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THE ROLE OF ALVEOLAR MACROPHAGES IN THE DEVELOPMENT OF AIRWAY RESPONSIVENESS TO BRONCHOSPASTIC AGONISTS IN BROWN NORWAY RATS

by

BING ZHANG ©

A thesis submitted to the **Faculty of Graduate studies and Research** in partial fulfilment of the requirements for the degree of **MASTER OF SCIENCE**

In

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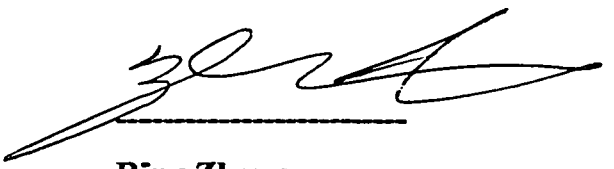

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **A ROLE OF ALVEOLAR MACROPHAGES IN THE DEVELOPMENT OF AIRWAY HYPERRESPONSIVENESS TO BRONCHOSPASTIC AGONISTS IN BROWN NORWAY RATS** submitted by **BING ZHANG** in partial fulfilment of the requirements of the degree of **MASTER OF SCIENCE** in **PHARMACEUTICAL SCIENCES**

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Abstract

We evaluated airway responsiveness to methacholine and serotonin in ovalbumin (OA)-immunized BN rats and controls. BN rats, sensitized to OA and adjuvants, developed airway hyperresponsiveness (AHR) after OA aerosol challenge. They showed three to five-fold increases in pulmonary flow resistance (R_L) compared with the adjuvant-injected controls ($p<0.05$). Statistically significant changes of pulmonary elastance (E_L) between the two groups have been observed ($p<0.05$).

We prepared liposome-encapsulated dichloromethylene diphosphonate (LE DMDP) with higher encapsulation efficiency than previously reported. We confirmed that more than 70% of AM were eliminated from the BN rats by treatment with liposomal DMDP compared to the normal controls. Neutrophil percentages transiently increased as AM were depleted.

We evaluated *in vivo* airway responsiveness in immunized and control rats using the AM-depleted model. The administration of LE DMDP leads to an influx of monocyte-like macrophages into airways as well as the elimination of resident AM. With the depletion of AM, immunized rats showed remarkably AHR to agonists relative to sham controls ($p<0.05$). The highest airway response in the AM-depleted and OA-immunized rats was observed at higher concentrations of the agonists injected; even higher than those in OA-immunized rats without AM depletion. These changes in hyperresponsiveness may result from functional dysregulation of AM in airways, which are perhaps exacerbated by the administration of LE DMDP. Based on the observations, we propose that depletion of AM leads to an imbalance of

functionally distinct macrophage subsets, which seem to play, at least in part, an important role in preventing the development of airway hyperresponsiveness. Increasing our understanding of AM regulation may allow us to increase their immunosuppressive activity in inflammatory responses, thereby preventing the development of secondary allergic responses.

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LIST OF ABBREVIATIONS AND SYMBOLS

APC	**Antigen presenting cells**
AHR	**Airway hyperresponsiveness**
AM	**Alveolar macrophages**
BAL	**Bronchoalveolar lavage**
BN	**Brown Norway**
BSA	**Bovine serum albumin**
DC	**Dendritic cells**
DMDP	**Dichloromethylene diphosphonate**
DMPC	**Dimyristylphosphatidylcholine**
DMPG	**Dimyristylphosphatidylglycerol**
EAR	**Early asthmatic response**
EE	**Encapsulation efficiency**
E_L	**Dynamic pulmonary elastance**
FcεRI	**Fc epsilon I receptor, a higher affinity IgE receptor**
FcεRII	**Fc epsilon II receptor, a lower affinity IgE receptor**
GM-CSF	**Granulocyte macrophage colony stimulating factor**
IFN	**Interferon**
IM	**Interstitial macrophages**
ip	**Intraperitoneal injection**
iv	**Intravenous injection**
Ig	**Immunoglobulin**
IL	**Interleukin**
LAR	**Late asthmatic response**
LE	**Liposome-encapsulated**
LT	**Leukotriene**
MCP	**Monocyte chemoattractant protein**

MIF	**Macrophage migration inhibitory factor**
NO	**Nitric oxide**
OA	**Ovalbumin**
PAF	**Platelet activating factor**
PBM	**Peripheral blood monocytes**
PBS	**Phosphate-buffered saline**
PG	**Prostaglandin**
PMN	**Polymorphonuclear leukocytes**
REV	**Reverse phase evaporation**
R_L	**Pulmonary flow resistance**
sc	**Subcutaneous injection**
SEM	**Standard error of mean**
TGF	**Transforming growth factor**
Th	**Helper T cells**
TNF	**Tumor necrosis factor**
Tx	**Thromboxane**

1. INTRODUCTION

1.1. Asthma

Asthma is a chronic lung disease characterized by airway obstruction, airway inflammation and airway hyperresponsiveness (AHR) to various stimuli (National Institutes of Health, 1991). Generally, the airway obstruction is reversible (spontaneously or with treatment). Asthma can be classified into extrinsic (atopic, allergic) and intrinsic (non-atopic, idiosyncratic) groups (Weiss and Stein, 1993).

The changes associated with airway obstruction in asthma are thought to be initiated by inflammatory events in the airways (Busse, 1989; Woolcock and Barnes, 1997). Furthermore, airway inflammation is also thought to be a primary mechanism responsible for AHR in asthma (Holtgate *et al*, 1987). Inflammation is a general process with cardinal signs of heat, redness, swelling and pain. Inflammatory responses are concerned with defence against invasion by organisms and with tissue repair, and are thus beneficial. However in asthma inflammatory response appears to have been mounted inappropriately, leading to adverse effects. The inflammatory response is involved many interacting cells which release a whole variety of inflammatory mediators that activate several target cells in the airway, resulting in bronchoconstriction, microvascular leakage and edema, mucus hypersecretion, and stimulation of neural reflexes (Laitinen *et al*, 1985; Djukanovic *et al*, 1990). It is not completely understood how inflammation leads to the symptoms of asthma. Inflammation

increases the responsiveness of the airways but inflammation may directly lead to symptoms, such as cough and chest tightness, through activation of sensitized sensory nerve endings.

AHR, a feature characteristic of asthma, is defined as an increase above the normal responsiveness of the airways to physiological, chemical and pharmacological stimuli (Cockcroft and O'Byrne, 1993). After exposure to the appropriate stimuli, subjects can develop an immediate early asthmatic response (EAR) within 10 - 20 min. A second, late asthmatic response (LAR) may occur within 4-6 h and peaks at 8-12 h. Not all patients with asthma experience EAR and LAR. In children and adults, "dual" responses occur in 75 and 50 percent, respectively (see Malo and Cartier, 1993). However, both children and adults may experience only an EAR, or only a LAR, after provocation of an asthmatic attack.

The pathogenesis of asthma is very complex rather than a single "cause". It has been suggested that it involves mechanical injury or changes e.g. epithelial damage, decreases in airway baseline caliber and alterations in airway smooth muscle, as well as neuronal and cellular abnormalities (Barnes *et al*, 1988). It is commonly accepted that asthma cannot be cured, only controlled. To find effective treatments for asthma, the pathogenesis of this disease must be investigated further.

The pathology of asthma extends to airway eosinophilia, epithelial damage, epithelial basement membrane thickening, mucosal plugging of the bronchioles and hypertrophy and hyperplasia of airway smooth muscle. More recent data obtained from biopsy specimens of human bronchus confirm the presence of airway inflammation in subjects with mild to moderately severe asthma, suggesting that the inflammation of the bronchi is an important

factor in asthma (Djukanovic *et al*, 1990; Lane *et al*, 1994). In addition, lung transplantation studies have suggested that there is a local airway mechanism driving asthmatic inflammation (Woolcock and Barnes, 1997).

Because the pathology of bronchial asthma involves a multicellular process, attempts to understand the pathogenesis of asthma must account for the mechanisms of cellular interactions. Infiltration of mononuclear cells, T-lymphocytes, eosinophils and mast cells is a known pathologic feature of asthma but their individual and collective roles in the pathogenesis of allergic airway disease are not well defined. Eosinophils, mast cells and certain subtypes of lymphocytes are usually only present in small numbers in the airway walls of normal people and of asthmatics in remission. Other cells such as alveolar macrophages (AM) and epithelial cells must be responsible for the recruitment of those inflammatory cells into the airways. In allergic asthma, an initial trigger may be cell activation from the interaction between inhaled allergen and cell-surface bound Immunoglobulin E (IgE). High affinity IgE receptors (Fc epsilon I receptor, FcεRI) are found in high density on mast cells, dendritic cells (DC) and Langerhan's cells (see Howarth, 1995). DC may represent the most potent antigen-presenting cells (APC) within the airways to initiate host immune response. Lower affinity IgE receptors (Fc epsilon II receptor, FcεRII) may become upregulated on macrophages, T-lymphocytes, platelets and eosinophils in response to cytokine stimulation (see Howarth, 1995).

Inflammatory mediators produced by activated bronchial mast cells, macrophages, epithelial cells and other cells induce the migration and activation of other inflammatory cells

such as T cells, eosinophils and neutrophils. The inflammatory alterations induce excessive amounts of mediators and sequentially cause disruption of epithelial integrity, abnormalities of the autonomic neural control of airway tone, changes in mucociliary function and increased airway smooth muscle response. Those finally lead to contraction of the smooth muscle and narrowing of the thickened airways (Bleecker *et al*, 1986; Metzger *et al*, 1987; Cockcroft DW and O'Byrne PM. 1993).

Cytokines, released from many cell types including mast cells, eosinophils, T- and B-lymphocytes, macrophages, epithelial cells, endothelial cells and fibroblasts, exert many different functions including modulation of cellular proliferation, chemotaxis of inflammatory cells, production of IgE, immunoregulation, angiogenesis and fibrosis. In asthma, cytokines play an important role in the coordination of the allergic response and the persistence of inflammation. Human mast cells are an important source of interleukin (IL)-3, IL-4, IL-5, IL-6, IL-8, granulocyte macrophage colony stimulating factor (GM-CSF), and tumor necrosis factor (TNF) (Bradding *et al*, 1993; Bradding *et al*, 1994; Howarth, 1995). Upon mast cell activation by inhaled antigen, these released products affect and activate epithelial cells, airway smooth muscle, macrophages, T- and B-lymphocytes (Bradding *et al*, 1994). Activated AM can generate a number of pro-inflammatory molecules (IL-1, IL-6, IL-8, IL-12, GM-CSF and TNF etc.) and anti-inflammatory molecules (IL-10, IL-1Ra and transforming growth factor-β, TGF- β), which can influence the activity of other cell types (Gosset *et al*, 1991 and 1992; Kelly *et al*, 1992; Hallsworth *et al*, 1994; Toews, 1997). Enhanced production of cytokines from macrophages may contribute to airways' inflammation (detailed below).

Different subsets of T-lymphocytes can be defined by their profile of cytokine production (Mosmann and Coffman, 1989). In helper T cells (Th), Th_2 clones produce IL-4, IL-5, IL-6 and IL-10 and are involved in promoting humoral immunity and IgE responses, while Th_1 produce IL-2 and interferon gamma (IFNγ) and are involved in developing cell-mediated immunity. Both types produce IL-3, GM-CSF and TNF. The generation of cytokines leads to the further recruitment of peripheral blood lymphocytes, monocytes and other inflammatory cells, and induces IgE synthesis by B-lymphocytes. In addition, activated eosinophils and epithelial cells also release cytokines that contribute to inflammation and AHR (see Howarth, 1995; Polite AJ and Proud D, 1997; Strek and Leff, 1997). The cytokine network in inflammation is complex and prediction of its effects cannot be based on the action of one cytokine. It is likely that asthma results from complex interactions among inflammatory cells, mediators, and other cells and tissues in the airways.

1.2. Alveolar macrophages in asthma

Macrophages may play an important role in both enhancing and inhibiting inflammation, dependent on homeostatic macrophage functioning in the lung (detailed below). AM recovered from asthmatic subjects differ functionally and metabolically from those from healthy subjects. AM in asthma have been described as "activated". The production of cytokines released by macrophages is increased in asthma and it has been suggested that cytokines derived from macrophages may act as the messengers for cellular communication

(Hallsworth *et al*, 1994). *In vivo*, AM and their precursors (monocytes and interstitial macrophages etc.) are exposed to a wide variety of cytokines and other mediators, which may prime other inflammatory cells, T-lymphocytes, DC and epithelial cells for enhanced or inhibited functions in inflammatory response. Thus, much attention has been recently devoted to the role of AM in bronchial asthma.

1.2.1. Alveolar macrophages

Pulmonary macrophages are the pulmonary representative of the mononuclear phagocyte system. They are the first line of pulmonary defence through their capacity to scavenge particulates, remove macromolecular debris, kill microorganisms, function as an accessory cell in immune responses, recruit and activate other inflammatory cells, maintain and repair the lung parenchyma, provide surveillance against neoplasms and modulate normal lung physiology (Bezdicek and Crystal, 1997). They are categorized as AM, interstitial macrophages (IM), monocytes, DC and pleural macrophages (Bezdicek and Crystal, 1997).

The term *alveolar macrophage* used in this thesis refers to the macrophages that are located on the epithelial surface of the airways and the alveoli, which can be recovered from bronchoalveolar lavage (BAL). AM thus constitute the majority of cells recovered by BAL both in normal and in asthmatic subjects (Eschenbacher *et al*, 1987). AM are directly exposed to inhaled stimuli. They are the most abundant non-parenchymal cells in the lung; there are 5-10 times more AM than lymphocytes (Hunninghake *et al*, 1979). There are far more AM in

the low respiratory tract than there are in the tracheobronchial tree, in proportion to the distribution of surface area. AM are mostly derived from blood monocytes and IM, only a small proportion (about 1%) of AM in the normal human lung are renewable by local proliferation (Bitterman et al, 1984). The lifespan of AM ranges from at least months to perhaps years (Bezdicek and Crystal, 1997).

Apart from their scavenger role, there is evidence that AM play a role in particle transportation to the lung-associated lymph nodes (Harmsen *et al*, 1985; Thepen *et al*, 1993). Furthermore, it is becoming clear that the AM can play a regulatory role in antigen presentation by DC in lung tissue or the lung-associated lymph nodes (Harmsen *et al*, 1985; Holt *et al*, 1987; Rochester *et al*, 1988; Holt *et al*, 1993), as well as in the antigen-specific activation of T and B lymphocytes (Holt, 1986; Thepen *et al*, 1989 and 1991; Strickland *et al*, 1993).

However, a given macrophage may not express all of these functional activities simultaneously. AM are a heterogeneous mixture of immature and mature cells. Poulter *et al* (1986) divided human lung macrophages into mature phagocytes ($RFD7^+$), antigen-presenting macrophages ($RFD1^+$) and suppressor macrophages ($RFD1^+RFD7^+$) using monoclonal antibodies. The $RFD1^+RFD7^+$ suppressor macrophages are normally present in relatively large numbers in the lung (45.2% of total macrophage at mucosal surface) compared to $RFD1^+$ APC (11.4%) (Poulter and Burke, 1996), which is consistent with the function of the cells in down-regulating the primed T cell-dependent immune response.

Alveolar macrophages originating from blood monocytes undergo functional,

morphological and phenotypic changes during the maturation process. Generally, peripheral blood monocytes (PBM) are morphologically smaller than the mature AM. AM showed a higher expression of adhesion molecules except CD11b (Mac-1) than PBM, whereas PBM showed higher expression of CD11b than AM (Striz *et al*, 1998). However, AM are less effective than monocytes in inducing proliferation of blood lymphocytes to soluble "recall" antigens and in activating T lymphocytes necessary for antibody production. The cytokines, receptors, and ligands that modulate monocyte traffic to the normal lung have not been well defined. GM-CSF and monocyte chemoattractant protein-1 (MCP-1) may be involved in the recruitment of monocytes (Sousa *et al*, 1993). IL-10 may also increase the differentiation of normal monocytes to macrophages and increase higher proportion of suppressive cells in cultures of asthmatic monocytes than that of normal monocytes (Tormey *et al*, 1998).

Interstitial macrophages, more closely resemble monocytes, are smaller than AM and have a higher nuclear to cytoplasm ratio than AM. They have markedly lower phagocytic capacity. IM should not only be regarded as a precursor to AM, but also as a highly immunocompetent cell (Johansson *et al*, 1997). They express higher MHC class Ia (OX-6) and CD54 than AM. Both receptors are important for the antigen presenting capacity of macrophages.

1.2.2. Observations from bronchoalveolar lavage and biopsies

Experimental observations from bronchoalveolar lavage (BAL) and biopsies

demonstrated that AM from asthmatic subjects are different from those from normal subjects. In asthmatic children, bronchial hyperresponsiveness has been correlated with numbers of AM (Ferguson and Wong, 1989). Other studies have reported no change or an increase in macrophage numbers in bronchial biopsy and in BAL from asthmatics (see Arm and Lee, 1992; Bousquet *et al*, 1990; Poston *et al*, 1992). Immunohistochemical studies of bronchial biopsies from asthmatic patients demonstrated a significantly increased submucosal infiltration of macrophages stained with HAM 56 (anti-CD68; pan-macrophage marker) into the airways, compared to normal individuals, as well as significant increases in the numbers of primed T cells, activated eosinophils and cells expressing the HLA class II antigen (LN3 positive) (Poston *et al*, 1992). Moreover, Poston and co-workers (1992) have found that in asthmatic subjects, there was also an increase in cells that stained with MAC387 and antimuramidase antibodies, markers for macrophages with phenotypic characteristics of monocytes and monocyte-like tissue macrophages. These results indicate that the macrophage population from human BAL fluid and in bronchial walls is heterogeneous (Poulter *et al*, 1986; Poston *et al*, 1992).

The heterogeneous macrophage population in lungs may suggest that the surface characteristics of airway macrophages obtained by BAL or biopsy vary according to the proportions of mature and immature (newly-arrived) cells. Poulter and Burke (1996) have revealed an increased proportion of $RFD1^{+}$ inductive macrophages (APC) from BAL in asthmatic patients and a decreased proportion of $RFD1^{+}RFD7^{+}$regulatory macrophages (suppressive cells) in asthmatic subjects peri-bronchial tissue, which implies a gross

imbalance within lung macrophage populations.

1.2.3. Activation of macrophages in asthma

Alveolar macrophages obtained from BAL retain the capacity to respond to antigen and have IgE on their surface (Fuller *et al*, 1986). This implies that they have the capacity to respond to antigen exposure, *in vivo*. Monocytes and macrophages of asthmatic patients appear to have enhanced expression of FcεRII that can be modulated by corticosteroid treatments (Melewiz *et al*, 1981).

Accumulated evidence suggests that macrophage secretory processes may be activated by allergens (see Lane *et al*, 1994). Normal AM can be activated by monoclonal IgE (anti-DNP) and specific antigen (DNP-HSA) to generate both leukotriene C_4 and leukotriene B_4 (Rankin *et al*, 1982 and 1986). Metzger and associates (1987) showed that the total number of peroxidase-positive AM obtained from BAL fluid is increased at 48 and 96 h after allergen challenge, suggesting that a population of monocytes has entered the lung from the local vascular compartment.

In vitro studies have shown that AM respond to challenge with stimuli dependent upon both IgE and IgG (see Fuller, 1992). The importance of IgE or IgG-mediated reaction is dependent on species and sensitizing procedures (see Hirshman and Downes, 1993). Ovalbumin (OA)-immunized highly inbred rats show an IgE-mediated reaction. In guinea pigs, some sensitizing procedures used to produce anaphylactic bronchoconstriction produce

IgG-type rather than IgE-type antibodies (see Hirshman and Downes, 1993).

Sousa *et al* (1993; 1994) showed that bronchial epithelial cells produce two cytokines, GM-CSF and monocyte chemoattractant protein-1 (MCP-1), which show specificity for monocytes. GM-CSF and MCP-1 may play a role in monocyte and macrophage recruitment and activation.

1.2.4. Alveolar macrophages and inflammatory mediators

Depending upon the type and intensity of stimulation, macrophages release a spectrum of mediators. Over 100 different secretory products from macrophages have been identified (Nathan, 1987; Crystal, 1997). Activated macrophages generate a number of pro- and anti-inflammatory molecules, which can influence the activity of other cell types. The cells have the capacity to release: bioactive lipids including thromboxane (Tx) A_2, leukotriene (LT), platelet activating factor (PAF), prostaglandin (PG) E_2 and D_2, hydrolytic enzymes, potentially damaging oxygen metabolites, complement fractions, growth factors (GM-CSF) and cytokines including pro-inflammatory cytokines such as IL-1, IL-6, IL-8, IL-12, TNF, IFN, macrophage migration inhibitory factor (MIF), MCP-1, and anti-inflammatory cytokines such as IL-10 and TGF- β (see Fuller, 1992; Cavaillon, 1994; Toews GB, 1997).

Production of cytokines by activated AM from allergic asthmatics is enhanced (Gosset *et al*, 1991 and 1992; Kelly *et al*, 1992; Hallsworth *et al*, 1994) and this may contribute to airways' inflammation. As discussed above, different subsets of AM can be identified with

monoclonal antibodies. These different subsets may exhibit quite different functions that are determined by the expression of different arrays of surface receptors and the release of different repertoires of cytokines (Spiteri and Poulter, 1991).

Tumor necrosis factor is expressed at mRNA level in higher amounts by mature AM than by monocytes (Martinet *et al*, 1988). TNF increases endothelial cell adhesion molecule expression (Garnble *et al*, 1985) and production of inflammatory mediators e.g., GM-CSF, PGs and PAF, in addition to its cytotoxic action (Carswell *et al*, 1975). The recent finding that TNF secretion is increased in the allergen-induced LAR supports the possible role of this cytokine in airway inflammation (Gosset *et al*, 1991).

Increased levels of GM-CSF and MCP-1 were detected using monoclonal or polyclonal antibodies in bronchial biopsies from asthmatic subjects. GM-CSF might be involved in the survival, differentiation and activation, and chemotaxis of inflammatory cells such as macrophages and eosinophils. MCP-1 is known to activate different inflammatory cells, such as monocytes, and can be produced by several different cell types, including monocytes, macrophages and AM (see Lane *et al*, 1994).

IL-8 is a potent neutrophil- activating chemotactic peptide (Peveri *et al*, 1988) and its expression can be increased by TNF and IL-1β (Strieter *et al*, 1990). IL-8 is also a chemoattractant of eosinophil but neutrophils respond much more rapidly to IL-8 than eosinophils (Warringa *et al*, 1993; see Toews, 1997). This molecule is relatively resistant to proteases compared to other cytokines, and may be involved in more prolonged inflammatory actions.

IL-12 can amplify the immune response by selectively inducing proliferation of activated CD4 and CD8 lymphocytes, as well as reorient the development of Th1 immune response. IL-12 from PBM is also a potent inhibitor of IL-4-induced IgE synthesis (see Toews, 1997).

IL-10 is crucially involved in the inhibition of Th1-type immune response and on the synthesis of several cytokines including IL-1, IL-6, IL-8, IL-10, IL-12 GM-CSF and TNF (see Toews, 1997). AM from asthmatic patients release lower amounts of IL-10 but more MIP-1, IFNγ, and GM-CSF. The inhalation of corticosteroid reduced the release of pro-inflammatory cytokines and increased IL-10 mRNA and protein expression from AM stimulated by lipopolysaccharide and IL-1β (John *et al*, 1998). Tormey and coworkers (1998) have also suggested that the reduced proportion of maturing AM with a T cells suppressive phenotype ($RFD1^{+}RFD7^{+}$) could be reversed by addition of IL-10 into the culture of PBM from asthmatic patients.

IL-1 is a hallmark of macrophages recently derived from either the PBM or from locally replicated tissue precursors. This cytokine induces the proliferation and activation of T cells (Mattoli *et al*, 1990). It also increases expression of adhesion molecules on endothelial cells (Bevilaqua *et al*, 1985), and may play a critical role in initiating leucocyte infiltration into asthmatic airways. However, Wewer *et al* (1984) demonstrated that human AM have a severely limited capacity for IL-1 production in relation to PBM tested in parallel. In addition, activated AM from asthmatics release an IL-1 inhibitory factor (Gosset et al, 1988). This finding may indicate that macrophages play a key role in the regulation and modulation in the

inflammatory responsiveness.

1.2.5. The regulation function of alveolar macrophages in immune responses in asthma

Poulter and Burke (1996) have revealed an imbalance in inductive macrophages (APC) and regulatory macrophages (suppressive cells) from BAL in asthma. AM have a role distinct from other types of APC. AM (suppressive macrophages) normally have a suppressive effect on T cell function. AM inhibit lymphocyte proliferation by releasing a variety of mediators including PGE2, superoxide anion, and vitamin D metabolites (see Toews, 1997). AM suppressive function restricts or downregulates immune responses within the pulmonary parenchyma to protect the lung from the damage of inflammatory reactions. However, AM suppressive function may be impaired in asthma after allergen exposure (Aubas *et al*, 1984; Gant *et al*, 1992). Support for this hypothesis has been provided by the results of experiments involving selective elimination of AM, *in vivo*. AM elimination was accomplished by the intratracheal administration of a cytotoxic drug encapsulated in liposomes, which are avidly phagocytosed by AM. The procedure greatly enhances the humoral immune response to T-cell-dependent antigens delivered to the deep lung by intratracheal intubation or by inhalation of an aerosol antigen (Thepen *et al*, 1989 and 1991).

Alveolar macrophages, relatively weak accessory cells in the normal human lung, become more efficient antigen-presenting cells in allergic individuals. AM from asthmatic

subjects present soluble antigens (tetanus toxoid and streptolysin-O) to freshly isolated T cells more efficiently than AM from nonatopic control subjects (Burastero *et al*, 1999). AM have additionally an ability to down-regulate the APC activity of local DC in the lung (Holt *et al*, 1993). AM also release mediators to recruit PBM into lungs and promote them to mature to resident AM. IL-10 can inhibit the synthesis of several pro-inflammatory cytokines and Th1-type immune response, while IL-12 can reorient the response to Th1 pattern. AM can enhance the Th2 response by turning off their secretion of IL-12, or tend to downregulate it by producing IL-10 and IL-12. The cytokines and possibly other mediators secreted by Th2 bring about either the specific accumulation and activation of eosinophils or IgE production by B lymphocytes. All of those may involve the amplification of antigen-specific T-cell response in the airways of asthmatic subjects and may contribute to the initiation and development of airway inflammation and airway hyperresponsiveness.

In summary, evidence for macrophage activation, phenotype change and loss or enhancement of certain regulatory functions indicates a central role for macrophages in the pathogenesis of bronchial asthma. Asthma, and related lung disorders such as airway hyperresponsiveness, may result from imbalance of homeostatic macrophage functioning in the lung. However, there is very little information from *in vivo* studies that helps us to understand the association between the AM regulatory functions and the development of airway hyperresponsiveness. Thus, elucidation of the role of macrophages in the development of airway hyperresponsiveness through their modulation and regulatory function on T cell-dependent allergic reaction is needed and may lead to new approaches in asthmatic therapy.

1.3. Elimination of alveolar macrophages

Depletion of macrophages has been widely used for the investigation of the functional aspects of macrophages in the host immune system. Macrophages can be depleted by the administration of silica (Pomeroy and Filice, 1988), asbestos (Kagan and Hartmann, 1984) and other treatments (Pinto *et al*, 1989). However, none of these treatments results in complete depletion of all macrophages without effects on other cells. A new approach has been developed recently to deplete alveolar macrophages, *in vivo*, using liposome-encapsulated dichloromethylene diphosphonate (LE DMDP) (Thepen *et al*, 1989). The depletion is selective for intra-airway and intra-alveolar macrophages, as the LE DMDP has no effect on the number and location of interstitial macrophages or other cells of pulmonary parenchyma (Thepen *et al*, 1989, 1991; Kradin *et al*, 1999).

DMDP ($CH_2Cl_2Na_2O_6P_2$, sodium clodronate, Bonefos) is a small, highly water-soluble molecule, which does not readily enter cells and is rapidly removed from circulation by the kidneys (Fleisch, 1991). It is considered a "safe" drug and used clinically for the treatment of osteolytic bone disease in human. DMDP inhibits the activity of osteoclasts in their bone-attached form and thus, cells other than osteoclasts are not affected. Liposomes containing phosphate-buffered saline elicit no effects on the elimination of macrophages, *in vivo* (Van Rooijen and Classen, 1988). *In vitro*, LE DMDP was 50 times more potent than free drug (non-liposome encapsulated) at inhibiting growth of RAW 264, a murine macrophage cell line (Mönkkönen and Heath, 1993). Insufflations of free DMDP caused cytoplasmic edema of

alveolar epithelial cells and resulted in depletion of AM (Berg *et al*, 1993). Encapsulation in biodegradable and immunological inert liposomes enables the drug to selectively target macrophages and minimizes its effects on non-phagocytic cells. Once LE DMDP is phagocytosed, the liposomal membranes are disrupted by the phospholipases of the lysosomes and DMDP is released into the cell. This leads to the rapid death of the cell. The exact mechanism of DMDP on the depletion of macrophages is still under investigation. It may be associated with the calcium-binding activity of the molecule, or the presence of chloride ions and intracellular iron (Van Rooijen, 1991 and 1992; Mönkkönen and Heath, 1993).

The successful destruction of macrophages is dependent on the DMDP concentration delivered and liposomal surface charge and size (Heath *et al*, 1985). Liposomes have been shown to be effective carriers of DMDP to macrophages. However, only two percent encapsulation efficiency (EE) was obtained with neutral multilamellar vesicles (MLVs) (Claassen and Van Rooijen, 1984, 1986). In general, negatively or positively charged liposomes bind to cells and become endocytosed by cells to a greater extent than neutral liposomes (Heath *et al*, 1985; Mönkkönen *et al*, 1994). Mönkkönen *et al* (1994) suggested that negatively charged unilamellar liposomes made by reverse-phase evaporation (REV) are far more effective in delivering DMDP to macrophages than neutral liposomes, *in vitro*. Although a variety of methods to prepare liposomes have been recommended, the hydration method initially reported is still widely used. The hydration method is the most convenient means of preparing mutilamellar liposomes. Hence, in the present study the hydration method was used to the preparation for the negatively charged LE DMDP.

Currently, no one animal model completely reproduces asthma processes found in human. However, animal models have been used to test hypotheses concerning mechanism of airway hyperresponsiveness that are untestable in humans. An IgE-mediated reaction is thought to play a major role in the pathogenesis of atopic human asthma, although other immunoglobulins (e.g. IgG *etc.*) are considered to be involved. It is easier to produce an IgE specific allergic reaction in specific rats than in other species such as guinea pigs. Highly inbred BN rats are high IgE-responders following active sensitization with ovalbumin and adjuvants (Bordetella pertussis etc.). BN rats have been well defined as an animal model of asthma (Pauwels *et al*, 1979; Eidelman *et al*, 1988; Elwood *et al*, 1991; Haczku *et al*, 1994, 1995). The BN rat model of AHR has been shown to possess features similar to human allergic asthma. These include early- and late-phase reactions after allergen challenge (Eidelman *et al*, 1988), the development of an IgE response to allergic sensitization (Pauwels *et al*, 1979; Haczku *et al*, 1995), AHR in response to acetylcholine or methacholine inhalation (Eidelman *et al*, 1988; Haczku *et al*, 1994), and accumulation of inflammatory cells and activation of T cells in the airways after exposure of sensitized animals (Elwood *et al*, 1991; Haczku *et al*, 1995). A major advantage of using a highly inbred strain of rats as a model of allergic asthma is in the genetic homogeneity of animals. Inbred strains would be expected to show less inter-individual variation in response to antigen challenge than outbred strains.

1.4. Hypothesis and Objectives

Allergic asthma is recognized as a chronic inflammatory lung disease with airway hyperresponsiveness characterized by peri-bronchial infiltration of T cells, macrophages and eosinophils. An IgE-mediated reaction may play a major role in the pathogenesis of atopic human asthma. IgE-mediated hyperactivity is regulated by T cell-mediated immune mechanisms. To regulate the T cell reactivity in the lung, a balance of functionally distinct macrophage subsets needs to be maintained. Increased proportions of inductive macrophages (APC) and decreased proportions of regulatory macrophages (suppressive cells) from BAL in human asthma imply a gross imbalance within the lung macrophage populations. Loss of, or changes in, macrophage functioning in the airways contribute to, or result in, the development of the pathologic signs of asthma. However, human studies addressing the association between elimination of lung macrophages and the development of pathologic signs are not available. Thus, it was hypothesized that in the BN rat model of asthma, the depletion of intra-airway macrophages by intra-airway administration of LE DMDP will prevent the normal negative homeostatic effects of macrophages on airways' hyperresponsiveness and result in increased airways' responsiveness to bronchospastic agonists compared to controls.

Using BN rats, the objectives of this research were:

1. To compare airways' responsiveness to bronchospastic agonists in immunized rats and controls.
2. To prepare and characterize liposome-encapsulated DMDP with high

encapsulation efficiency.

3. To confirm the effect of liposome encapsulated DMDP on elimination of alveolar macrophages in rats.

4. To examine the changes in cell populations in the lavage fluid in rats treated with liposome-encapsulated DMDP.

5. To evaluate airway hyperresponsiveness in alveolar macrophage-depleted and ovalbumin-immunized rats and controls.

2 MATERIALS AND METHODS

2.1. Materials

2.1.1. Chemicals and reagents

Dichloromethylene diphosphonate (DMDP, sodium clodronate) was obtained from Rhône-Poulenc-Rorer Canada Inc. Montreal, Québec. Bovine serum albumin (BSA), ovalbumin (OA, chicken egg, grade V), methacholine chloride, and serotonin sulphate were purchased from Sigma Chemical Co., St. Louis, MO. Murexide (5,5'- nitrilodibarbituric acid), L-α-dimyristylphosphatidylcholine (DMPC), L-α-dimyristylphosphatidylglycerol (DMPG), and cholesterol were purchased from Sigma Chemical Co., St. Louis, MO. Bordetella pertussis was obtained from Connaught Laboratories Ltd., Willowdale, Ont.. Solvents, buffers and laboratory reagents were obtained from various suppliers and were reagent grade or better.

2.1.2. Animals

Male, Brown Norway rats, 200-250 g, were obtained from Charles River Inc., St. Constant, Québec. A maximum of three animals were conventionally housed in each cage in a temperature-controlled room, maintained on a 12-hr light / dark cycle. Regular chow and water were available, *ad libitum*. The rats were monitored for at least 1 wk before being used

in experiments.

2.2. Experimental design

Studies were carried out in accordance with the guidelines of the Canadian Council on Animal Care and approved by Health Sciences Animal Welfare Committee, University of Alberta.

2.2.1. Experiment one: Preparation of the animal asthma model

To set up an effective animal asthma model and to compare airway response, eight male BN rats were randomly assigned to two groups (n=4). One group was immunized with a single intraperitoneal injection (ip) of OA (1 mg, grade V, Sigma) and aluminium hydroxide (200 mg) in 1 mL sterile saline and a single subcutaneous injection (sc) of 1 mL Bordetella pertussis containing about $6x10^9$ heat-killed bacilli. The other group was sham treated with the same amount of Bordetella pertussis and aluminium hydroxide (adjuvant-immunized controls).

Twelve days later, on each of 3 consecutive days, animals were challenged with OA or saline aerosol. OA-immunized rats were exposed to an OA aerosol (2%, in saline, VIX Acorn™ nebulizer, compressed air at 15 psi) for up to 5 min and adjuvant-immunized rats were exposed to a saline aerosol under the similar conditions.

Animals were carefully observed for signs of respiratory distress. If anaphylaxis developed, animals were removed from the exposure chamber and given an adrenalin chloride injection (0.1 mg).

The choice of 12 days from immunization to OA challenge was based on published observations of serum IgE titers in immunized BN rats, which develop progressive increases in serum IgE and dual airway responses during approximately 12 to 14 days after immunization (Pauwels *et al*, 1979; Eidelman *et al*, 1988; Haczku *et al*, 1995).

Twenty-four hours after the last antigen exposure, the rats were anesthetized with sodium pentobarbital (35-40 mg kg^{-1}, ip) and airway responsiveness to methacholine and serotonin was evaluated in each animal (see 2.9.). Dose-response curves to methacholine and serotonin (iv) were established.

After the evaluation of airway responsiveness, the lungs were lavaged with phosphate-buffered saline (PBS) containing 1% BSA and total and differential cell counts were carried out (see 2.3.). Immediately after the experiment all of the rats were euthanized with an overdose of sodium pentobarbital according to approved procedures.

2.2.2. Experiment two: The effect of liposome encapsulated dichloromethylene diphosphonate on alveolar macrophage depletion

To confirm the effect of liposome encapsulated dichloromethylene diphosphonate on the alveolar macrophage depletion, 27 male BN rats were randomly divided into three groups

(n=9). Group A was dosed intratracheally with LE DMDP (see 2.4.). Group B and C were treated with normal saline and LE saline ("empty" liposome), respectively, at similar volumes and concentration.

At days1, 3 and 7 after treatment, three rats from each group were anesthetized. The lungs were lavaged and total and differential cell counts were performed (see 2.3.). Another three rats that had not received any form of treatment were lavaged as normal controls at day 0. All the rats were sacrificed with an overdose of pentobarbital immediately after the experiment.

2.2.3. Experiment three: Evaluation of airway hyperresponsiveness in alveolar macrophage-depleted rats

To evaluate airway hyperresponsiveness in alveolar macrophage-depleted and ovalbumin-immunized rats compared to controls, 16 male BN rats were randomly assigned to four groups (n=4) and treated as described in Table 2.2.1. At day 0, group OLO and OSO were immunized with OA and adjuvants while SSS and SLS were treated with saline alone following the procedure described in section 2.2.1.. Twelve days later, rats were injected intratracheally (see 2.8.) with LE DMDP (AM-depleted groups SLS and OLO) or saline (non AM-depleted groups SSS and OSO). Twenty-four hours later, the rats were exposed to an OA (2%) aerosol or saline aerosol for up to 5 min on each of 3 consecutive days.

Table 2.2.1 Protocol for the depletion of AM and the immunization of rats

Groups	Sensitization (Day 0)	Intratracheal injection (Day 12)	Aerosol Challenge (Day 13-15)
SSS	Saline	Saline	Saline
OSO	OA & adjuvants	Saline	OA
SLS	Saline	LE DMDP	Saline
OLO	OA & adjuvants	LE DMDP	OA

Eighteen to 24 hours after the last aerosol exposure, immunized rats were anesthetized and airway responses to methacholine and serotonin were evaluated (see 2.9.). The rat lungs were lavaged and then total and differential cell counts were carried out. All of animals were euthanized with an overdose of pentobarbital immediately after the experiment.

2.3. Bronchial alveolar lavage and cellular analysis

The rats were placed in an upright position under total anesthetization with sodium pentobarbitol. The anesthetized rats' tracheas were cannulated close to the larynx. A hypodermic needle (18-gauge) was inserted into the cannula (4 cm, PE240) and sealed in place via a short length of flexible tubing (Masterflex 96400-14). With a 5 mL Luer lock syringe, the lungs were lavaged with 2 x 5 mL of PBS containing 1% BSA at 37^{0}C. The two aliquots were combined. The total recovered medium was 7 to 8 mL. Three washings of the recovered cells were carried out by adding PBS (containing 1% BSA) and centrifugation (2000 rpm for 5 min, Dynac II centrifuge, Adams).

After viability of cells was determined by trypan blue exclusion, slides were prepared with a Cytospin^{R} III using 125 μL of the resuspended cells. The viable cells were stained with Leukostat^{TM}, allowed to dry and cover-slipped. The total number of viable lavage fluid cells, excluding erythrocytes, was counted with a hemocytometer (Neubauer).

Differential cell counts were performed on 200 consecutively counted cells under nonoverlapping high-power fields. Macrophages, neutrophils and eosinophils were morphologically identified and calculated from those totals and the differential counts. Lymphocytes and other leukocytes were counted and grouped as "others".

2.4. Preparation of Liposomes

Liposomes were prepared by hydration (Sehgal, 1994) at DMPC:cholesterol:DMPG (3:1:0.5 mole ratio). 74 mg of DMPC, 12.5 mg of DMPG and 14 mg of cholesterol were dissolved in 5 mL of chloroform and methanol (3:1). A dried film of the lipids was formed on the walls of a round-bottom flask by low vacuum evaporation at 37°C; the film was further dried overnight in a vacuum desiccator at 40°C. Subsequently, the film was hydrated with a solution of 270 mg DMDP in 5 mL saline under vortexing for 10 min. The formed liposomes were allowed to enlarged 2-3 hours at room temperature. The multilamellar vesicles were subjected to five freeze-thaw cycles (in dry ice and acetone for 8 s and then at room temperature for 2-3 hours). Untrapped drug was removed by ultracentrifugation (Beckman Model L8-55 Ultracentrifuge 100,000 x g, 15°C, 30 min). Following washing the pellet was washed with 5 mL of PBS and resuspended in 5 mL saline or PBS. The final liposome preparation was either used immediately or stored overnight under nitrogen at 4°C.

2.5. Measurement of particle size

Samples (100 μL) of LE DMDP were diluted to 3 mL with saline or PBS and the samples were measured by laser light scattering (Brookhaven Instruments BI-90 Particle Sizer). The dust factor was maintained at 0.01 for all measurements.

2.6. Determination of dichloromethylene diphosphonate and encapsulation efficiency

The amount of DMDP entrapped in liposomes was determined on the basis of the competition for calcium between DMDP and Murexide (Classen and Van Rooijen, 1986).

A murexide stock solution was prepared by dissolving 100 mg 5,5'-nitrilodibarbituric acid mono-ammonium salt in 2 mL deionized water and 10 mL ethanol (95%) (indicator solution). A sufficient volume of this solution was added to 200 mL 0.05 M NaOH to obtain an absorbance of 0.50 at 545 nm against water as reference. A solution of $CaCl_2$ (5 mL, 2×10^{-3} M) was added to another 95 mL of the indicator solution for a sample cuvette. Aliquots (1.9 mL) were diluted with a 100 μL LE DMDP sample. A 10-point calibration curve of DMDP (with LE saline) ranging from 1-40 mM DMDP was determined at 510 nm. Before analysis, liposomes were first disrupted by the addition of 1% Triton-X100.

The concentration of DMDP in liposomes was determined from the calibration curve as described above. The EE was expressed as a ratio of the amount of DMDP in the pellet to the initial amount of DMDP added during liposome preparation.

2.7. Administration of liposome encapsulated dichloromethylene diphosphonate

BN rats were placed in an upright position following anesthetization with pentobarbitol (35-40 mg/kg) and intubated using a 16-gauge intravenous catheter with the

assistance of an otoscope. LE DMDP (0.2 mL) was injected into the airways through the intratracheal catheter and then followed by 2 mL of air. Auscultation of the chest with a stethoscope was done to ensure that the drug was insufflated into the lungs. LE saline (0.2 mL) or saline was intratracheally injected into control animals. After rats had recovered from the effect of anesthesia, they were housed as the same group and given free access to water and food.

2.8. Measurement of airway hyperresponsiveness

Resistance and elastance of the pulmonary system were measured 24 h after the last antigen exposure in experiment one and three. BN rats were anesthetized with sodium pentobarbital (35-40 mg kg^{-1}, ip). After shaving the neck, the trachea of rats were cannulated into (PE240) and attached to a rodent ventilator (Ugo Basile) with a tidal volume of 10 mL kg^{-1} (Haczku *et al*, 1994) at a breath rate of 20 breath/min based on a preliminary study. A jugular vein was cannulated (PE50) for drug injections. All animals received succinylcholine (0.1 mg kg^{-1}, iv) to prevent spontaneous breathing that would interfere with measurements. Serotonin and methacholine were injected (iv) in an increasing order of dose with recovery to baseline between injections of doses until R_L increased by at least 150% over baseline. Serotonin was always given first. Sufficient time was allowed for recovery to baseline between doses until the dose-response curves were established. Recovery to baseline was aided by briefly inflating the lungs with twice tidal volumes.

Airflow rate was measured breath-by-breath via a Fleisch 0000 pneumotachograph (Gould Godart BV, Bilthoven, The Netherlands) with its ports attached across a Validyne MP45 differential pressure transducer (Validyne Engineering Corp., Northridge, CA). Intratracheal pressure, which approximates transpleural pressure, was measured via one port of Validyne MP pressure transducer with the other port open to atmosphere (via solenoid valve). Excitation for the transducers was supplied by preamplifiers (Validyne preamplifier, Buxco Electronics Inc.). The portion of breath examined was determined by an adjustable gate driven by a digital timing device. Signals were collected only during the inspiratory phase of the of the pump cycle. Also, the timing device operated a solenoid valve that opened the system to atmosphere during the expiratory phase. This prevented back flow through the pneumotachograph. Signals from the transducers were collected and digitized using a Metrabyte DAS 20 data acquisition board controlled by Viewdac™ , Version 2 software (Asyst Software Technologies Inc., Rochester, NY) running under Windows™ on a 386-based PC clone. Digitized signals were processed to yield breath-by-breath values of pulmonary flow resistance (R_L) and dynamic pulmonary elastance (E_L). A subroutine averaged data for the first 5 breaths as a " control " and calculated changes in R_L and E_L as percent of control. The Viewdac™ software presents the user with a sequence of panels that provide visual feedback and enable mouse control. Pressure and flow rate signals, and measurements of R_L and E_L breath by breath were displayed in a strip chart format in a real time. Calibration data, timing information, pressure measurements and values of R_L and E_L in absolute values and as percent control were stored on disk and then were printed out.

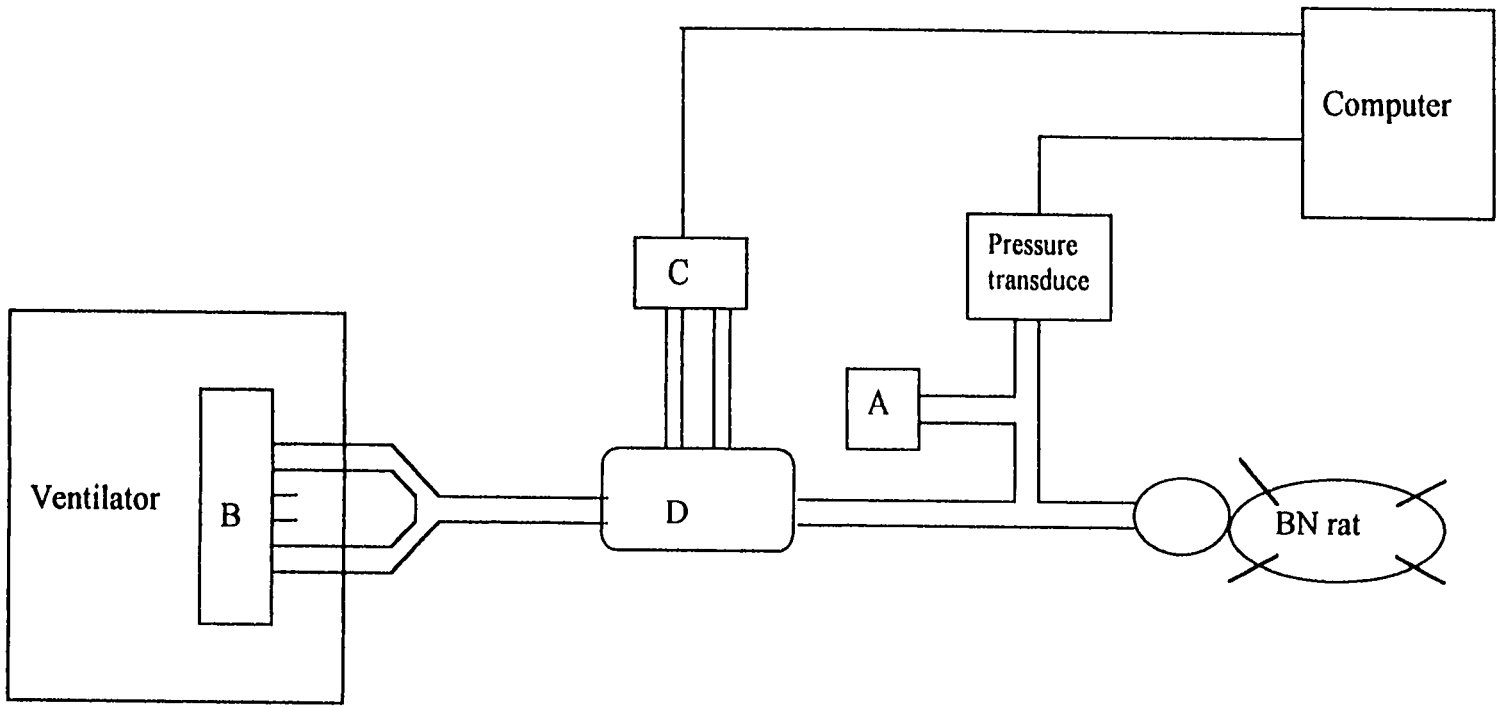

Figure 2.8.1 Schematic representation of the system for measuring pulmonary flow resistance and dynamic pulmonary elastance. A: solenoid; B: cylinder; C: differential pressure transducer; D: pneumotachograph.

2.9. Statistical analyses

Data were analyzed using SigmaStat software. Differences among groups were compared by Student's "t" test, ANOVA and Student-Newman-Keuts' test. Data were expressed as average values ± standard error of mean (SEM). Significance was assumed at $p \leq 0.05$.

3. RESULTS AND DISCUSSION

3.1. Experiment one: Preparation of the animal asthma model

On each of 3 consecutive days, OA-immunized and adjuvant-immunized rats (immunized) were challenged with OA aerosol and the adjuvant-immunized and saline-challenged rats (controls). All the test animals tolerated up to 5 min of aerosol exposure. Care was taken during first aerosol challenge to avoid severe anaphylactic reaction.

After aerosol exposure, OA-immunized rats appeared to have a dyspnea. OA-immunized rats were usually quiet and moved less than controls. Some of them showed "hair roughed up" and others walked with a laboured, jerky movement with audible wheezing after OA exposures. None of the rats, however, showed severe dyspnea or died. In the recovery period of 24 hr, the rats gradually recovered from respiratory stress. The controls did not show these symptoms of respiratory stress and anaphylactic responses.

3.1.1. Cellular content of bronchoalveolar lavage

Table 3.1.1 and Figure 3.1.1 show the results of total and differential cell counts done on lavage fluid recovered after measuring airway responses. Total viable leucocyte counts were not significantly different between the two groups. Differential cell counts, however, revealed a significant increase in eosinophils (0.6×10^5 mL^{-1}) in the OA-immunized rats compared to those in adjuvant-immunized animals (0.03×10^5 mL^{-1}) ($p<0.001$). There were no significant changes in macrophages and neutrophils between

the groups. Other cells (others) counted, consisting largely of lymphocytes, increased in the OA-immunized rats ($p < 0.05$).

The increase in eosinophil numbers is typical of allergic reactions. OA-immunized BN rats challenged by a higher concentration of OA (10%) aerosol exhibit anaphylactic reaction characterized by eosinophil increase, which is maximal at 24-48 hours after the challenge (Tarayer *et al,* 1992). Elwood *et al* (1991) also demonstrated a significant increase in eosinophils and lymphocytes in OA-sensitized BN rats challenged by a single exposure or repeated exposure to OA (1%) aerosol compared to the sensitized rats with saline exposure. Their results of eosinophil and lymphocyte increase are similar to current observations, although the protocols and controls used are not completely identical. Haczku *et al* (1994) further found that long-term (3 wk to 8 wk) repeated exposure to OA challenge leads to suppression of AHR and suppression of eosinophil numbers. This suppression is accompanied by increased numbers of AM, lymphocytes, and neutrophils. Table 3.1.1 and Figure 3.1.1 show that neutrophil counts apparently tend to increase but without statistical significance. This may have resulted from the influence of several factors; 1) the degree of variation of individual cell counts; 2) the decline of neutrophil count from the transient increase due to a later recovery of BAL cells (24 hr after last OA exposure). Tarayer *et al* (1992) have reported that neutrophil population transiently increased and gradually recovered 24 hr after OA exposure; 3) other factors (agonists, adjuvant, or saline) might influence the increase the neutrophil numbers in

Table 3.1.1. Cell counts in lavage fluid in immunized BN rats and sham treated controls 24 hr after 3 d of OA challenge or sham challenge

Groups	Leukocyte Counts ($x10^6$ mL^{-1})				
	Total	Macrophages	Eosinophils	Neutrophils	Others
Controls	2.7 ± 0.4	2.4 ± 0.3	0.003 ± 0.003	0.007 ± 0.004	0.3 ± 0.02
Immunized	2.3 ± 0.6	1.7 ± 0.4	0.07 ± 0.01#	0.02 ± 0.007	0.5 ± 0.03*

OA- and adjuvant-immunized BN rats (n=4) were challenged with OA (immunized, see 2.2.1.). The controls were treated with adjuvants alone and then challenged with saline. After measuring airway responses, lungs were lavaged and cells were recovered. Values represent mean ± SEM. " * " denotes $p < 0.05$ and " # " denotes $p < 0.001$.

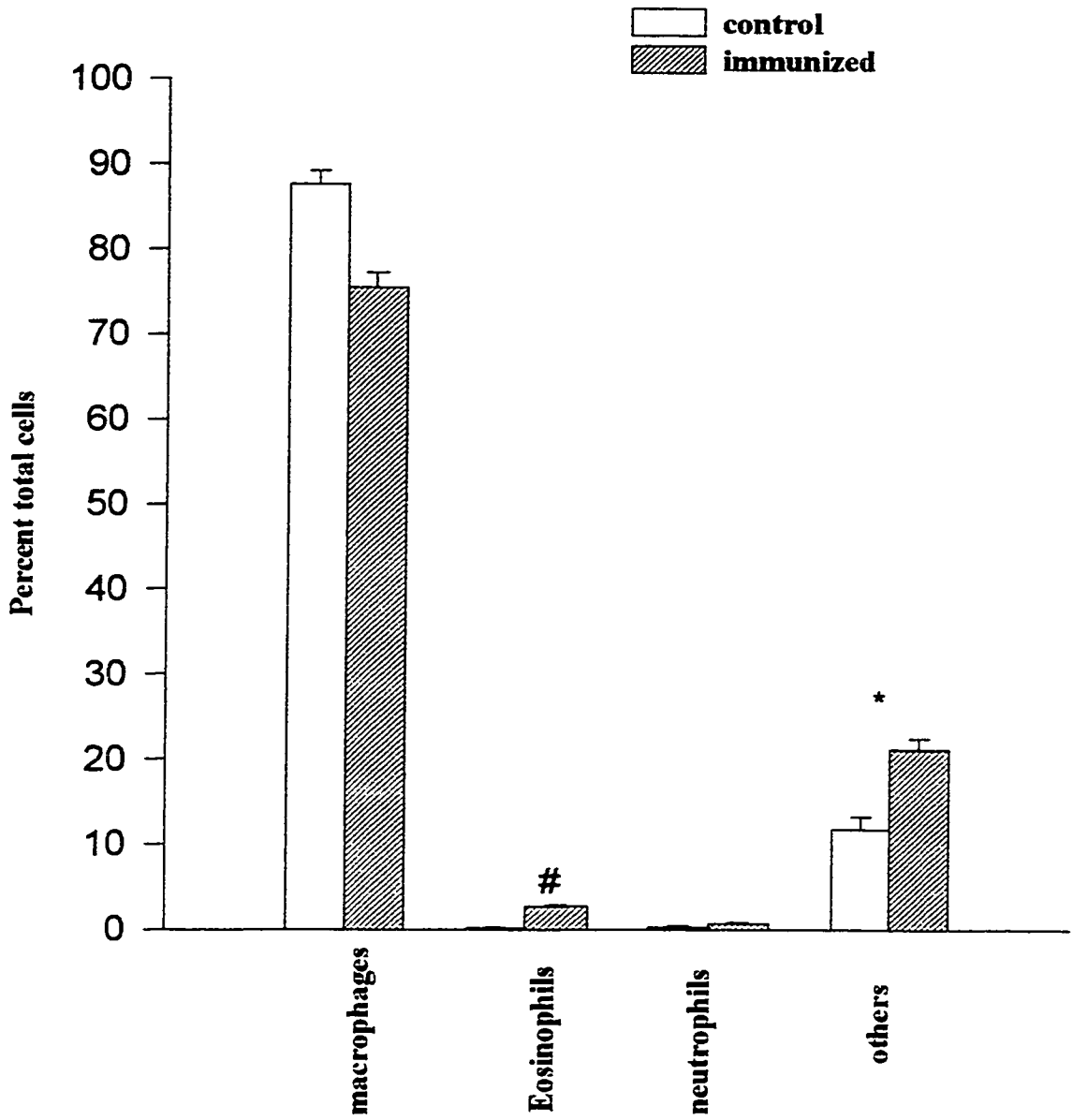

Figure 3.1.1 Differential cell counts, as percent total cells, found in lavaged fluid from OA-immunized rats (immunized) and adjuvant-immunized rats (control) immediately after the measurement of airway responses. Each bar presents the mean ± SEM of counts from 4 animals. Lymphocytes comprised the majority of other cell types (others). In the immunized group, eosinophils significantly increased (# $p<0.001$). Neutrophils in the immunized rats showed no significant change, whereas lymphocytes increased (* $p<0.05$)

controls; Elwood *et al* (1991) have found a small, but significant, increase in inflammatory cell infiltration (i.e., eosinophils, lymphocytes, and neutrophils) in animals from 3 groups (i.e., adjuvant-sensitized with no aerosol exposure, OA-sensitized and saline-challenged, and 1% OA exposure only, respectively) when compared to saline–only treated animals. This increase may represent a non-specific response of the immune system or of the lungs to adjuvants or to aerosolized allergen exposure. In addition, some investigators have also suggested that BAL neutrophils may result from an artifact of saline instillation (Liu *et al*, 1991) rather than specific inflammatory reaction to allergen.

The recovered total cell numbers from BAL in our study were approximately equivalent to those in other studies (Blythe *et al*, 1986; Sapienza *et al*, 1992; Watanabe *et al*, 1995), whether they used the same (10 mL) or different (25 to 50 mL) amounts of lavage fluid compared to our procedure. In OA-immunized rats, the total leukocyte numbers and AM have been reported to show no significant changes (Blythe *et al*, 1986) during a short period (within 24 h) after OA aerosol exposure. After 24 h, the total leukocytes increased compared to saline-immunized controls (Blythe *et al*, 1988; Elwood *et al*, 1991). The present study showed an apparent decrease in AM although without statistical significance, similar to other studies (Blythe *et al*, 1986; Tarayre *et al*, 1992; Haczku *et al*, 1994). To summarise, the present study has shown a significant increase in inflammatory cell numbers (eosinophils, $p<0.001$ and lymphocytes, $p<0.05$) recovered from OA-immunized BN rats.

It is difficult to compare BAL results obtained by different laboratories because varying procedures are used for evaluation of BAL cells, including the use of different sensitization protocols, different BAL protocols and dilution factors. Even in the same

laboratory using the same protocol, variances of 4 to 5 fold exist in the differential cell counts from BAL (Elwood *et al,* 1991; Haczku *et al,* 1994). In addition, it is not possible to explain whether the profile of cells recovered from the BAL represents a true reflection of submucosal airway inflammation without histological examination of the airway. BAL cell counts also cannot represent dynamic cell movement between peripheral blood circulation and airway lumen. Blythe *et al* (1986) revealed the presence of mononuclear inflammatory cells on histological examination but not in BAL samples in OA-immunized rats. Inflammation is a dynamic process best accessed by a combination of techniques that reflect both static (histopathology) and cumulative (BAL) events. In addition, the immunocytochemical technique is considered by some to be more reliable in the identification of cells than conventional staining.

3.1.2. Airway responsiveness

Serotonin is a major important bronchoactive mediator in rat anaphylaxis. Methacholine has been used to induce hyperreactivity in BN rats (Eidelman *et al*, 1988). Histamine does not contract rat airway smooth muscle even in massive doses (Hirshman and Downes, 1993), an observation confirmed in preliminary studies. Furthermore, experiments have shown that rats experience higher airway reactivity to serotonin and methacholine but less sensitivity to histamine. Thus, methacholine and serotonin were chosen to measure airway responsiveness.

Figures 3.1.2-3.1.5 show airway responses to methacholine or serotonin in the OA-immunized group and in the adjuvant-treated group. The OA-immunized rats showed

three to five-fold increases in R_L airway responses to serotonin (Figure 3.1.4) compared to the adjuvant-treated rats. The differences were statistically significant ($p<0.05$). A 3-4 fold increase in R_L was also observed, which is statistically significant at the higher concentration of methacholine (Figure 3.1.2). Significant increases in E_L (Figure 3.1.3) were observed in the immunized rats in response to methacholine ($p<0.05$) while increases in E_L in response to serotonin were noted only at the higher concentration (Figure 3.1.5).

Airway hyperresponsiveness has been demonstrated in BN rats after active sensitization with ovalbumin and adjuvants. After aerosol exposure on three consecutive days, OA-immunized rats had a slight dyspnea. OA-immunized rats also showed 3-5 fold increases in airway responses to both serotonin and methacholine compared to adjuvant-immunized rats. Repeat challenge (in a short term) of sensitized BN rats with OA aerosol has been shown to cause persistent AHR to acetylcholine or methacholine aerosol (Bellofiore and Martin, 1998; Elwood *et al*, 1991; Haczku *et al*, 1994), whereas after only a single OA challenge, the hyperreactivity was not present 5 days later (Elwood *et al*, 1991). On the other hand, long-term (8 wk) and repeat OA aerosol exposure leads to suppression of AHR, which may be involved in immunologic tolerances (Haczku *et al*, 1994). Furthermore, higher mortality in repeated exposure prevents further methacholine response measurements (Eidelman *et al*, 1988). Thus, repeated aerosol exposure over 3 days was done. In addition, inhaled aerosol of agonist may preclude measuring responsiveness to several agonists in the same animal. The amount of inhaled agonist actually reaching the lower airways is unknown, because constriction of the upper

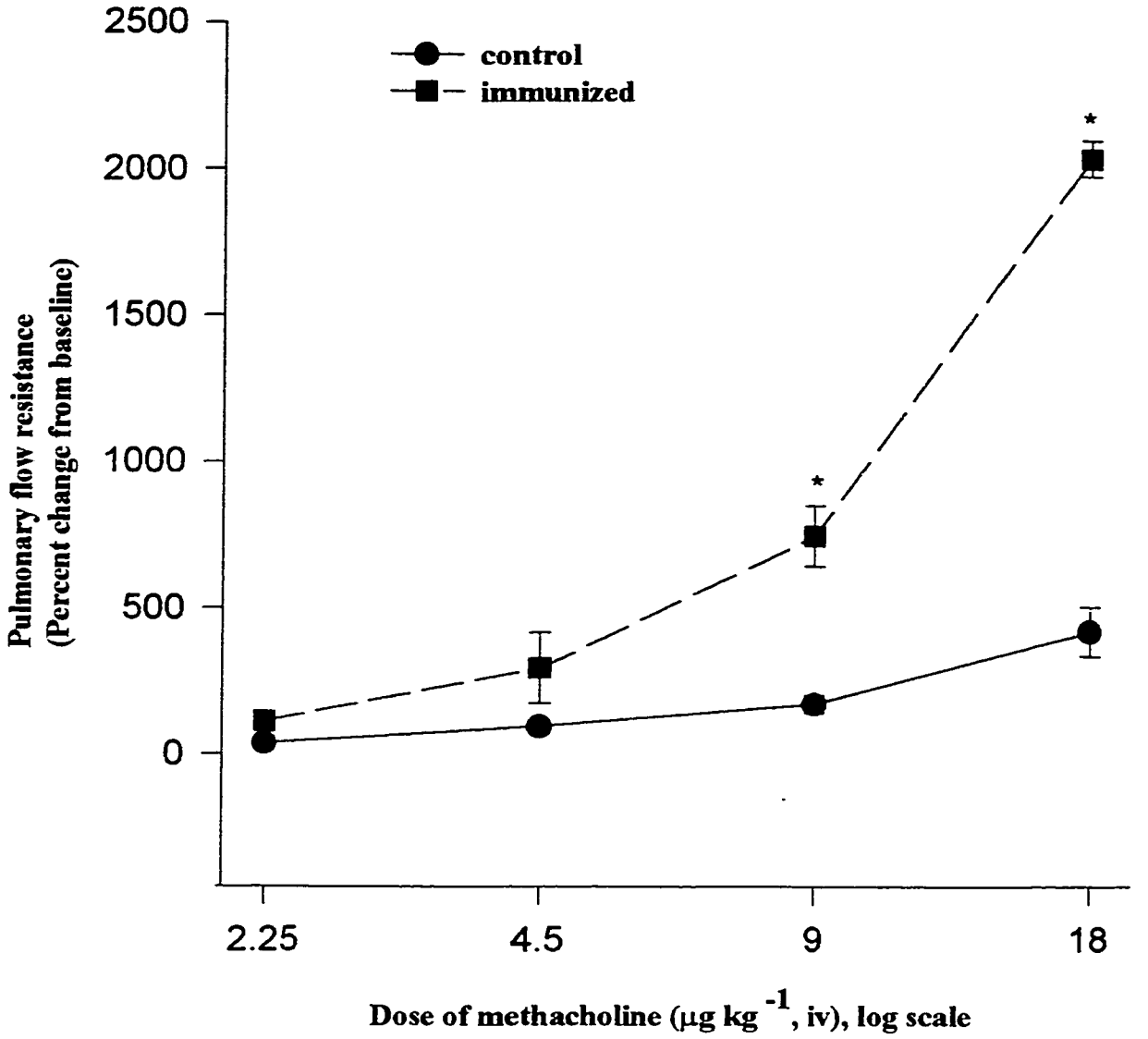

Figure 3.1.2 Changes in pulmonary flow resistance (R_L) in response to various doses of methacholine (iv) in anesthetized and mechanically ventilated BN rats immunized with OA and adjuvants (immunized). The controls were treated with adjuvant only. Each point presents percent change from baseline and values are mean ± SEM of four animals. The challenged rats showed three to four-fold increase in R_L at higher concentrations of methacholine compared to the controls. * denotes $p<0.05$.

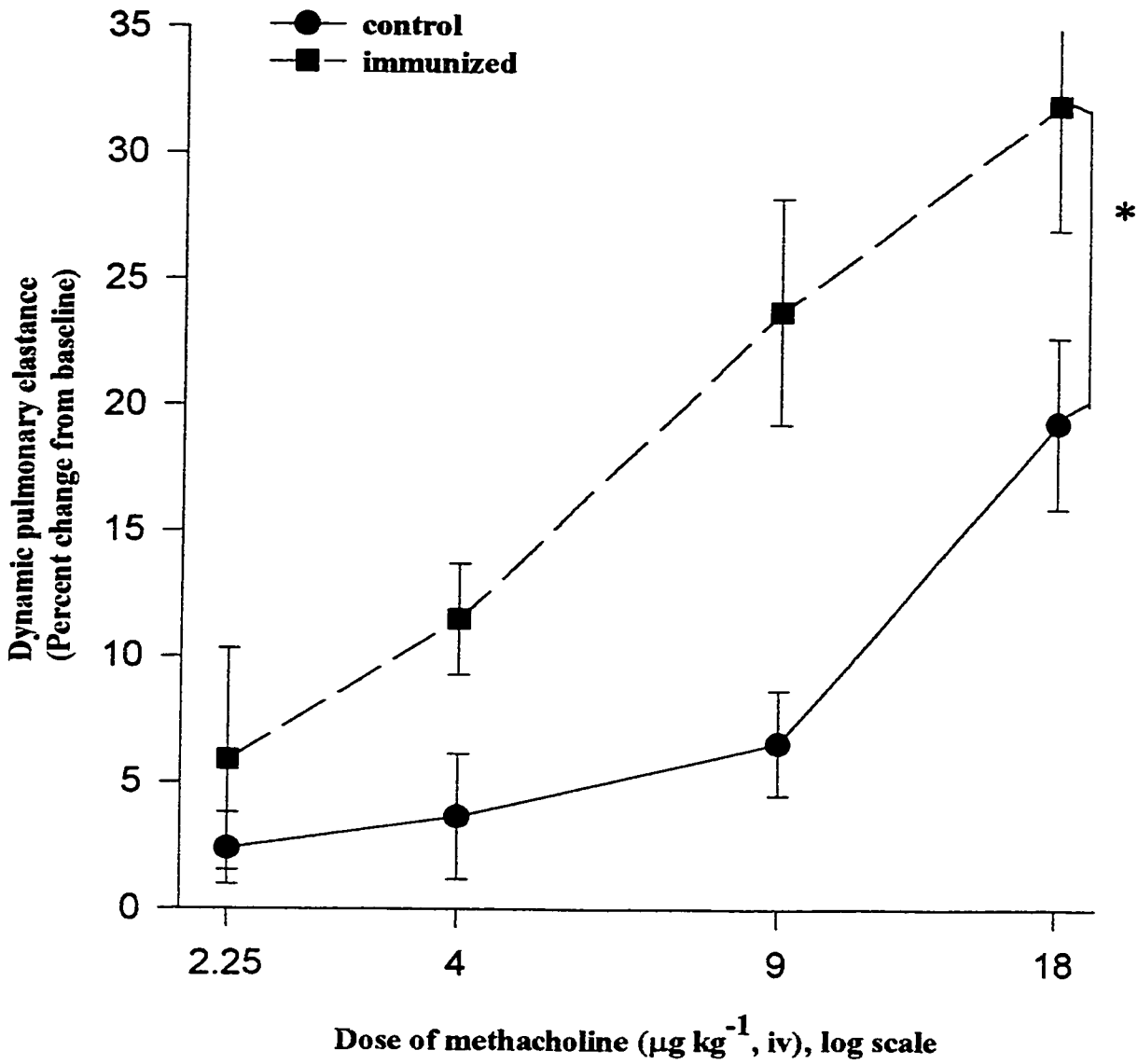

Figure 3.1.3 Changes in dynamic pulmonary elastance (E_L) in response to various doses of methacholine (iv) in anesthetized and mechanically ventilated BN rats immunized with OA and adjuvants (immunized). The controls were treated with adjuvant only. Each point presents percent change from baseline and values are mean ± SEM of four animals. The increases in E_L in the immunized rats were statistically significant ($p<0.04$) compared to the controls denoted by *.

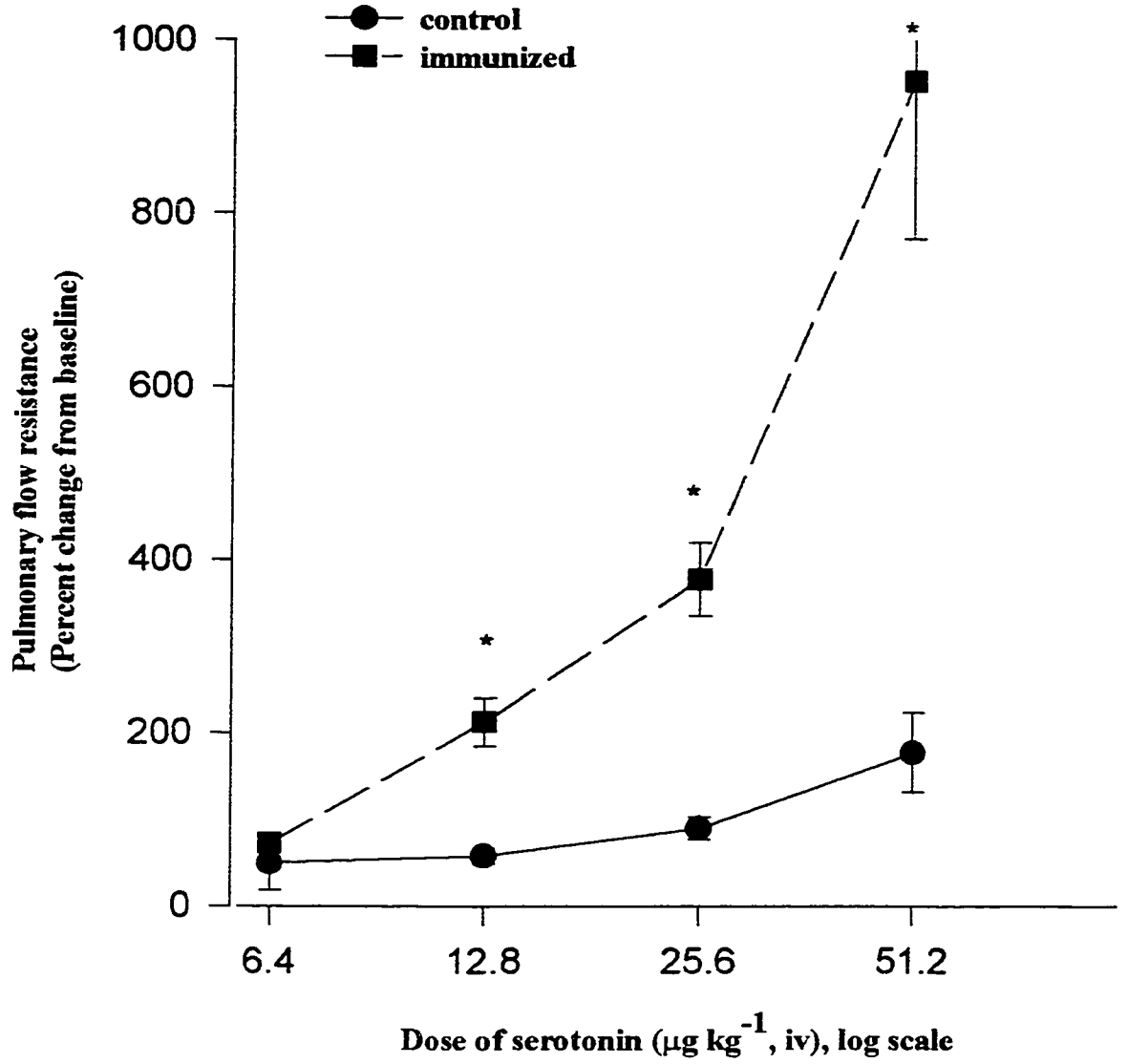

Figure 3.1.4 Changes in pulmonary flow resistance (R_L) in response to various doses of serotonin (iv) in anesthetized and mechanically ventilated BN rats immunized with OA and adjuvants (immunized). The controls were treated with adjuvant only. Each point represents percent change from baseline and values are mean ± SEM of four animals. Three to five-fold increase in R_L in the immunized group was observed compared to the controls. * denotes $p<0.05$.

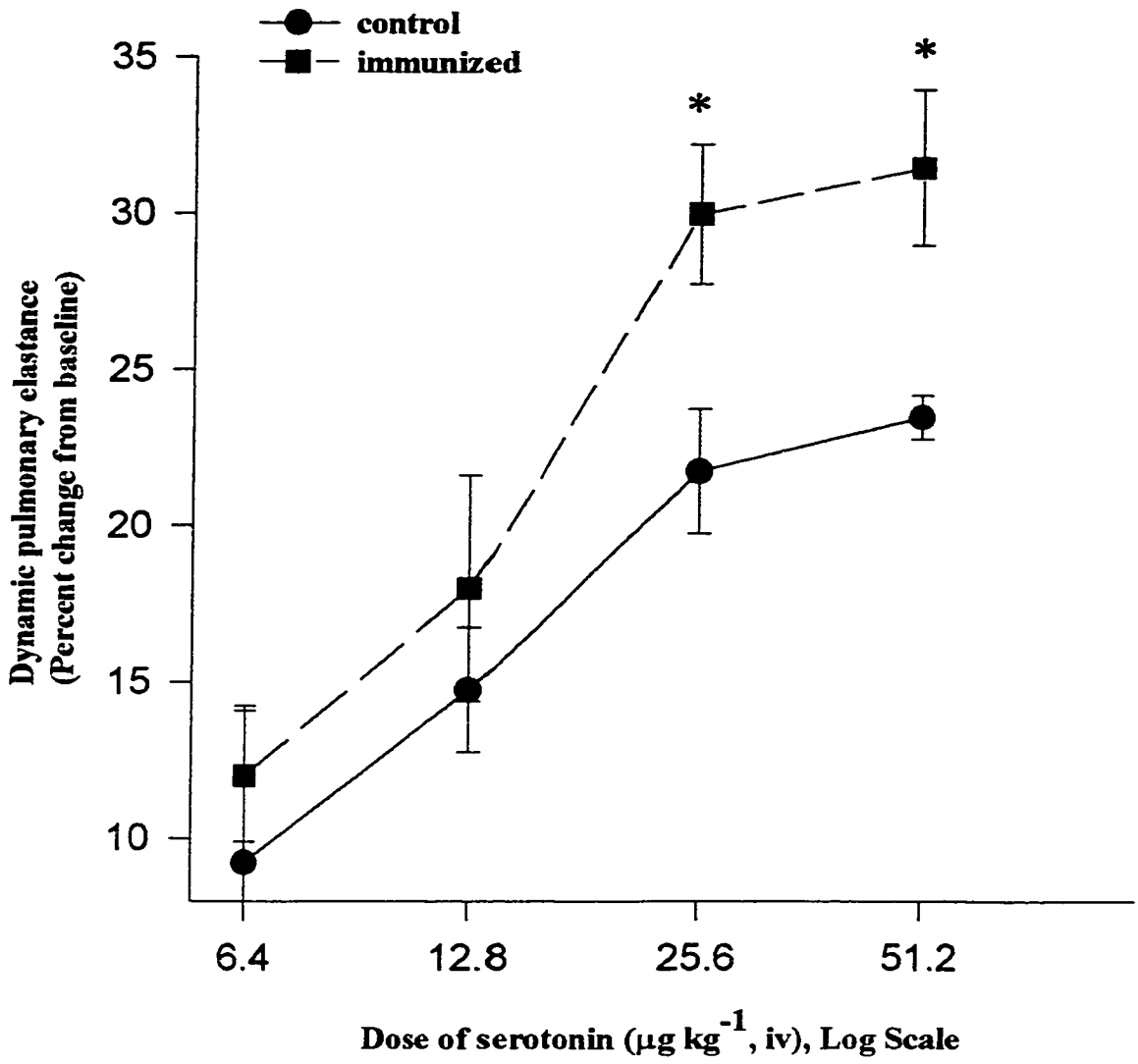

Figure 3.1.5 Changes in dynamic pulmonary elastance (E_L) in response to various doses of serotonin (iv) in anesthetized and mechanically ventilated BN rats immunized with OA and adjuvants (immunized). The controls were treated with adjuvant only. Each point presents percent change from baseline and values are mean ± SEM of four animals. * denotes $p<0.05$ compared to the controls.

airways prevents agonists from reaching to the lower airways. Therefore, an intravenous delivery of the agonists was chose to effectively induce airway hyperreactivity to the agonists compared to the adjuvant-immunized controls. Elwood *et al* (1991) performed control studies (e.g. the adjuvant used, sensitization with OA and adjuvant, or exposure of unsensitized rats to OA aerosol) to determine whether the treatments had any effect on airway responsiveness or inflammatory changes compared to saline-only treated group. These procedures caused no significant changes in AHR and no large increase in inflammatory cell recovery found in the BAL fluid after allergen exposure. Based on observations, adjuvants-immunized controls were used to exclude possible non-specific response of the immune system or of the lungs to adjuvants.

In summary, OA-immunized BN rats showed airway hyperresponsiveness as well as signs and symptoms of allergic reaction e.g. dyspnea, increased eosinophils and lymphocytes. This inbred rat strain offers promise as a convenient model of antigen-induced airway hyperresponsiveness.

3.2. Experiment two: The effect of liposome encapsulated dichloromethylene diphosphonate on alveolar macrophage depletion

3.2.1. Preparation of liposome encapsulated dichloromethylene diphosphonate

The concentration of LE DMDP ranged from 12.5 to 20 mM, and the particle size was 1.42 ± 0.18 μm (n = 6). The encapsulation efficiency was 10.5% ± 2.5%. Difficulty in obtaining raw DMDP required using the commercially available intravenous drug

preparation. The initial concentration of DMDP from this source was only 54 mg/mL, much lower than the initial concentration (1890 mg/mL) used in other studies. On the other hand, the encapsulation efficiency (8% to 13%) was 4-6 fold higher than from neutral liposomes of DMDP (2%) prepared by Claassen and Van Rooijen (1984 and 1986) and higher than that of the negatively-charged liposome (7.1%) prepared by Mönkkönen (1993).

Based on the method of Claassen and Van Rooijen, the composition of liposomes was modified to employ the process of freeze-thaw cycling. The use of freeze-thaw cycling results in repeated rupture and fusion of the vesicles and a reduction in the number of liposomal lamellae. This process can lead to increased EE of hydrophilic solutes and improve size homogeneity (Mayer, 1985). Furthermore, MLVs possessing negatively or positively charged bilayer surfaces have larger aqueous compartments than their neutral counterparts due to the electrostatic repulsion of bilayers, and, hence, can achieve higher encapsulation efficiency. This was achieved by adding charged DMPG into liposome. The increased EE of DMPC:Cholesterol:DMPG is attributed to large aqueous compartments between bilayers in multilamellar vesicles.

In general, negatively-charged liposomes are bound to and endocytosed by cells to a greater extent than neutral liposomes (Heath *et al*, 1985). The successful elimination of macrophages is dependent on the DMDP concentration in liposome and liposomal size and surface charge (Heath *et al*, 1985; Mönkkönen *et al*, 1994). Mönkkönen *et al* (1994) indicated that neutral multilamellar vesicles over 5000 nm were not effective in the delivery of DMDP to cells *in vitro* in a study of the inhibition of the growth of RAW264 cells. Reduction of vesicle size to 120 nm strongly increased the growth inhibitory

potency of DMDP but also broadened the effects of DMDP to other cell types such as fibroblasts. In comparison, a negatively-charged liposomes effectively inhibited RAW264 cells. Unfortunately, details of size of LE DMDP were not provided (Claassen and Van Rooijen, 1986; Thepen *et al*, 1992).

3.2.2. Studies on alveolar macrophage depletion with liposome encapsulated dichloromethylene diphosphonate

Using the modified method liposome preparation described in 2.4. and 3.2.1., negatively-charged multilamellar vesicles were an appropriate size (1.42 ± 0.18 μm), which resulted in successful elimination of more than 70% of AM, assessed by direct cell count (Figure 3.2.1). Berg *et al* (1993) showed that the depletion of AM in BAL fluid was 70% by direct counting of AM whereas counting of AM in immunohistochemical tissue sections was 83%.

Figure 3.2.1 shows the number of viable AM as a percent of control AM found in alveolar lavage fluid at 0, 1, 3 and 7 days after intratracheal administration of LE DMDP. After days 1 and 3 AM comprised, on average, <30% of viable cells recovered. This increased to around 43.8% after 7 days. Interestingly, at days 1, 3 and 7 after administration of similar volumes and concentrations of saline alone or LE saline, the recovered viable AM was also reduced (Table 3.2.1). At days 1, 3 and 7 the percent of

Table 3.2.1 Cells ($x10^6 mL^{-1}$) recovered from lavaged fluid at different times after administration of LE DMDP or control treatments

Cells	Day 1 (n=3/group)			Day 3 (n=3/group)			Day 7 (n=3/group)			Day 0 (n=3/group)
	Sal	Lip	LE	Sal	Lip	LE	Sal	Lip	LE	Controls
Total	1.2 ± 0.3	1.2 ± 0.4	0.5 ± 0.04*	1.4 ± 0.1	1.1 ± 0.6	0.6 ± 0.2	1.6 ± 0.2	1.4 ± 0.6	0.9 ± 0.3	2.1 ± 0.8
AM	1.0 ± 0.04 (89 ± 3.8)	1.1 ± 0.02 (91 ± 1.7)	0.4 ± 0.03* (75 ± 5.9)	1.2 ± 0.02 (91 ± 1.2)	1.0 ± 0.06 (91 ± 6.2)	0.5 ± 0.03 (83 ± 4.7)	1.5 ± 0.02 (94 ± 1.1)	1.3 ± 0.02 (94 ± 1.6)	0.8 ± 0.01 (90 ± 1.3)	1.9 ± 0.02 (94 ± 1.6)
N	0.01 ± 0.006 (1.3 ± 0.6)	0.01 ± 0.001 (0.8 ± 0.1)	0.05 ± 0.002* (8.5 ± 0.5)	0.01 ± 0.001 (0.6 ± 0.1)	0.02 ± 0.01 (1.8 ± 1.3)	0.009 ± 0.005 (1.3 ± 0.8)	0.002 ± 0.002 (0.16 ± 0.1)	0.002 ± 0.002 (0.16 ± 0.1)	0.001 ± 0.001 (0.2 ± 0.1)	0.003 ± 0.003 (0.2 ± 0.1)
Other	0.1 ± 0.09 (9.0 ± 7.8)	0.1 ± 0.06 (8.0 ± 5.1)	0.1 ± 0.005* (16 ± 0.8)	0.1 ± 0.02 (8.5 ± 1.7)	0.08 ± 0.004 (7.2 ± 0.4)	0.1 ± 0.01 (15 ± 1.6)	0.09 ± 0.04 (6.0 ± 2.5)	0.07 ± 0.001 (5.1 ± 0.1)	0.10 ± 0.001 (9.8 ± 0.1)	0.1 ± 0.001 (5.8 ± 0.001)

The number of viable total leucocytes (Total), alveolar macrophages (AM), neutrophils (N) and other cells (Other), found from lavaged fluid at 0 (Controls), 1, 3 and 7 days after the intratracheal administration of LE DMDP (LE), LE saline (lip) or saline (sal). Values represent Mean ± SEM from three Brown Norway rats. Values in parentheses represent the percent total cells. * denotes $p<0.05$ compared to the controls.

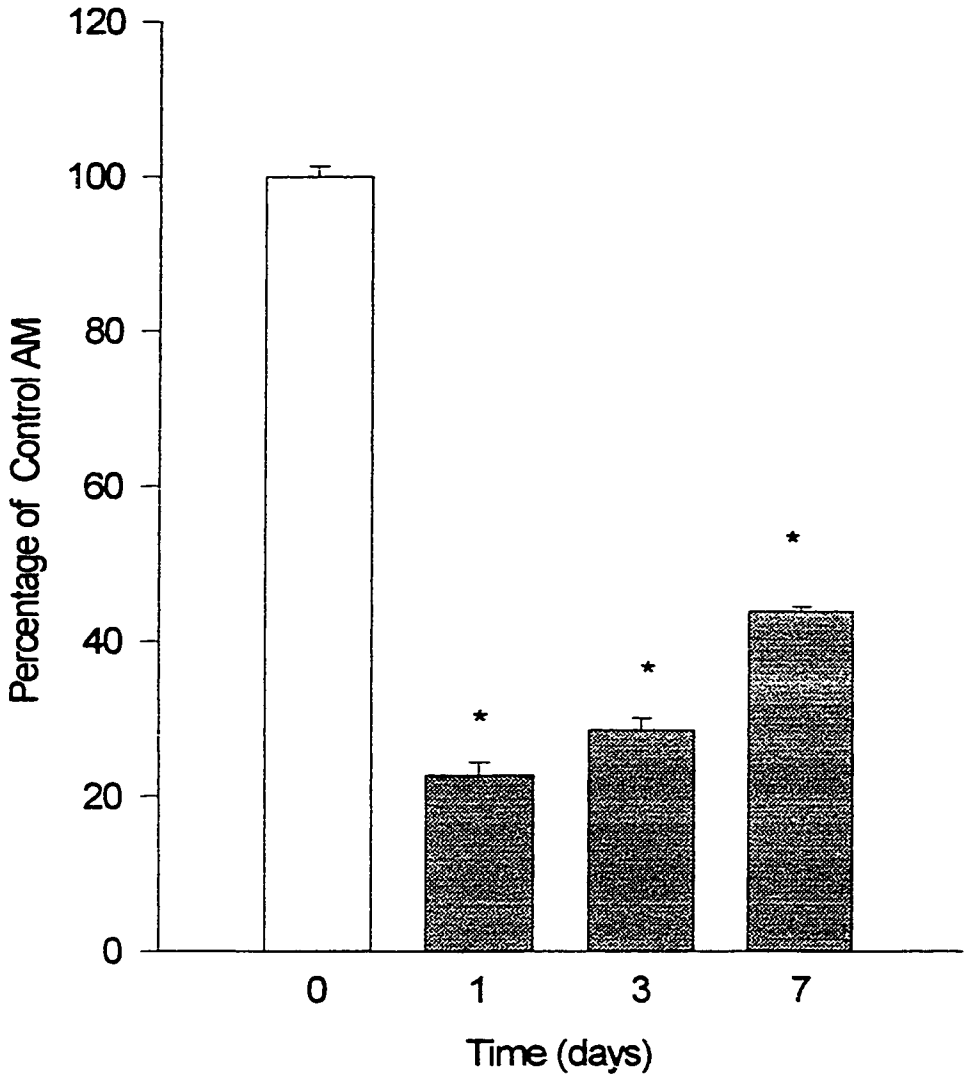

Figure 3.2.1 The number of viable alveolar macrophages (AM) recovered as a percentage of control AM, found in alveolar lavage fluid at 0, 1, 3 and 7 days after the intratracheal administration of liposome-encapsulated (LE) DMDP. * denotes $p<0.05$ compared to controls at day 0 (3 BN rats without any treatments as a controls).

AM recovered was always significantly less after LE DMDP administration than after saline, or LE saline, administration. Although the total viable leukocytes in other groups indicated a tend to decrease, only the reduction of the total leucocytes in the LE DMDP group is statistically significant ($p<0.05$) (Table 3.2.1). After administration of LE DMDP, macrophages recovered from lavage fluid were heavily vacuolated and became gigantic morphologically, implying that the LE DMDP was phagocytosed by AM. Eosinophil counts yielded no significant changes among the groups (data not shown).

At day 1 after administration, neutrophil population in the LE DMDP group increased remarkably ($p<0.05$) as AM and total leukocytes decreased. Transient increases in the fraction of neutrophils may have resulted from a reactive inflammation either to LE DMDP-induced destruction of AM or to direct irritation from intratracheal injection. Berg *et al* (1993) also reported a transient increase in neutrophil number after the depletion of AM but gave no explanation. They further reported that the LE DMDP pre-treatment markedly reduced the number of AM and, consequently, reduced endotoxin-induced increase in neutrophils and the release of TNF into the alveolar space 4 hours after endotoxin insufflation in rats.

Compared to the normal controls (day 0), AM comprised <30% of viable cells recovered following the administration of LE DMDP. Unexpectedly, saline and LE saline also induced a decrease of AM and total leukocytes compared to normal controls, nevertheless, the absolute cell numbers showed no statistical differences. On the other hand, no relevant morphological changes to AM, such as vacuolization and death of AM, were observed as usually shown in the LE DMDP-insufflated group (Berg *et al*, 1993). The mechanism of these changes is unknown. Non-cytotoxic saline or LE saline seem

unlikely to directly induce the destruction of AM. Additionally, *in vitro* culturing of AM in the presence of saline or LE saline did not induce AM death, (Van Rooijen N *et al*, 1988; Mönkkönen and Heath, 1993; Mönkkönen *et al*, 1994). In other studies with AM-depleted rats (Thepen *et al* 1989 and 1992; Berg *et al*, 1993), there were no data available on BAL cells from normal rats to compare cell changes from rats with treatments of PBS, LE PBS or LE DMDP.

LE DMDP is effective only for cells with high phagocytic capacity. As discussed in 1.3., the precise molecular mechanism of DMDP on the depletion of macrophages is unclear. It may be possible that the calcium-binding activity of the molecule, or the presence of chlorine groups or intracellular iron play a role in the depletion of macrophages (Rooijen, 1991 and 1992; Mönkkönen and Heath, 1993). A recent study suggests that LE DMDP can induce *in vitro* apoptosis in osteoclasts and macrophages (Selander *et al*, 1996). Furthermore, DMDP-induced destruction of macrophages can be partially prevented by the addition of several inhibitors of the apoptotic cascade (staurosporine or homocysteine).

In summary, the production of negatively-charged liposomes of appropriate size and containing DMDP was achieved. Our procedure produced a product with higher encapsulation efficiency of DMDP than previously reported. The LE DMDP prepared by the modified procedure was effective in the depletion of AM *in vivo*. This provides a useful AM-depleted animal model for an *in vivo* study of the functions of AM and their role in various physiological and pathological conditions.

3.3. Experiment three: Evaluation of airway hyperresponsiveness in alveolar macrophage-depleted rats

The four groups of BN rats in (OLO, OSO, SLS, and SSS, see Table 2.2.1) showed different symptoms after aerosol exposure. OA-immunized rats (OLO and OSO) showed slight respiratory stress (laboured breath) after OA aerosol exposure. OA-immunized rats were usually quiet and moved less than controls. They walked with laboured, jerky movements with a pant. Some showed "hair roughed up" appearance and audible wheezing. After first exposure, one rat in the OLO group developed severe dyspnea, seizures and made a "croaking" sound (signalled severe bronchospasm). Despite an injection of adrenaline chloride (0.1 mg, sc), the rat still showed cyanotic, severe respiratory stress and finally died of anaphylaxis. The data analysis for this group is therefore derived from the surviving three rats. Saline-immunized rats showed no specific reaction to saline aerosol challenge and tolerated the whole challenge procedure.

Cell changes in lavaged fluid are summarized in Table 3.3.1. Immunized rats were lavaged after the evaluation of airway responses. The cell changes represent the transient changes 4 days after AM depletion. Neutrophils and other cells showed no significant changes among groups. It is notable that at day 4 after administration of LE DMDP, AM remained at a low population in AM-depleted groups (OLO, 1.4×10^6 mL $^{-1}$ and SLS, 1.0×10^6 mL $^{-1}$) than those in non-depleted groups (SSS and OSO) (2.1×10^6 mL $^{-1}$and 2.3×10^6 mL^{-1}). In addition, after administration of LE DMDP (groups OLO and SLS), macrophages recovered from lavage fluid were heavily vacuolated and were gigantic morphologically.

Table 3.3.1 Cells ($x10^6$ mL^{-1}) recovered from lavaged fluid after immunization and AM depletion

Cells	SSS	OSO	OLO	SLS
Total	2.5 ± 0.5	2.5 ± 0.8	1.6 ± 0.3	1.2 ± 0.2
AM	2.3 ± 0.02^{cd} (92 ± 0.9)	2.1 ± 0.1^{cd} (83 ± 4.6)	1.4 ± 0.02^{abd} (83 ± 1.2)	1.0 ± 0.001^{abc} (81 ± 0.3)
N	0.004 ± 0.004 (0.2 ± 0.2)	0.03 ± 0.01 (1.2 ± 0.4)	0.005 ± 0.005 (0.3 ± 0.3)	0.04 ± 0.02 (3.0 ± 1.5)
E	0.00 ± 0.00^{bcd} (0.00 ± 0.00) ^{bcd}	0.1 ± 0.02^{acd} (3.5 ± 0.4) ^{ad}	0.04 ± 0.007^{abd} (2.6 ± 0.4) ^{ad}	0.01 ± 0.003^{abc} (0.8 ± 0.3) ^{abc}
Others	0.2 ± 0.03 (8.1 ± 1.1)	0.3 ± 0.1 (12 ± 3.9)	0.2 ± 0.02 (13 ± 1.2)	0.2 ± 0.01 (15 ± 1.0)

The number of viable total leucocytes (Total), alveolar macrophages (AM), neutrophils (N), eosinophils (E) and other cells (Others) found from lavaged fluid immediately after the measurement of airway response (mean ± SEM). AM-depleted rats were immunized with OA (OLO) or saline (SLS). Rats that were not AM depleted were also immunized with OA (OSO) or saline (SSS) (see text 2.2.3.). Values in parenthesis denote percent total cells. Significant differences ($p<0.05$) are denoted as "a" compared to SSS; "b" compared to OSO; "c" compared to OLO and "d" compared to SLS.

Three days after the administration of LE DMDP some macrophages were a smaller size and had monocyte-like characteristics, which may imply that those macrophages were newly recruited from peripheral blood.

Viable eosinophil counts and percentage in total leucocytes significantly increased in OA-immunized groups (OLO and OSO) compared to saline-immunized groups (SSS and SLS). It is interesting that in AM-depleted groups, OA-immunization (OLO) led to increased AM and eosinophil populations relative to those in the saline-treated group (SLS). This implied elimination of AM promotes the cellular reactions to OA challenge. Kradin *et al* (1999) recently showed that eliminating AM *in vivo* promotes pulmonary cell-mediated immunity to heat-killed Listeria and increases in AM compared to saline controls. In addition, Thepen *et al* (1992) observed that the OA-challenged, AM-depleted BN rats developed large mononuclear cell infiltration in lung and airways, which included a substantial CD4 T cells component accessed by cytofluorographic analysis. However, they did not observe eosinophil changes in the AM-depleted animals.

Changes in pulmonary flow resistance and dynamic pulmonary elastance in response to graded doses of methacholine or serotonin (both iv) in the four groups are shown in Figures 3.3.1 - 3.3.4. In saline-immunized and saline-challenged rats, no significant differences in R_L and E_L were noted between AM-depleted group (SLS) and non AM-depleted group (SSS) for either methacholine or serotonin. In OA-immunized rats (OLO and OSO), R_L to methacholine and serotonin was remarkably enhanced over those in saline-immunized rats (SSS and SLS).

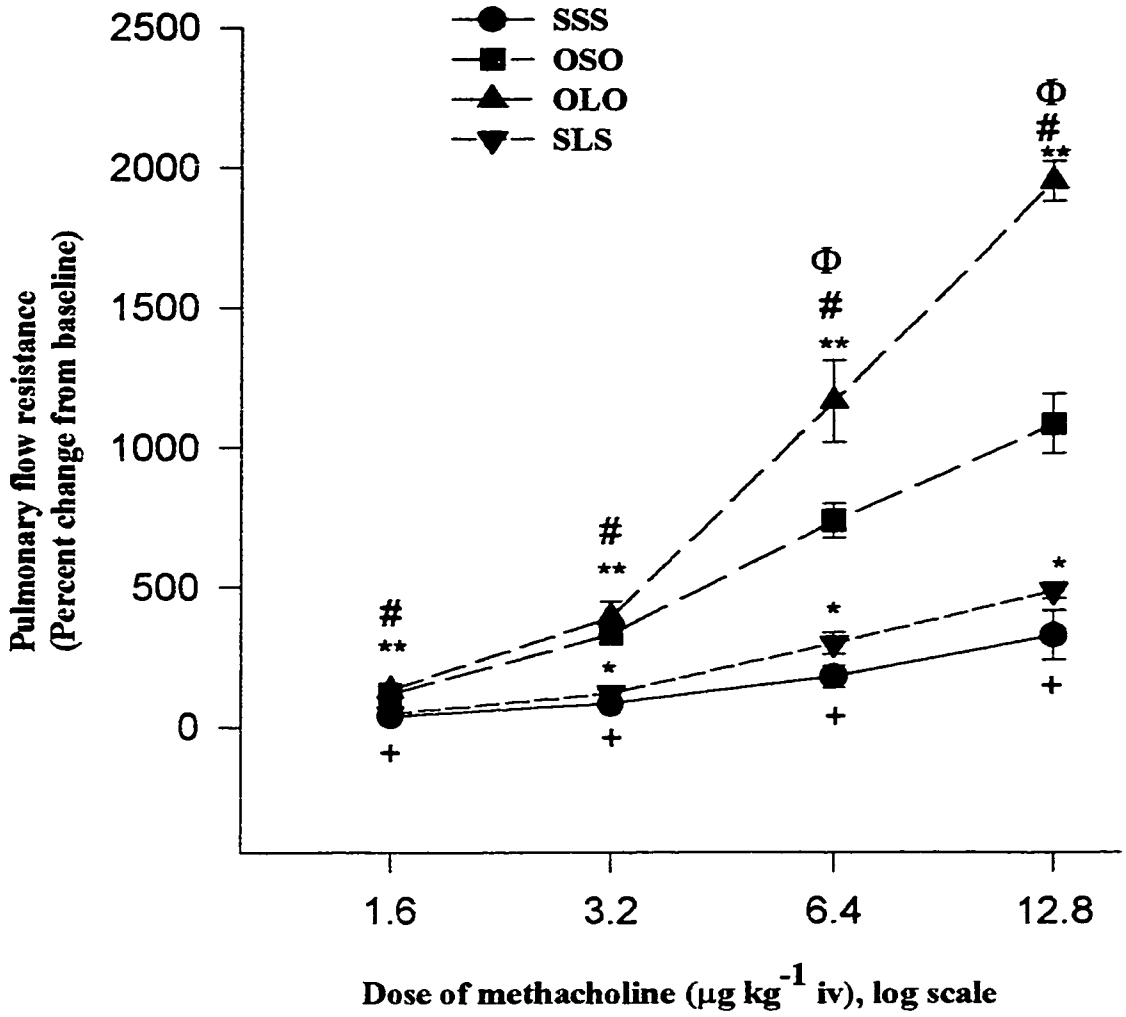

Figure 3.3.1 Changes in pulmonary flow resistance (R_L) to various doses of methacholine (iv) in anesthetized and mechanically ventilated BN rats immunized with OA and adjuvants (challenged). Alveolar macrophage (AM)-depleted rats were immunized with OA (OLO) or saline (SLS). Rats with normal AM were immunized with OA (OSO) and saline (SSS), respectively (see text 2.2.3.). Each point represents percent change from baseline and values are mean ± SEM of 3 to 4 rats. (# $p<0.05$ - SSS *vs* OLO; + $p<0.05$ - SSS *vs* OSO; * $p<0.05$ - SLS *vs* OSO; ** $p<0.05$ - SLS *vs* OLO; Φ $p<0.05$ - OSO *vs* OLO).

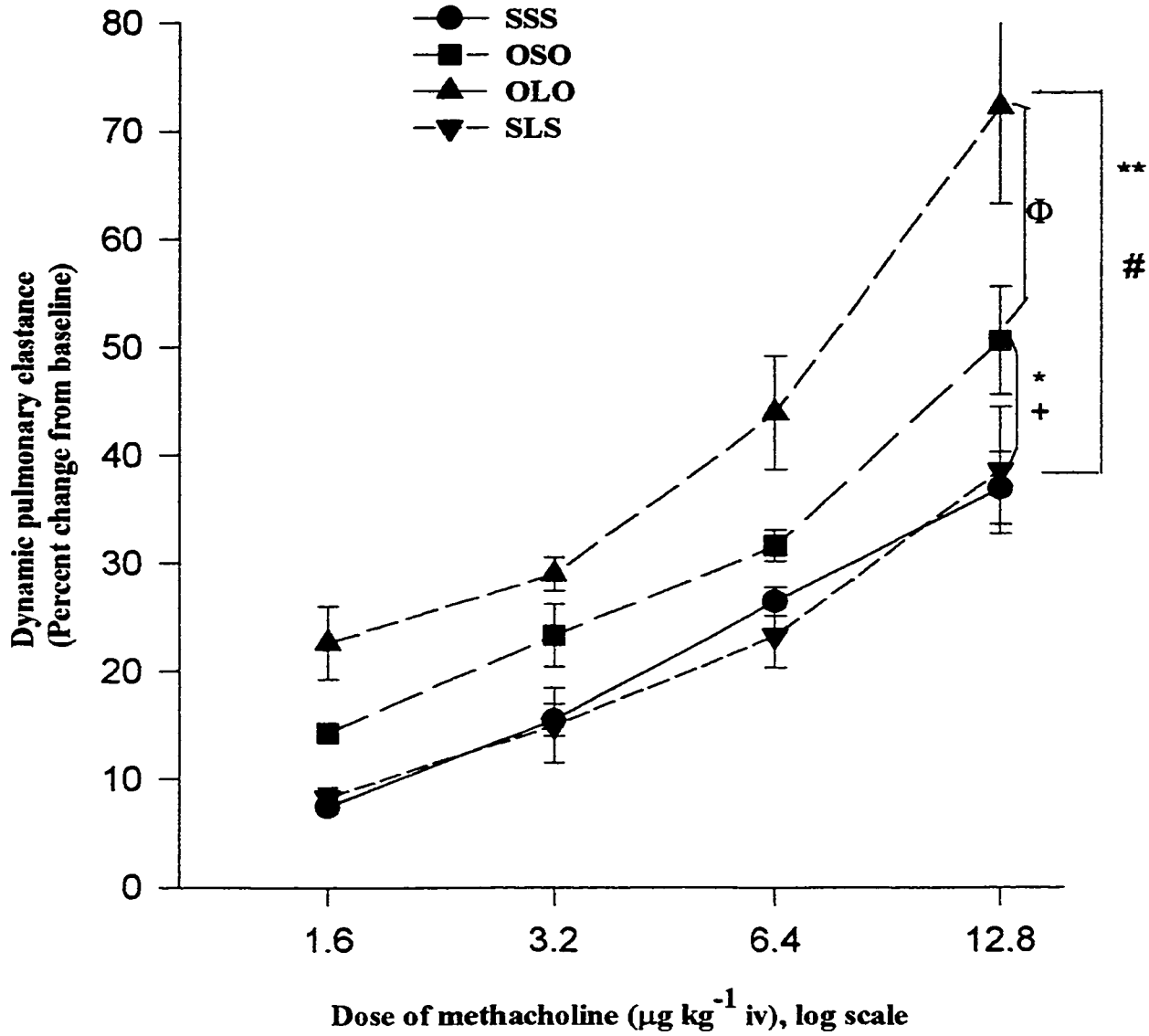

Figure 3.3.2 Changes in dynamic pulmonary elastance (E_L) to various doses of methacholine (iv) in anesthetized and mechanically ventilated BN rats immunized with OA and adjuvants (the groups described as Figure 3.3.1). Each point represents percent change from baseline and values are mean ± SEM of 3 to 4 rats. (# $p<0.05$ - SSS *vs* OLO; + $p<0.05$ - SLS *vs* OSO; * $p<0.05$ - SLS *vs* OSO; ** $p<0.05$ - SLS *vs* OLO; Φ $p<0.05$ - OSO vs OLO).

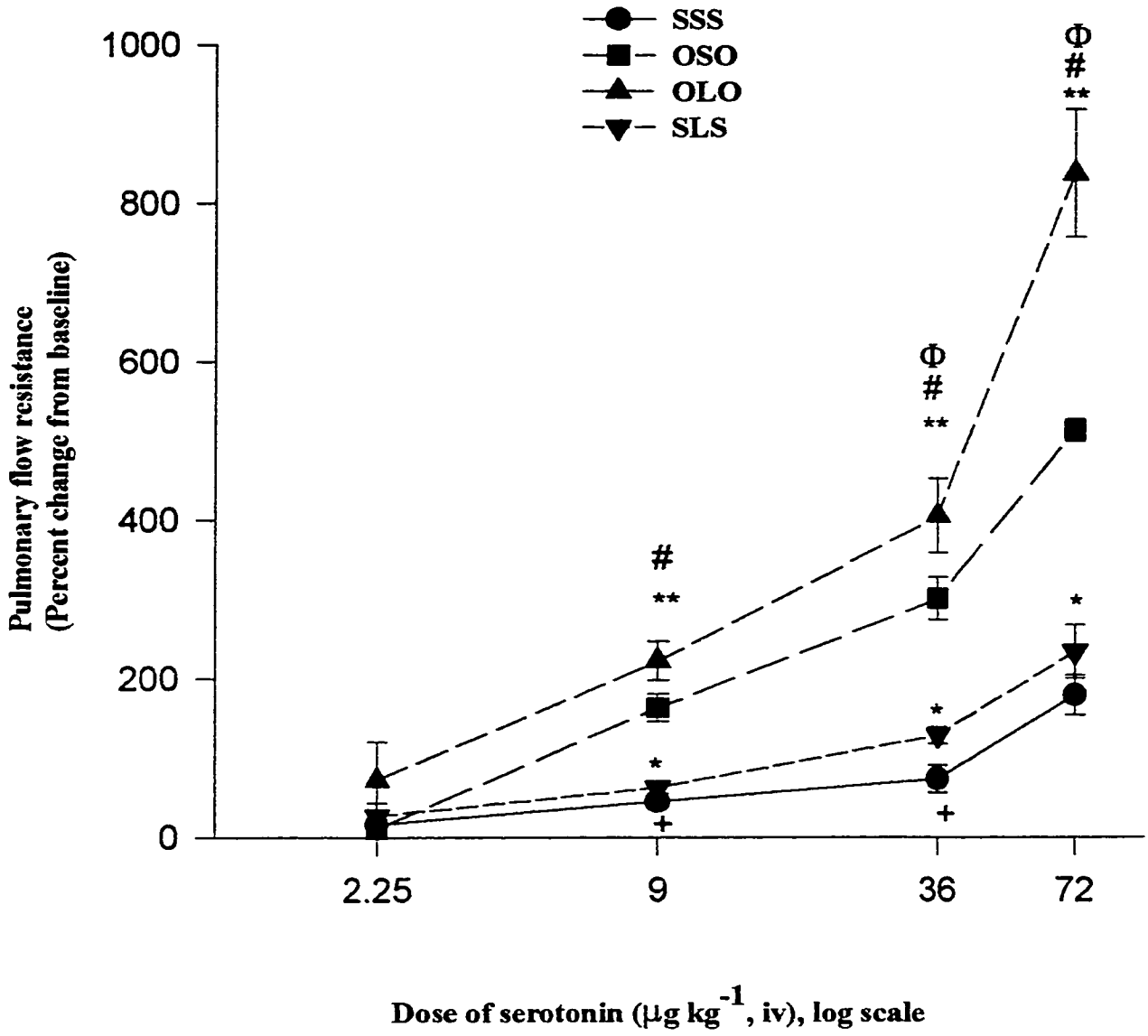

Figure 3.3.3 Changes in pulmonary flow resistance (R_L) to various doses of serotonin (iv) in anesthetized and mechanically ventilated BN rats immunized with OA and adjuvants (the groups described as Figure 3.3.1). Each point represents percent change from baseline and values are mean ± SEM of 3 to 4 rats. (# $p<0.05$ - SSS *vs* OLO; + $p<0.05$ - SSS *vs* OSO; * $p<0.05$ - SLS *vs* OSO; ** $p<0.05$ - SLS *vs* OLO; Φ $p<0.05$ - OSO *vs* OLO).

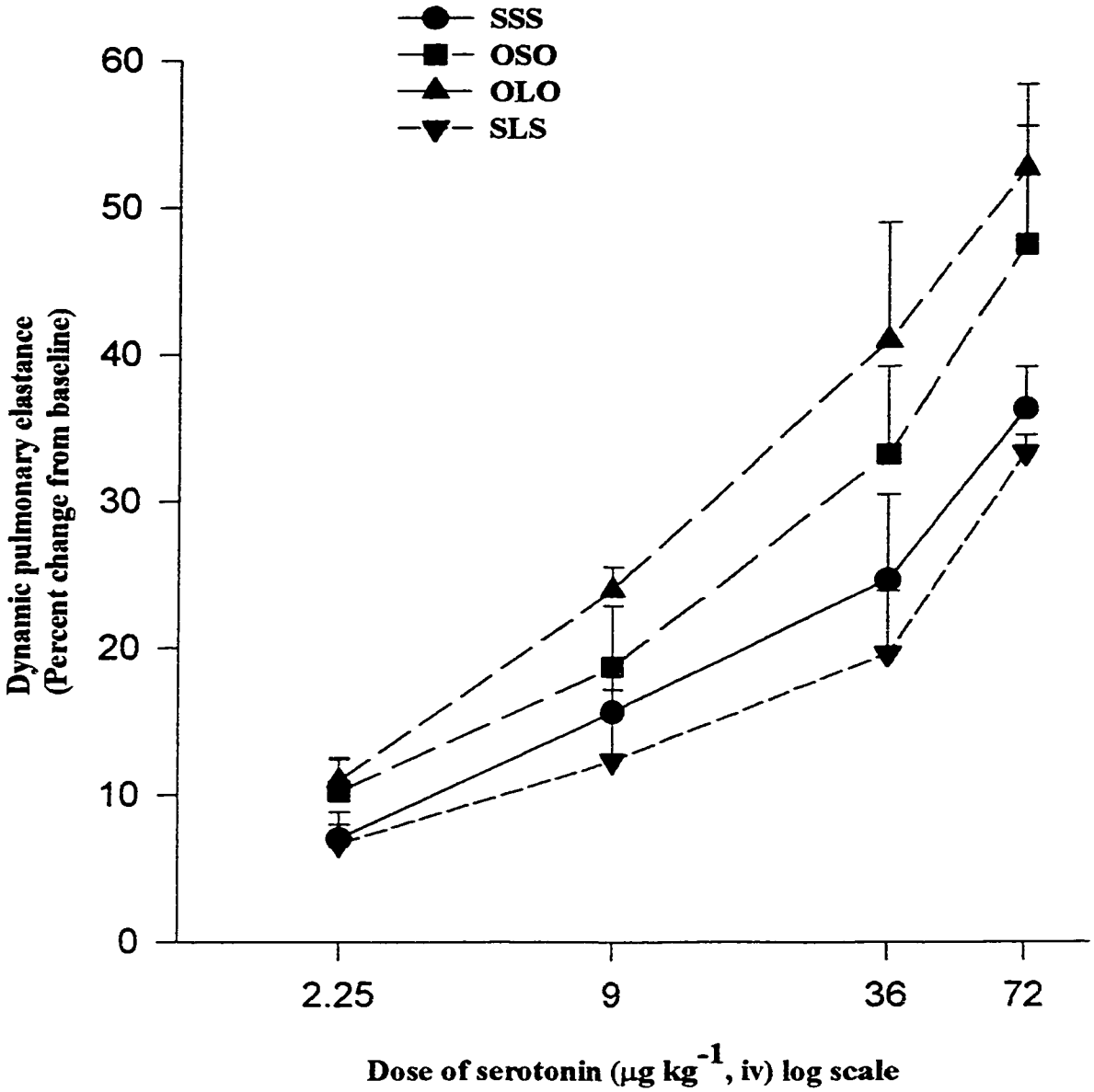

Figure 3.3.4 Changes in dynamic pulmonary elastance (E_L) to various doses of serotonin (iv) in anesthetized and mechanically ventilated BN rats immunized with OA and adjuvants (the groups described as Figure 3.3.1). Each point represents percent change from baseline and values are mean ± SEM of 3 to 4 rats. There were no significant differences among groups.

Furthermore, at higher concentrations of methacholine (Figure 3.3.1) and serotonin (Figure 3.3.3), R_L in AM-depleted rats (OLO) was significantly enhanced over that in rats without AM depletion (OSO). This shows that the OA-immunized and AM-depleted rats (OLO) exhibit the highest airway responsiveness to the agonists.

OA-immunized rats (OLO and OSO) have enhanced E_L to methacholine compared to saline-immunized rats (SSS and SLS) (Figure 3.3.2). Following administration of serotonin, no significant changes in E_L among the groups were noted (Figure 3.3.4) likely as a result of the degree of variation in these measurements and also the lower sensitivity of this parameter.

Allergic lung diseases such as atopic asthma are now recognized as chronic inflammatory diseases promoted by dysregulation of T cell-mediated immune mechanisms. Although the mechanism remains unclear, accumulating evidence demonstrates that T cells are associated with the development of AHR. Adoptive transfer of allergen-specific CD4 T cells, but not CD8 T cells, did induce AHR and airway eosinophilia in rats (Watanbe *et al*, 1995). CD8 T cells depletion in BN rats showed that CD8 T cells have probably a protective role in allergen-induced AHR and eosinophils inflammation, probably through activation of the Th1 cytokine response (Huang *et al*, 1999). These observations suggested the presence of an imbalance of functionally distinctive T cells. Interestingly, rat AM were revealed to express CD8α, using the anti-CD8 antibody MRC OX-8, to detect the CD8 hinge region, in over 60% of AM (Hirji *et al*, 1997). Actually, the imbalance of functionally distinctive T cell subsets may be regulated by AM. AM have both the "negative" and "positive" regulation functions on T cell-dependent allergic reaction in the lungs. "Negative" or down-regulation functions

include 1) down-regulation of the function of Th2 in controlling the synthesis of IgE by B-lymphocytes; 2) inhibition of the accessory function of DC to present antigen to lymphocytes; 3) suppression of the infiltration of PBM in lung. PBM are more effective than AM in inducing proliferation of lymphocytes to antigen.

Selective elimination of AM *in vivo* has resulted in increased T cell reactivity and pulmonary immune response via promoting IgE production and other mechanisms (Poulter *et al*, 1986; Thepen *et al*, 1989, 1992). Nevertheless, there is little information available on the *in vivo* effect of AM depletion on the development of AHR in intact animals.

We first showed that AM depletion induces *in vivo* airway hyperresponsiveness to agonists. OA-immunized rats showed a significant increase of airway responsiveness to agonists compared to sham controls ($p<0.05$). In both OA-immunized groups, higher airway responses in the AM-depleted group were observed in comparison to the group without AM depletion. The differences are statistically significant at higher concentrations of the bronchospasm agonists.

A question that arises is why do not all atopic individuals who are sensitized to inhaled allergens develop asthma. It must be a delicate protective system to control lung immune response to allergen. Current studies have paid more attention to the special role of AM in determining the local immune responses provoked by allergen exposure in atopic subjects. The "negative" regulative function of AM is thought to have been compromised in asthma. Another explanation is that AM in asthmatics may consist of a dynamic system of functional suppressive AM and enhancer AM subpopulations. This postulate suggests that the suppressive AM, an AM subset, is inhibited and loses its

inhibitory effects in asthma but does not show a generalized decrease in functional activity.

The selective elimination of AM increases the functional imbalance thought to exist in asthma. Following AM depletion, monocytes (or enhancer AM) come from the peripheral blood or from local proliferation. However, the incoming monocytes have to take time to mature or reacquire the "negative" regulatory functions equivalent to that of resident (mature) AM (Bilyk and Holt, 1995). It is postulated that AM (enhancer AM) mainly up-regulate immune responses to foreign allergen through enhancing T cell-dependent allergic responses. Thepen's studies (1989 and 1992) confirmed that *in vivo* selective elimination of AM promotes local IgE production and humoral response to inhaled antigen. Bilyk and Holt (1995) indicated that resident AM are poor APC and suppress the activities of both DC and T lymphocytes. Haczku *et al* (1994) made another observation of interest. In the 8 wk exposed BN rats long-term OA-exposed rats showed suppression of AHR, accompanied by increased numbers of AM. It is possible that the increased AM may play an inhibitory role in the development of AHR. *In vivo* experiments to measure airway hyperresponsiveness in the OA-immunized and AM-depleted BN rats support those proposals. The data including the cell counts and the morphological observations, also suggest that after the administration of LE DMDP, the remaining AM demonstrated monocyte-like and immature cell characteristics (renewed enhancer AM). The inhibition of T cell-suppressing activity of resident AM appears to be only transient, as resident AM reacquire their suppressive property in a short time (Bilyk and Holt, 1995). AM depletion further extends the "transient" inhibition of resident AM. The incoming macrophages skew the AM population into a predominant "accessory

cells" phenotype with T cell-activating activity equivalent to monocytes. In other words, newly arrived monocytes "dilute" the mature resident AM population resulting in a functional imbalance of AM subsets.

The ability to down-regulate immune responses may be possessed by a subset of AM (e.g. suppressive AM) rather than by the entire AM population. Thus, a gross imbalance among functionally distinct macrophage subsets becomes an important factor to regulate T cell-dependent immune responses in lungs, which finally leads to the development of AHR. Further studies are needed to identify precise functions of each AM subset in the development of airway hyperresponsiveness.

The exact mechanism of AM dysregulation on T cell reactivity is unclear. AM from atopic asthmatic subjects but not atopic nonasthmatic subjects, play a significant role in airway immunity by enhancing Th2-type cytokine production (IL-4, IL-5 and IFN) (Tang *et al*, 1998). In fact, in atopic nonasthmatic subjects there was a significant decrease in production of both IL-4 and IL-5 in allergen-stimulated AM-CD4+ T cell cocultures compared to the parallel polymorphonuclear leukocytes (PMN) cocultures, which may reflect a protective mechanism offered by AM from these subjects (Tang *et al*, 1998). This illustrated that AM inhibit T cell proliferative responses in normal but not asthmatic subjects. Poulter and Burke (1996) showed that some cytokines that come from AM such as IL-5, IL-3 and GM-CSF might be involved in the regulatory function. In addition, IL-10 and IL-12 that can be derived from AM can deactivate T cells and stimulate the differentiation of naive T cells to Th1 cells (in turn to inhibit serum levels of IgE synthesis). It is speculated that AM from asthmatic subjects may lose their function or they may turn off the secretion of these cytokines.

In addition, resident AM suppress the activities of both DC and T cells probably through the release of nitric oxide (NO) and other soluble inhibitors (TGF-β, IL-10 and prostaglandins) (Bilyk and Holt, 1995; Kradin *et al*, 1999). Kradin *et al* (1999) eliminated AM with LE DMDP in Lewis rats and found that the elimination reduces local production of NO and promotes pulmonary cell-mediated immunity to heat-killed *Listeria*. These suggests that AM are the major source of NO production in the lung during the inflammation. Furthermore, NO released by AM has a direct bronchodilatous effect in humans. (Bilyk and Holt, 1995). The elimination of AM *in vivo* reduced local production of NO. Those observations suggested NO from AM and other cells is probably involved in the initiation of AHR.

The elimination of AM also influences the function of AM to up-regulate pulmonary immune responses, for instance, inhibition of producing pro-inflammatory mediators. However, the depletion of AM with LE DMDP is incomplete. It is not clear whether the remaining AM after LE DMDP treatment have ability to allocate the development of inflammatory responses. In addition, LE DMDP does not influence other macrophages including IM, vascular macrophages and DC (Thepen *et al*, 1989, 1991; Strickland *et al*, 1993; Kradin *et al*, 1999), because liposomes have limited capacities to penetrate the alveolar wall. In addition, incoming PBM might execute a positive regulation on T cell-mediated immune response and the development of AHR after the depletion of AM. Thus, whether or not AHR develops depends on the role of balance of enhanced and inhibited regulation on inflammation by AM subsets and T cell subsets and other inflammatory cells.

3.4. Limitation and future works

More experiments performed with this experimental model would allow more precise evaluation airway inflammatory responses to inhaled allergen as discussed in 3.3.1. (histological and immunochemical examination and improvement of techniques in collection and analysis of inflammatory cells). In addition, the number of animals used in each experiment was small. There are a variety of further studies that would contribute to our understanding of the role of AM in secondary immune responses to inhaled antigen. Adoptive transfer experiment may help to clarify some postulates and to remedy a defect of the current study, in which there was not a reproducible design for the effect of AM depletion in the development of AHR. Following the depletion of AM, in the BN rat model of asthma, adoptive transfer of AM or monocytes separated from OA-challenged or normal BN rats would help to validate which macrophage subset plays the protective role against allergen airway challenge. It is important to elucidate which macrophage secretory products have important *in vivo* effects in recruitment and regulation of other inflammatory cells and the development of AHR. This can be confirmed by evaluating changes in secretion products of AM (e.g. IL-8, TNF, IL-10, IL-12, PAF and NO etc.) following AM depletion and OA immunization. The pattern of T cell-mediated immune responses to inhaled allergen following AM depletion can be determined by measuring Th2 cell and Th1 cell secretory products and the level of OA-specific IgE produced by B cells, which is regulated by Th2 cells and AM.

3.5. Conclusion

The loss of AM regulatory function and aberrant activation of macrophages may account for their involvement in inflammatory diseases such as asthma. Studies are now beginning to reveal that AM from asthmatic subjects have a decreased suppressor function. It has been demonstrated that OA-immunized and OA-aerosol-challenged BN rats yielded a three to five-fold increase in R_L airway responses to serotonin compared to the adjuvant-treated rats, which suggested that there was a deficiency of the down-regulating function of AM in the allergic response. More importantly, AM-depleted and OA-immunized BN rats exhibited the highest airway responsiveness, even higher than OA-immunized BN rats without the AM depletion. Although the precise mechanisms responsible for the observed bronchoconstriction are not clear, the results highlight the important role of AM in the AHR. The elimination of AM and the subsequent recruitment of peripheral blood monocytes appear to disturb the homeostasis of the AM pool in lungs. It is postulated that this dysregulation aggravates the deficiency of the down-regulating function of AM on T cell-dependent allergic response and airway inflammation, which finally induces AHR. Thus, AM homeostasis is one of important factors for *in vivo* control of the development of airway hyperresponsiveness to specific allergens through their regulation of T cell-dependent allergic responses. It is proposed that depletion of AM leads to an imbalance of functionally distinct macrophage subsets, which seem to play, at least in part, an important role in preventing the development of airway hyperresponsiveness.

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