ovalbumin-exposed mice treated with IP CBN had a 29-fold increase in IM relative to saline-instilled controls.

There was no significant difference in the amount of IM between untreated ovalbumin-exposed mice and ovalbumin-exposed mice treated with IN instillations of CBN (amounts were not significantly different from the Vs in the ovalbumin instilled group not treated with IP CBN in Figure 3.4).

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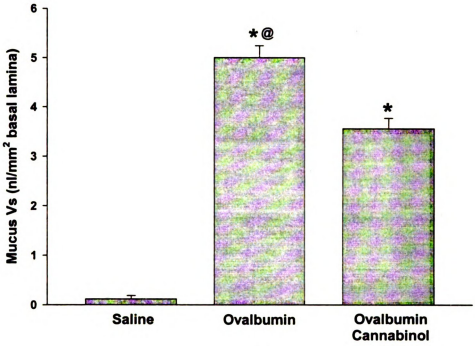

Figure 3.4: Morphometric quantification of AB/PAS-stained material in the surface epithelium lining the main axial airway of the left lung lobe 48 hr after double challenge with saline, ovalbumin, or ovalbumin plus co-treatment with IP CBN. Bars represent the Volume Density (Vs) of intraepithelial mucosubstances ± standard error of the mean (n = 5 or 6/ group). * = significantly greater than saline-instilled group($p<0.05$). @ = significantly greater than IP CBN-treated group ($p<0.05$).

Bronchoalveolar lavage fluid (BALF)

The total number of cells in the BALF was determined at 48 hr after the second challenge in ovalbumin-exposed mice that were co-treated with IN CBN. Ovalbumin-exposed mice had a 14.5-fold increase in the number of lymphocytes, an 83-fold increase in the number of eosinophils, and a 26-fold increase in the number of neutrophils in the BALF compared to saline-treated mice (Figure 3.5). The predominant cell type in the BALF of ovalbumin-exposed mice was the neutrophil. IN instillations of CBN markedly attenuated the ovalbumin-induced increase in all three cell types. IN CBN caused a 7.5-fold decrease in the number of lymphocytes, a 5.5-fold decrease in the number of eosinophils, and a 2.7-fold decrease in the number of neutrophils in the BALF.

Total Serum IgE

Total serum IgE levels were determined at 48 hr after the second challenge in ovalbumin-exposed mice that were co-treated with IP injections of CBN and in mice co-treated with IN instillations of CBN. Mice sensitized and challenged with ovalbumin in the IP CBN study had a 1.7–fold increase in ovalbumin-specific serum IgE (Figure 3.6A) and no change in total serum IgE (Figure 3.6B) relative to the saline-exposed mice. IP CBN significantly reduced the ovalbumin-induced increase in ovalbumin-specific IgE by 1.7-fold. Mice sensitized and challenged to ovalbumin in the IN CBN study had a 16-fold increase in ovalbumin-specific serum IgE (Figure 3.7A) and a 1.9-fold

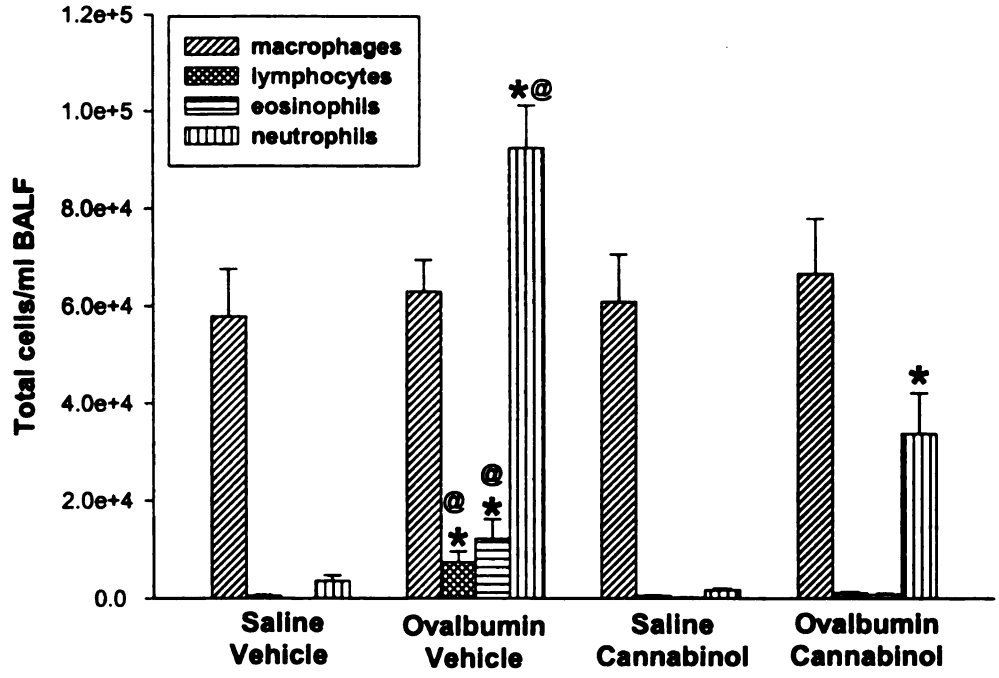

Figure 3.5: Total number of macrophages, lymphocytes, eosinophils, and neutrophils in BALF from the left lung lobes of mice treated with IN instillations of EA/OO vehicle and instilled with saline, mice treated with IN instillations of EA/OO vehicle and instilled with ovalbumin, mice treated with IN instillations of CBN and instilled with saline, or mice treated with IN instillations of CBN and instilled with ovalbumin. Bars represent mean number of cells per ml BALF ± standard error of the mean (n = 6/group). * = significantly greater than saline-instilled controls ($p<0.05$). @ = significantly greater than ovalbumin-instilled group treated with IN CBN ($p<0.05$).

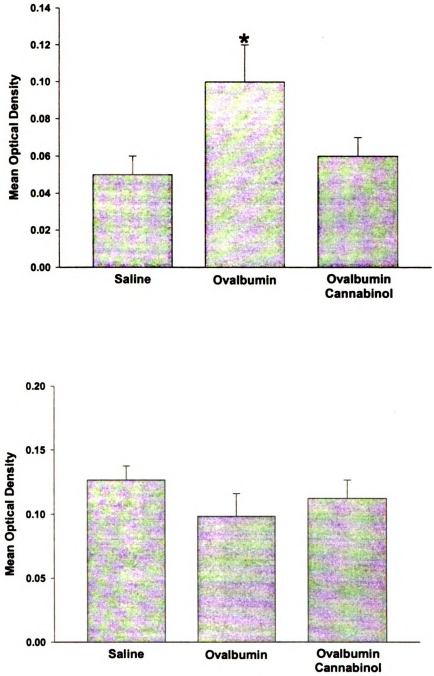

Figure 3.6: Ovalbumin-specific (upper panel) and Total IgE (lower panel) levels in mouse serum isolated 48 h hr after double challenge with saline, ovalbumin, or ovalbumin plus co-treatment with IP CBN. Bars represent average optical density ± standard error of the mean (n = 5 or 6/group). * = significantly greater than all other groups.

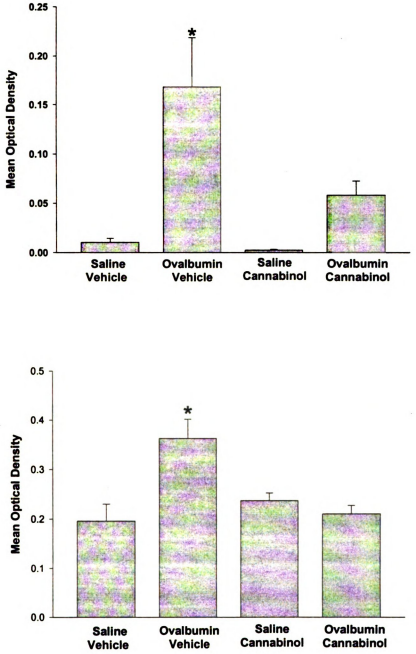

Figure 3.7: Ovalbumin-specific (upper panel) and Total IgE (lower panel) levels in mouse serum isolated 48 hr after mice were treated with IN instillations of EA/OO vehicle and instilled with saline, IN instillations of EA/OO vehicle and instilled with ovalbumin, IN instillations of CBN and instilled with saline, IN instillations of CBN and instilled with ovalbumin. Bars represent average optical density ± standard error of the mean (n = 6/group). * = significantly greater than all other groups.

increase in total serum IgE (Figure 3.7B) relative to saline-exposed control mice. IN CBNreduced the ovalbumin-induced increase in ovalbumin-specific serum IgE by 2.8-fold and the increase in total serum IgE by 1.7-fold.

Cytokine Gene Expression

Quantitative RT-PCR: Semi-quantitative RT-PCR for the cytokine IL-4 was performed on right lung lobe RNA from mice 48 hr after they were sensitized and then challenged with ovalbumin and co-treated with IP CBN. Mice sensitized and challenged with ovalbumin had an 11.4-fold increase in IL-4 mRNA expression within the lung relative to saline-exposed control mice (Figure 3.8). IP CBN co-treatment significantly reduced the ovalbumin-induced increase in IL-4 mRNA by 2.5-fold.

Real-Time RT-PCR: Real-Time RT-PCR was performed for the Th2 cytokines IL-4, IL-5, IL-10, IL-13, and the Th1 cytokine IFN-γ on RNA isolated from the right lung lobes of mice 48 hrs after they were sensitized and challenged with ovalbumin and co-treated with intranasal instillations of CBN. Mice sensitized and challenged with ovalbumin had a 4.5-fold increase in lung-derived IL-4 mRNA expression relative to saline-exposed controls (Figure 3.9). IN CBN co-treatment in ovalbumin-exposed mice had no significant effect on the ovalbumin-induced increase in IL-4. Mice sensitized and challenged with ovalbumin had a 7.7-fold increase in lung-derived IL-5 mRNA expression relative to saline-exposed controls. IN CBN co-treatment reduced the ovalbumin-induced increase in IL-5 mRNA by 1.5-fold, although

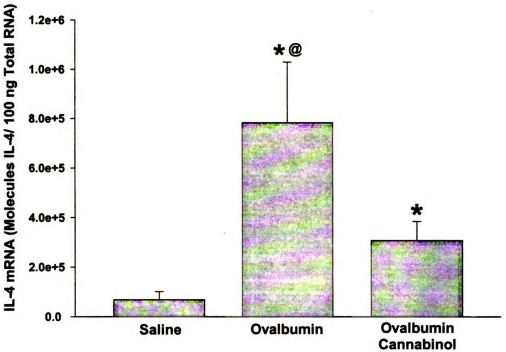

Figure 3.8: Quantification of IL-4 mRNA in right lung lobes 48 hr after double challenge with saline, ovalbumin, or ovalbumin plus co-treatment with IP CBN using semi-quantitative RT-PCR. Bars represent molecules of IL-4 mRNA per 100 ng of total RNA isolated ± standard error of the mean (n = 5 or 6/group). * = significantly greater saline-instilled control ($p<0.05$). @ - significantly greater than ovalbumin instilled group treated with IN CBN ($p<0.05$).

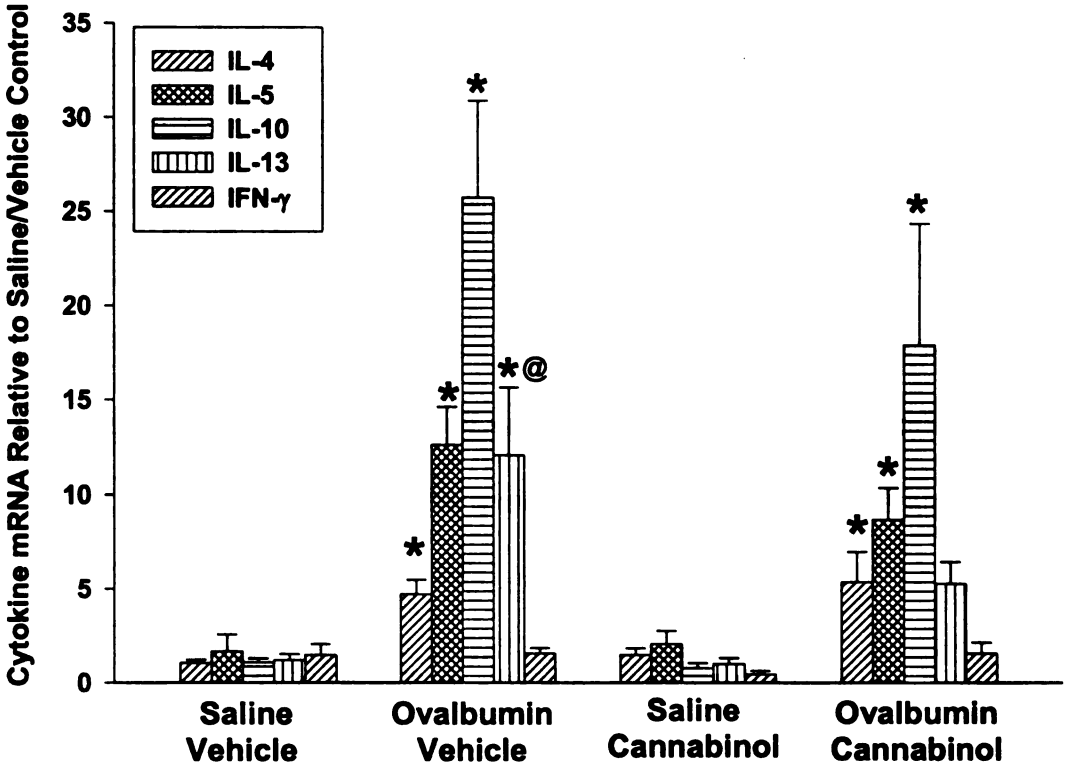

Figure 3.9: Relative quantification using real-time PCR of IL-4, IL-5, IL-10, IL-13, and IFN-γ mRNA in right lung lobes 48 hr after mice were treated with IN instillations of EA/OO vehicle and instilled with saline, IN instillations of EA/OO vehicle and instilled with ovalbumin, IN instillations of CBN and instilled with saline, IN instillations of CBN and instilled with ovalbumin. Bars represent cytokine mRNA relative to the saline/vehicle control ± standard error of the mean (n = 6/group). * = significantly greater than saline-instilled controls ($p<0.05$). @ = significantly greater than IL-13 levels in the ovalbumin-instilled group treated with IN CBN ($p<0.05$).

this difference was not deemed statistically significant. Mice sensitized and challenged with ovalbumin had a 23.8-fold increase in lung-derived IL-10 mRNA expression relative to saline-exposed controls. IN CBN co-treatment reduced the ovalbumin-induced increase in IL-10 mRNA by 1.4-fold, although this difference was also not statistically significant. Mice sensitized and challenged with ovalbumin had a 10.1-fold increase in lung-derived IL-13 mRNA expression relative to saline-exposed controls. IN CBN co-treatment significantly reduced the ovalbumin-induced increase in IL-13 mRNA by 2.3-fold. Ovalbumin and/or IN CBN did not have any effect on the expression of IFN-γ within the lung.

DISCUSSION

The primary objective of this study was to determine if CBN administered either via IP injection or IN instillation would suppress the hallmark pathologic and immunologic features of allergic airway disease in the pulmonary airways of mice sensitized and challenged via IN instillation of ovalbumin. Another objective was to demonstrate a strong link between Th2 cytokine expression and the ovalbumin-induced allergic airway response in this model by co-treating with CBN, an inhibitor of Th2 cytokine expression. Morphologic changes in the pulmonary airways were correlated with changes in cytokine gene expression in the lung after ovalbumin and/or CBN treatment.

Several reports describe the effects of cannabinoids on T cell cytokine gene expression. Cannabinoids have been shown to inhibit the expression of the T cell cytokines IL-2, IL-4 and IL-5 using *in vitro* T cell cultures systems (Condie et al, 1996; Herring et al, 1998; Berdyshev, 2000; Jan and Kaminski, 2000). The inhibitory effects of cannabinoids were also demonstrated in an *in vivo* model of allergic airway disease. In a previous study, Jan et al, 2003, demonstrated that IP cannabinoid treatment suppressed the ovalbumin-induced increase in IL-2 expression and the expression of the Th2 cytokines IL-4, IL-5, IL-10 and IL-13 within the lungs of A/J mice (Jan et al, 2003). These findings suggest that cannabinoids may inhibit Th2 cytokine-mediated allergic airway diseases and thus possess potential therapeutic/prophylactic utility. The development of cannabinoids as a class of potential therapeutics/prophylactics for the treatment of allergic airway disease must be done in parallel with the development of a

method of intra-airway delivery of these drugs in order to produce a high local concentration of the drug in the lungs, the site of inflammation in asthma, and minimize systemic toxicity. A method of intra-airway delivery of CBN was achieved in the present study by intranasal instillation. CBN was dissolved in an EA/OO vehicle and then administered via intranasal instillation before each intranasal ovalbumin instillation during both sensitization and challenge.

The airways of asthmatics are characterized by the presence of chronic inflammation with infiltration of lymphocytes, plasma cells, eosinophils, and mast cells in the bronchial mucosa and epithelial changes that include MCM (Holgate, 2000, Wills-Karp, 1999). These effects are mediated, in part, by Th2 cytokines including IL-4, IL-5, and IL-13. A histopathologic assessment of the pulmonary airways of ovalbumin-exposed mice co-treated with IP injections or IN instillations of CBN was made to determine if CBN inhibited some of the characteristic features of allergic airway disease. IP CBN co-treatment significantly reduced the ovalbumin-induced increase in IM in the surface epithelium lining the main axial airway of the left lung lobe. The inhibition of these pathologic features is consistent with our previous findings that showed that IP CBN attenuated many of the pathologic features in the airways of A/J mice that resulted from IP sensitization and airway challenge with ovalbumin. In the IN CBN study, an assessment of the BALF was made. Ovalbumin-exposed mice had a large increase in the number of eosinophils, lymphocytes, and neutrophils in the BALF. Interestingly, the neutrophil was by far the most numerous cell type in the BALF. IN CBN significantly reduced the ovalbumin-induced increase in eosinophils,

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lymphocytes, and neutrophils in the BALF. IN CBN co-treatment in mice sensitized and challenged with ovalbumin, however, did not significantly alter the ovalbumin-induced increase in intraepithelial mucus. BALF cellularity may thus be more sensitive to the effects of CBN than changes in IM. The lack of an inhibition on the ovalbumin-induced increase in IM by IN instillations of CBN may be due to the dose of CBN that was used and/or the vehicle that CBN was dissolved in. The exact amount of CBN that entered the lungs after either route of administration is unknown as no pharmacokinetic studies were conducted to determine the amount of CBN that entered the target tissue. The route of CBN exposure that led to a higher local concentration of CBN in the lung was thus not known. Although the dose of CBN that was administered via IN instillation, 500 μM, was much less than that administered via IP injection, i.e., 50 mg/kg, the proportion of each dose that entered the lung is not known and thus the conclusion that the dose may partly explain the differences in response observed with the two routes of administration cannot be made.

The effect of intranasal CBN instillations on the allergic airway response in appears to have been largely influenced by the vehicle that was used to dissolve it. The viscous nature of EA/OO likely limited access of the lung to CBN, thus limiting the effect of CBN on the inflammatory response within the lung. In pilot studies designed to assess the distribution of the EA/OO after IN instillation, I intranasally instilled the dye Sudan Black dissolved in EA/OO (refer to Chapter 4) and determined that most of the instilled volume ended up in the nasal airways and gut and very little in the lung. One method that can be potentially used to

improve CBN distribution with intranasal instillation is the development of a less viscous vehicle that is also non-volatile and non-irritating to the airway epithelium. An alternative method to improve distribution with intranasal instillation of CBN is the development of a water-soluble formulation of CBN with equal immune-modulating potency. This would allow the dissolution of CBN in an aqueous vehicle, which when intranasally instilled, distributes to the lung, thus increasing the local concentration of CBN in the lung. Methods of intra-airway delivery other than intranasal instillation may alternatively be used to determine if intra-airway delivery of CBN will inhibit the allergic airway response. They include aerosol delivery of CBN or intratracheal instillation.

The preponderance of evidence suggests that Th2 cytokines play a crucial role in the pathogenesis of allergic airway diseases such as asthma. Lung tissue from mice sensitized and challenged with ovalbumin and co-treated with IP injections of CBN or IN instillations of CBN was assessed for Th2 cytokine mRNA expression to determine if CBN influenced the ovalbumin-induced expression of these cytokines. Mice sensitized and challenged to ovalbumin in the IP CBN study had a large increase in lung-derived IL-4 mRNA expression, the only cytokine measured in that study. IP CBN co-treatment significantly attenuated the ovalbumin-induced increase in IL-4 mRNA. This data is consistent with the inhibitory effects of cannabinoids on cytokine gene expression demonstrated using in vitro models (Condie et al, 1996; Herring et al, 1998; Berdyshev, 2000; Jan and Kaminski, 2000) and an *in vivo* model (Jan et al, 2003). IP CBN-mediated inhibition of the lung-derived increase in IL-4 expression further

supports the data presented in Chapter 2 linking the ovalbumin-induced pathologic changes to enhanced Th2 cytokine expression and suggests that Th2 cytokines are required for the elicitation of the pathologic changes in this model. This finding also suggests that a chemical that causes a local increase in Th2 cytokine expression within the airways, as in the studies described in Chapters 4 and 5 of this dissertation, may have allergenic potential in the respiratory tract. The requirement of Th2 cytokines in the allergic airway response in this model may have alternatively been tested by treatment with antibodies against specific Th2 cytokines or using mice of the same strain that had one of their Th2 cytokine genes such as IL-4 'knocked out.' The rationale for using CBN was that its therapeutic/prophylactic potential was also tested.

In the IN CBN study, the expression of the cytokines IL-4, IL-5, IL-10, IL-13 and IFN-γ mRNA was assessed. Mice sensitized and challenged with ovalbumin in the IN CBN study had significant increases in the expression of the Th2 cytokines IL-4, IL-5, IL-10, and IL-13 but no change in the expression of the Th1 cytokine IFN-γ. IN CBN did not affect the ovalbumin-induced increase in lung-derived IL-4 mRNA expression. IN instillation of CBN did reduce the ovalbumin-induced increase in IL-5 and IL-10, although these differences were not statistically significant. IN CBN significantly reduced the ovalbumin-induced increase in lung-derived IL-13 mRNA expression, which may be more sensitive to CBN-mediated effects. CBN, therefore, had a partial effect on the expression of Th2 cytokines within the lung, but the CBN-induced inhibition of Th2 cytokine expression was less than that resulting from IP CBN administration in this study

and in our previous study where CBN inhibited the ovalbumin-induced increase in all the Th2 cytokines measured. The less pronounced effect on Th2 cytokine expression by IN instillation of CBN may also be due to the ineffectiveness of the vehicle EA/OO in distributing the drug to the lower airways.

IgE antibodies are critical effector molecules in the pathogenesis of allergic airway disease that are largely controlled by the Th2 cytokines IL-4 and IL-13. IgE molecules bind receptors on the surface of mast cells in sensitized individuals and upon re-exposure to the offending allergen bind the allergen triggering release of a number of mediators from the mast cell. Mast cell mediators contribute to the vasodilation, bronchoconstriction and mucus hypersecretion common in allergic airway diseases such as asthma. Total and antigen-specific serum IgE levels were measured in mice sensitized and challenged with ovalbumin and co-treated with IP CBN or IN CBN to determine if CBN influenced the allergen-induced increase in IgE levels. Mice sensitized and challenged with ovalbumin in the IP CBN study had a significant increase in ovalbumin-specific serum IgE but no increase in total serum IgE. IP CBN co-treatment significantly reduced the ovalbumin-induced increase in ovalbumin-specific serum IgE. Mice sensitized and challenged with ovalbumin in the IN CBN study had significant increases in both ovalbumin-specific IgE and total serum IgE. IN CBN significantly reduced the ovalbumin-induced increase in both ovalbumin-specific and total serum IgE. This is consistent with data from our previous study that showed that IP CBN administration attenuated the ovalbumin-induced increases in both ovalbumin-specific and total serum IgE (Jan

et al, 2003). Thus, IN CBN is at least as effective in inhibiting allergen-induced increases in IgE as IP CBN administration. The fact that both routes of CBN administration similarly affected IgE levels and not cytokine levels or mucus production may be related to the fact that serum IgE levels is a systemic response that is in turn more sensitive than other more local aspects of the allergic response.

The present study illustrated that CBN administered IP attenuated the increase in IM, serum IgE, and lung-derived IL-4 mRNA expression elicited after intranasal sensitization and challenge with ovalbumin. In addition, CBN administered via IN instillation with an EA/OO vehicle attenuated the ovalbumin-induced increase in total and antigen-specific serum IgE, the number of inflammatory cells in BALF, and lung-derived IL-13 mRNA expression. Intranasal CBN did not, however, affect the ovalbumin-induced mucus response in the pulmonary airways, or lung-derived IL-4, IL-5, and IL-10 mRNA expression. The failure to influence the ovalbumin-induced airway mucus response and the expression of the other Th2 cytokines by intranasal instillation of CBN may be due to the failure of the CBN to distribute into the lung using an EA/OO vehicle. This suggests that improved distribution of CBN into the lung by intranasal instillation may lead to more significant attenuation of the allergen-induced airway pathology and the Th2 cytokine increase. Nevertheless, the suppression of some features of the ovalbumin-induced allergic airway response by intranasal instillation of CBN supports further investigation of the intranasal delivery of CBN.

CHAPTER 4

PATHOLOGIC AND CYTOKINE RESPONSES IN THE RESPIRATORY TRACT OF A/J MICE AFTER INTRANASAL SENSTIZATION AND CHALLENGE WITH TOLUENE DIISOCYANATE

ABSTRACT

Occupational asthma is the most frequently diagnosed respiratory disease in the workplace and is often linked to low molecular weight (LMW) chemicals such as toluene diisocyanate (TDI) that sensitize the respiratory tract. Occupational asthma is characterized by smooth muscle hypertrophy, mucus hypersecretion, epithelial desquamation and an inflammatory influx consisting of lymphocytes, eosinophils, and neutrophils in the airways. Many experimental models have linked the Th2 cytokine phenotype to LMW chemical-induced occupational asthma. Most murine models of occupational asthma, however, use systemic administration (e.g., dermal) to sensitize mice. The present study was designed to test the hypothesis that intranasal sensitization and challenge to TDI will induce the immunologic and pathologic responses in the lung that are characteristic of LMW chemical-induced occupational asthma. A major obstacle in the intranasal instillation of TDI is the selection of an appropriate vehicle to dissolve the chemical in. TDI was intranasally instilled in a 1:1 ethyl acetate/olive oil vehicle. Only TDI sensitized and challenged mice had an infiltration of lymphocytes and plasma cells in the nasal airway, an increase in lung-derived IL-4, IL-5, and IL-13 mRNA 48 hr after the final TDI instillation, and an increase in total serum IgE 48 h after the final TDI instillation that persisted at that level 2 weeks after final TDI instillation. No pulmonary lesions were found in mice of any group. This study is the first to demonstrate that intranasal administration of an LMW chemical is an effective method of sensitization and results in an up-

regulation of critical mediators of allergic airway disease, IL-4, IL-5, and IL-13 within the lung and serum IgE after challenge.

INTRODUCTION

Toluene diisocyanate (TDI) is a leading cause of occupational asthma and allergic rhinitis (Petsonk, 2002; WHO, 1999). TDI is used to manufacture a variety of different products including polyurethanes and varnishes and is a part of a class of highly reactive LMW chemicals with a molecular weight of less than 1 kDa that sensitize the respiratory tract (Petsonk, 2002). Murine models have been developed to assess the allergenic effects of LMW chemicals. Some murine models of LMW chemical-induced allergic airway disease sensitize the animals by dermal application and successfully induce features characteristic of LMW chemical-induced allergic airway disease after intra-airway challenge. However, inhalation and not dermal exposure, is the most common and natural route of exposure to LMW chemical respiratory allergens in occupational settings. Intranasal instillation is a method of administration that more closely resembles inhalation than dermal exposure. In a previous study, I found that intranasal sensitization and challenge with the protein ovalbumin in a saline vehicle was a simple, inexpensive, and noninvasive method of inducing IgE-mediated allergic airway disease in the upper and lower respiratory tract of the A/J mouse (refer to Chapter 3 of this dissertation, Farraj et al, 2003). It is not clear, however, whether intranasal sensitization and challenge with known LMW chemical respiratory allergens will induce the characteristic features of LMW chemical-induced allergic airway disease.

One of the difficulties associated with the intranasal instillation of LMW chemicals is the selection of a suitable, nontoxic vehicle in which to deliver these

chemicals to the airways. Most LMW chemicals linked to occupational asthma and allergic rhinitis are lipophilic and hydrolyze in aqueous solutions, which precludes the use of aqueous vehicles (Ebino et al, 1999). Also, many organic solvents such as ethyl acetate and acetone are very irritating to the airway mucosa. Ideally, a vehicle should allow complete dissolution of the chemical and should not induce cytotoxicity and/or inflammation in the respiratory tract. In the present study, two vehicles, one consisting of ethyl acetate alone and the second consisting of ethyl acetate and olive oil in a 1:1 combination, were tested for their abilities to dissolve the chemical TDI and to distribute to the lower airways with minimal induction of a cytotoxic/inflammatory response in the airway mucosa. Ethyl acetate has been used in other models of LMW chemical-induced allergic airway disease that used intranasal instillation to challenge dermally-sensitized mice to TDI (Scheerens et al, 1999).

The mechanism(s) of LMW chemical-induced allergic airway disease are currently being elucidated. Th2 cytokines, however, have been implicated in LMW chemical-induced allergic airway disease both in humans and in experimental animal models. For example, the production of IL-4 and IL-5 proteins was increased in bronchial biopsies of patients with TDI-induced asthma (Maestrelli et al, 1997). In addition, the selective enhancement of Th2 cytokines was detected in several murine models of TDI-induced asthma (Herrick et al, 2002; Hayashi et al, 2001; Dearman et al, 1996). The methods by which cytokine measurements are made vary in murine models of LMW chemical-induced allergic airway disease. They include the assessment of changes in

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cytokine expression by measuring the secreted product of stimulated local draining lymph nodes draining the site of dermal application or by analyzing bronchoalveolar lavage fluid (BALF) for protein or mRNA expression. In the present study, the local mRNA expression of Th2 cytokines was assessed in the right lung lobe.

One objective of this study was to determine whether ethyl acetate alone or 1:1 ethyl acetate and olive oil are suitable vehicles for the intranasal instillation of TDI. A second objective of this study was to test the hypothesis that intranasal sensitization and challenge with the known chemical respiratory allergen TDI will induce the characteristic features of LMW chemical-induced allergic airway disease in the nasal and pulmonary airways. The pathologic changes in the nasal and pulmonary airways of mice after intranasal sensitization and challenge with TDI were assessed. In addition, the right lung lobes were assessed for changes in cytokine mRNA expression. This was done to examine the utility of measuring local cytokine gene expression as a biomarker for the respiratory allergenicity of LMW chemicals.

MATERIALS AND METHODS

Animals and Chemical Sensitization and Challenge

A total of 54 male A/J mice (Jackson Laboratories, Bar Harbor, Maine), six weeks of age, were randomly assigned to one of nine experimental groups. Mice were free of pathogens and respiratory disease, and used in accordance with guidelines set forth by the All University Committee on Animal Use and Care at Michigan State University. Animals were housed six per cage in polycarbonate boxes, on Cell-Sorb Plus bedding (A&W Products, Cincinnati, OH) covered with filtered lids, and had free access to water and food. Room lights were set on a 12 hr light/dark cycle beginning at 6:00 am, and temperature and relative humidity were maintained between 21-24°C and 40-55 % humidity, respectively.

Selection of Vehicle: A pilot study was performed to select a vehicle for the purpose of sensitizing and challenging mice with TDI by the intranasal route. Mice were anesthetized with 4% halothane and 96% oxygen and then intranasally instilled with 30 μl of ethyl acetate alone or 1:1 ethyl acetate/olive oil once per day for 5 consecutive days and sacrificed 24 h after the final instillation. Nasal and pulmonary airways were examined histopathologically to determine whether either vehicle caused any irritant effects (cytotoxicity and/or inflammation). Mice were also intranasally instilled with 30 μl of 10 % TDI in either ethyl acetate or 1:1 ethyl acetate/olive oil once per day for 5 consecutive days and sacrificed 24 h after the final instillation to determine if the chemical dissolved in either vehicle would distribute to the lung. The 10 % TDI concentration was adapted from the work of Sugawara et al, 1993 who

administered 10 % TDI by aerosol exposure. A vehicle was selected after examining nasal and pulmonary tissue sections for any histologic change and assessing the BALF for changes in lung cellularity (procedures described below).

Selection of TDI Concentration: A pilot study was also performed to select a concentration of TDI with which to use for the intranasal sensitization and challenge of mice. The 10 % concentration of TDI that was used to determine the distribution of the chemical in the selection of vehicle study caused severe toxicity to the airway epithelium. A second pilot study was performed in which mice were anesthetized with 4% halothane and 96% oxygen and then intranasally instilled with 30 μl of ethyl acetate alone or TDI dissolved in ethyl acetate alone at concentrations of 0.05, 0.1, or 0.5 % once per day for 5 consecutive days and sacrificed 24 h after the final instillation. Nasal and pulmonary airways were examined histopathologically to determine whether either TDI concentration caused any irritant effects. The dose that was least irritating to the airway after 5 intranasal instillations was selected. This was done to distinguish the irritating effects of the chemical from any potential adaptive immune response normally associated with TDI-induced allergic airway disease. A concentration of 0.1 % was selected after examining nasal and pulmonary tissue sections for any histologic change and assessing the BALF for changes in lung cellularity (procedures described below).

Sensitization and challenge with TDI: The vehicle 1:1 ethyl acetate/olive oil was used to dissolve TDI at a concentration of 0.1 %. Mice were anesthetized with 4% halothane and 96% oxygen. The mice were then exposed to toluene

diisocyanate (TDI) (Sigma-Aldrich, St. Louis, MO) at a concentration of 0.1 % that was dissolved in a 1:1 ethyl acetate to olive oil vehicle. The animals were treated by intranasal instillations consisting of a volume of 30 μl. Figure 4.1 depicts the exposure regimen utilized for intranasal sensitization and challenge of mice with TDI. The mice were sensitized by a single intranasal instillation on Days 1 and 3. This sensitization regimen differed slightly from that used in the ovalbumin studies described in Chapters 2 and 3 of this dissertation in terms of the number of instillations. In the ovalbumin studies, five instillations were used to sensitize the mice; in the present study only two instillations given one day apart were used to sensitize the mice to TDI. This was because even with the dose of 0.1 %, two animals died after just two consecutive one-day instillations. The mice were then challenged with a single intranasal instillation on Days 17 and 27. There were 5 different treatment groups; 1) an untreated naïve group; 2) mice sensitized and challenged with the vehicle alone; 3) mice sensitized with the vehicle alone and then challenged the first time with the vehicle and then the second time ten days later with TDI in vehicle; 4) mice sensitized with TDI in vehicle and then challenged with the vehicle alone; and 5) mice sensitized and challenged with TDI in vehicle. Mice were sacrificed 48 h or 2 wk (Day 29 or Day 41) after the final instillation. One set of animals was sacrificed 48 hr after the final challenge because in a previous study (refer to Chapters 2 and 3, Farraj et al, 2003), we determined that in this period of time after challenge there was a robust cytokine and morphologic response to ovalbumin. The second set was sacrificed 2 weeks after the final challenge to histopathologically assess the

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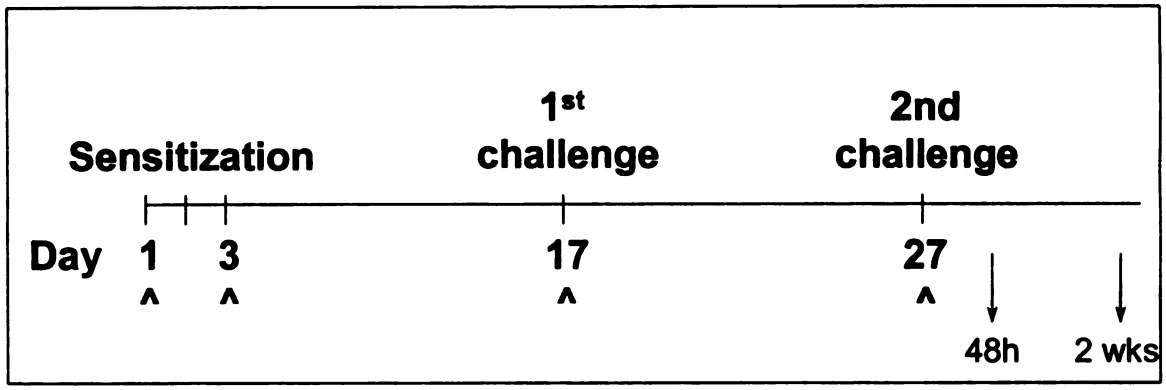

Figure 4.1: Timeline of the exposure regimen used to sensitize and challenge A/J mice with 0.1 % TDI in 1:1 ethyl acetate/olive oil vehicle. Mice were sensitized on days 1 and 3 with intranasal instillations of 30 μl of TDI in 1:1 ethyl acetate/olive oil vehicle or vehicle alone. Mice were then challenged two weeks later on day 17 with a similar volume of TDI in vehicle and challenged a second time 10 days later on day 27 followed by sacrifice 48 hr or 2 weeks after double challenge. ^ = 30 μl of TDI in 1:1 ethyl acetate/olive oil vehicle or vehicle alone. ↓ = time after challenge when animals were sacrificed.

longer-term remodeling that takes place in the nasal airway in response to the irritant and/or adaptive immune effects of TDI.

Necropsy, Blood Sample Collection and Tissue Preparation

Before sacrifice, mice were deeply anesthetized via intraperitoneal injection of 0.1 ml of 12% pentobarbital in saline. Blood samples (0.1 to 0.5 ml) were taken from the abdominal aorta and collected in Beckton Dickinson vacutainers. Serum samples were collected after the blood samples were centrifuged to remove cells and the supernatants were frozen and stored at -20°C. The abdominal aorta and renal artery were then severed to exsanguinate the animals. Immediately after death, the trachea was cannulated and the heart/lung block removed. A syringe was then attached to the cannula and BALF was collected from the whole lung via two sequential changes of 1 ml saline. Differential cell counts were determined by cytocentrifuging cells onto glass slides and then staining with Diff-Quik (DADE Behring, Newark, DE). Neutrophils, macrophages, eosinophils, lymphocytes other cell types were microscopically identified and their numbers were determined by multiplying the percentage of each cell type from a total of 200 cells by the total number of cells per ml of lavage fluid. The right bronchus was clamped using suture thread. All four right lung lobes were placed in 2 ml Tri reagent (Molecular Research Center, Cincinnati, OH), homogenized and stored at -80°C.

After removal of the right lung lobes, the left lung lobe was intratracheally perfused with 10% neutral buffered formalin at a constant pressure of 30 cm of

fixative. After 1 hr, the trachea was ligated, and the inflated left lung lobe was immersed in a large volume of the same fixative for 24 hr. After fixation, the left lung lobe was microdissected along the axial airways, and two sections were then excised at the level of the fifth (proximal airway) and eleventh (distal airway) airway generation (Figure 4.2), as has been described previously in detail (Steiger et al, 1995). Tissue sections were embedded in paraffin, at a thickness of 5 microns, and stained with hematoxylin and eosin for histopathologic assessment or with Alcian blue (pH 2.5) and periodic acid Schiff's (AB/PAS) reagent, which stains neutral and acidic mucosubstances.

The head of each mouse was removed from the carcass, and the lower jaw and skin were removed. The heads were then immersed in 10% neutral buffered formalin for 24 hr. After fixation, the heads were decalcified in 13% formic acid for 7 days and then rinsed in tap water for at least 4 hr. The nasal cavity of each mouse was transversely sectioned at three specific anatomic locations according to a modified method of Young (1981) and processed for light microscopy and image analysis. The most proximal nasal section was taken immediately posterior to the upper incisor teeth (proximal); the middle section was taken at the level of the incisive papilla of the hard palate (middle); the most distal nasal section was taken at the level of the second palatal ridge (distal) (Figure 4.2). Tissue sections were embedded in paraffin, at a thickness of 5 microns, and stained with hematoxylin and eosin for histopathologic assessment. Paraffin sections were also stained with AB/PAS to identify intraepithelial mucosubstances (IM).

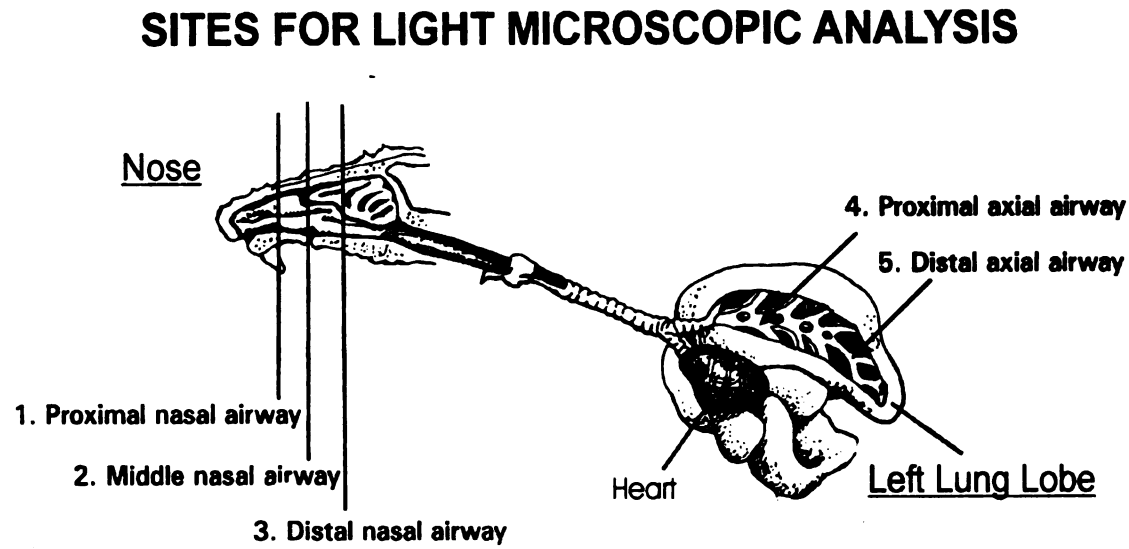

Figure 4.2: Sites of airway tissue selection for morphometric analysis. The cartoon depicts the lateral wall of one nasal passage with the septum removed of the murine respiratory tract and the main axial airway of the left lung lobe. Transverse sections of the nasal cavity were taken 1) immediately posterior to the upper incisor teeth (T1), 2) at the level of the incisive papilla of the hard palate (T2), and 3) at the level of the second palatal ridge (T3). The left lung lobe was microdissected along the axial airways, and two sections were then excised at the level of the fifth (proximal airway) and eleventh (distal airway) airway generation.

ELISA for Total serum IgE

Total serum IgE was measured using a 96-well Immulon ELISA plate (Dynex, Technologies, Chantilly, Virginia) coated with 2 □g/ml anti-mouse IgE (Purified Rat Anti-Mouse IgE Monoclonal Ab, Pharmingen, San Diego, CA) and incubated overnight at 4°C. After washing, the plates were incubated in 3 % Bovine Serum Albumin (3 % BSA) at 37°C for 1 hr. (BSA, CALBIOCHEM, La Jolla, CA). Serum samples at 1:10 dilution were then added followed by incubation at 37°C for 1 hr. After washing, biotinylated anti-mouse IgE (Biotin-conjugated Rat Anti-Mouse IgE Monoclonal Ab, Pharmingen, San Diego, CA) was then added at 2 µg/ml and allowed to incubate at 25°C for 1 hr. After washing, 1.5 µg/ml of streptavidin peroxidase was added followed by incubation at 25°C for 1 hr. After washing, TMB substrate (12.5ml citric-phosphate buffer + 200 µl of TMB stock solution (6mg/ml in DMSO)) was added to produce a color reaction. The reaction was terminated by the addition of 6 N H_2SO_4. Optical density was determined at 450 nm using an EL-808 microplate reader (Bio-Tek instruments, Winooski, Vermont).

Real-time RT-PCR

Total RNA was isolated from the right lung lobes using the TRI REAGENT method (Molecular Research Center, Cincinnati, OH) following the manufacturer's protocol. The evaluation of the relative expression levels of the cytokines IL-4, IL-5, IL-10, IL-13, and IFN-γ mRNA was determined using the TaqMan one-step real-time multiplex RT-PCR with TaqMan pre-developed

primers and probe using the manufacturer's recommended protocol (Applied Biosystems, Foster City, CA). Briefly, aliquots of isolated tissue RNA (100 ng total RNA) were added to the RT-PCR reaction mixture, which included the target gene (IL-4, IL-5, IL-10, IL-13, or IFN-γ) primers and probe, endogenous reference primers and probe (18S ribosomal RNA), AmpliTaq DNA polymerase and Multiscribe reverse transcriptase (MuLV). The probes are designed to exclude detection of genomic DNA. RNA samples were first reverse transcribed and then immediately amplified by PCR. Following the PCR, amplification plots (change in dye fluorescence versus cycle number) were examined and a dye fluorescence threshold within the exponential phase of the reaction was set separately for the target gene and the endogenous reference (18S). The cycle number at which each amplified product crosses the set threshold represents the C_T value. The amount of target gene normalized to its endogenous reference was calculated by subtracting the endogenous reference C_T from the target gene C_T (ΔC_T). Relative mRNA expression was calculated by subtracting the mean ΔC_T of the control samples from the ΔC_T of the treated samples ($\Delta\Delta C_T$). The amount of target mRNA, normalized to the endogenous reference and relative to the calibrator (i.e., RNA from control) is calculated by using the formula $2^{-\Delta\Delta C}{}_T$.

Statistics

The data obtained from each experimental group were expressed as a mean group value ± the standard error of the mean (SEM). The differences among groups were determined by one way or two-way analysis of variance

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(ANOVA) and an All Pairwise Post-hoc Comparison Test (Tukey), using SigmaStat software from Jandel Scientific Co. (San Rafael, CA).

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RESULTS

Short-term Pilot Studies

Airway pathology and BALF data

A pilot study was performed to assess the irritant effects of ethyl acetate and 1:1 ethyl acetate/olive oil and their abilities to distribute TDI into the lungs after intranasal instillation. Ethyl acetate was irritating to the nasal airway mucosa indicated by mild epithelial necrosis accompanied by a mild neutrophilic infiltrate (Figure 4.3). Ethyl acetate when combined with olive oil was less irritating to the nasal airway epithelium than ethyl acetate alone (Figure 4.3). The intranasal instillation of 10 % TDI in ethyl acetate and or 1:1 ethyl acetate/olive oil resulted in similar increases in neutrophils and macrophages in the BALF relative to either vehicle alone indicating that intranasal instillation of TDI with either vehicle resulted in distribution of TDI into the lung (Figure 4.4). TDI at a concentration of 10 % in either vehicle caused an acute to severe necrotizing rhinitis characterized by tremendous neutrophilic infiltration and light pink proteinacious exudates that almost entirely occluded the nasal passages and loss of turbinate bone (Figure 4.5). Additional evidence that TDI distributed into the lung was the presence of necrosis and sloughing of the airway epithelium that lined the main axial airway of the left lung lobe accompanied by neutrophilic infiltration (Figure 4.6).

A second pilot study was performed to select a concentration of TDI that was minimally irritating to the airway mucosa. A much lower range of

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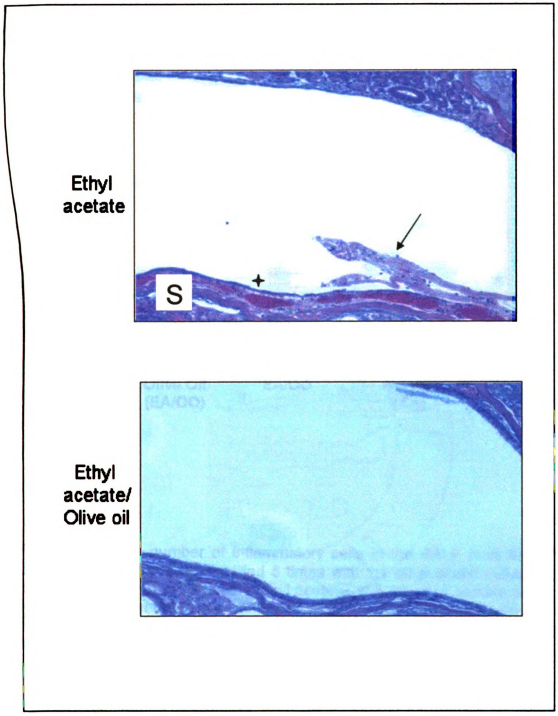

Figure 4.3: Light photomicrographs of the proximal septum from the proximal nasal airways of mice intranasally instilled with ethyl acetate or 1:1 ethyl acetate and olive oil. Tissues stained with hematoxylin and eosin. Arrow = sloughed off epithelial cells and neutrophils. Star = basal lamina with epithelium stripped off.

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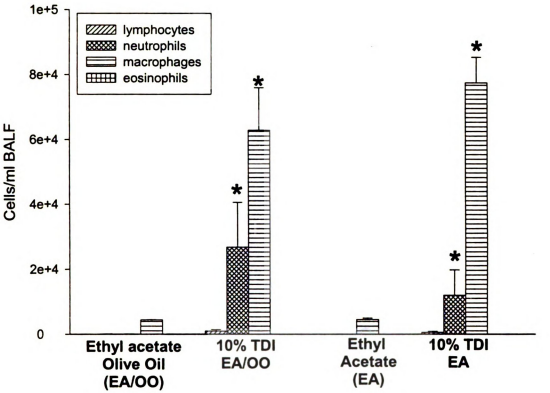

Figure 4.4: Total number of inflammatory cells in the BALF from the left lung lobes of mice intranasally instilled 5 times with 1:1 ethyl acetate/olive oil, 10 % toluene diisocyanate (TDI) in 1:1 ethyl acetate/olive oil, ethyl acetate alone, or 10 % TDI in ethyl acetate alone and sacrificed 24 hr after the final instillation. Bars represent mean number of inflammatory cells per ml BALF ± standard error of the mean (n = 5/group).

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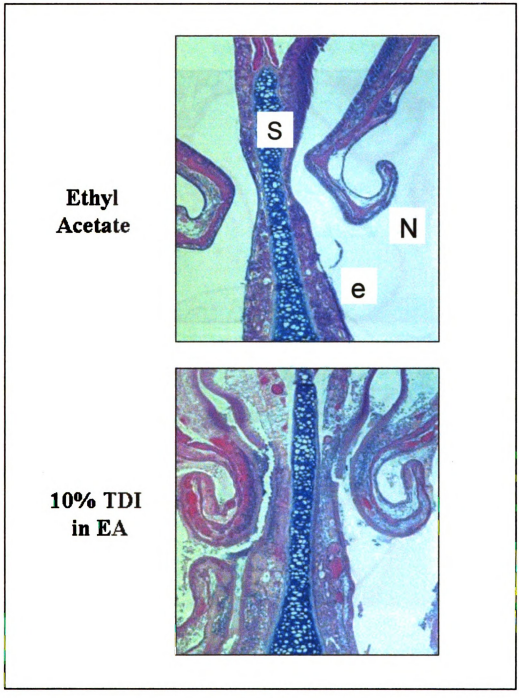

Figure 4.5: Light photomicrographs of the proximal nasal airway of mice intranasally instilled with ethyl acetate alone or 10 % toluene diisocyanate (TDI) in ethyl acetate. Tissues stained with hematoxylin and eosin. S = nasal septum, N = nasoturbinate, e = surface epithelium.

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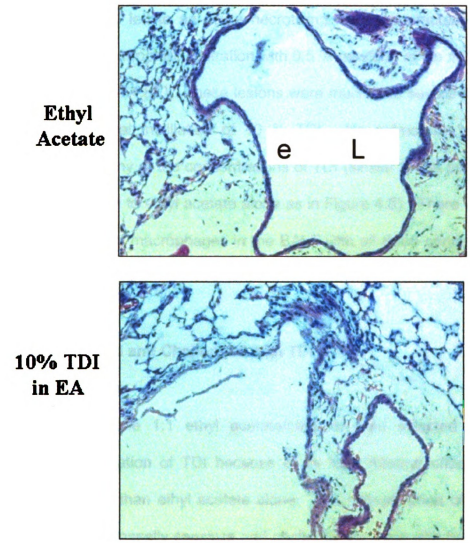

Figure 4.6: Light photomicrographs of the main axial airway of the left lung lobe of mice intranasally instilled with ethyl acetate alone or 10 % toluene diisocyanate (TDI) in ethyl acetate. Tissues stained with hematoxylin and eosin. e = airway epithelium, L = airway lumen.

concentrations of TDI were selected in order to minimize toxicity to the airway mucosa caused by 10% TDI. Intranasal instillation of 0.05, 0.1 and 0.5 % TDI in ethyl acetate all led to an acute necrotizing rhinitis characterized by epithelial necrosis and neutrophilic infiltration with 0.5 % resulting in the most severe lesion (similar to Figure 4.8E). These lesions were much less severe than that caused by the intranasal instillation of 10 % TDI. No pulmonary lesions resulted, however, with any of the concentrations of TDI (similar to the pulmonary airways of mice exposed to ethyl acetate alone as in Figure 4.6). There was an increase in the number of macrophages in the BALF with all three concentrations of TDI (Figure 4.7).

Mice Sensitized and Challenged with TDI

Airway Pathology

The vehicle 1:1 ethyl acetate/olive oil was selected for use in the intranasal instillation of TDI because of its less irritating effects on the nasal airway mucosa than ethyl acetate alone. The concentration of 0.1 % TDI was selected to intranasally sensitize and challenge the mice with because of its less toxic effects on the nasal airway mucosa than 0.5 %.

Mice that were intranasally instilled with TDI in vehicle had an acute, necrotizing rhinitis at 48 hr post instillation. At 48 hr post-instillation, there was extensive necrosis of the transitional and respiratory mucosa lining the medial and lateral meatus in the proximal nasal passages (Figure 4.8). Though the airway injury was bilateral, often the nasal passage was more severely affected.

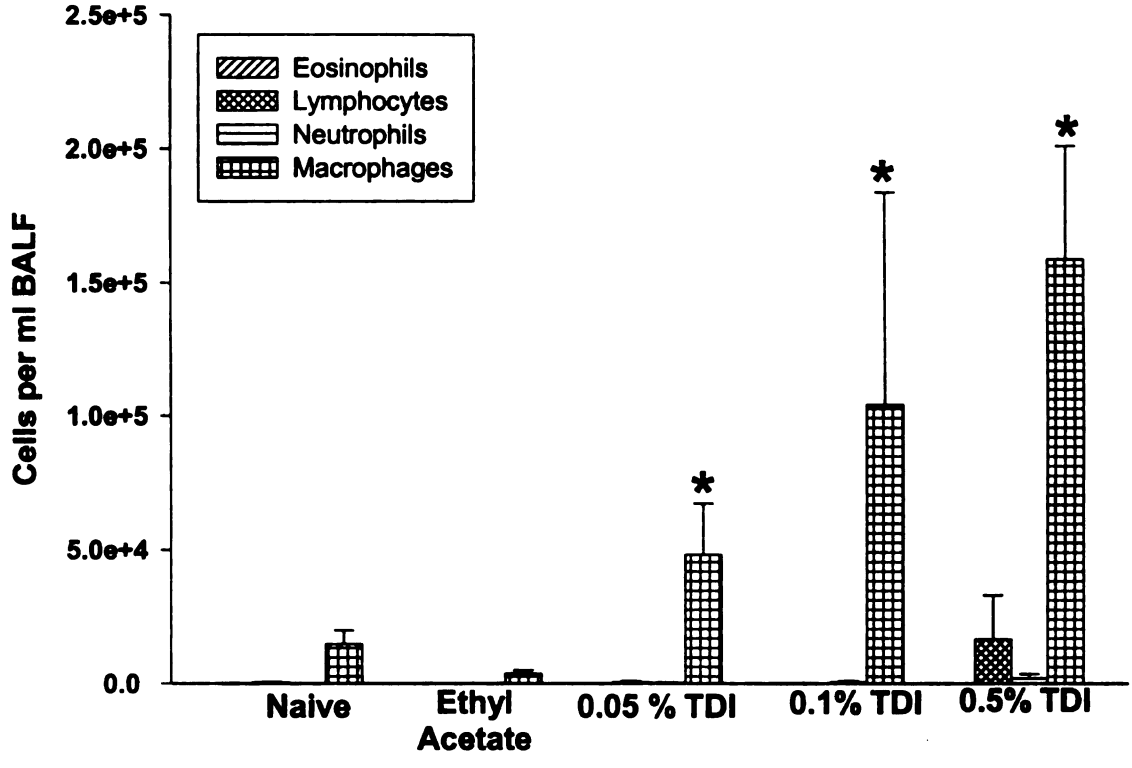

Figure 4.7: Total number of inflammatory cells in the BALF from the left lung lobes of mice intranasally instilled 5 times with ethyl acetate/olive oil, 10 % toluene diisocyanate (TDI) in ethyl acetate/olive oil, ethyl acetate alone, or 10 % TDI in ethyl acetate alone and sacrificed 24 hr after the final instillation. Bars represent mean number of inflammatory cells per ml BALF ± standard error of the mean (n = 5/group). *-significantly greater than naïve group ($p<0.05$).

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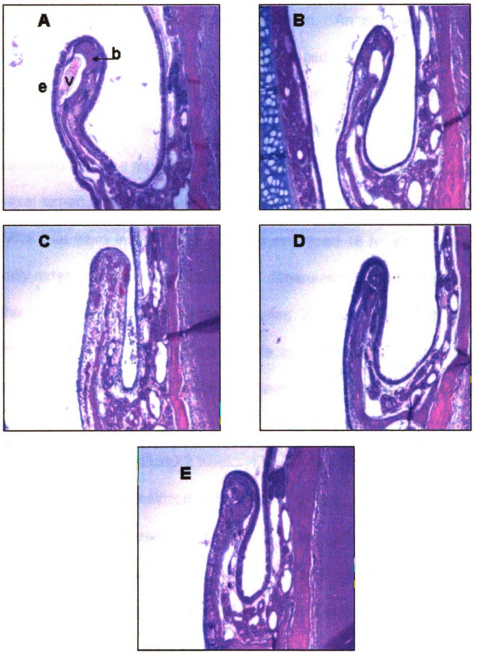


Figure 4.8: Light photomicrographs of the maxilloturbinates from the proximal nasal airways of naïve mice (A), mice sensitized and challenged with 1:1 ethyl acetate/olive oil vehicle (B), mice sensitized with 0.1 % TDI in vehicle and challenged with vehicle (C), mice that were sensitized with vehicle and challenged the first time with vehicle and the second time with 0.1 % TDI (D), and mice sensitized and challenged with 0.1 % TDI (E). Tissues stained with hematoxylin & eosin. e = surface epithelium; b = turbinate bone; v = blood vessel in the lamina propria of the nasal mucosa.

Surface epithelium lining the mid-septum, maxilloturbinates and lateral wall were most severely affected with extensive exfoliation. An attenuated regenerative cuboidal epithelium (one or two cells thick) replaced some of the affected airway surfaces. Degeneration and necrosis of the underlying lamina propria was present in some areas (full thickness necrosis). A marked influx of neutrophils was associated with the mucosal injury. Aggregates of neutrophils were present in the nasal lumen and throughout the nasal mucosa in the affected regions.

Mice that were instilled with TDI and sacrificed 48 hr after instillation also had locally extensive areas of necrosis and degeneration of the olfactory mucosa lining the nasal septum and ethmoturbinates in the middle and distal nasal passages. Unlike the TDI-induced lesions in the transitional and respiratory mucosa, no inflammatory cell influx was associated with these lesions in the olfactory mucosa.

Mice that were sacrificed 2 weeks after the last TDI instillation irrespective of whether they were sensitized with TDI or vehicle had a mild to moderate rhinitis with minimal mucosal degeneration. The surface epithelium lining the middle and lateral meatus was a regenerative stratified cuboidal epithelium with few ciliated cells. The epithelium covering some of these areas was moderately to markedly hyperplastic.

In addition, conspicuous changes were found in the ethmoturbinates of mice sacrificed 2 weeks after the last TDI instillation irrespective of whether they were sensitized with TDI or vehicle. There was a marked remodeling of ethmoturbinates characterized by loss and fusion of turbinates, proliferation of

the turbinate bone, interstitial fibrosis of mucosal tissue, loss of subepithelial glands and nerve bundles, and marked respiratory metaplasia (i.e., replacement of normal olfactory epithelium by ciliated respiratory epithelium). Often, large accumulations of PAS-positive mucoid partially filled the luminal airspaces in the affected ethmoid region of the nose.

Sensitization and challenge with TDI did not cause a marked increase of lymphoid cells or eosinophils in the nasal mucosa. However, there were small aggregates of plasma cells and Russel bodies in the areas of the lamina propria, in the areas of ethmoturbinate 48 h after the final TDI instillation (data not shown).

Interestingly, no conspicuous pulmonary changes suggestive of airway injury or an allergic immune response were microscopically detected in the lungs of TDI-treated mice (similar to the pulmonary airways in mice instilled with ethyl acetate alone as in Figure 4.6).

BALF

The total number of cells in the BALF was determined at 48 hr and 2 weeks after the second challenge. There were no statistically significant differences in the total number of cells in the lavage fluid in any of the treatment groups relative to the untreated naïve control at either 48 hr or 2 weeks after the second challenge (levels similar to the levels in the lungs of the naïve mice depicted in Figure 4.7). The predominant cell type in all of the groups including the untreated naïve group was the macrophage.

Total Serum IgE

Mice sensitized and then challenged twice with TDI had a significant increase in serum IgE that was approximately 6-fold the level of the naïve control mice at 48 hr after the final challenge and persisted at that level at least until 2 weeks after the final challenge (Figure 4.9). Mice in all of the control groups exhibited comparable levels of total serum IgE that were not statistically different from the naïve untreated mice.

Cytokine Gene Expression

Mice intranasally sensitized and challenged with TDI had significant increases in the lung-derived mRNA expression of IL-4, IL-5, and IL-13 48 h after the final TDI instillation relative to the naive controls. TDI sensitized and challenged mice had a 6.7-fold increase in IL-4 mRNA expression, a 5.9-fold increase in IL-5 mRNA expression, and an 11.9-fold increase in IL-13 expression compared to the untreated naïve group (Figure 4.10). TDI sensitized and challenged mice also had a 15.3-fold increase in IL-10 mRNA expression within the lung but this was not deemed statistically significant. There was no increase in the mRNA expression of the Th1 cytokine IFN-γ in mice sensitized and challenged with TDI. There were also no significant increases in the mRNA expression of any of the Th2 cytokines or IFN-γ in any of the other treatment groups relative to the untreated naïve group. No significant changes in the expression of any of the Th2 cytokines or IFN-γ were detected in the lungs of any mice 2 weeks after the second challenge (Table 4.1).

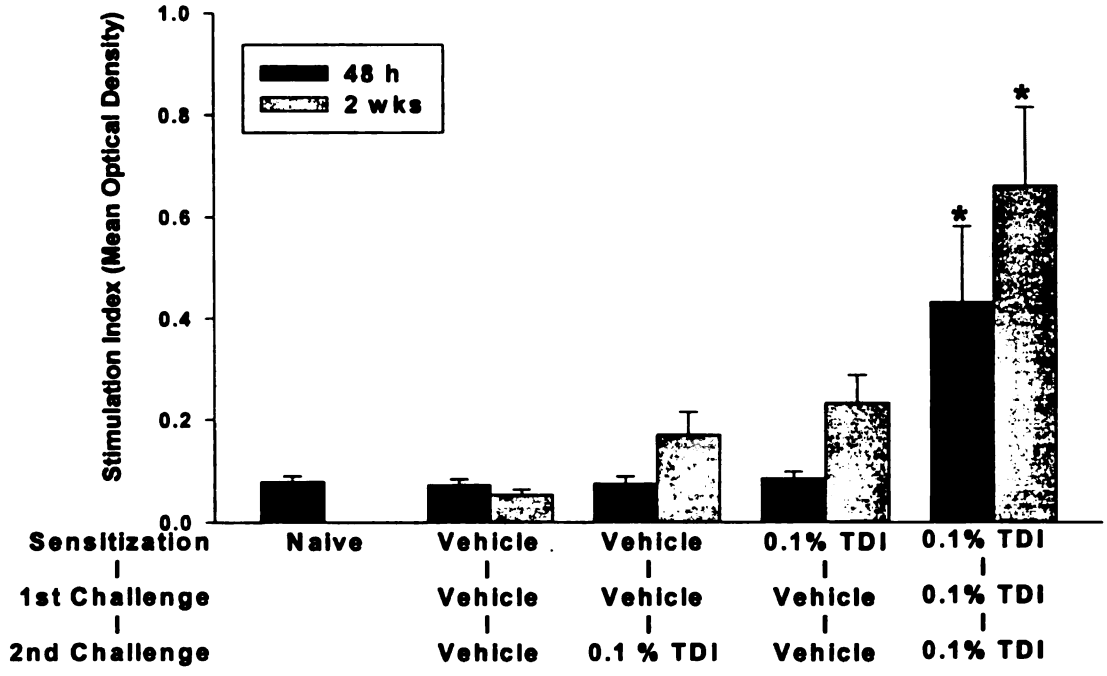


Figure 4.9: Total IgE levels in mouse serum isolated 48 hr or 2 weeks after double challenge with 0.1 % TDI. Mice were sensitized with intranasal instillations of 30 μl of 0.1 % TDI in 1:1 ethyl acetate/olive oil vehicle. Mice were then challenged two weeks later with a similar volume and challenged a second time 10 days later followed by sacrifice 48 hr or 2 weeks after double challenge. Bars represent average optical density ± standard error of the mean (n = 6/group). * = significantly greater than all other groups sacrificed at same time after challenge.

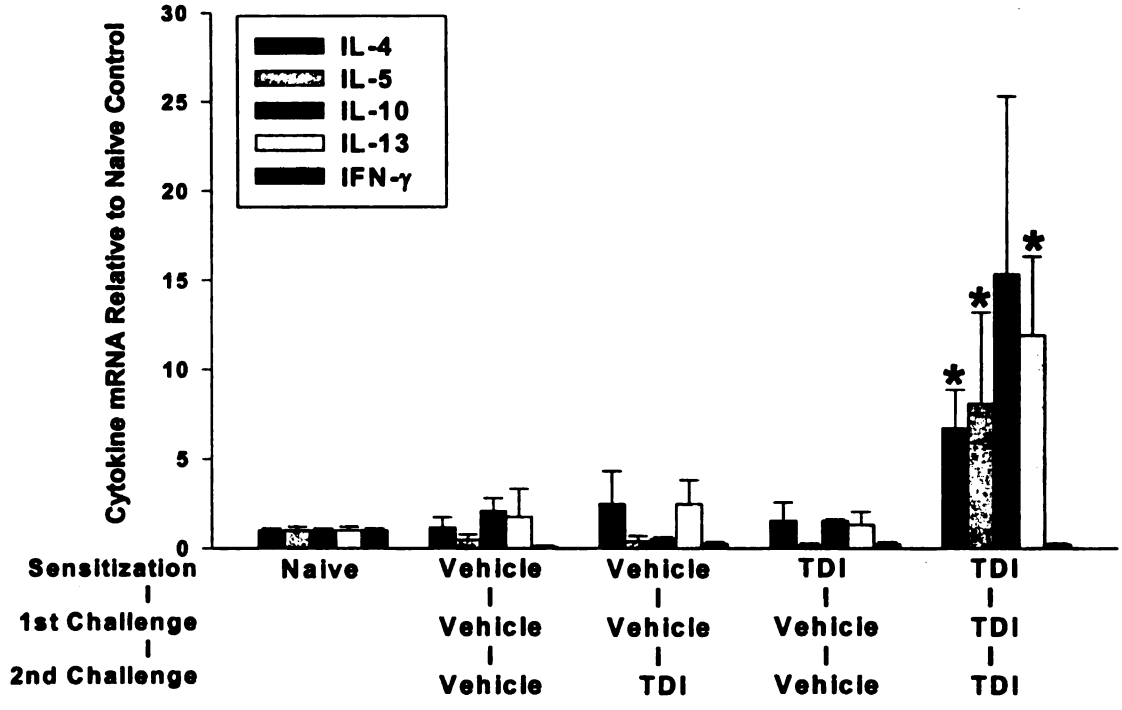


Figure 4.10: Relative quantification of IL-4, IL-5, IL-10, IL-13, and IFN-γ mRNA in right lung lobes 48 hr after double challenge with 0.1 % TDI. Mice were sensitized with intranasal instillations of 30 μl of 0.1 % TDI in 1:1 ethyl acetate/olive oil vehicle. Mice were then challenged two weeks later with a similar volume and challenged a second time 10 days later followed by sacrifice 48 hr after double challenge. Bars represent cytokine mRNA relative to the naïve control ± standard error of the mean (n = 6/group). * = significantly greater than all other groups at similar time-point ($p<0.05$).

Table 4.1: Summary of Th2 and Th1 cytokine mRNA expression changes within the right lung lobes after TDI sensitization and challenge.

Cytokine	Change 48 h post TDI sensitization/challenge	Change 2 wk post TDI sensitization/challenge
IL-4	Significant Increase	No significant change
IL-5	Significant Increase	No significant change
IL-10	Increase not significant	No significant change
IL-13	Significant Increase	No significant change
IFN-γ	No significant change	No significant change

DISCUSSION

One objective of this study was to determine whether or not ethyl acetate alone or 1:1 ethyl acetate and olive oil are suitable vehicles for the intranasal instillation of the known chemical respiratory allergen, TDI. A second objective was to test the hypothesis that intranasal sensitization and challenge with TDI will induce the characteristic features of LMW chemical-induced allergic airway disease in the nasal and pulmonary airways. Morphologic changes in the nasal and pulmonary airways were assessed in addition to changes in cytokine gene expression in the lung after TDI treatment to determine if local cytokine gene expression may be used as a biomarker for the respiratory allergenicity of selected LMW chemicals.

One major obstacle in the intranasal instillation of LMW chemicals has been the selection of an appropriate vehicle that is not irritating to the airway epithelium and that allows distribution of the chemical into the lower airways. In the present study, the vehicles ethyl acetate and 1:1 ethyl acetate/olive oil were compared to determine which vehicle was most suitable for use in the intranasal sensitization and challenge of mice with TDI. The vehicle ethyl acetate alone when intranasally instilled caused more stripping of the airway epithelium and neutrophilic inflammation in the nasal airways than the vehicle 1:1 ethyl acetate/olive olive. The addition of olive oil to ethyl acetate limited the irritant effects of ethyl acetate. Ethyl acetate has been documented as an irritant to the eyes and the mucous membrane of the respiratory passages of humans (Mackison et al, 1981). Ethyl acetate has also been shown to cause toxicity in

the nasal airways of Sprague Dawley rats with the lesions being most severe in the olfactory epithelium (Hardisty et al, 1999). In addition, both vehicles appeared equally capable of distributing 10 % TDI into the lungs after intranasal instillation indicated by the induction of similar increases in inflammatory cells in BALF. Ethyl acetate/olive oil in a 1:1 combination was thus selected as a vehicle to dissolve TDI in this study because of the less irritating effects on the airway epithelium. A concentration of 0.1 % TDI was selected to sensitize and challenge the mice because it caused less airway irritation than 0.5 %.

Individuals afflicted with occupational asthma often suffer from concurrent allergic rhinitis. Some studies suggest that allergic rhinitis due to workplace exposure to LMW chemicals usually coexists with occupational asthma (Leynaert et al, 2002). The pathologic features of TDI-induced allergic rhinitis are similar to non-occupational allergic rhinitis and include plasma exudation, hyper-secretion of mucus, and cellular infiltrates consisting of T- and B-lymphocytes, eosinophils, and plasma cells in the nasal airway (WHO, 1999). The nasal airways of mice intranasally sensitized and challenged to TDI were examined histologically to determine if the airway response was similar to TDI-induced allergic rhinitis in humans. Both TDI-sensitized and non-sensitized mice had an acute rhinitis, which indicates that they were caused by the irritating effects of the chemical and were not immune-mediated. However, only mice that were intranasally sensitized and challenged with TDI had small aggregates of plasma cells and Russel bodies in the lamina propria of the ethmoturbinate 48 h after the final TDI instillation, indicative of an immune-mediated effect.

The Th2 subtype of the $CD4^+$ T lymphocyte produces a unique profile of cytokines that has been linked to the pathogenesis of LMW chemical-induced allergic airway disease (Maestrelli et al, 1997; Herrick et al, 2002; Hayashi et al, 2001; Dearman et al, 1996). Th2 cells produce IL-4, IL-5, IL-10, and IL-13 (Yssel and Groux, 2000). IL-4 promotes T cell activation and differentiation into the Th2 subtype while both IL-4 and IL-13 promote IgE production in B cells and mucus production in the airways (Shim et al, 2001; Frew, 1996). IL-5 is a chemotactic factor for eosinophils (Frew, 1996). Various methodologies for cytokine analysis are used in murine models of LMW chemical-induced allergic airway disease including the assessment of changes in BALF-derived cytokine content and the analysis of local draining lymph node cells that have been re-stimulated with a mitogen *in vitro*. In the present study, I assessed Th2 or Th1 cytokine mRNA expression in RNA isolated from the lungs of mice intranasally instilled with TDI. Only TDI sensitized and challenged mice had significant increases in lung-derived IL-4, IL-5, and IL-13 mRNA expression 48 h after final TDI instillation. There was no significant change in the expression of the Th1 cytokine IFN-γ in any of the treatment groups. These findings support the possibility that LMW chemicals linked to occupational asthma selectively express Th2 cytokines and that Th2 cytokine expression within the lung may be used to identify chemical respiratory allergens.

The role of IgE antibodies in the pathogenesis of LMW chemical-induced occupational asthma and allergic rhinitis is unclear and controversial. Only 10 to 30 % of all cases of TDI-induced occupational asthma, for example, exhibit

increases in IgE antibodies (Weissman and Lewis, 2000). Investigators have suggested that the failure to establish a consistent link between high circulating IgE levels and LMW chemical-induced allergic airway disease is due to the deficiencies in IgE detection methods (Kimber et al, 2002) while others have demonstrated that non-IgE-dependent mechanisms of LMW chemical-induced allergic airway disease may exist (Herrick et al, 2002; Larsen et al, 2001; Bernstein, 2002). Nevertheless, we found that mice intranasally sensitized and challenged with TDI exhibited increases in total serum IgE suggesting that IgE may play a role in TDI-induced allergic airway disease.

In the present study, no pulmonary lesions were found in any group instilled with TDI. The absence of pathologic changes, whether irritant-induced or immune-mediated in the pulmonary airways, suggests that there may have been poor distribution of the chemical into the lung or that the airway mucosa of the pulmonary airways is less sensitive to the effects of TDI. The viscosity of ethyl acetate and olive oil as a vehicle may have prevented effective aspiration of the chemical and distribution of the chemical into the lower airways. The distribution of ethyl acetate and 1:1 ethyl acetate/olive oil into the lower airways was also determined after the intranasal instillation of the dye Sudan black in either vehicle and it was found that most of the solution distributed to nasal airways and gut. In addition, the inherent high reactivity of the chemical may have played a role in limiting the distribution of the chemical to the lower airways. A majority of the chemical administered via intranasal instillation may have reacted with proteins in the upper airways of these mice thus serving to limit

distribution of the chemical into the lower airways. Greenberg et al, 1994, reported that after aerosolized toluene diisocyanate exposure in guinea pigs, the vast majority of the chemical remained in the nasal airway.

The source of the TDI-induced increase in lung-derived Th2 cytokine mRNA expression is unknown given the absence of pulmonary airway inflammation with an associated lymphocyte infiltrate in the regions of the lung examined. Several possibilities exist that may explain this increase. One is that the increase originated from infiltrating lymphocytes in areas of the lung not examined histologically such as the right lung lobes or more proximal regions of the left lung lobe. Another possibility is that the source of the cytokines was the bronchial-associated lymphoid tissue or the airway epithelium. Or perhaps a systemic immune response was elicited that originated in some other region of the respiratory tract that caused an increase in lymphocytes in the circulation and likewise the pulmonary vasculature. Further studies are required to determine the exact source.

The vehicle used in this study, 1:1 ethyl acetate/olive oil, although less irritating to the nasal airway epithelium than ethyl acetate alone, did irritate the airway epithelium. The vehicle, in some instances, caused stripping of the nasal airway epithelium, which was accompanied by a mild neutrophilic infiltrate. The toxicity of the vehicle made it difficult, in some cases, to distinguish between vehicle-induced effects from TDI-induced effects. In addition, a single intranasal instillation of TDI at a concentration of 0.1 % elicited substantial toxicity to the nasal airway that was not immune-mediated. The severity of the toxic response

made it difficult to distinguish irritant-induced effects from immune-mediated effects of TDI. Most cases of LMW chemical-induced asthma result from immune-mediated mechanisms (Beach et al, 2000). The use of lower concentrations of the chemical will limit the irritant/toxic effects of the chemical and facilitate the discernment of the immune-mediated effects.

The present study illustrated that the intranasal instillation of TDI resulted in some of the characteristic pathologic and immunologic features of TDI-induced allergic airway disease. TDI sensitization and challenge caused lymphocyte and plasma cell infiltration in the nasal airway, an increase in lung-derived Th2 cytokine mRNA expression, and an increase in serum IgE suggesting that the analysis of local cytokine gene expression in the respiratory tract after intranasal exposure may prove useful in the identification of other allergenic chemicals. However, the vehicle-induced airway toxicity, the likely insufficient distribution of the chemical into the lower airways after intranasal instillation, and the irritant effects of TDI on the airway epithelium are all major obstacles that must be overcome before this approach can be used to evaluate the allergenic potential of LMW chemicals in the respiratory tract.

CHAPTER 5

PATHOLOGIC AND CYTOKINE RESPONSES WITHIN THE NASAL AND PULMONARY AIRWAYS OF A/J MICE AFTER INTRANASAL SENSITIZATION AND CHALLENGE WITH TRIMELLITIC ANHYDRIDE, DINITROCHLOROBENZENE, OR OXAZOLONE

ABSTRACT

Sensitization of the respiratory tract to low molecular weight (LMW) chemicals including trimellitic anhydride (TMA) is a leading cause of occupational asthma and allergic rhinitis in industrial settings. Mucus hypersecretion and airway inflammation consisting of lymphocytes and eosinophils are pathologic features of allergic airway diseases. Many experimental models have linked LMW chemical-induced allergic airway disease to Th2 cytokine expression. Most murine models, however, use systemic administration (e.g., dermal) to sensitize mice. The present study tests the hypothesis that intranasal sensitization and challenge with TMA will induce the immunologic and pathologic responses characteristic of LMW chemical-induced allergic airway disease in the nasal and pulmonary airways. A/J mice were intranasally sensitized and then intranasally challenged twice with TMA in a 1:4 ethyl acetate/olive oil vehicle or vehicle alone. The response to TMA was compared to the responses after intranasal sensitization and challenge to the contact sensitizers, dinitrochlorobenzene (DNCB) and oxazolone (OXA), chemicals not known to elicit occupational asthma or allergic rhinitis. Only mice that were intranasally sensitized and challenged with TMA had a marked allergic rhinitis characterized by an influx of eosinophils, lymphocytes and plasma cells, 24 hr after the final challenge. By 96 hr, the nasal airway epithelium exhibited increases in stored mucus and a regenerative hyperplasia. No nasal lesions were found in the airways of mice sensitized and challenged to DNCB or OXA. TMA sensitized and challenged mice exhibited an increase in lung-derived IL-5 mRNA expression. There were

no changes in the mRNA expression of any of the cytokines measured in the lungs of DNCB or OXA treated mice. TMA sensitized and challenged mice exhibited an increase in total serum IgE. However, mice sensitized and challenged to DNCB also exhibited an increase in total serum IgE, whereas OXA treated mice did not. No pulmonary lesions were found in mice treated with TMA, DNCB, or OXA. A subsequent assessment of cytokine mRNA expression in the nasal airway of TMA treated mice revealed that TMA sensitization and challenge increased nasal airway-derived mRNA expression of the Th2 cytokines IL-4, IL-5 and IL-13 but caused no change in the expression of the Th1 cytokine IFN-γ. This study is the first to demonstrate that intranasal administration of a known chemical respiratory allergen is an effective method of sensitization resulting in the hallmark features of allergic rhinitis with a concomitant increase in Th2 cytokine mRNA expression in the nasal airway and an increase in lung-derived IL-5 mRNA and total serum IgE after intranasal challenge. This is in contrast to the chemicals DNCB and OXA, which failed to elicit the pathologic changes in the nasal and pulmonary airways and cytokine changes in the lung, that are characteristic of allergic airway disease. This model may be useful for identifying other chemical respiratory allergens.

INTRODUCTION

The inhalation of LMW chemicals is a leading cause of occupational asthma and allergic rhinitis (Petsonk, 2002; WHO, 1999). LMW chemicals that sensitize the respiratory tract are highly reactive chemicals that have a molecular weight of less than 1 kDa (Petsonk, 2002). An example of a LMW chemical associated with occupational asthma and allergic rhinitis in industrial settings is trimellitic anhydride (TMA). TMA is used to make plasticizers, resins, polymers, dyes, and printing inks. Murine models have been developed to assess the allergenic effects of LMW chemicals. Some murine models of LMW chemical-induced allergic airway disease sensitize the animals by dermal application and successfully induce features characteristic of LMW chemical-induced allergic airway disease after intra-airway challenge. Although the skin may represent an actual route of exposure to chemical respiratory allergens, the most common route of human exposure to such chemicals is inhalation (Lovik et al, 1996; Kimber et al, 1996). Also, there is recent evidence that the route of sensitization may affect the quality of the immune response in the lung after allergen challenge. Vohr and coworkers, 2002, showed in rats that topical induction followed by intra-airway challenge led to a qualitatively different complement of immunocompetent cells in bronchoalveolar lavage fluid (BALF) than aerosol induction followed by aerosol challenge. Another method of intra-airway administration that more closely resembles inhalation is intranasal instillation. In a previous study, we found that intranasal sensitization and challenge with the protein ovalbumin in a saline vehicle was a simple, inexpensive, and noninvasive

method of inducing IgE-mediated allergic airway disease in the upper and lower respiratory tract of the A/J mouse (refer to Chapter 2 of this dissertation, Farraj et al, 2003). It is not clear, however, whether intranasal sensitization and challenge with known LMW chemical respiratory allergens will induce the characteristic features of LMW chemical-induced allergic airway disease.

The study presented in Chapter 4 of this dissertation described an attempt to elicit the characteristic immunologic and pathologic features of TDI-induced allergic airway disease by intranasal sensitization and challenge with TDI. Although some features of TDI-induced allergic airway disease were elicited with intranasal instillation of TDI (i.e., lymphocytic influx in the nasal airways, increased lung-derived Th2 cytokine mRNA, and increased serum IgE), several problems remained regarding the intranasal instillation of TDI. Principal among them was the toxicity of the vehicle. Ethyl acetate and olive oil in a 1:1 combination when intranasally instilled caused toxicity to the nasal airway mucosa. In the present study, the irritant effects of 1:1 ethyl acetate/olive oil and 1:4 ethyl acetate/olive oil were compared for the purpose of selecting a minimally-irritating vehicle for intranasally sensitizing and challenging mice with TMA. An additional problem with the TDI study was the failure to elicit any pathologic changes in the pulmonary airways after intranasal instillation, which may have been due to insufficient distribution of TDI into the lung. Southam et al, 2002, determined that by increasing the volume of an intranasally instilled saline solution in mice (up to 75 μl), the distribution to the lung increases. In the

present study, the volume of the solution that was intranasally instilled was increased from 30 μl to 60 μl in an attempt to improve distribution into the lung.

Th2 cytokines have been implicated in LMW chemical-induced allergic airway disease both in humans and in experimental animal models. For example, the production of IL-4 and IL-5 proteins was increased in bronchial biopsies of patients with TDI-induced asthma (Maestrelli et al, 1997). In addition, the selective enhancement of Th2 cytokines was detected in several murine models of TMA-induced asthma (Betts et al, 2002; Dearman et al, 2002, Vandebriel et al, 2000). The methods by which cytokine measurements are made vary in murine models of LMW chemical-induced allergic airway disease. They include the assessment of changes in cytokine expression by measuring the secreted product of stimulated local draining lymph node cells that drain the site of dermal application or by analyzing bronchoalveolar lavage fluid (BALF) for cytokine protein or mRNA expression. In the present study, I examined the local mRNA expression of Th2 cytokines in both pulmonary and nasal tissue after intranasal administration of TMA.

One objective of this study was to determine whether 1:4 ethyl acetate/olive oil was a more suitable vehicle for intranasal delivery of TMA than 1:1 ethyl acetate/olive oil. The second objective of this study was to test the hypothesis that intranasal sensitization and challenge with the known chemical respiratory allergen TMA but not the non-respiratory sensitizers DNCB and OXA will induce the characteristic features of LMW chemical-induced allergic airway disease in the nasal and pulmonary airways of mice. DNCB and OXA are known

LMW chemical contact allergens that elicit contact hypersensitivity in the skin mediated by Th1 cytokines such as IFN-γ and do not cause occupational asthma or allergic rhinitis (Dearman et al, 2001). The pathologic changes in the nasal and pulmonary airways of mice after intranasal sensitization and challenge with TMA were compared to the responses after intranasal sensitization and challenge to DNCB or OXA. In addition, the right lung lobes were assessed for any changes in Th2 and Th1 cytokine mRNA expression. The nasal airways of mice exposed to TMA alone were also assessed for changes in Th2 and Th1 cytokine mRNA expression. This was done to examine the utility of measuring local cytokine gene expression as a biomarker for the respiratory allergenicity of low molecular weight chemicals.

MATERIALS AND METHODS

Animals and Chemical Sensitization and Challenge

Male A/J mice (Jackson Laboratories, Bar Harbor, Maine), six weeks of age, were randomly assigned to one of 27 experimental groups (n = 6). Mice were free of pathogens and respiratory disease, and used in accordance with guidelines set forth by the All University Committee on Animal Use and Care at Michigan State University. Animals were housed six per cage in polycarbonate boxes, on Cell-Sorb Plus bedding (A&W Products, Cincinnati, OH) covered with filtered lids, and had free access to water and food. Room lights were set on a 12 hr light/dark cycle beginning at 6:00 am, and temperature and relative humidity were maintained between 21-24°C and 40-55 % humidity, respectively. Figure 5.1 depicts the exposure regimen used for the intranasal sensitization and challenge of the mice.

Pilot study for selection of vehicle: The irritating effects of the vehicles 1:4 ethyl acetate/olive oil and 1:1 ethyl acetate/olive oil in the nasal airway mucosa were compared for the purpose of selecting a vehicle for use in the intranasal sensitization and challenge of mice with TMA. Mice were anesthetized with 4% halothane and 96% oxygen and then intranasally instilled with 60 μl of 1:4 ethyl acetate/olive oil once per day for three consecutive days and then sacrificed 24 hr after the final instillation. The nasal and pulmonary airways were histopathologically examined for any evidence of airway irritation.

Sensitization and challenge: Mice were anesthetized with 4% halothane and 96% oxygen and then exposed to one of three chemicals: trimellitic

anhydride (TMA) (Sigma-Aldrich, St. Louis, MO), 2,4-dinitro-1-chlorobenzene (DNCB) (Sigma-Aldrich, St. Louis, MO) or 4-ethoxymethylene-2-phenyl-2-oxazolin-5-one (oxazolone, OXA) (Fisher Scientific-Acros Organics, Pittsburgh, PA). The chemicals were dissolved in a 1:4 ethyl acetate to olive oil vehicle that was non-irritating to the nasal and pulmonary airways. The mice were sensitized via single intranasal instillations of 60 μl of each chemical separately on Days 1 and 3 and then challenged with single intranasal instillations on Days 17 and 27. The concentrations of the chemicals used were 0.125 % TMA, 0.5 % DNCB, or 0.1 % OXA. The concentrations of each of these chemicals were selected after performing a pilot study that screened a range of concentrations of the test chemical for airway irritation. The highest dose that was not irritating to the airway (determined histologically) after 3 intranasal instillations, was selected. This was done to distinguish the irritating effects of the chemical from any potential adaptive immune effect. For each chemical, there were 5 different treatment groups: 1) untreated naïve group; 2) mice sensitized and challenged with the vehicle alone; 3) mice sensitized with the vehicle alone and then challenged once with vehicle and then the second time with the LMW chemical in vehicle; 4) mice sensitized with the LMW chemical in vehicle and then challenged with the vehicle alone; and 5) mice sensitized and challenged with the LMW chemical in vehicle. Mice were sacrificed 24 or 96 h (Day 28 or Day 31) after the final instillation.

An additional set of mice was obtained for the purpose of assessing cytokine mRNA expression in the nasal airway of mice sensitized and challenged

Exposure Regimen

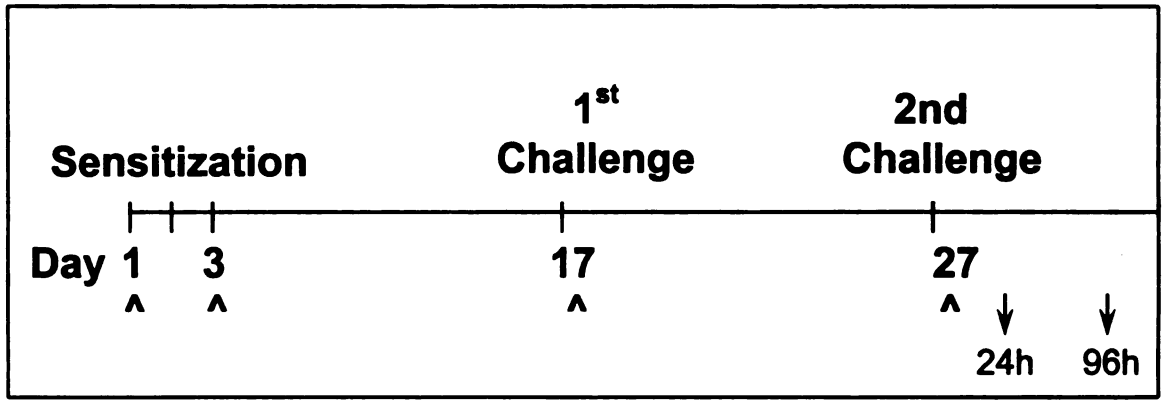


Figure 5.1: Timeline of the exposure regimen used to sensitize and challenge A/J mice with 0.125 % TMA, 0.5 % DNCB, or 0.1 % OXA in a 1:4 ethyl acetate/olive oil vehicle. Mice were sensitized on days 1 and 3 with intranasal instillations of 60 μl of TMA, DNCB, or OXA in a 1:4 ethyl acetate/olive oil vehicle or vehicle alone. Mice were then challenged two weeks later on day 17 with a similar volume of TMA, DNCB, or OXA and challenged a second time 10 days later on day 27 followed by sacrifice 24 or 96 hr after double challenge. ^ = 60 μl of TMA, DNCB, or OXA in 1:4 ethyl acetate/olive oil vehicle or vehicle alone. ↓ = time after challenge when animals were sacrificed.

with TMA alone. The mice were sensitized and challenged with TMA as described above but were sacrificed 48 h after the final instillation.

Necropsy, Blood Sample Collection and Tissue Preparation

At the designated sacrifice times, mice were deeply anesthetized via intraperitoneal injection of 0.1 ml of 12% pentobarbital in saline. Blood samples (0.1 to 0.5 ml) were taken from the abdominal aorta and collected in Beckton Dickinson vacutainers. Serum samples were collected and stored at -20°C after the blood samples were centrifuged to remove cells. The abdominal aorta and renal artery were then severed to exsanguinate the animals. Immediately after death, the trachea was cannulated and the heart/lung block removed. A syringe was then attached to the cannula and brochoalveolar lavage fluid was collected from the whole lung via two sequential changes of 1 ml saline. Differential cell counts were determined by cytocentrifuging cells onto glass slides and then staining with Diff-Quik (DADE Behring, Newark, DE). Neutrophils, macrophages, eosinophils and other cell types were microscopically identified and their numbers were determined by multiplying the percentage of each cell type from a total of 200 cells by the total number of cells per ml of lavage fluid. The right bronchus was then clamped using suture thread. All four right lung lobes were placed in 2 ml Tri reagent (Molecular Research Center, Cincinnati, OH), homogenized and stored at -80°C.

After removal of the right lung lobes, the left lung lobe was intratracheally perfused with 10% neutral buffered formalin at a constant intra-airway pressure

of 30 cm of fixative. After 1 hr, the trachea was ligated, and the inflated left lung lobe was immersed in a large volume of the same fixative for 24 hr. After fixation, the left lung lobe was microdissected along the axial airways, and two sections were then excised at the level of the fifth (proximal airway) and eleventh (distal airway) airway generation (Figure 5.2), as has been described previously in detail (Steiger et al, 1995). Paraffin sections (5 μm) were stained with Alcian blue (pH 2.5) and periodic acid Schiff's (AB/PAS) reagent, which stains both neutral and acidic mucosubstances within airway moucous (goblet) cells.

The head of each mouse was excised from the carcass, and the eyes, skin, skeletal muscle, and lower jaw were removed from the head. The heads were then immersed in 10% neutral buffered formalin for 24 hr. After fixation, the heads were decalcified in 13% formic acid for 7 days and then rinsed in tap water for at least 4 hr. The nasal cavity of each mouse was transversely sectioned at three specific anatomic locations according to a modified method of Young (1981). The most proximal nasal section was taken immediately posterior to the upper incisor teeth (proximal, T1); the middle section was taken at the level of the incisive papilla of the hard palate (middle, T2); the most distal nasal section was taken at the level of the second palatal ridge (distal, T3) (Figure 5.2). These tissue blocks were embedded in paraffin, sectioned at a thickness of 5 microns, and then stained with hematoxylin and eosin for light microscopic examination. Other paraffin sections were stained with AB/PAS to identify intraepithelial mucosubstances (IM).

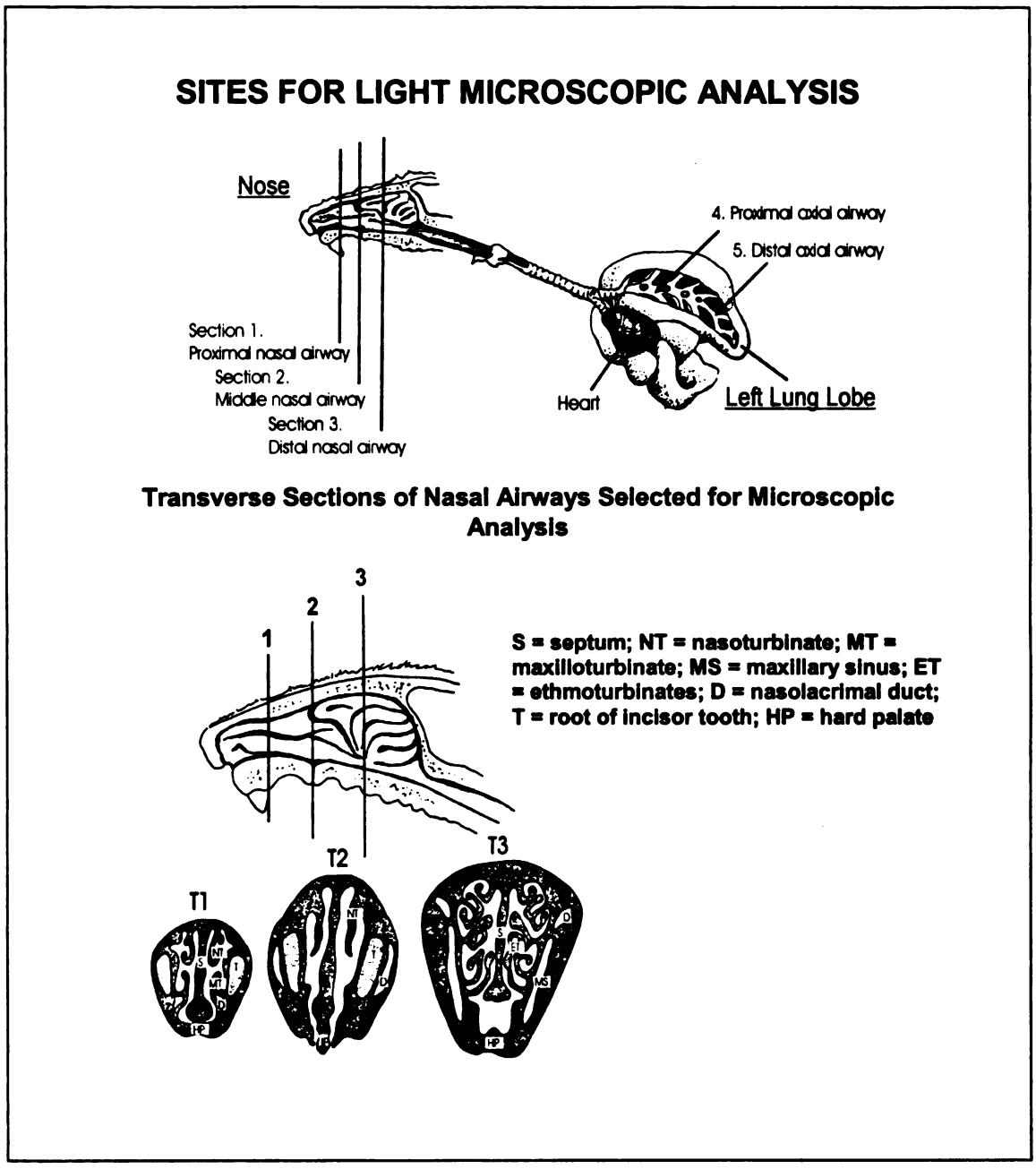


Figure 5.2: Sites of airway tissue selection for morphometric analysis. The cartoon depicts the lateral wall of one nasal passage with the septum removed of the murine respiratory tract and the main axial airway of the left lung lobe. The left lung lobe was microdissected along the axial airways, and two sections were then excised at the level of the fifth (proximal airway) and eleventh (distal airway) airway generation. Transverse sections of the nasal cavity (cartoon on bottom) were taken 1) immediately posterior to the upper incisor teeth (T1), 2) at the level of the incisive papilla of the hard palate (T2), and 3) at the level of the second palatal ridge (T3).

Sections of the spleen, jejunum and duodenum were also fixed in formalin or placed in TRI Reagent to determine if the LMW chemical treatment elicited any pathologic or cytokine responses in these tissues.

For the analysis of cytokine mRNA expression in the nasal mucosa, the heads of mice were split in a sagittal plane adjacent to the midline exposing the mucosal surfaces lining the nasal lateral wall and septum. The nasoturbinate and maxilloturbinate from each nasal airway and the proximal septum were micro-dissected. The excised tissues from each animal were placed in 1 ml Tri Reagent, homogenized, and stored at -80°C.

Morphometry of Stored Intraepithelial Mucosubstances in Nasal Airways

The amount of stored mucosubstances in the respiratory epithelium lining the mid-septum in the most proximal nasal section was estimated by quantifying the volume density of AB/PAS-stained mucosubstances using computerized image analysis and standard morphometric techniques. The area of AB/PAS-stained mucosubstances was calculated by circumscribing the perimeter of the stained material using the Scion Image program (Scion Corporation, Frederick, MD). The length of the basal lamina underlying the surface epithelium was calculated from the contour length of the digitized image of the basal lamina. The volume of stored mucosubstances (volume density, Vs) per unit of surface area was estimated using the method described in detail by Harkema et al (Harkema et al, 1987) and is expressed as nanoliters of IM per square millimeter basal lamina.

ELISA for Total serum IgE

Total serum IgE was measured using a 96-well Immulon ELISA plate (Dynex, Technologies, Chantilly, Virginia) coated with 2 μg/ml anti-mouse IgE (Purified Rat Anti-Mouse IgE Monoclonal Ab, Pharmingen, San Diego, CA) and incubated overnight at 4°C. After washing, the plates were incubated in 3 % Bovine Serum Albumin (3 % BSA) at 37°C for 1 hr. (BSA, CALBIOCHEM, La Jolla, CA). Serum samples at 1:10 dilution were then added followed by incubation at 37°C for 1 hr. After washing, biotinylated anti-mouse IgE (Biotin-conjugated Rat Anti-Mouse IgE Monoclonal Ab, Pharmingen, San Diego, CA) was then added at 2 μg/ml and allowed to incubate at 25°C for 1 hr. After washing, 1.5 μg/ml of streptavidin peroxidase was added followed by incubation at 25°C for 1 hr. After washing, TMB substrate (12.5 ml citric-phosphate buffer + 200 μl of tetramethylbenzidine (TMB) stock solution (6mg/ml in DMSO) + 100 μl 1 % H_2O_2 (Fluka Chemical Co., Ronkonkoma, NY)) was added to produce a color reaction. The reaction was terminated by the addition of 6 N H_2SO_4. Optical density was determined at 450 nm using an EL-808 microplate reader (Bio-Tek instruments, Winooski, Vermont).

Real-time RT-PCR

Total RNA was isolated from lung, nasal airway tissue, small intestine, or spleen using the TRI REAGENT method (Molecular Research Center, Cincinnati, OH) following the manufacturer's protocol. The evaluation of the relative expression levels of the cytokines IL-4, IL-5, IL-10, IL-13, and IFN-γ mRNA was

determined using the TaqMan one-step real-time multiplex RT-PCR with TaqMan pre-developed primers and probe using the manufacturer's recommended protocol (Applied Biosystems, Foster City, CA). Briefly, aliquots of isolated tissue RNA (100 ng total RNA) were added to the RT-PCR reaction mixture, which included the target gene (IL-4, IL-5, IL-10, IL-13, or IFN-γ) primers and probe, endogenous reference primers and probe (18S ribosomal RNA), AmpliTaq DNA polymerase and Multiscribe reverse transcriptase (MuLV). The probes are designed to exclude detection of genomic DNA. RNA samples were first reverse transcribed and then immediately amplified by PCR. Following the PCR, amplification plots (change in dye fluorescence versus cycle number) were examined and a dye fluorescence threshold within the exponential phase of the reaction was set separately for the target gene and the endogenous reference (18S). The cycle number at which each amplified product crosses the set threshold represents the C_T value. The amount of target gene normalized to its endogenous reference was calculated by subtracting the endogenous reference C_T from the target gene C_T (ΔC_T). Relative mRNA expression was calculated by subtracting the mean ΔC_T of the control samples from the ΔC_T of the treated samples ($\Delta\Delta C_T$). The amount of target mRNA, normalized to the endogenous reference and relative to the calibrator (i.e., RNA from control) is calculated by using the formula $2^{-\Delta\Delta C_T}$.

Statistics

The data obtained from each experimental group were expressed as a mean group value ± the standard error of the mean (SEM). The differences among groups were determined by one way or two-way analysis of variance (ANOVA) and an All Pairwise Comparison Test (Tukey), using SigmaStat software from Jandel Scientific (San Rafael, CA).

RESULTS

Selection of Vehicle Study

The vehicle 1:4 ethyl acetate/olive oil did not cause any airway irritation including epithelial necrosis and neutrophilic inflammation in the nasal airway (Figure 5.3A) unlike the vehicle 1:1 ethyl acetate/olive oil (see Chapter 4). The vehicle 1:4 ethyl acetate/olive oil was thus selected for use in the intranasal sensitization and challenge of mice to TMA, DNCB, and OXA.

Airway Pathology

Only mice sensitized and challenged with TMA had airway lesions associated with intranasal instillations. TMA-induced airway alterations in these animals were restricted to the nasal airways. No exposure-related alterations were microscopically evident in the lungs of any of these mice. The principal morphologic alteration in the mice sensitized and challenged with TMA and sacrificed 24 hr after the last instillation was a moderate-marked allergic rhinitis characterized by a conspicuous influx of mixed inflammatory cells predominated by eosinophils and accompanied by lesser numbers of mononuclear cells (lymphocytes and plasma cells) (Figure 5.3). The inflammatory cell influx was bilateral and most severe in the nasal mucosa lining the proximal lateral meatus and the midseptum in T1. Accompanying the nasal inflammation was a moderate to marked regenerative hyperplasia with areas of degeneration and individual cell necrosis of the nasal transitional epithelium in these proximal nasal airways.

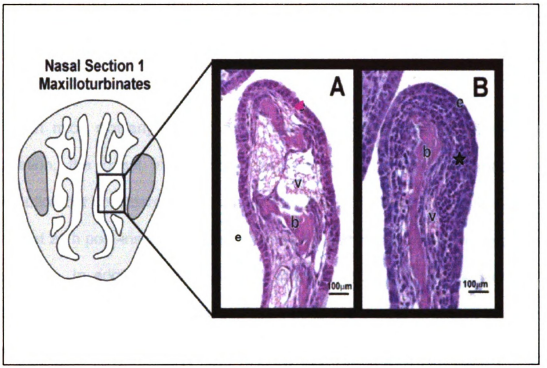


Figure 5.3: Light photomcrographs of the maxillotubinates from the proximal nasal airways of mice sensitized and challenged with vehicle alone (A) or TMA in vehicle (B). Numerous eosinophils present only in the lamina propria of the maxillotubinate from the TMA-exposed mouse (star in B). Tissues stained with hematoxylin & eosin. e = surface epithelium; b = turbinate bone; v = blood vessel in the lamina propria of the nasal mucosa.

Similar but less severe mucosal inflammation was present in the more distal nasal airways in T2 and T3. In these latter two sections the inflammation was restricted to the respiratory mucosa lining the middle and lateral meatus in T2 and the nasopharyngeal meatus in T3. In addition, there was moderate lymphoid hyperplasia of the nasal associated lymphoid tissue (NALT) in T3 of these mice.

In mice similarly instilled with TMA but sacrificed 96 hr after the last instillation, the nasal inflammatory response was similar in character and distribution but considerably attenuated from that in the mice that were sacrificed earlier at 24 h post-instillation. These mice also had mild lymphoid hyperplasia of the NALT. In addition, the respiratory epithelium lining the mid-septum in T1 underwent mucous cell metaplasia (Figure 5.4). No exposure-related alterations were microscopically evident in the nasal or pulmonary airways of DNCB or OXA-instilled mice (Table 5.1). In addition, there were no morphologic alterations in the gut or spleen of all treated mice relative to the naïve controls (Table 5.1).

Stored Intraepithelial Mucosubstances in Nasal Airways

The amount of IM in the airway epithelium lining the proximal septum was estimated in mice sensitized and challenged with TMA and sacrificed 96 hr after the final challenge using image analysis and standard morphometric techniques. Intranasal sensitization and challenge with TMA in vehicle resulted in slightly more than a 2-fold increase in the amount of IM in the respiratory epithelium

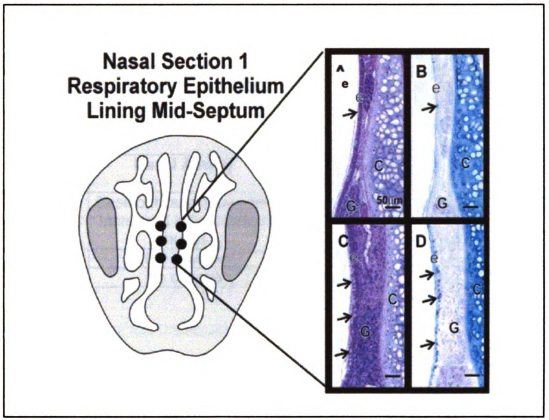


Figure 5.4: Light photomicrographs of the nasal mucosa lining the mid-septum from the proximal nasal airways of mice exposed to vehicle alone (control, A & B) or TMA in vehicle (C & D). Nasal tissues were stained with hematoxylin & eosin (A & C) or Alcian blue (pH 2.5)/periodic acid Schiff (AB/PAS; B & D). More mucous goblet cells with AB/PAS-stained mucosubstances (arrows) are present in the surface epithelium (e) in the TMA-exposed mice (C & D) compared to the vehicle-control mouse (A & B). G = subepithelial glands in the nasal mucosa; C = septal cartilage.

Table 5.1: Summary of the morphologic changes in the nasal airways, pulmonary airways, small intestine, or gut after sensitization and challenge with TMA, DNCB, or OXA.

Chemical	Nasal Airway	Pulmonary Airway	Small Intestine	Spleen
TMA	Eosinophils/lymphocytes Increased intraepithelial mucus	No change	No change	No change
DNCB	No change	No change	No change	No change
OXA	No change	No change	No change	No change

lining the proximal septum in the proximal nasal airway as compared to the vehicle controls (Figure 5.5). There were no such increases evident in mice intranasally instilled with DNCB or OXA (Table 5.1).

Bronchoalveolar Lavage Fluid (BALF)

The total number of cells in the BALF was determined at 24 and 96 hr after the second challenge. A modest but statistically significant increase (2-fold) in macrophages in the BALF was observed in TMA-sensitized and challenged mice that were sacrificed 24 hr after the second challenge relative to the naïve control (Figure 5.6). A similar trend in macrophages recovered from the BALF (~ 2-fold increase) was observed in the group that was sensitized with vehicle, received a first vehicle challenge and a second challenge with TMA. Mice that received only TMA sensitizations or only a TMA challenge did not exhibit significant increases in macrophages recovered from the BALF. There were no increases detected at 96 hr in any of the treatment groups. Mice sensitized and challenged with DNCB or OXA did not exhibit any significant increases in macrophages recovered from the BALF (data not shown).

Total Serum IgE

Total serum IgE levels were determined both at 24 and 96 hr after the final challenge. Mice sensitized and challenged with TMA exhibited a significant increase in total serum IgE (5-fold) relative to naïve controls both 24 and 96 hr

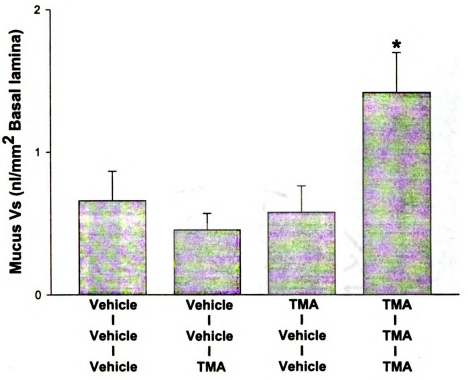


Figure 5.5: Morphometric quantification of AB/PAS-stained material in the surface epithelium lining the mid-septum in nasal airway region T1 of mice 96 hr after double challenge with TMA. Mice were sensitized and then challenged twice with TMA in vehicle or vehicle alone. Bars represent the volumetric density (Vs) of intraepithelial mucosubstances +/- the standard error of the mean (n = 6/group). * - significantly greater than all other groups ($p<0.05$).

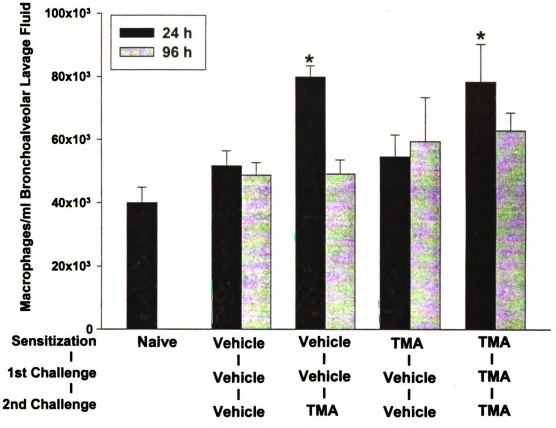


Figure 5.6: Total number of macrophages in BALF from the left lung lobes of mice sensitized and challenged with TMA. Mice were sensitized with intranasal instillations of 60 μl of 0.125% TMA in a 1:4 ethyl acetate/olive oil vehicle. Mice were then challenged two weeks later with a similar volume and challenged a second time 10 days later followed by sacrifice 24 or 96 hr after double challenge. Bars represent mean number of macrophages per ml BALF ± standard error of the mean (n = 6/group). * = significantly greater than the naïve group ($p<0.05$).

after the final challenge (Figure 5.7A). No significant increase in total serum IgE was observed in the vehicle control group relative to the naïve control. Mice that received only TMA sensitizations or only a TMA challenge did not exhibit significant increases in total serum IgE. Mice sensitized and challenged with DNCB also exhibited a significant increase in total serum IgE (8-fold) relative to the naïve control both 24 and 96 hr after the final challenge (Figure 5.7B). Conversely, mice sensitized and challenged with OXA did not exhibit a significant increase in total serum IgE relative to naïve controls both at 24 and 96 hr after the final challenge (Figure 5.7C).

Cytokine Gene Expression

Lung: Real-time RT-PCR was performed on right lung lobe-derived RNA from mice that were sensitized and then challenged twice with TMA, DNCB, or OXA. TMA sensitized and challenged mice had a 30-fold increase in IL-5 mRNA expression 24 hr after the second challenge as compared to the vehicle controls that returned to control levels by 96 hr after challenge (Figure 5.8A). There were no significant increases in the mRNA expression of any of the other Th2 cytokines measured or the Th1 cytokine IFN-γ at 24 or 96 hr after the final challenge. Mice that received only TMA sensitizations or only a TMA challenge did not exhibit significant increases in the mRNA expression of any of the measured cytokines within the lung. Intranasal instillation with DNCB or OXA did not result in any significant increase in the expression of any cytokine measured within the lung tissue (Figures 5.8B and 5.8C).

Figure 5.7A

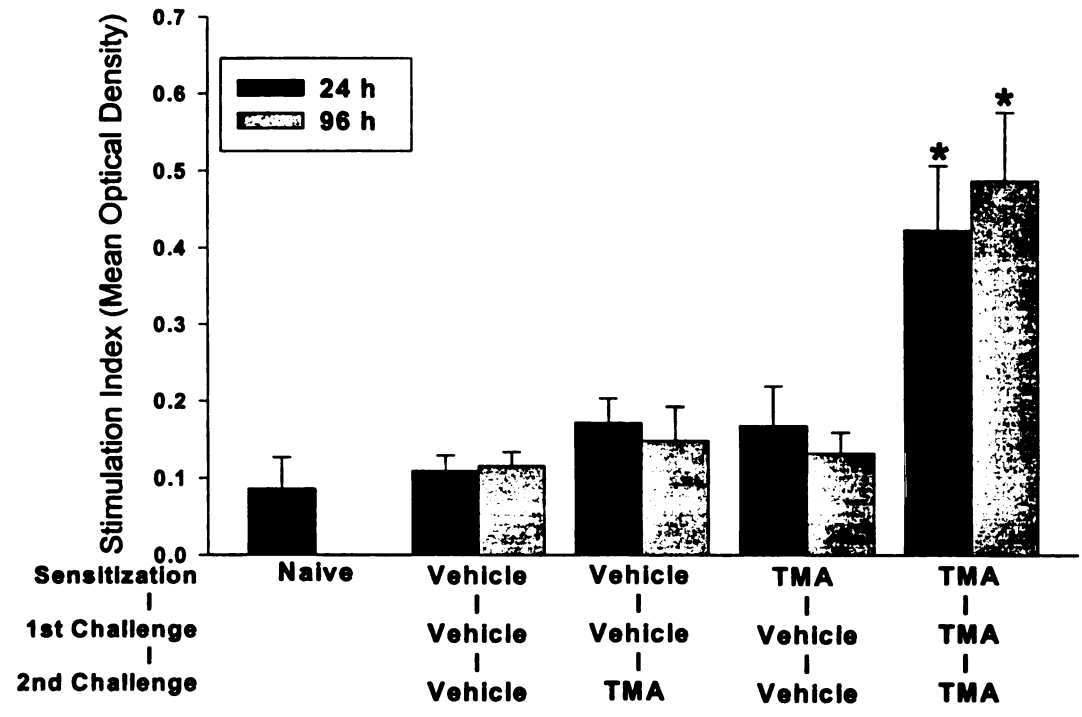


Figure 5.7: Total IgE levels in mouse serum isolated 24 or 96 h after double challenge with TMA (A), DNCB (B), or OXA (C). Mice were sensitized with intranasal instillations of 60 μl of 0.125% TMA, 0.5 % DNCB, or 0.1 % OXA in a 1:4 ethyl acetate/olive oil vehicle. Mice were then challenged two weeks later with a similar volume and challenged a second time 10 days later followed by sacrifice 24 or 96 hr after double challenge. Bars represent average optical density ± standard error of the mean (n = 6/group). * = significantly greater than all other groups.

Figure 5.7B

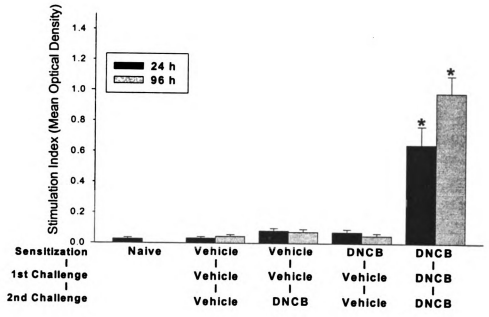


Figure 5.7C

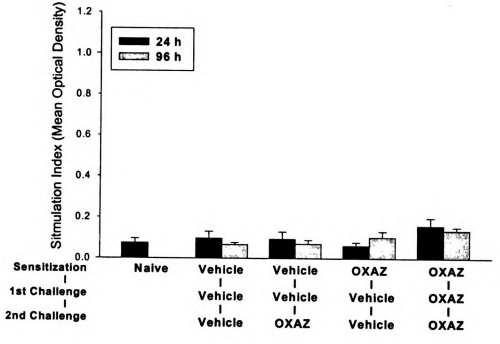


Figure 5.8A

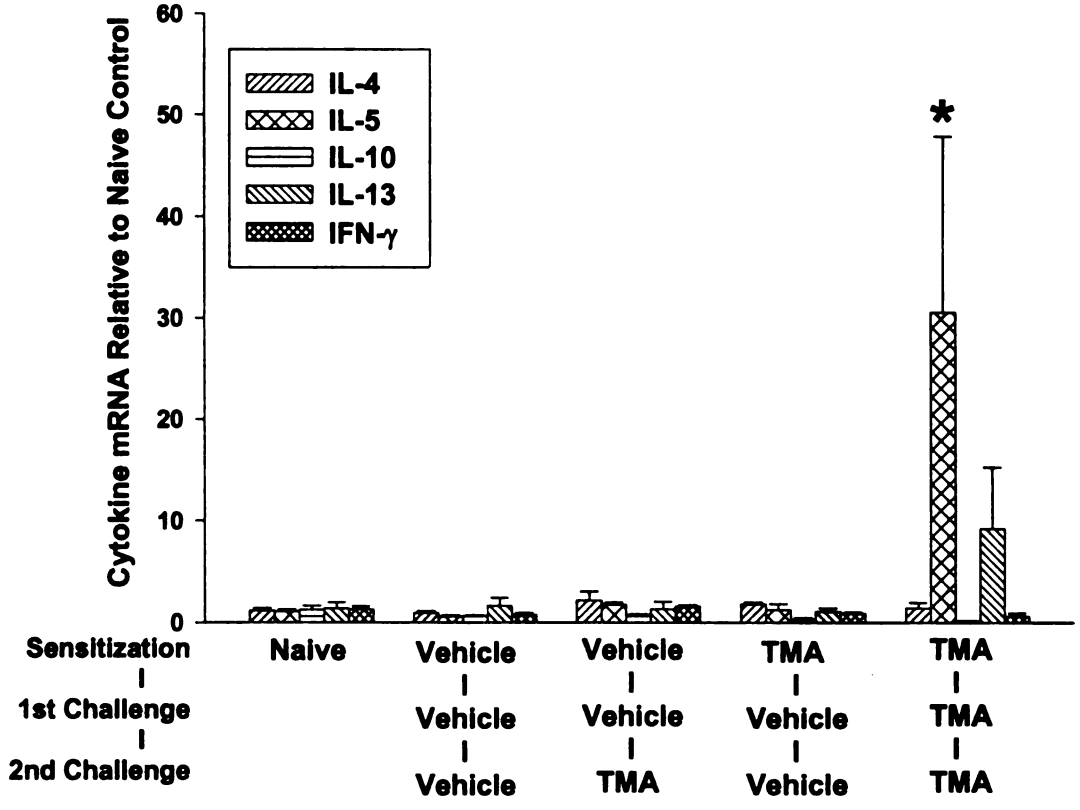


Figure 5.8: Relative quantification of IL-4, IL-5, IL-10, IL-13, and IFN-γ mRNA in right lung lobes 24 hr after double challenge with TMA (A), DNCB (B), or OXA (C) using real-time RT-PCR. Mice were sensitized with intranasal instillations of 60 μl of 0.125% TMA, DNCB, or OXA in 1:4 ethyl acetate/olive oil vehicle or vehicle alone. Mice were then challenged two weeks later with a similar volume and challenged a second time 10 days later followed by sacrifice 24 hr after double challenge. Bars represent cytokine mRNA relative to the naïve control ± standard error of the mean (n = 6/group). * = significantly greater than all other groups at similar time-point ($p<0.05$).

Figure 5.8B

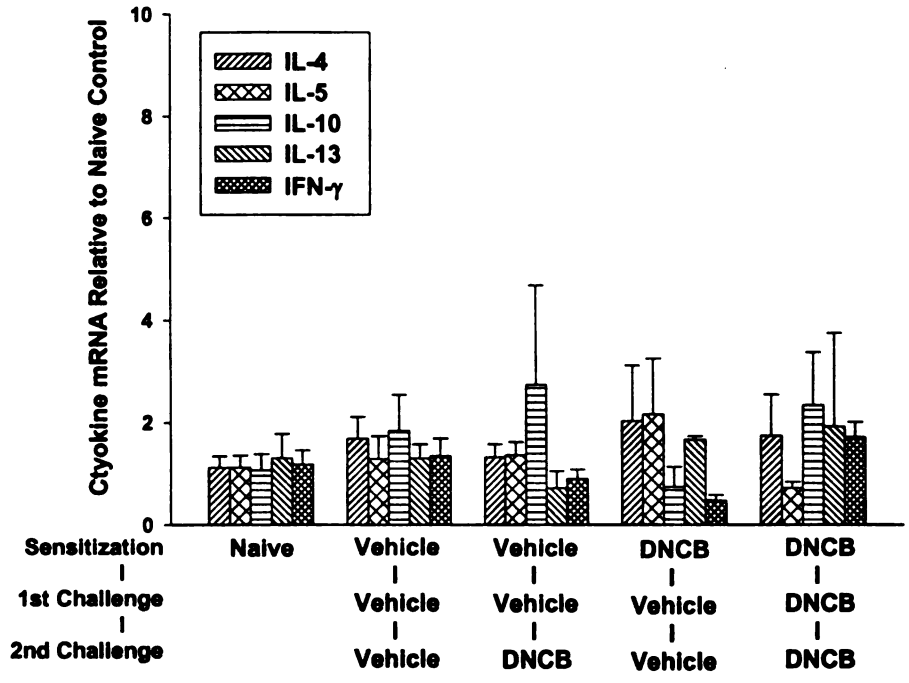
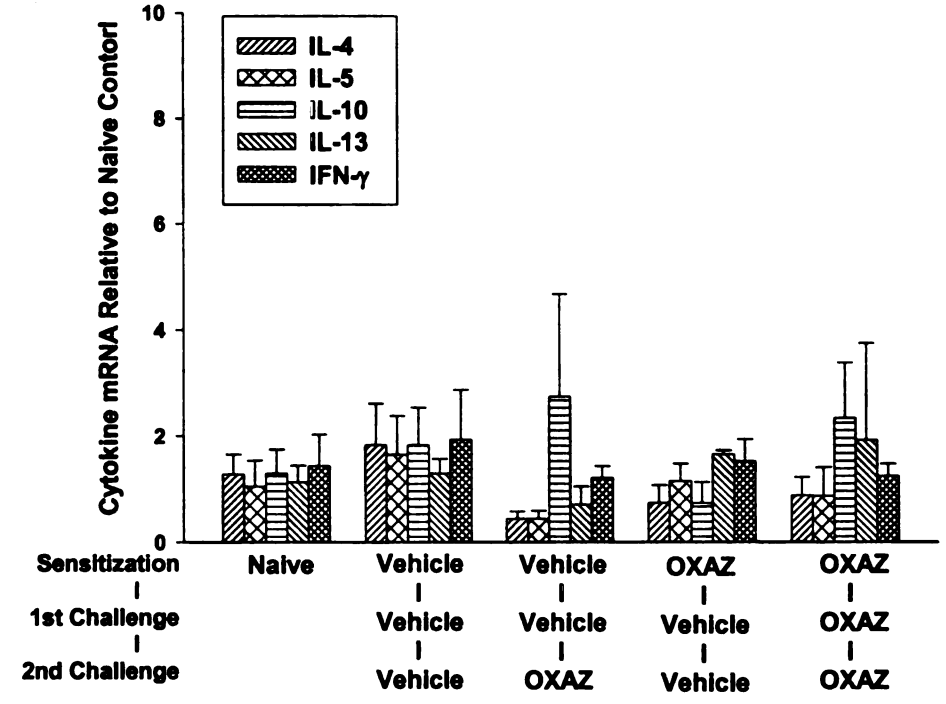


Figure 5.8C



Nasal Airway: Real-time RT-PCR was also performed on nasal airway tissue RNA from mice that were sensitized and then challenged twice with TMA. TMA sensitized and challenged mice exhibited a 4.5-fold increase in IL-4 mRNA expression, a 3-fold increase in IL-5 mRNA expression, and a 7-fold increase in IL-13 mRNA expression, 48 hr after the second challenge as compared to the vehicle controls (Figure 5.9). There were no significant increases in the mRNA expression of IL-10 or the Th1 cytokine IFN-γ. Mice that received only TMA sensitizations or only a TMA challenge did not exhibit significant increases in the mRNA expression of any of the measured cytokines within the nasal airway tissue.

No assessment of cytokine mRNA expression was made in the nasal airways of mice instilled with DNCB or OXA because of the lack of DNCB or OXA-induced nasal airway histopathology.

Spleen and Small Intestine: There were no increases in the mRNA expression of any of the Th2 cytokines or the Th1 cytokine IFN-γ in the gut or spleen of any TMA, DNCB, or OXA treated mice relative to the naïve controls (Table 5.2).

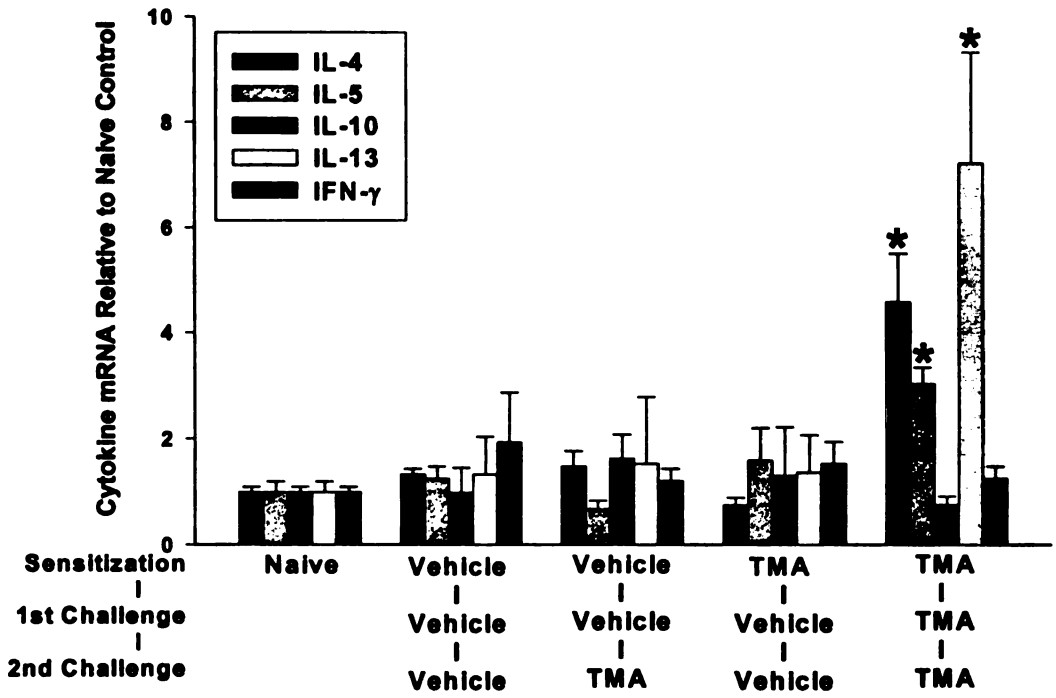


Figure 5.9: Relative quantification of IL-4, IL-5, IL-10, IL-13, and IFN-γ mRNA in nasal airway tissue 24 hr after double challenge with TMA using real-time RT-PCR. Mice were sensitized with intranasal instillations of 60 μl of 0.125% TMA in a 1:4 ethyl acetate/olive oil vehicle or vehicle alone. Mice were then challenged two weeks later with a similar volume and challenged a second time 10 days later followed by sacrifice 24 hr after double challenge. Bars represent cytokine mRNA relative to the naïve control ± standard error of the mean (n = 6/group). * = significantly greater than all other groups at similar time-point ($p<0.05$).

Table 5.2: Summary of cytokine mRNA expression changes in the nasal airway, lung, small intestine, and spleen after sensitization and challenge with TMA, DNCB, or OXA.

Chemical	Nasal Airway	Pulmonary Airway	Small Intestine	Spleen
TMA	Increased IL-4, IL-5, and IL-13 mRNA	Increased IL-5 mRNA	No change	No change
DNCB	No change	No change	No change	No change
OXA	No change	No change	No change	No change

DISCUSSION

One objective of this study was to determine whether 1:4 ethyl acetate/olive oil was a more suitable vehicle for intranasal delivery of TMA than 1:1 ethyl acetate/olive oil. The second objective of this study was to test the hypothesis that intranasal sensitization and challenge with the known chemical respiratory allergen TMA but not the non-respiratory sensitizers DNCB and OXA will induce the characteristic features of LMW chemical-induced allergic airway disease in the nasal and pulmonary airways of mice. Morphologic changes in the nasal and pulmonary airways of TMA-sensitized and challenged mice were compared with the nasal and pulmonary airways of mice sensitized and challenged with the contact sensitizers DNCB and OXA. In addition, the right lung lobes of TMA, DNCB, and OXA exposed mice were assessed for any changes in Th2 and Th1 cytokine mRNA expression. The nasal airways of mice exposed to TMA alone were also assessed for changes in Th2 and Th1 cytokine mRNA expression. This was done to examine the utility of measuring local cytokine gene expression as a biomarker for the respiratory allergenicity of low molecular weight chemicals.

Individuals afflicted with occupational asthma often suffer from concurrent allergic rhinitis (Leynaert et al, 2000). Some studies suggest that allergic rhinitis due to workplace exposure to TMA precedes and may be more common than occupational asthma (Bernstein, 1993; Grammer et al, 2002). The main symptoms of TMA-induced allergic rhinitis are itching, sneezing, watery discharge, and obstructed nose (WHO, 1999). The pathologic features of TMA-

induced allergic rhinitis are similar to non-occupational allergic rhinitis and include plasma exudation, hypersecretion of mucus, and cellular infiltrates consisting of T- and B-lymphocytes, eosinophils, and plasma cells in the nasal airway (WHO, 1999). In the present study, only mice that were intranasally sensitized and challenged with TMA had a marked allergic rhinitis characterized by an influx of eosinophils, lymphocytes and plasma cells, 24 h after the final challenge. The predominant cell type was the eosinophil with fewer numbers of lymphocytes. This is slightly different from the inflammatory infiltrate in humans which also consists of mast cells and neutrophils and more lymphocytes (WHO, 1999). The reason for this difference may have to do with the species differences between humans and mice and the level of exposure. The TMA exposure in humans may have elicited an irritant/adaptive immune response in the nasal airway. In the present study, the concentrations of the LMW chemicals used were not irritating to the nasal airway mucosa. By 96 h, the nasal airway epithelium exhibited increases in stored mucus and a regenerative hyperplasia. This is similar to human studies that report increased mucus production. In contrast, intranasal sensitization and challenge with DNCB or OXA did not result in any lesions within the nasal airways.

Th2 cells produce IL-4, IL-5, IL-10, and IL-13 (Yssel and Groux, 2000). IL-4 promotes T cell activation and differentiation into the Th2 subtype while both IL-4 and IL-13 promote IgE production in B cells and mucus production in the airways (Shim et al, 2001; Frew, 1996). The mechanism of non-occupational allergic rhinitis is dependent on Th2 cytokines (Andersson et al, 2000). Th2

cytokines have also been linked to the pathogenesis of LMW chemical-induced asthma. Several groups have shown that lymph node cells draining the site of dermal application in murine models of TMA-induced asthma preferentially secrete Th2 cytokines including IL-4, IL-10, and IL-13 (Dearman et al, 2003, Plitnick et al, 2002). Limited evidence exists, however, that demonstrates a link between LMWC-induced allergic rhinitis and the up-regulation of Th2 cytokines. Nevertheless, the similarity in pathogenesis between LMW chemical-induced allergic rhinitis and non-occupational allergic rhinitis and the evidence that Th2 cytokines are up-regulated in TMA-induced asthma suggests a critical role for Th2 cytokines in LMW chemical-induced allergic rhinitis. There are many murine models of LMW chemical-induced allergic airway disease that vary in the method used for cytokine analysis with some focusing on changes in BALF-derived cytokine content while others analyze local draining lymph node cells that have been re-stimulated with a mitogen *in vitro*. In the present study, RNA isolated from nasal airway tissue of TMA sensitized and challenged mice was assessed for changes in Th2 and Th1 cytokine mRNA expression to determine if the TMA-induced allergic rhinitis is accompanied by local changes in cytokine mRNA expression within the nasal airway. TMA-sensitized and challenged mice had an increase in the nasal airway-derived mRNA expression of the Th2 cytokines IL-4, IL-5, and IL-13 and no change in the expression of the Th1 cytokine IFN-γ. This is the first report that demonstrates, in a murine model, a link between TMA-induced allergic rhinitis and the selective enhancement of Th2 cytokines within the nasal airway. The nasal airways of DNCB or OXA treated mice were not

assessed for Th2 cytokine expression. Although the rationale for not doing so was the lack of any DNCB- or OXA-induced nasal airway histopathology, an assessment of the expression levels of Th2 and Th1 cytokines in mice treated with DNCB or OXA would have been appropriate to confirm the selectivity of Th2 cytokine expression with known chemical respiratory allergens.

In the present study, no pulmonary lesions were found in any group instilled with TMA, DNCB, or OXA. The lack of any airway pathology suggests that the amount of chemical that entered the lung may not have been sufficient to elicit an irritant/adaptive immune response in the lung. This may have been due to the viscosity of the ethyl acetate and olive oil vehicle, which may have prevented effective aspiration of the chemical and distribution of the chemical into the lower airways. The increase in the volume intranasally instilled did not result in any pathology. In addition, the inherent high reactivity of these chemicals may have played a role in limiting the distribution of these chemicals to the lower airways. It is likely that a large portion of the chemical reacted with proteins in the nasal airways of these mice. Greenberg et al, 1994, reported that after aerosolized toluene diisocyanate exposure in guinea pigs, the vast majority of the chemical remained in the nasal airway. The small increase in macrophages in the BALF after sensitization and challenge with TMA, however, suggests that there was some distribution of the chemical into the lung. This increase was not an immune-mediated effect as it also took place after just a single instillation of the chemical and may be due to the inherent irritating properties of the chemical.

The lipophilic and highly reactive characteristics of LMW chemicals hinder sufficient distribution of such chemicals into the lung via IN instillation. Increasing the concentration of the chemical instilled is one method of increasing distribution into the lung via IN instillation. The direct acute toxicity in the airway that subsequently results, as in the study described in Chapter 4 of this dissertatation, precludes the use of such high concentrations. This remains a tremendous obstacle in the elicitation of features characteristic of LMW chemical-induced asthma when administered via IN instillation. An alternative to IN instillation is intratracheal instillation of LMW chemicals using the same non-irritating vehicle 1:4 EA/OO.

I attempted to determine if any potential airway pathology in the pulmonary airways after instillation of the chemicals was accompanied by an increase in local Th2 or Th1 cytokine gene expression within the lung. Despite the lack of any pathologic changes in the lungs of mice sensitized and challenged to TMA, TMA sensitized and challenged mice had a 30-fold increase in lung-derived IL-5 mRNA expression 24 hr after the final TMA instillation. There was no increase in the expression of any other Th2 cytokine or the Th1 cytokine IFN-γ. In contrast, intranasal sensitization and challenge with DNCB or OXA did not result in a significant change in the expression of any of the measured cytokines including IFN-γ. These findings support other reports that link LMW chemical-induced occupational asthma with the selective expression of Th2 cytokines while known non-sensitizers of the respiratory tract do not. Several possibilities exist that may explain the TMA-induced increase in lung-

derived IL-5 expression in the absence of pulmonary airway inflammation. One is that the increase originated from infiltrating lymphocytes in areas of the lung not examined histologically such as the right lung lobes or more proximal regions of the left lung lobe. Another possibility is that the source of the cytokines was the bronchial-associated lymphoid tissue or the airway epithelium. Or perhaps a systemic immune response was elicited that originated in some other region of the respiratory tract that caused an increase in lymphocytes in the circulation and likewise the pulmonary vasculature. Further studies are required to determine the exact source.

The role of IgE antibodies in the pathogenesis of LMW chemical-induced occupational asthma and allergic rhinitis is unclear and controversial. Only 10 to 30 % of all cases of TDI-induced occupational asthma, for example, exhibit increases in IgE antibodies (Weissman and Lewis, 2000). There is a stronger link between IgE antibodies and TMA-induced allergic airway disease (Dearman, 2002), but not wholly consistent. Some investigators have suggested that the failure to establish a consistent link between high circulating IgE levels and LMW chemical-induced allergic airway disease is due to the deficiencies in IgE detection methods (Kimber et al, 2002) while others have demonstrated that non-IgE-dependent mechanisms of LMW chemical-induced allergic airway disease may exist (Herrick et al, 2002; Larsen et al, 2001; Bernstein, 2002). Nevertheless, we found that mice intranasally sensitized and challenged with TMA exhibited increases in total serum IgE. The increase in serum IgE, however, was not restricted to mice exposed to chemical respiratory allergens as

DNCB sensitized and challenged mice exhibited a similar increase in total serum IgE. This was despite the fact that DNCB failed to elicit any pathologic response in the nasal and pulmonary airways and did not elicit a cytokine response in the lung. Our report is not the first to demonstrate that DNCB elicits an IgE response. Others have shown that DNCB elicits an increase in serum IgE while failing to sensitize the respiratory tract (Ban et al, 2001). The IgE data from this study has two opposing implications. One is that serum IgE levels is not a good biomarker of LMW chemical-induced allergic airway disease as non-sensitizers of the respiratory tract (i.e., DNCB) induce increases in serum IgE. There is data that supports this possibility. One group showed that IgE levels are inversely correlated with LMW chemical-induced airway inflammation in a murine model of LMW chemical-induced asthma (Herrick et al, 2002). Another implication is that DNCB, unlike most non-sensitizers of the respiratory tract, has a unique capacity to elicit an IgE response despite its inability to induce airway inflammation. This possibility is supported by the finding that intranasal sensitization and challenge with another non-sensitizer of the respiratory tract, OXA, failed to induce an increase in serum IgE. The exact role of IgE antibodies in this study is unclear. Further studies with other LMW chemical respiratory sensitizers and non-sensitizers need to be conducted in order to determine the relevance of enhanced circulating IgE levels in LMW chemical-induced allergic airway disease.

Despite the fact that a pathologic response was not elicited in the pulmonary airways after intranasal instillation, this model of allergen-induced

effects in the nasal airway may be used to assess the allergenic effects of a chemical agent on the entire respiratory tract, including the pulmonary airways. Allergic rhinitis and asthma used to be thought of as separate disease entities, but there is increasing support for the concept of "one airway, one disease," i.e., that both these diseases are really a "continuum of inflammation within one airway (Grossman, 1997)." Several lines of evidence support this theory and the use of this model of nasal airway changes as a concurrent model to assess the allergenic effects of chemicals in the lower airways. Epidemiologic data suggests that allergic rhinitis and asthma coexist. In one study, it was found that 92 % of subjects with occupational asthma experienced symptoms of rhinitis (Leynaert et al, 2000). In addition to their association, allergic rhinitis and asthma share pathologic characteristics including the fact that they are both immediate type hypersensitivity reactions characterized by the infiltration of mast cells, eosinophils, and lymphocytes into the airways and mucus hypersecretion and both are elicited by the same inflammatory mediators such as histamine (WHO, 1999). In addition, the airway epithelia lining the nasal and pulmonary airways are similar. Both the nasal and pulmonary airways contain respiratory epithelium that includes ciliated cells and mucous cells that respond similarly to allergen exposure (Harkema et al, 2000). Also, a common therapeutic approach is used in the treatment of both diseases. For example, inhaled corticosteroids are used to ameliorate symptoms of both allergic rhinitis and asthma (Grossman, 1997).

The present study illustrated that intranasal sensitization and challenge with TMA, but not DNCB or OXA, was effective in generating some of the

hallmark pathologic features of allergic airway disease in the nasal airways of mice. In addition, the TMA-induced allergic rhinitis was accompanied by local increases in Th2-specific cytokine mRNA within the nasal airway. The analysis of local cytokine gene expression in the nasal airway after intranasal exposure may prove useful in the identification of other allergenic chemicals.

CHAPTER 6

SUMMARY AND CONCLUSIONS

The driving hypothesis for this dissertation was that local Th2 cytokine gene expression in the nasal and/or pulmonary airways may be used to identify chemicals with the potential to elicit a Type I allergic response in the respiratory tract of humans. This main hypothesis was tested with a number of studies that used an in vivo approach utilizing a murine model of allergic airway disease to examine the histopathologic and immunologic responses to known chemical respiratory sensitizers and non-sensitizers after intra-airway administration.

Results from Chapter 2 defined a novel murine model of human allergic airway disease that resembled human exposure to aeroallergens and was the first to demonstrate that intranasal instillation is an effective method of sensitization to aeroallergens. Intranasal sensitization and challenge with adjuvant-free ovalbumin in A/J mice elicited many of the hallmark pathologic and immunologic features of IgE-mediated allergic rhinitis and asthma in humans. The pathologic changes in the pulmonary airways including mucous cell metaplasia and a lymphocytic and eosinophilic inflammatory infiltrate correlated with an increase in lung-derived Th2 cytokine gene expression and increased serum IgE. Taken together, the data support the hypothesis that intranasal sensitization and challenge is effective in exposing the upper and lower respiratory tract of mice to protein agents and generating the characteristic features of allergic airway disease. Also, the pathologic response was likely mediated by increased Th2 cytokine gene expression. Increased Th2 cytokine gene expression may be used to predict the respiratory allergenicity of other

proteins and chemicals in this model. This murine model is a sensitive model for determining the allergenicity of protein agents.

One of the benefits of having a sensitive murine model of allergic airway disease that responds robustly to aeroallergen exposure is that the effects of pharamacologic agents on the allergic response can be readily determined. Prior murine studies demonstrated successful attenuation of several features of the allergic airway response by IP injection of CBN, but no study has reported inhibition by intra-airway delivery of CBN. Intra-airway delivery of CBN would minimize systemic toxicity and ensure a high local concentration of CBN in the respiratory tract, which would likely increase its effectiveness. In Chapter 3, I designed a study that tested the hypothesis that both IP and intranasal administration of CBN in A/J mice would attenuate the hallmark pathologic and immunologic features of allergic airway disease. Results from Chapter 3, support the hypothesis that the systemic administration of CBN attenuates the characteristic pathologic and immunologic features of allergic airway disease elicited by intranasal sensitization and challenge with ovalbumin in A/J mice. IP injection of CBN suppressed the OVA-induced increase in intraepithelial mucus (IM) in the pulmonary airways, serum IgE, and lung-derived IL-4 mRNA expression. The intranasal delivery of CBN, however, wasn't as effective as IP CBN in attenuating the allergic airway response. Despite inhibition of total and antigen-specific IgE, the number of inflammatory cells in the BALF, and lung-derived IL-13 mRNA expression, intranasal CBN did not affect the ovalbumin-induced increase in IM in the pulmonary airways or the lung-derived increase in

IL-4, IL-5, and IL-10 mRNA expression. The failure to affect the IM and cytokine responses in the lung may have been due to the inability of the intranasal route of administration to result in sufficient distribution of CBN into the lung. This suggests that improved lung distribution using intranasal instillation will enhance intranasal CBN instillation as a method of inhibiting the pulmonary allergic response.

IP CBN-mediated inhibition of the lung-derived increase in IL-4 expression further supports the data presented in Chapter 2 linking the ovalbumin-induced pathologic changes to enhanced Th2 cytokine expression and suggests that Th2 cytokines are required for the elicitation of the pathologic changes in this model. This finding also suggests that a chemical that causes a local increase in Th2 cytokine expression within the airways, as in the studies described in Chapters 4 and 5 of this dissertation, may have allergenic potential in the respiratory tract.

CBN has potential therapeutic/prophylactic utility for allergic diseases of the respiratory tract because of its immunosuppressive effects and data from human and animal studies that suggest an inhibition of some features of the allergic airway response. The development of CBN as a therapeutic/prophylactic to be used by humans requires that CBN be delivered via the intra-airway route to avoid the debilitating side effects that may result from systemic administration. Despite the failure to inhibit the ovalbumin-induced pathologic and cytokine responses in the lung, this is the first study to demonstrate successful inhibition of some features of the allergic airway response by intra-airway delivery of CBN. This study has thus narrowed the gap in the development of CBN as a

therapeutic by demonstrating that intra-airway delivery of CBN may potentially be a viable alternative to systemic administration of CBN. Improved distribution of CBN to the lower respiratory tract by intra-airway delivery and the corresponding inhibition of the pulmonary allergic airway response will further enhance the development of CBN as a human therapeutic to combat allergic airway disease.

Although, CBN appeared not to distribute effectively into the lung using an EA/OO vehicle, it likely distributed in a sufficient amount to the upper airways including the nasal airway. This finding suggests that intranasal instillation of CBN may be an effective method of attenuating the pathologic and immunologic features of allergic rhinitis. The studies with TMA demonstrated that intranasal sensitization and challenge with TMA was effective in generating the characteristic features of allergic rhinitis. The co-treatment of TMA sensitized and challenge mice with intranasal instillations of CBN would be a good way of determining whether CBN is effective in the inhibition of allergic rhinitis.

The results described in Chapter 2, i.e., successful induction of features of allergic airway disease with intranasal instillation of a protein allergen, presented a model with which to determine the effects of LMW chemicals on the respiratory tract of A/J mice. This study is the first to demonstrate that intranasal administration of an LMW chemical is an effective method of sensitization and results in an up-regulation of critical mediators of allergic airway disease, IL-4, IL-5, and IL-13 within the lung after challenge. The study described in Chapter 4 was designed to test the hypothesis that intranasal sensitization and challenge with TDI will induce the immunologic and pathologic responses in the lung that

are characteristic of LMW chemical-induced occupational asthma. Intranasal sensitization and challenge with TDI elicited some of the characteristic pathologic features of TDI-induced allergic rhinitis, thus supporting the hypothesis. TDI sensitized and challenged mice had an infiltration of plasma cells in the nasal airway, a characteristic pathologic feature of immune-mediated TDI-induced rhinitis. The tremendous irritant effects of the chemical were more conspicuous, however, as all TDI-exposed mice, whether TDI-sensitized or not, had an acute rhinitis with associated epithelial necrosis and exfoliation. This hampered the discernment of the immune-mediated effects suggesting that a less irritating dose of TDI should have been used. The irritant effect was also due to the vehicle 1:1 EA/OO, suggesting that a less irritating vehicle such as 1:4 EA/OO would have been more suitable. In addition, no pulmonary lesions were found in mice of any group. The absence of pulmonary lesions does not support the hypothesis as intranasal instillation with TDI failed to elicit the pathologic features of allergic airway disease in the lung. The absence of pulmonary lesions is likely due to the limited lung distribution of the chemical due to the viscosity of the vehicle, as in the intranasal CBN study and the high reactivity of the chemical, which may have predominantly bound to proteins in the upper airways. Also, only mice that were intranasally sensitized and challenged to TDI had an increase in lung-derived IL-4, IL-5, and IL-13 mRNA and total serum IgE. The lung-derived increase in Th2 cytokine gene expression could not be correlated with a pathologic response in the lung as intranasal instillation of TDI did not result in any pulmonary lesions leaving uncertainty about the source of these cytokines within the lung.

The results from the study described in Chapter 4 with TDI led me to change the vehicle combination that was used to dissolve the low molecular weight chemicals that were used in the study described in Chapter 5. EA/OO was modified from a 1:1 combination that was irritating to the airway epithelium to a non-irritating 1:4 EA/OO combination. In addition, the volume that was instilled was doubled from 30 to 60 μl in an attempt to improve lung distribution of the chemical and concentrations of the chemicals used in the study were not irritating to the airway epithelium as in the TDI study. The study in Chapter 5 compared the effects of a known chemical respiratory allergen TMA to two contact allergens. The study tested the hypothesis that intranasal sensitization and challenge with TMA, but not the contact allergens DNCB and OXA, will induce the immunologic and pathologic responses characteristic of LMW chemical-induced allergic airway disease in the nasal and pulmonary airways. The strong nasal airway response with TDI in the study described in Chapter 4 convinced me to assess the nasal airway after TMA, DNCB, and OXA exposure for Th2 and Th1 cytokine expression. The study described in Chapter 5 is the first to demonstrate that the intranasal administration of the known chemical respiratory allergen TMA, but not the chemical contact allergens DNCB and OXA, was an effective method of sensitization resulting in the hallmark pathologic features of allergic rhinitis with a concomitant increase in Th2 cytokine mRNA expression within the nasal airway, thus supporting the hypothesis. The absence of an airway response to DNCB and OXA is consistent with other experimental models of allergic airway disease. Only mice that were intranasally

sensitized and challenged with TMA had a marked allergic rhinitis characterized by an influx of eosinophils, lymphocytes and plasma cells and increased stored mucus. The TMA-induced nasal airway pathology correlated with a local increase in Th2 cytokines and not Th1 cytokine expression and an increase in serum IgE. This is consistent with data from the study described in Chapter 2 where the ovalbumin-induced pathologic changes in the lung correlated with a local increase in lung-derived Th2 cytokine gene expression. The results from both studies support the use of Th2 cytokine gene expression as a predictor of the potential of chemicals to elicit allergic airway disease. However, no pulmonary lesions were found in any group treated with TMA. This does not support the hypothesis as intranasal instillation with TMA failed to elicit the pathologic features of allergic airway disease in the lung. The absence of pulmonary lesions is likely due to the limited lung distribution of the chemical due to the viscosity of the vehicle, as in the CBN and TDI studies, and the high reactivity of the chemical. DNCB and OXA did not induce any nasal or pulmonary airway lesions or increases in Th2 cytokine gene expression in the pulmonary airways. DNCB sensitization and challenge did, however, elicit an increase in serum IgE. The importance of the increase in serum IgE with DNCB remains to be determined, and may undervalue the use of serum IgE levels to identify chemical respiratory allergens. TMA sensitized and challenged mice exhibited an increase in lung-derived IL-5 mRNA expression. There were no changes in the mRNA expression of any of the cytokines measured in the lungs of DNCB or OXA treated mice. The lung-derived increase in Th2 cytokine gene

expression could not be correlated with a pathologic response in the lung as the intranasal instillation of TMA did not result in any pulmonary lesions leaving uncertainty about the source of these cytokines within the lung, as in the TDI study.

Despite the fact that a pathologic response was not elicited in the pulmonary airways after intranasal instillation, this model of allergen-induced effects in the nasal airway may be used to assess the allergenic effects of a chemical agent on the entire respiratory tract, including the pulmonary airways. A number of facts support the use of chemical-induced nasal airway effects as a surrogate for and accurate predictor of the allergenic effects of the chemical in the pulmonary airways. For example, Individuals afflicted with asthma usually suffer from concurrent allergic rhinitis, especially in occupational settings. Allergic rhinitis and asthma also share similar pathologic features and are initiated by similar inflammatory mediators. In addition, the nasal airway and pulmonary airways share similar structural features including similar respiratory epithelium.

There is a need for identifying LMW chemical respiratory sensitizers. There is currently no well validated and widely accepted method for identifying LMWC respiratory allergens. Such a method would ideally be analogous to the murine Local Lymph Node Assay (LLNA) developed by Kimber and colleagues, 1997, which is a reliable method for the identification of chemicals that have the ability to cause skin sensitization and allergic contact dermatitis. The LLNA is considered a 'stand-alone method' for skin sensitization hazard identification.

The studies presented in this dissertation collectively provide two major findings. The first is that intranasal sensitization and challenge of protein or chemical allergens successfully induces many of the characteristic features of allergic airway disease. The second is that Th2 cytokine gene expression and potentially serum IgE levels may be used to predict the potential of protein or chemical agents to elicit allergic airway disease. These findings may facilitate the identification of LMW chemical respiratory allergens.

The data from this dissertation show that the intranasal administration of LMW chemicals is a viable route of exposure for testing the allergenicity of LMW chemicals in the upper and lower airways if coupled with measurements within the nasal airways including the analysis of Th2 cytokine gene expression and histopathology. These measurements should be combined with the use of a murine strain sensitive to aeroallergens such as the A/J strain to provide a reliable and sensitive method for identifying LMW chemical respiratory allergens. This method uses a simple and inexpensive method of allergen administration, i.e., intranasal instillation, which is a relevant route of administration. It also focuses on changes within the respiratory tract and assesses a very sensitive endpoint, i.e., cytokine gene expression, which is critical to the development of allergic airway disease. These facts make this method a practical, inexpensive, safe, and reliable alternative for identifying LMW chemical respiratory allergens that may be used by chemical and pharmaceutical manufacturers and others to facilitate the screening of chemical agents with unknown potential for inducing allergic airway responses such as asthma and allergic rhinitis.

BIBLIOGRAPHY

Adamko DJ, Yost BY, Gleich GJ, Fryer AD, and Jacoby DB (1999). Ovalbumin Sensitization Changes the Inflammatory Response to Subsequent Parainfluenza Infection: Eosinophils Mediate Airway Hyperresponsiveness, M_2 Muscarinic Receptor Dysfunction, and Antiviral Effects. *J Exp Med.* 190(10):1465-77.

Andersson M, Greiff L, Svensson C and Persson CGA (2000). Allergic and non-allergic rhinitis. In: Busse WW and Holgate ST: *Asthma and Rhinitis*, Volume 1 Second Edition. Blackwell Science Ltd., Osney Meade, Oxford, pp. 232-244.

Arts JH, Kuper CF, Spoor SM, and Bloksma N (1998). Airway morphology and function of rats following dermal sensitization and respiratory challenge with low molecular weight chemicals. *Toxicol Appl Pharmacol.* 152(1):66-76.

Ban M, and Hettich D (2001). Relationship between IgE positive cell numbers and serum total IgE levels in mice treated with trimellitic anhydride and dinitrochlorobenzene. *Toxicol Lett.* 118:129-137.

Beach JR (2000). Immunologic Versus Toxicologic Mechanisms in Airway Responses. In: *Occupational Medicine: State of the Art Reviews,* Volume 15 No. 2 Hanley and Belfus, Inc., Philadelphia, pp. 455-469.

Bellinghausen I, Knop J, and Saloga J (2001). The Role of Interleukin 10 in the Regulation of Allergic Immune Responses. *Int. Arch. Allergy Immunol.* 126:97-101.

Berdyshev EV (2000). Cannabinoid receptors and the regulation of immune response. *Chem Phys Lipids.* 108:169-190.

Bernstein DI, Cartier A, Cote J, Malo J-L, Boulet L-P, Wanner M, Milot J, L'Archeveque J, Trudeau C, and Lummus Z (2002). Diisocyanate Antigen-stimulated Monocyte Chemoattractant Protein-1 Synthesis Has Greater Test Efficiency then Specific Antibodies for Identification of Diisocyanate Asthma. *Am J Respir Crit Care Med.* 166:445-450.

Bernstein IL, and Brooks SM (1993). Metals, in Bernstein IL, Chan-Yeung M, Malo JL, & Bernstien DI (Eds) *Asthma in the Workplace*, New York: Marcel Dekker, pp.459-479.

Betts CJ, Dearman RJ, Flanagan BF, and Kimber I (2002). Temporal Changes in cytokine gene expression profiles induced in mice by trimellitic anhydride. *Toxicol Lett.* 136:121-132.

Blaikie L, and Basketter DA (1999). Experience with a Mouse Intranasal Test for the Predictive Identification of Respiratory Sensitization Potential of Proteins. *Food and Chemical Toxicology.* 37: 889-896.

Bruijnzeel-Koomen CA, Geert M and Holt P (2000). Antigen Presentation and IgE Synthesis, in Holgate ST, Church MK, and Lichtenstein LM (2nd Ed) *Allergy*, London:Mosby, pp. 265-281.

Condie R, Herring A, Koh WS, Lee M, and Kaminski NE (1996). Cannabinoid inhibition of adenylate cyclase-mediated signal transduction and interleukin 2 (IL-2) expression in the murine T-cell line, EL4.IL-2. *J. Biol. Chem.* 271(22): 13175-83.

Corry DB, Folkesson HG, Warnock ML, Erle DJ, Matthay MA, Wiener-Kronish JP, and Locksley RM (1996). Interleukin-4, but not Interleukin-5 or eoinophils, is required in a murine model of acute airway hyperreactivity. *J Exp Med.* 183:109-17.

Day BW, Jin R, Basalyga DM, Kramarik JA, and Karol MH (1997). Formation, Solvolysis, and Transcarbamoylation Reactions of Bis(S-glutathionyl) Adducts of 2,4- and 2,6-Diisocyanatotoluene. *Chem Res Toxicol.* 10:424-431.

Dearman RJ, Betts CJ, Humphreys N, Flanagan BF, Gilmour NJ, Basketter DA, and Kimber I (2003). Chemical Allergy: Considerations for the Practical Application of Cytokine Profiling. *Toxicol Sci.* 71:137-145.

Dearman RJ, Basketter DA, Evans P and Kimber I (1999). Comparison of cytokine secretion profiles provoked in mice by glutaraldehyde and formaldehyde. *Clin Exp Allergy.* 29(1):124-32.

Dearman RJ, Basketter DA and Kimber I (1996). Characterization of Chemical Allergens as a Function of Divergent Cytokine Secretion Profiles Induced in Mice. *Toxicol Appl Pharmacol.* 138:308-16.

Dearman RJ, Filby A, Humphreys IR, and Kimber I (2002). Interleukins 5 and 13 Characterize Immune Responses to Respiratory Sensitizing Ahydrides. *J Appl Toxicol.* 22:317-325.

Dearman RJ and Kimber I (2001). Cytokine Fingerprinting and Hazard Assessment of Chemical Respiratory Allergy. *J Appl Toxicol.* 21:153-163.

Dearman RJ, Warbrick EV, Humphreys IR, and Kimber I (2000). Characterization in mice of the immunological properties of five allergenic acid anhydrides. *J Appl Toxicol.* 20(3):221-30.

Denzeler KL, Farmer SC, Crosby JR, Borchers M, Cieslewicz G, Larson KA, Cormier-Regard S, Lee NA, and Lee JJ (2000). Eosinophil Major Basic Protein-1 Does Not Contribute to Allergen-Induced Airway Pathologies in Mouse Models of Asthma. *J. Immunol.* 165: 5509-5517.

Deschamps F, Prevost A, Lavauds F, and Kochman S (1998). Mechanisms of Occupational Asthma Induced by Isocyanates. *Ann Occup Hyg.* 42(1): 33-36.

Ebino K, Lemus R, and Karol M (1999). The Importance of the Diluent For Airway Transport of Toluene Diisocyanate following Intranasal Dosing of Mice. *Inhalation Toxicology.* 11:171-185.

Foster PS, Ming Y, Matthei KI, Young IG, Temelkovski J, and Kumar RK (2000). Dissociation of Inflammatory and Epithelial Responses in a Murine Model of Chronic Asthma. *Lab Invest.* 80:655-62.

Farraj AK, Harkema JR, Jan T-R, and Kaminski NE (2003). Immune Responses in the Lung and Local Lymph Node of A/J Mice to Intranasal Sensitization and Challenge with Adjuvant-Free Ovalbumin. *Toxicol Path.* 31:5.

Foster PS, Ming Y, Matthei KI, Young IG, Temelkovski J, and Kumar RK (2000). Dissociation of Inflammatory and Epithelial Responses in a Murine Model of Chronic Asthma. *Lab. Invest.* 80: 655-62.

Frew AJ (1996). The immunology of respiratory allergies. *Toxicol Lett.* 86:65-72.

Gelfand EW (2002). Mice are a Good Model of Human Airway Disease. *Am J Respir Crit Care Med.* 166:5-8.

Gilliland G, Perrin S, Blanchard K, and Bunn HF (1990). Analysis of cytokine mRNA and DNA: detection and quantitation by competitive polymerase chain reaction. *Proc Natl Acad Sci USA.* 87(7): 2725-9.

Grammer LC, Ditto AM, Tripathi A, and Harris KE (2002). Prevalence and onset of rhinitis in subjects with occupational asthma caused by trimellitic anhydride. *J Occup Environ Med.* 44(12):1179-81.

Greenberg, MM (1994). Isocyanates and nasal toxicity: Risk assessment issues. *Inhalation Toxicology.* 6(Suppl):458-464.

Griffin P, Allan L, Beckett P, Elms J and Curran AD (2001). The development of antibodyto trimellitic anhydride. *Clin Exp Allergy.* 31:453-457.

Grossman J (1997). One Airway, One Disease. *Chest.* 111:11S-16S.

Grunig G, Warnock M, Wakil AE, Venkayaa R, Brombacher F, Rennick DM, Sheppard D, Mohrs M, Donaldson DD, Locksley RM, Corry DB (1998). Requirement of IL-13 Independently of IL-4 in Experimental Asthma. *Science* 282:2261-62.

Hamelman E, Oshiba A, Loader J, Larsen GL, Gleich G, Lee J, and Gelfand EW (1997). Antiinterleukin-5 Antibody Prevents Airway Hyperresponsiveness in a Murine Model of Airway Sensitization. *Am J. Respir Crit Care Med.* 155:819-825.

Hardisty JF, Garman RH, Harkema JR, Lomax LG and Morgan KT (1999). Histopathology of Nasal Olfactory Mucosa from Selected Inhalation Toxicity Studies Conducted with Volatile Chemicals. Toxic Pathology. 27(6):618-627.

Harkema JR, Plopper CG, Hyde DM, St. George JA, and Dungworth DL (1987). Effects of an ambient level of ozone on primate nasal epithelial mucosubstances. Quantitative histochemistry. *Am J Pathol.* 127: 90-96.

Harkema JR, Plopper CG and Pinkerton KE (2000). Comparative Structure of The Respiratory Tract: Airway Architecture in Humans and Animals. In: Cohen MD, Zelikoff JT and Schlesinger RB, *Pulmonary Immunotoxicology*, Kluner Academic Press Publishers, Boston. Chapter 1:1-59.

Hayashi M, Higashi K, Kato H, and Kaneko H (2001). Assessment of preferential Th1 or Th2 induction by low-molecular-weight compounds using a reverse transcription-polymerase chain reaction method: comparison of two mouse strains, C57BL/6 and BALB/c. *Toxicol Appl Pharmacol.* 177(1):38-45.

Herrick CA, Xu L, Wisnewski AV, Das J, Redlich CA, and Bottomly K (2002). A novel mouse model of diisocyanate-induced asthma showing allergic-type inflammation in the lung after inhaled antigen challenge. *J Allergy and Clin Immunol.* 109:873-8.

Herring AC and Kaminski NE (1999). Cannabinol-Mediated Inhibition of Nuclear Factor-κB, cAMP Response Element-Binding Protein, and Interleukin-2 Secretion by Activated Thymocytes. *J Pharmacol Exp Ther.* 291(3):1156-1163.

Herring AC, Koh WS, and Kaminski NE (1998). Inhibition of the Cyclic AMP Signaling Cascade and Nuclear Factor Binding to CRE and κB Elements by Cannabinol, a Minimally CNS-Active Cannabinoid. *Biochemical Pharmacol.* 55:1013-1023.

Holgate ST (2000). Inflammatory and structural changes in the airways of patients with asthma. *Resp Med.* 94(Suppl D):S3-S6.

Jagannath C, Hoffman H, Sepulveda E, Actor JK, Wetsel RA, and Hunter JR RL (2000). Hypersusceptibility of A/J Mice to Tuberculosis is in Part Due to a Deficiency of the Fifth Complement Component (C5). *Scand J Immunol.* 52:369-379.

Jajosky RA, Harrison R, Reinisch F, Flattery J, Chan J, Tumpowsky C, Davis L, Reilly MJ, Rosenman KD, Kalinowski D, Stanbury M, Schill DP, and Wood J

(1999). Surveillance of work-related asthma in selected U.S. states using surveillance guidelines for state health departments--California, Massachusetts, Michigan, and New Jersey, 1993-1995. *Morb Mortal Wkly Rep Surveill Summer.* 48(3):1-20 (1999).

Jan T-R, and Kaminski NE (2002). Cannabinol Inhibition of interleukin-4 expression in EL4.IL-2 cells is mediated through down-regulation of NF-AT. *Toxicologist.* 54:57.

Jan T-R, Farraj AK, Harkema JR, and Kaminski NE (2003). Attenuation of the ovalbumin-induced allergic airway response by cannabinoid treatment in A/J mice. *Toxicol Appl Pharmacol.* 188:24-35.

Jan T-R, Rao GK and Kaminski NE (2002). Cannabinol Enhancement of Interleukin-2 (IL-2) Expression by T Cells is Associated with an Increase in IL-2 distal Nuclear Factor of Activated T Cell Activity. *Molec Pharmacol.* 61(2):446-454.

Karol MH (2001). Bonding and transfer: do epithelial conjugates have a role in chemical asthma? *Clin Exp Allergy.* 31:357-360.

Karol MH, Macina OT, and Cunningham A (2001). Cell and Molecular biology of chemical allergy. *Ann Allergy Asthma Immunol.* 87(Suppl):28-32.

Karras JG, McGraw K, McKay, Cooper SR, Lerner D, Lu T, Walker C, Dean NM, and Monia BP (2000). Inhibition of antigen-induced eosinophilia and late phase hyperresponsiveness by an IL-5 antisense oligonucleotide in mouse models of asthma. *J. Immunol.* 164: 5409-15.

Kay AB (2001). Allergy and Allergic Diseases. *N Engl J Med.* 344(1): 30-37.

Kimber I (1996). The role of the skin in the development of chemical respiratory hypersensitivity. *Toxicol Lett.* 86:89-92.

Kimber I and Dearman R (1997). Predicitve Assessment of Respiratory Sensitizing Potential of Chemicals in Mice. In: Kimber I and Dearman RJ, *Toxicology of Chemical Respiratory Hypersensitivity.* Taylor and Francis Limited, Bristol, PA, pp. 121-134.

Kimber I, and Dearman RJ (2002). Chemical Respiratory Allergy: role of IgE antibody and relevance of route of exposure. *Toxicology.* 181-182:311-315.

Kraneveld AD, van der Kleij HPM, Kool M, van Houwelingen AH, Weitenberg ACD, Redegeld FAM, and Nijkamp FP (2002). Key Role for Mast Cells in Nonatopic Asthma. *J Immunol.* 169:2044-2053.

Lange RW, Day BW, Lemus R, Tyurin VA, Kagan VE, and Karol MH (1999) Intracellular S-Glutathionyl Adducts in Murine Lung and Human Bronchoepithelial Cells after Exposure to Diisocyanatotoluene. *Chem Res Toxicol.* 12:931-936.

Lange RW, Lantz RC, Stolz DB, Watkins SC, Sundareshan P, Lemus R and Karol MH (1999). Toluene Diisocyanate Colocalizes with Tubulin on Cilia of Differentiated Human Airway Epithelial Cells. *Toxicol Sci.* 50:64-71.

Larsen CP, Regal RR, and Regal JF (2001). Trimellitic Anhydride-Induced Allergic Response in the Guinea Pig Involves Antibody-Dependent and -Independent Complement System Activation. *J Pharm Exper Ther.* 296(2):284-292.

Lee CG, Homer RJ, Cohn L, Link H, Jung S, Craft JE, Graham BS, Johnson TR, and Elias JA (2002). Transgenic Overexpression of Interleukin (IL)-10 in the Lung Causes Mucus Metaplasia, Tissue inflammation, and Airway Remodeling via IL-13-dependent and –independent Pathways. *J. Biol Chemistry.* 277(38):35466-35474.

Leynaert B, Neukirch F, Demoly P, and Bousquet J (2000). Epidemiologic evidence for asthma and rhinitis comorbidity. *J Allergy Clin Immunol.* 106:S201-5.

Lombardo LJ and Balmes JR (2000). Occupational Asthma: A Review. *Environ Health Persp.* 108(S4):697-704.

Longphre M, Li D, Gallup M, Drori E, Ordonez CL, Redman T, Wenzel S, Bice DE, Fahy JV, and Basbaum C (1999). Allergen-induced IL-9 directly stimulates mucin transcription in respiratory epithelial cells. *J Clin Invest.* 104(10):1375-82.

Lovik M, Dybing E, and Smith E (1996). Environmental Chemicals and respiratory hypersensitization: A synopsis. *Toxicology Letters.* 86:211-222.

Mackison FW, Stricoff RS and Partridge Jr. LJ (1981). NIOSH/OSHA Occupational Health Guidelines for Chemical Hazards. DHHS (NIOSH) Publication No. 81-123 (3 Vols). Washington, DC: U.S. Government Printing Office.

Maestrelli P, Occari P, Turato G, Papiris SA, Di Stefano A, Mapp CE, Milani GF, Fabbri LM, and Saetta M (1997). Expression of interleukin (IL)-4 and IL-5 proteins in asthma induced by toluene diisocyanate (TDI). *Clin Exp Allergy.* 27(11)1292-8.

Makela MJ, Kanehiro A, Borish L, Dakhama A, Loader, J, Joetham A, Xing Z, Jordana M, Larsen GL, and Gelfand EW (2000). IL-10 is necessary for the

expression of airway hyperresponsiveness but not pulmonary inflammation after allergic sensitization. *Proc Natl Acad Sci USA*. 97(11): 6007-6012.

Malo J-L and Chan-Yeung M (2001). Occupational asthma. *J Allergy and Clin Immunol*. 108:317-328.

Matheson JM, Lange RW, Lemus R, Karol MH, and Luster MI (2001). Importance of inflammatory and immune components in a mouse model of airway reactivity to toluene diisocyanate (TDI). *Clin Exp Allergy*. 31:1067-1076.

Matsuda LA, Lolait SJ, Brownstein MJ, Young AC, and Bonner TI (1990). Structure of a cannabinoid receptor and functional expression of the cloned cDNA. *Nature*. 346:561-564.

Meyer JD, Holt DL, Chen Y, Cherry NM, and McDonald JC (2001). Sword '99:surveillance of work-related and occupational respiratory disease in the UK. *Occup Med (Lond)*. 51(3):204-208.

Munro S, Thomas KL, and Abu-Shaar M (1993). Molecular characterization of a peripheral receptor for cannabinoids. *Nature*. 365:61-65.

Occupational Safety and Health Administration (1993). OSHA General Industry Standard (29 CFR 1910.1000). Tables Z-1, Z-2 and Z-3.

Okano M, Nishizaki K, Abe M, Wang M-M, Yoshino T, Satoskar AR, Masuda Y, and Harn Jr. DA (1999). Strain-dependent induction of allergic rhinitis without adjuvant in mice. *Allergy*. 54: 593-60.

Peterson JD, Herzenberg LA, Vasquez K, Waltenbaugh C (1998). Glutathione levels in antigen-presenting cells modulate Th1 versus Th2 response patterns. *Proc Natl Acad Sci USA*. 95:3071-3076.

Petsonk EL (2002). Work-Related Asthma and Implications for the General Public. *Environ Health Perspect*. 110 Suppl 4:569-72.

Plitnick LM, Loveless SE, Ladics GS, Holsapple MP, Selgrade MJ, Sailstad DM, and Smialowicz RJ (2002). Cytokine Profiling for Chemical Sensitizers: Application of the Ribonuclease Protection Assay and Effect of Dose. *Toxicol Appl Pharm*. 179:145-154.

Rajananthanan P, Attard GS, Sheikh NA, and Morrow WJW (1999). Evaluation of novel aggregate structures as adjuvants: composition, toxicity studies and humoral responses. *Vaccine*. 17: 715-30.

Regal JF, Mohrman ME, and Sailstad DM (2001). Trimellitic Anhydride-Induced Eosinophilia in a Mouse Model of Occupational Asthma. *Toxicol Appl Pharm.* 175:234-242.

Robinson MK, Babcock LS, Horn PA, and Kawabata TT (1996). Specific Antibody Responses to Subtilisin Carslberg (Alcalase) in Mice: Development of an Intranasal Exposure Model. *Fundam Appl Toxicol.* 34: 15-24.

Satoh T, Kramarik JA, Tollerud DJ, and Karol MH (1995). A murine model fro assessing the respiratory hypersensitivity potential of chemical allergens. *Toxicol Lett.* 78(1):57-66.

Schatz AR, Lee M, Condie RB, Pulaski JT and Kaminksi NE (1997). Cannabinoid receptors CB1 and CB2: A Characterization of Expression and Adenylate Cyclase Modulation within the Immune System. *Toxicol Appl Pharmacol.* 142:278-287.

Scheerens H, Buckley TL, Muis TL, Garssen J, Dormans J, Nijkamp FP and Van Loveren H (1999). Long-term Topical Exposure to Toluene Diisocyanate in Mice Leads to Antibody Production and In Vivo Airway Hyper-responsiveness Three Hours after Intranasal Challenge. *Am J Respir Crit Care Med.* 159(4) 1074-80.

Serafin WE (1996). Drugs Used In The Treatment of Ashtma, in Hardman JG and Limbird LE (9th Ed) *Goodman and Gilman's The Pharmacological Basis of Therapeutics*, New York: McGraw-Hill, pp. 6

Shen H, Liu J, Liu F, Wang X, Lin X, Xu z Z, Huang G, and Yuan S (2002). The observation of local immune response in the lungs from antigen-sensitized and challenged mice. (article in Chinese) *Zhonghua Jie He HE Hu Xi Za Zhi.* 25(3):156-158

Shim JJ, Dabbagh K, Ueki IF, Dao-Pick T, Burgel P-R, Takeyama K, Tam DC-W, and Nadel JA (2001). IL-13 induces mucin production by stimulating epidermal growth factor receptors and by activating neutrophils. *Am J Physiol Lung Cell Mol Physiol.* 280:L134-L140.

Southam DS, Dolovich M, O'Byrne PM and Inman MD (2002). Distribution of intranasal instillations in mice: effects of volume, time, body position, and anesthesia. *Am J Physiol Lung Cell Mol Physiol.* 282:L833-L839.

Stampfli MR, Wiley RE, Neigh GS, Gajewska BU, Lei X-F, Snider DP, Xing Z, and Jordana M (1998). GM-CSF Transgene Expression in the Airways Allows Aerosolized Ovalbumin to Induce Allergic Sensitization in Mice. *J Clin Invest.* 102(9): 1704-1714.

Steiger D, Hotchkiss J, Baja L, Harkema J, and Basbaum C (1995). Concurrent increases in the storage and release of mucin-like molecules by rat airway

epithelial cells in response to bacterial endotoxin. *Am J Respir Cell Mol Biol.* 12: 307-314.

Sugawara Y, Okamoto Y, Sawahata T and Tanaka K (1993). An Asthma Model Developed in the Guinea Pig by Intranasal Application of 2,4-Toluene Diisocyanate. *Int Arch Allergy Immunol.* 101:95-101.

Tashkin DP, Shapiro BJ and Frank IM (1974). Acute effects of smoked marijuana and oral delta9-tetrahydrocannabinol on specific airway conductance in asthmatic subjects. *Am Rev Respir Dis.* 109:420-428.

Tashkin DP, Shapiro BJ, Lee YE, and Harper CE (1975). Effects of smoked marijuana in experimentally induced asthma. *Am Rev Respir Dis.* 112:377-386.

Tee RD, Cullinan P, Welch J, Burge PS, and Newman-Taylor AJ (1998). Specific IgE to isocyanates: A useful diagnostic role in occupational asthma. *J Allergy Clin Immunol.* 101:709-15.

Traidl C, Jugert F, Krieg T, Merk H and Hunzelmann N (1999). Inhibition of Allergic Contact Dermatitis to DNCB But Not to Oxazolone in Interleukin-4 Deficient Mice. *J Invest Dermatol.* 112:476-482.

Ulrich P, Grenet O, Bluemel J, and Vohr HW (2001). Cytokine expression profiles during murine contact allergy: T helper 2 cytokines are expressed irrespective of the type of contact allergen. *Arch Toxicol.* 75:470-479.

van Houwelingen AH, Kool M, de Jager SCA, Redegeld FAM, van Heuven-Nolson D, Kraneveld AD and Nijkamp FP (2002). Mast Cell-Derived TNF-α Primes Sensory Nerve Endings in a Pulmonary Hypersensitivity Reaction. *J Immunol.* 168:5297-5302.

Vandebriel R, De Jong WH, Spiekstra SW, Van Dijk M, Fluitman A, Garssen J, and Van Loveren H (2000). Assessment of Preferential T-Helper 1 or T-Helper 2 Induction by Low Molecular Weight Compounds Using the Local Lymph Node Assay in Conjunction with RT-PCR and ELISA for Interferon-γ and Interleukin-4. *Toxicol Appl Pharm.* 162:77-85.

Vanden Heuvel JP, Clark GC, Thompson CL, McCoy Z, Miller CR, Lucie GW, and Bell DA (1993). CYP1A1 mRNA levels as a human exposure biomarker: use of quantitative polymerase chain reaction to measure CYP1A1 expression in human peripheral blood lymphocytes. *Carcinogenesis.* 14(10): 2003-6.

van de Rijn M, Mehlhop PD, Judkins A, Rothenberg ME, Luster AD, and Oettgen HC (1998). A murine model of allergic rhinitis: Studies on the role of IgE in pathogenesis and analysis of the eosinophil influx elicited by allergen and eotaxin. *J Allergy Clin Immunol.* 102: 65-74.

Vohr H-W, Pauluhn J, and Ahr HJ (2002). Respiratory hypersensitivity to trimellitic anhydride in Brown Norway rats: evidence for different activation pattern of immune cells following topical and respiratory induction. *Arch Toxicol.* 76:538-544.

Warbrick EV, Dearman RJ, Basketter DA and Kimber I (1999). Analysis of interleukin 12 protein production and mRNA expression in mice exposed topically to chemical allergens. *Toxicology.* 132(1):57-66.

Weissman DN, and Lewis DM. Is Specific Antibody Determination Diagnostic For Asthma Attributable to Low-Molecular-Weight Agents? *Occupational Medicine: State of the Art Reviews.* Vol. 15, No. 2, April–June, 2000, pp. 385-397. Philadelphia, Hanley, & Belfus, Inc.

Wills-Karp M (1999). Immunologic Basis of Antigen-Induced Airway Hyper-responsiveness. *Annu. Rev. Immunol.* 17: 255-81.

Wills-Karp M (1999). Immunologic Basis of Antigen-Induced Airway Hyper-responsiveness. *Annu Rev Immunol.* 17:255-81.

Wills-Karp M, and Ewart SL (1997). The genetics of allergen-induced airway hyperresponsiveness in mice. *Am J Respir Crit Care Med.* 154(4 Pt 2):S89-96.

Wills-Karp M, Luyimbazi J, Xu X, Schofield B, Neben TY, Karp CL, and Donaldson DD (1998). Interleukin-13: Central Mediator of Allergic Asthma. *Science.* 282:2258-2261.

Wisnewski AV, Sirvastava R, Herick C, Xu L, Lemus R, Cain H, Magoski NM, Karol MH, Bottomly K, and Redlich CA (2000). Identification of Human Lung and Skin Proteins Conjugated with Hexamethylene Diisocyanate *In Vitro and In Vivo. Am J Respir Crit Care Med.* !62:2230-36.

Woolhiser MR, Munson AE, and Meade BJ (2000). Immunological Responses of Mice Following Administration of Natural Rubber Latex Proteins by Different Routes of Exposure. *Toxicological Sciences.* 55:343-351.

World Health Organization (1999). Clinical Aspects of the Most Important Allergic Diseases: Allergic Rhinitis and Conjunctivitis. *Principles and Methods for Assessing Allergic Hypersensitization Associated with Exposure to Chemicals, International Program on Chemical Safety, Environmental Health Criteria 212,* pp. 181-186. Geneva.

Yang X, Wang S, Fan Y, and Han X (2000). IL-10 deficiency prevents IL-5 overproduction and eosinophilic inflammation in a murine model of asthma-like reaction. *Eur J. Immunol.* 30(2): 382-91.

Yea SS, Yang KH and Kaminski NE (2000). Role of nuclear factor of activated T-cells and activator protein-1 in the inhibition of Interleukin-2 gene transcription by cannabinol in EL4 T-cells. *J Pharmacol Exp Ther.* 292:597-605.

Young JT (1981). Histopathologic examination of the rat nasal cavity. *Fundam Appl Toxicol.* 1: 309-312.

Yssel H, and Groux H (2000). Characterization of T Cell Subpopulations Involved in the Pathogenesis of Asthma and Allergic Diseases. *Int Arch Allergy Immunol.* 121: 10-18.

Zhou C, Crocker C, Koenig G, Romero,FA, Townley RG (1997). Anti-interleukin-4 Inhibits Immunoglobulin E Production in a Murine Model of Atopic Asthma. *J Asthma.* 34(3):195-201.