Low molecular weight chemical-induced occupational asthma
The focus on alveolar macrophages

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Low molecular weight chemical-induced occupational asthma
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Beroepsastma geïnduceerd door chemische stoffen met een laag molecuulgewicht
De focus op alveolaire macrofagen

(met een samenvatting in het Nederlands)

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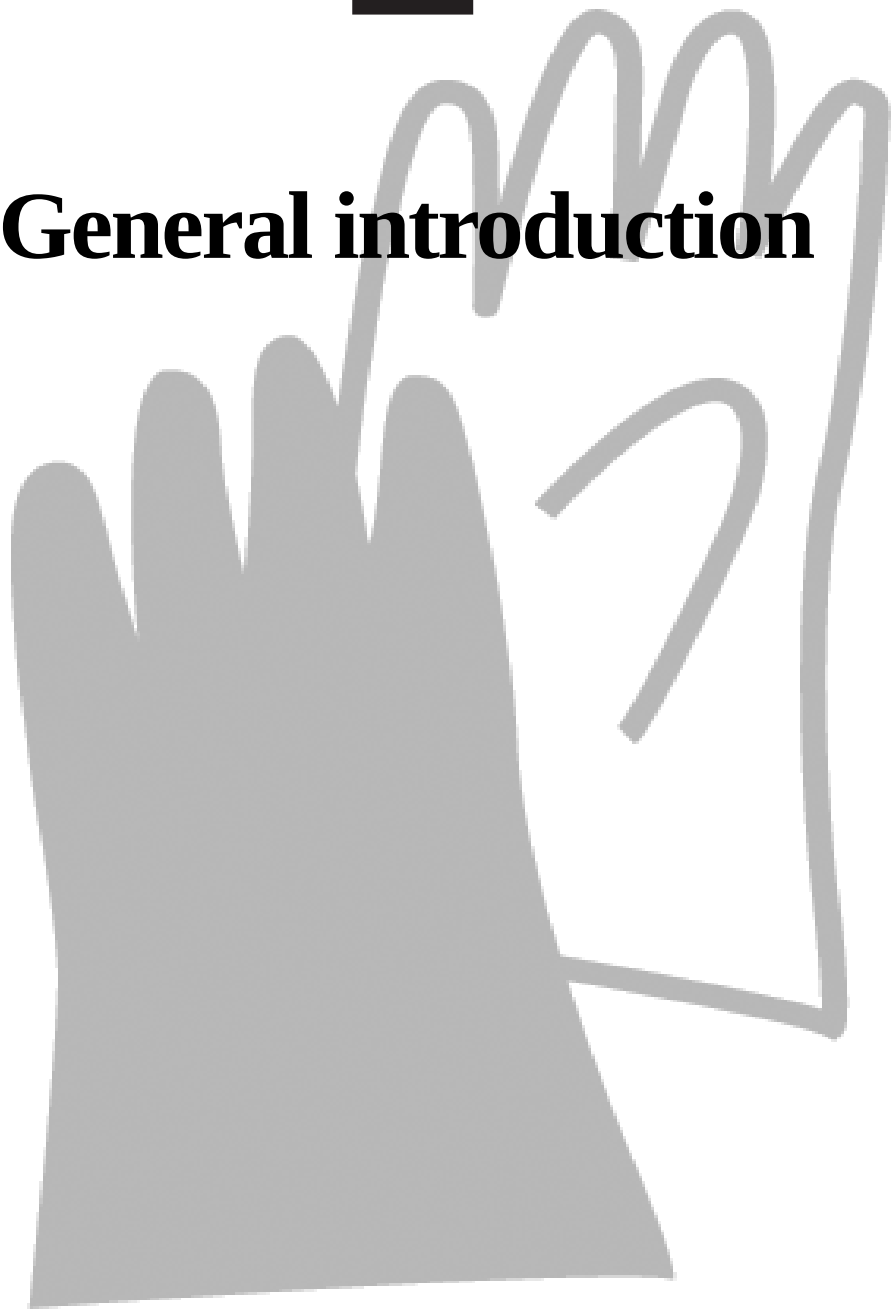
Contents

1

General introduction



Asthma

Asthma is the most common chronic respiratory disease in the world. An estimated 300 million people of all ages and all ethnic backgrounds suffer from asthma and the morbidity and mortality associated with it are increasing in industrialized nations (Global burden of Asthma, 2004, www.ginasthma.com). The major clinical symptoms of asthma include coughing, wheezing, shortness of breath, and chest tightness, alone or in combination and these symptoms may vary from mild to life threatening. The symptoms are due to reversible airflow obstruction, persistent airway hyperresponsiveness, airway inflammation, mucus hypersecretion, and airway remodeling (1). In general, asthma can be divided in two groups. The majority of asthmatics (approximately 70%) has atopic asthma and display increased IgE levels in their serum, the remainder has nonatopic asthma and exhibit normal IgE levels. A recent study, however, provided evidence of local IgE synthesis in the bronchial mucosa of both atopic and nonatopic asthmatics (2). Furthermore, increased numbers of Fcε-receptor-bearing cells were identified in bronchial biopsies from atopic and nonatopic asthmatics (3) indicating that the underlying mechanisms of both types of asthma might be very similar.

The development of asthma requires sensitization to environmental allergens, like house dust mite and animal dander (4-6). Allergens are taken up and processed by antigen-presenting cells, like the dendritic cells that are abundantly present in the submucosa of the airways (7). These cells then migrate to the local lymph node where the processed allergen is presented to allergen-specific T cells, inducing T cell differentiation and memory (8). These T cells then secrete cytokines, like interleukin 4 (IL-4) and IL-13, that induce proliferation of, and IgE production by allergen-recognizing B cells (9). Allergen-specific IgE will then bind to FcεRI on the surface of mast cells lining the airways (10). Cross-linking of mast cell-bound IgE upon re-exposure to the allergen will lead to the release of mediators, like histamine and leukotrienes, by these cells (10). In general, these mast cell-derived mediators induce airway constriction, increase vascular permeability, enhance airway responsiveness, induce mucus secretion, and promote the recruitment of inflammatory cells, like eosinophils, T cells, and macrophages, into the airways (11). In addition to resident cells, like epithelial cells, endothelial cells, and smooth muscle cells, these inflammatory cells can produce a vast array of inflammatory mediators, like chemokines, cytokines and leukotrienes (12), promoting the chronic characteristic of the airway inflammation after repeated allergen exposure. As a result of this chronic inflammation, airway tissue is continuously being injured and healed, leading to structural changes of the airways that may account for the decline in airway function seen in patients over the years (13).

Occupational asthma

Occupational asthma is defined as "a disease characterized by either or both variable airflow limitation and bronchial hyperresponsiveness due to causes and conditions attributable to a particular working environment and not to stimuli encountered outside the workplace" (14). The proportion of cases of asthma attributable to occupational exposure is unknown, but it is widely accepted that ~10% of adult-onset asthma is caused by agents encountered in the workplace (15). The actual prevalence of this disease may even be higher as many symptomatic workers leave their jobs prior to a definitive diagnosis of occupational asthma (16). The total duration of exposure, the duration of symptoms and the severity of asthma at the time of diagnosis are important determinants of the outcome of the disease. Persistence of occupational asthma has been consistently associated with a longer duration of work-related symptoms before removal and with a more severe asthma at the time of diagnosis (17). Therefore, withdrawal from exposure is one of the first measures after occupational asthma has been diagnosed. Follow-up data have shown that approximately 70% of affected workers still experience asthma symptoms and retain airway hyperresponsiveness several years after cessation of exposure to the causative agent (17).

About 250 agents capable of causing occupational asthma have been reported and some of the most common agents are shown in Table 1. This number of agents is expected to increase over the next years since new chemicals are introduced in the industry every year. The agents that cause occupational asthma can be divided into two categories according to their molecular weight: high molecular weight (HMW) compounds (> 5 kD), which usually are proteins derived from animals, plants, and other organisms and low molecular weight (LMW) compounds (< 1 kD). HMW compounds induce occupational asthma by specific IgE antibody-dependent reactions (15, 18). With some LMW compounds a strong association between IgE antibody and clinical symptoms exists, like for acid anhydrides and platinum salts (15, 19-21). However, for most of the LMW compounds, and notably diisocyanates, it has been reported that symptomatic individuals may lack detectable IgE antibodies (22-25). The presence of IgE antibodies in atopic asthma and the absence of these antibodies in occupational asthma induced by LMW compounds have been related to different T cell subset involvement. In atopic asthma CD4+ T cell orchestrate the inflammatory reactions via the release of IL-4 and IL-5, which promote the production of IgE and induce eosinophilia, respectively. In contrast, CD8+ T cells producing IL-5 and interferon-gamma, but not IL-4, appeared predominant in diisocyanate-induced occupational asthma (26, 27). In line with this, an increased percentage of IL-5 producing CD8+ T cells has also been reported in nonatopic asthmatics (28, 29).

In some cases asthma symptoms can occur without a latency period after single exposure to a high concentration of irritating dust, mist, vapor, or fume. These high concentrations most probably have toxic and irritation effects on the respiratory tract resulting in airway obstruction. This type of airway obstruction is often referred to as 'Reactive Airways Dysfunction Syndrome' (15, 18).

Table 1. Selected major causes of occupational asthma and workers at risk**.**

Agent	Workers at risk
High molecular weight agents	
Animal-derived antigen (dander, urine)	Animal handlers
Flour	Bakers, millers
Enzymes	Detergent industry workers, pharmaceutical workers
Latex	Health care professionals
Seafood	Seafood processors
Low molecular weight agents	
Diisocyanates	Spray painters, workers with polyurethane, plastics, and varnish
Acid anhydrides	Workers with epoxy resins, alkyd resins, and plastics
Metals	Platinum-refining and metal-plating workers, hard-metal grinders
Wood dusts	Sawmill workers, carpenters
Reactive dyes	Textile workers
Complex amines	Photographers, shellac workers, painters

Occupational asthma due to LMW compounds

LMW compounds are the most common agents causing occupational asthma. In contrast to HMW compounds, which can serve as complete antigens, conjugation of LMW compounds to proteins, either endogenous or exogenous, is necessary to serve as antigens. Since these LMW compounds are typical electrophiles, they are capable of binding to nucleophilic structures, like hydroxyl, amino, and thiol groups on proteins (30, 31). LMW compound conjugates have been identified for several different endogenous structures, including hemoglobin, glutathione, laminin, tubulin, and serum albumin (31-36).

Diisocyanates have been the most commonly identified cause of LMW compound-induced occupational asthma (25, 37) and among these compounds toluene diisocyanate (TDI) is thought to be the number one cause (38). It is a highly volatile compound and it is used in the production of polyurethane foams, elastomers, adhesives, and coatings due to the capacity to form polymers because

of the reactive isocyanate groups (-N=C=O). Although only in ~5-20% of the workers that have developed asthma TDI-specific IgE antibodies have been detected, occupational asthma due to TDI is thought to be immunologically mediated (39-41). Another group of LMW compounds that has frequently been associated with occupational asthma is the group of acid anhydrides. These compounds share the reactive group O=C-O-C=O and are used as curing agents in the production of epoxy and alkyl resins, which have widespread applications in paints, plastics, and adhesives. Among these acid anhydrides, trimellitic anhydride (TMA) is one of the most commonly used anhydride to study IgE-mediated occupational respiratory disease in animal models (42-45).

Alveolar macrophages

Alveolar macrophages (AMs) are long-lived cells belonging to the family of mononuclear phagocytes. They are the most prominent cells in the lower respiratory tract and in healthy individuals 95% of the cells obtained by lung lavage are AMs. They represent a non-specific cellular host defense mechanism and can do so by binding and phagocytosis of microorganisms and macromolecules via pattern recognition receptors, and the secretion of a broad repertoire of mediators that regulate inflammatory and immune reactions in the lung (46-49). AMs appear to play an important role in decreasing pulmonary immune responses to inhaled protein-allergens (50-52). They were found to do so by secreting mediators that inhibit allergen-induced T cell activation and by inducing T cell anergy by activating T cells in the absence of costimulatory signals (53-56). However, evidence exists that AMs potentiate allergic inflammation, since AMs were found to be increased in number and have an activated phenotype in asthmatic patients (57, 58). They exhibit enhanced surface expression of the IgE receptors, FcεRI and CD23, MHC class I and II, and co-stimulatory molecules, making them more effective in antigen presentation to allergen-specific T cells (59, 60). Furthermore, AMs from asthmatics were found to secrete increased amounts of proinflammatory mediators, such as, TNF-α, IL-6, and nitric oxide (61-63). These mediators have been shown to directly or indirectly contribute to the local inflammation and tissue damage. TNF-α promotes inflammation by inducing synthesis and release of other inflammatory mediators and stimulates inflammatory cell infiltration via up-regulating adhesion molecule expression on epithelium, endothelium and inflammatory cells (64, 65). IL-6 is a pleiotropic cytokine with growth regulatory effects on many cells, is involved in T cell activation, growth, and differentiation, and is a terminal differentiation factor for B cells (13, 61, 66). Nitric oxide has both beneficial and deleterious effects in asthma and may be related to the amount of nitric oxide that is induced by an allergen challenge (see for review (67)).

Macrophage depletion

The role of the AM in various disease models has been studied by depleting these cells from the airways using clodronate (dichloromethylene diphosphonate)-containing liposomes (50, 51, 68-71). AMs, as the normal professional phagocytes in the airway lumen, will phagocytose and digest the liposomes leading to the intracellular release of clodronate and subsequent induction of apoptosis (72). The capacity of liposomes to target a particular type of cell is highly defined by their physicochemical characteristics, like size and charge. Large multilamellar liposomes are taken up by macrophages more efficiently than smaller unilamellar counterparts (72). Moreover, negatively charged liposomes associate more effectively and deliver their content more efficiently to macrophages than neutral liposomes (72, 73). By contrast, removal of liposomes by macrophages can be delayed by making the surface of the liposomes highly hydrophilic by covalently attaching hydrophilic polymers (74).

Aim and scope of the thesis

In the previous paragraphs, it has become clear that a number of LMW compounds can cause occupational asthma. AMs are among the first cells that encounter these compounds in the airways. Furthermore, these cells have an activated phenotype in atopic asthmatics and the pulmonary immune response was shown to be increased after depletion of these cells in animal models for atopic asthma. However, not much is known about the role of the AM in asthmatic responses due to inhalation of LMW compounds. Therefore, the goal of this thesis was to study the role of the AM in animal models for LMW compound-induced occupational asthma. To study the mechanisms of action of LMW compounds, two rodent models of occupational asthma have been used, a rat model for TMA-induced IgE-dependent asthma (44) and a mouse model for TDI-induced IgE-independent asthma (75). In chapter 2, the optimization of conditions for AM depletion is described. Neutral and negatively charged clodronate-containing liposomes prepared under conventional and aseptic conditions have been compared for their ability to deplete macrophages in both mice and rats. Chapter 3 describes the effects of AM depletion by negatively charged clodronate-containing liposomes prior to inhalation challenge with TMA in the rat model for TMA-induced occupational asthma. Conjugation of TMA to proteins is thought necessary to induce immunologically mediated effects, therefore the effects of AM depletion prior to an inhalation challenge with TMA conjugated to bovine serum albumin (BSA) were studied and described in chapter 4. Chapter 5 deals with the question whether both TMA and TMA-BSA have direct effects on mediator production by the AM cell line NR8383 and primary AMs derived from control and TMA-sensitized rats. Furthermore,

the effects of an intratracheal instillation with TDI conjugated to glutathione in the mouse model for TDI-induced occupational are described in chapter 6. The results of the experimental studies as a whole are summarized and discussed in chapter 7.

References

1. Maddox, L., and D. A. Schwartz. 2002. The pathophysiology of asthma. *Annu Rev Med 53:477*.
2. Ying, S., M. Humbert, Q. Meng, R. Pfister, G. Menz, H. J. Gould, A. B. Kay, and S. R. Durham. 2001. Local expression of epsilon germline gene transcripts and RNA for the epsilon heavy chain of IgE in the bronchial mucosa in atopic and nonatopic asthma. *J Allergy Clin Immunol 107:686*.
3. Humbert, M., J. A. Grant, L. Taborda-Barata, S. R. Durham, R. Pfister, G. Menz, J. Barkans, S. Ying, and A. B. Kay. 1996. High-affinity IgE receptor (FcepsilonRI)-bearing cells in bronchial biopsies from atopic and nonatopic asthma. *Am J Respir Crit Care Med 153:1931*.
4. Custovic, A., S. C. Taggart, H. C. Francis, M. D. Chapman, and A. Woodcock. 1996. Exposure to house dust mite allergens and the clinical activity of asthma. *J Allergy Clin Immunol 98:64*.
5. Noertjojo, K., H. Dimich-Ward, H. Obata, J. Manfreda, and M. Chan-Yeung. 1999. Exposure and sensitization to cat dander: asthma and asthma-like symptoms among adults. *J Allergy Clin Immunol 103:60*.
6. Lundback, B. 1998. Epidemiology of rhinitis and asthma. *Clin Exp Allergy 28 Suppl 2:3*.
7. Holt, P. G. 2000. Antigen presentation in the lung. *Am J Respir Crit Care Med 162:S151*.
8. Lambrecht, B. N., and H. Hammad. 2003. Taking our breath away: dendritic cells in the pathogenesis of asthma. *Nat Rev Immunol 3:994*.
9. Corry, D. B., and F. Kheradmand. 1999. Induction and regulation of the IgE response. *Nature 402:B18*.
10. Hart, P. H. 2001. Regulation of the inflammatory response in asthma by mast cell products. *Immunol Cell Biol 79:149*.
11. Bradding, P., and S. T. Holgate. 1999. Immunopathology and human mast cell cytokines. *Crit Rev Oncol Hematol 31:119*.
12. Bousquet, J., P. K. Jeffery, W. W. Busse, M. Johnson, and A. M. Vignola. 2000. Asthma. From bronchoconstriction to airways inflammation and remodeling. *Am J Respir Crit Care Med 161:1720*.
13. Elias, J. A. 2000. Airway remodeling in asthma. Unanswered questions. *Am J Respir Crit Care Med 161:S168*.
14. Bernstein, I. L., M. Chan-Yeung, J. L. Malo, and D. Bernstein. 1999. *Asthma in the workplace*. Marcel Dekker Inc., New York.

15. Malo, J. L., and M. Chan-Yeung. 2001. Occupational asthma. *J Allergy Clin Immunol 108:317*.
16. Cullinan, P., S. Tarlo, and B. Nemery. 2003. The prevention of occupational asthma. *Eur Respir J 22:853*.
17. Vandenplas, O., K. Toren, and P. D. Blanc. 2003. Health and socioeconomic impact of work-related asthma. *Eur Respir J 22:689*.
18. Venables, K. M., and M. Chan-Yeung. 1997. Occupational asthma. *Lancet 349:1465*.
19. Kimber, I., and R. J. Dearman. 2002. Chemical respiratory allergy: role of IgE antibody and relevance of route of exposure. *Toxicology 181-182:311*.
20. Venables, K. M. 1989. Low molecular weight chemicals, hypersensitivity, and direct toxicity: the acid anhydrides. *Br J Ind Med 46:222*.
21. Bolm-Audorff, U., H. G. Bienfait, J. Burkhard, A. H. Bury, R. Merget, G. Pressel, and G. Schultze-Werninghaus. 1992. Prevalence of respiratory allergy in a platinum refinery. *Int Arch Occup Environ Health 64:257*.
22. Park, H. S., S. K. Lee, H. Y. Kim, D. H. Nahm, and S. S. Kim. 2002. Specific immunoglobulin E and immunoglobulin G antibodies to toluene diisocyanate-human serum albumin conjugate: useful markers for predicting long-term prognosis in toluene diisocyanate-induced asthma. *Clin Exp Allergy 32:551*.
23. Cullinan, P. 1998. Occupational asthma, IgE and IgG. *Clin Exp Allergy 28:668*.
24. Tee, R. D., P. Cullinan, J. Welch, P. S. Burge, and A. J. Newman-Taylor. 1998. Specific IgE to isocyanates: a useful diagnostic role in occupational asthma. *J Allergy Clin Immunol 101:709*.
25. Bernstein, J. A. 1996. Overview of diisocyanate occupational asthma. *Toxicology 111:181*.
26. Maestrelli, P., G. F. Del Prete, M. De Carli, M. M. D'Elios, M. Saetta, A. Di Stefano, C. E. Mapp, S. Romagnani, and L. M. Fabbri. 1994. CD8 T-cell clones producing interleukin-5 and interferon-gamma in bronchial mucosa of patients with asthma induced by toluene diisocyanate. *Scand J Work Environ Health 20:376*.
27. Maestrelli, P., M. Saetta, C. Mapp, and L. M. Fabbri. 1997. Mechanisms of occupational asthma. *Clin Exp Allergy 27 Suppl 1:47*.
28. Walker, C., E. Bode, L. Boer, T. T. Hansel, K. Blaser, and J. C. Virchow, Jr. 1992. Allergic and nonallergic asthmatics have distinct patterns of T-cell activation and cytokine production in peripheral blood and bronchoalveolar lavage. *Am Rev Respir Dis 146:109*.
29. Shiota, Y., H. Arikita, N. Horita, J. Hiyama, T. Ono, and M. Yamakido. 2002. Intracellular IL-5 and T-lymphocyte subsets in atopic and nonatopic bronchial asthma. *J Allergy Clin Immunol 109:294*.
30. Karol, M. H., O. T. Macina, and A. Cunningham. 2001. Cell and molecular biology of chemical allergy. *Ann Allergy Asthma Immunol 87:28*.
31. Karol, M. H. 2001. Bonding and transfer: do epithelial conjugates have a role in chemical asthma? *Clin Exp Allergy 31:357*.
32. Griffin, P., L. Allan, P. Beckett, J. Elms, and A. D. Curran. 2001. The development of an antibody to trimellitic anhydride. *Clin Exp Allergy 31:453*.

33. Lange, R. W., R. C. Lantz, D. B. Stolz, S. C. Watkins, P. Sundareshan, R. Lemus, and M. H. Karol. 1999. Toluene diisocyanate colocalizes with tubulin on cilia of differentiated human airway epithelial cells. *Toxicol Sci 50:64*.
34. Jin, R., B. W. Day, and M. H. Karol. 1993. Toluene diisocyanate protein adducts in the bronchoalveolar lavage of guinea pigs exposed to vapors of the chemical. *Chem Res Toxicol 6:906*.
35. Day, B. W., R. Jin, and M. H. Karol. 1996. In vivo and in vitro reactions of toluene diisocyanate isomers with guinea pig hemoglobin. *Chem Res Toxicol 9:568*.
36. Kennedy, A. L., T. R. Wilson, M. F. Stock, Y. Alarie, and W. E. Brown. 1994. Distribution and reactivity of inhaled ^{14}C-labeled toluene diisocyanate (TDI) in rats. *Arch Toxicol 68:434*.
37. Tarlo, S. M., and G. M. Liss. 2002. Diisocyanate-induced asthma: diagnosis, prognosis, and effects of medical surveillance measures. *Appl Occup Environ Hyg 17:902*.
38. Diller, W. F. 2002. Frequency and trends of occupational asthma due to toluene diisocyanate: a critical review. *Appl Occup Environ Hyg 17:872*.
39. Karol, M. H., D. J. Tollerud, T. P. Campbell, L. Fabbri, P. Maestrelli, M. Saetta, and C. E. Mapp. 1994. Predictive value of airways hyperresponsiveness and circulating IgE for identifying types of responses to toluene diisocyanate inhalation challenge. *Am J Respir Crit Care Med 149:611*.
40. Raulf-Heimsoth, M., and X. Baur. 1998. Pathomechanisms and pathophysiology of isocyanate-induced diseases— summary of present knowledge. *Am J Ind Med 34:137*.
41. Wisnewski, A. V., and C. A. Redlich. 2001. Recent developments in diisocyanate asthma. *Curr Opin Allergy Clin Immunol 1:169*.
42. Pauluhn, J., P. Eidmann, A. Freyberger, G. Wasinska-Kempka, and H. W. Vohr. 2002. Respiratory hypersensitivity to trimellitic anhydride in Brown Norway rats: a comparison of endpoints. *J Appl Toxicol 22:89*.
43. Warbrick, E. V., R. J. Dearman, and I. Kimber. 2002. IgG and IgE antibody responses following exposure of Brown Norway rats to trimellitic anhydride: comparison of inhalation and topical exposure. *Toxicology 172:157*.
44. Arts, J. H., N. Bloksma, T. Leusink-Muis, and C. F. Kuper. 2003. Respiratory allergy and pulmonary irritation to trimellitic anhydride in Brown Norway rats. *Toxicol Appl Pharmacol 187:38*.
45. Plitnick, L. M., S. E. Loveless, G. S. Ladics, M. P. Holsapple, M. J. Selgrade, D. M. Sailstad, and R. J. Smialowicz. 2002. Cytokine profiling for chemical sensitizers: application of the ribonuclease protection assay and effect of dose. *Toxicol Appl Pharmacol 179:145*.
46. Laskin, D. L., B. Weinberger, and J. D. Laskin. 2001. Functional heterogeneity in liver and lung macrophages. *J Leukoc Biol 70:163*.
47. van Rooijen, N., O. L. Wijburg, G. P. van den Dobbelsteen, and A. Sanders. 1996. Macrophages in host defense mechanisms. *Curr Top Microbiol Immunol 210:159*.
48. Dorger, M., S. Munzing, A. M. Allmeling, K. Messmer, and F. Krombach. 2001. Phenotypic and functional differences between rat alveolar, pleural, and peritoneal macrophages. *Exp Lung Res 27:65*.

49. Peiser, L., S. Mukhopadhyay, and S. Gordon. 2002. Scavenger receptors in innate immunity. *Curr Opin Immunol 14:123*.

50. Tang, C., M. D. Inman, N. van Rooijen, P. Yang, H. Shen, K. Matsumoto, and P. M. O'Byrne. 2001. Th type 1-stimulating activity of lung macrophages inhibits Th2- mediated allergic airway inflammation by an IFN-gamma-dependent mechanism. *J Immunol 166:1471*.

51. Thepen, T., N. Van Rooijen, and G. Kraal. 1989. Alveolar macrophage elimination in vivo is associated with an increase in pulmonary immune response in mice. *J Exp Med 170:499*.

52. Thepen, T., C. McMenamin, B. Girn, G. Kraal, and P. G. Holt. 1992. Regulation of IgE production in pre-sensitized animals: in vivo elimination of alveolar macrophages preferentially increases IgE responses to inhaled allergen. *Clin Exp Allergy 22:1107*.

53. Blumenthal, R. L., D. E. Campbell, P. Hwang, R. H. DeKruyff, L. R. Frankel, and D. T. Umetsu. 2001. Human alveolar macrophages induce functional inactivation in antigen-specific CD4 T cells. *J Allergy Clin Immunol 107:258*.

54. Strickland, D. H., T. Thepen, U. R. Kees, G. Kraal, and P. G. Holt. 1993. Regulation of T-cell function in lung tissue by pulmonary alveolar macrophages. *Immunology 80:266*.

55. Holt, P. G., J. Oliver, N. Bilyk, C. McMenamin, P. G. McMenamin, G. Kraal, and T. Thepen. 1993. Downregulation of the antigen presenting cell function(s) of pulmonary dendritic cells in vivo by resident alveolar macrophages. *J Exp Med 177:397*.

56. Kawabe, T., K. I. Isobe, Y. Hasegawa, I. Nakashima, and K. Shimokata. 1992. Immunosuppressive activity induced by nitric oxide in culture supernatant of activated rat alveolar macrophages. *Immunology 76:72*.

57. Lensmar, C., J. Prieto, B. Dahlen, A. Eklund, J. Grunewald, and A. Roquet. 1999. Airway inflammation and altered alveolar macrophage phenotype pattern after repeated low-dose allergen exposure of atopic asthmatic subjects. *Clin Exp Allergy 29:1632*.

58. Viksman, M. Y., B. S. Bochner, R. S. Peebles, R. P. Schleimer, and M. C. Liu. 2002. Expression of activation markers on alveolar macrophages in allergic asthmatics after endobronchial or whole-lung allergen challenge. *Clin Immunol 104:77*.

59. Vignola, A. M., P. Chanez, P. Paul-Lacoste, N. Paul-Eugene, P. Godard, and J. Bousquet. 1994. Phenotypic and functional modulation of normal human alveolar macrophages by histamine. *Am J Respir Cell Mol Biol 11:456*.

60. Balbo, P., M. Silvestri, G. A. Rossi, E. Crimi, and S. E. Burastero. 2001. Differential role of CD80 and CD86 on alveolar macrophages in the presentation of allergen to T lymphocytes in asthma. *Clin Exp Allergy 31:625*.

61. Tang, C., J. M. Rolland, C. Ward, X. Li, R. Bish, F. Thien, and E. H. Walters. 1999. Modulatory effects of alveolar macrophages on CD4+ T-cell IL-5 responses correlate with IL-1beta, IL-6, and IL-12 production. *Eur Respir J 14:106*.

62. Lassalle, P., P. Gosset, Y. Delneste, A. Tsicopoulos, A. Capron, M. Joseph, and A. B. Tonnel. 1993. Modulation of adhesion molecule expression on endothelial cells during the late asthmatic reaction: role of macrophage-derived tumour necrosis factor-alpha. *Clin Exp Immunol 94:105*.

63. Barnes, P. J., and F. Y. Liew. 1995. Nitric oxide and asthmatic inflammation. *Immunol Today 16:128*.

64. Shah, A., M. K. Church, and S. T. Holgate. 1995. Tumour necrosis factor alpha: a potential mediator of asthma. *Clin Exp Allergy 25:1038*.

65. Godding, V., J. M. Stark, J. B. Sedgwick, and W. W. Busse. 1995. Adhesion of activated eosinophils to respiratory epithelial cells is enhanced by tumor necrosis factor-alpha and interleukin-1 beta. *Am J Respir Cell Mol Biol 13:555*.

66. Akira, S., T. Taga, and T. Kishimoto. 1993. Interleukin-6 in biology and medicine. *Adv Immunol 54:1*.

67. Nevin, B. J., and K. J. Broadley. 2002. Nitric oxide in respiratory diseases. *Pharmacol Ther 95:259*.

68. Zhang-Hoover, J., A. Sutton, N. Van Rooijen, and J. Stein-Streilein. 2000. A critical role for alveolar macrophages in elicitation of pulmonary immune fibrosis. *Immunology 101:501*.

69. Cheung, D. O., K. Halsey, and D. P. Speert. 2000. Role of pulmonary alveolar macrophages in defense of the lung against *Pseudomonas aeruginosa*. *Infect Immun 68:4585*.

70. Sekine, Y., L. K. Bowen, K. M. Heidler, N. Van Rooijen, J. W. Brown, O. W. Cummings, and D. S. Wilkes. 1997. Role of passenger leukocytes in allograft rejection: effect of depletion of donor alveolar macrophages on the local production of TNF-alpha, T helper 1/T helper 2 cytokines, IgG subclasses, and pathology in a rat model of lung transplantation. *J Immunol 159:4084*.

71. Hickman-Davis, J. M., S. M. Michalek, J. Gibbs-Erwin, and J. R. Lindsey. 1997. Depletion of alveolar macrophages exacerbates respiratory mycoplasmosis in mycoplasma-resistant C57BL mice but not mycoplasma-susceptible C3H mice. *Infect Immun 65:2278*.

72. Van Rooijen, N., and A. Sanders. 1994. Liposome mediated depletion of macrophages: mechanism of action, preparation of liposomes and applications. *J Immunol Methods 174:83*.

73. Ahsan, F., I. P. Rivas, M. A. Khan, and A. I. Torres Suarez. 2002. Targeting to macrophages: role of physicochemical properties of particulate carriers—liposomes and microspheres—on the phagocytosis by macrophages. *J Control Release 79:29*.

74. Metselaar, J. M., E. Mastrobattista, and G. Storm. 2002. Liposomes for intravenous drug targeting: design and applications. *Mini Rev Med Chem 2:319*.

75. Scheerens, H., T. L. Buckley, E. M. Davidse, J. Garssen, F. P. Nijkamp, and H. Van Loveren. 1996. Toluene diisocyanate-induced in vitro tracheal hyperreactivity in the mouse. *Am J Respir Crit Care Med 154:858*.

2

Depletion of alveolar macrophages by liposome-encapsulated clodronate in mice and rats

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Abstract

Alveolar macrophages (AMs) play an important role in the physiology of the lung. Studying the effects of their depletion could increase insight into AM function and their role in disease. Therefore, we tested the efficacy of clodronate encapsulated in both neutral and negatively charged liposomes in mice and rats to selectively deplete AMs after intratracheal instillation. Conventionally prepared neutral clodronate-containing liposomes depleted the spleen of macrophages in mice after intravenous injection. After intratracheal instillation, however, these neutral clodronate-containing liposomes were not able to deplete AMs in the airways and even increased neutrophil numbers in the airway lumen. Clodronate-containing neutral liposomes prepared with pyrogen-free compounds under aseptic conditions depleted AMs by 40% and did not increase neutrophil numbers in the airway lumen of mice and rats after either single or duplicate intratracheal instillation. Compared to the latter liposomes, negatively charged aseptically prepared clodronate-containing liposomes caused the highest degree of AM depletion. A single instillation of negatively charged clodronate-containing liposomes depleted AMs in the rat by 84%, whereas a duplicate instillation of the same liposomes depleted AMs both in mice and rats by 70%. Therefore, negatively charged clodronate-containing liposomes made under aseptic conditions are the most suitable for depleting the airways of AMs.

Introduction

Alveolar macrophages (AMs) are long-lived cells belonging to the family of mononuclear phagocytes and represent a major non-specific cellular host defense mechanism. AMs are located at the interface between air and lung tissue where they are the first cells to encounter and ingest particles and microorganisms. AMs secrete a broad repertoire of chemical mediators that regulate inflammatory and immune reactions in the lung (1-3).

The role of the AM in various disease models has been studied by depleting these cells from the airways (4-9). In these studies AMs were depleted with clodronate (dichloromethylene diphosphonate) encapsulated in liposomes. AMs, as the normal professional phagocytes in the airway lumen, will phagocytose and digest the liposomes leading to the intracellular release of clodronate. Clodronate will stay intracellularly and cause AMs to go into apoptosis (10). Apoptosis of non-phagocytic cells by leaky liposomes or release of free clodronate by apoptotic AMs is considered minimal because of the extremely short half-life of clodronate in body fluids (10). Therefore, primarily AMs will be depleted by instillation of liposome-encapsulated clodronate intratracheally.

The capacity of liposomes to target a particular type of cell is highly defined by their physicochemical characteristics, like size and charge. Large multilamellar liposomes are taken up by macrophages more efficiently than smaller unilamellar counterparts (10). Moreover, negatively charged liposomes associate more effectively and deliver their content more efficiently to macrophages than neutral liposomes (10, 11). By contrast, removal of liposomes by macrophages can be delayed by making the surface of the liposomes highly hydrophilic by covalently attaching hydrophilic polymers (12).

Most of the published studies, like the studies cited above (4-9), used neutral clodronate-containing liposomes provided by or made according to the protocol of N. van Rooijen to deplete macrophages (10). However, most of these studies do not extensively discuss bystander effects of clodronate-containing liposome treatment, like inflammatory cell influx into the target organ. Therefore, the objective of this study was to deplete AMs using neutral and negatively charged clodronate-containing liposomes prepared with pyrogen-free compounds under aseptic conditions and compare the results of these liposomes with the conventionally made neutral liposomes. The different liposome preparations were tested in mice and rats.

Materials and methods

Animals

Male BALB/cJIco mice (6-8 weeks of age) and male Brown Norway rats (BN/CrlBR; 7-8 weeks of age) were obtained from Charles River (Maastricht, the Netherlands). All animals were housed in macrolon cages under filter tops. Tap water and food (Hope Farms, Woerden, The Netherlands) were allowed *ad libitum*. All animal procedures were conducted in accordance with the Animal Ethics Committee of Utrecht University (Utrecht, The Netherlands).

Materials

Clodronate (dichloromethylene diphosphonate) was either a gift of Roche Diagnostics GmbH (Mannheim, Germany), or obtained from Schering (Weesp, The Netherlands). Phosphatidylcholine and egg-phosphatidylglycerol were purchased from Lipoid GmbH (Ludwigshafen, Germany). Cholesterol was obtained from Sigma (St Louis, MO). Midazolam was purchased from Roche B.V. (Mijdrecht, The Netherlands), fentanyl citrate and fluanisone from Janssen Pharmaceutica (Beerse, Belgium), ketamine from Cassot (Hasselt, Belgium), xylazine from Eurovet (Bladel, The Netherlands), and sodium pentobarbitone from Sanofi B.V. (Maassluis, The Netherlands). Diff-Quick was obtained from Merz and Dade (Düdingen, Switzerland).

Liposomes

Conventionally made neutral liposomes were prepared at the department of Molecular Cell Biology, Faculty of Medicine, Vrije Universiteit, Amsterdam, The Netherlands (10). Briefly, the liposomes contained phosphate-buffered saline (PBS) or clodronate (Roche Diagnostics) encapsulated in phosphatidylcholine and cholesterol in a molar ratio of 5.5:1, respectively. After swelling, sonication, and washing in sterilized PBS, the liposomes were resuspended in PBS. The concentration of clodronate in the liposome suspension was specified to be about 5 mg/ml (10).

Neutral and negatively charged liposomes were prepared with pyrogen-free components under aseptic conditions at the department of Pharmaceutics, Utrecht Institute of Pharmaceutical Sciences, Utrecht University, Utrecht, The Netherlands, as described earlier (13). Briefly, for negatively charged liposomes a mixture of egg-phosphatidylcholine, cholesterol, and egg-phosphatidylglycerol was dissolved in ethanol in a molar ratio of 10:1.5:1, respectively. For neutral liposomes egg-phosphatidylglycerol was omitted. After evaporation to dryness by rotation under

reduced pressure, the lipid film was hydrated in pyrogen-free PBS containing either or not 60 mg/ml of clodronate (Schering). Unencapsulated clodronate was removed by repeated washing with PBS by means of ultracentrifugation (Beckman Optima LE-80K, Palo Alto, CA) at 200,000 x *g* for 30 min. After the last washing step, the pellet was resuspended in PBS at a concentration of 90 mM phospholipid. The phospholipid concentration was determined according to Fiske and Subbarov as modified by King (14). The concentration of clodronate was determined spectrophotometrically at a wavelength of 238 nm after extraction and binding to Cu^{2+}. The final clodronate concentration of the liposome formulations was about 2.5 mg/ml.

To distinguish the liposome preparations prepared under conventional (C) and aseptic (AP) conditions they will be indicated hereafter as neutral (C) and neutral (AP) or negatively charged (AP) liposomes, respectively.

Macrophage depletion in the spleen

Mice were treated i.v. with 50 µl PBS, empty or clodronate-containing neutral (C) liposomes and killed by an overdose of sodium pentobarbitone 24 h after the treatment. Spleens were isolated and frozen in liquid nitrogen to prepare 5 µm sections that were stained for acid phosphatase, a specific marker for macrophages, and counter-stained with haematoxylin.

Responses of the airways after liposome treatment

Animals were anaesthetized (mice: a mixture of ketamine 30 mg/kg and xylazine 5 mg/kg i.m.; rats: a mixture of midazolam 0.6 mg/kg, fluanisone 1.2 mg/kg, and fentanyl citrate 40 µg/kg i.m.) and received an intratracheal instillation of either PBS, empty liposomes, or clodronate-containing liposomes (mice 50 µl; rats 150 or 300 µl). Animals were killed with an overdose of sodium pentobarbitone 24 h to 96 h after liposome instillation as indicated in the legends of the figures. For determination of broncho-alveolar cells, a cannula was inserted into the trachea, and the lungs were lavaged 5 times with 1 ml (mice) or 8 ml (rats) aliquots of saline warmed to 37°C. The cells were collected by centrifugation (390 x *g*, 10 min, 4ºC), and resuspended in 150 µl (mice) or 2 ml (rats) ice cold PBS. A Bürker-Türk chamber was used to count the total number of lavaged cells. For differential cell counts cytospin preparations were made and stained with Diff-Quick. Per cytospin 200 cells were counted and differentiated into AMs, lymphocytes, neutrophils, and eosinophils by standard morphology.

Statistical analysis

All data are expressed per individual plus group mean. Cell counts were statistically analyzed using the Mann-Whitney *U* test. Differences were considered statistically significant if $p<0.05$. Analyses were performed by the usage of Graphpad Prism (version 3.0, San Diego, U.S.A.).

Results

Depletion of spleen macrophages in mice with neutral (C) liposomes

Using the acid phosphatase staining method to detect macrophages, spleens of mice that had received PBS i.v. showed a normal distribution of macrophages in the red pulp one day after treatment (Fig. 1a). Compared to PBS, injection of empty neutral (C) liposomes induced an increase in red pulp macrophages (Fig. 1b). Clodronate-containing neutral (C) liposome treatment markedly reduced the number of acid phosphatase-positive cells in the red pulp compared to PBS and empty neutral (C) liposomes (Fig. 1c).

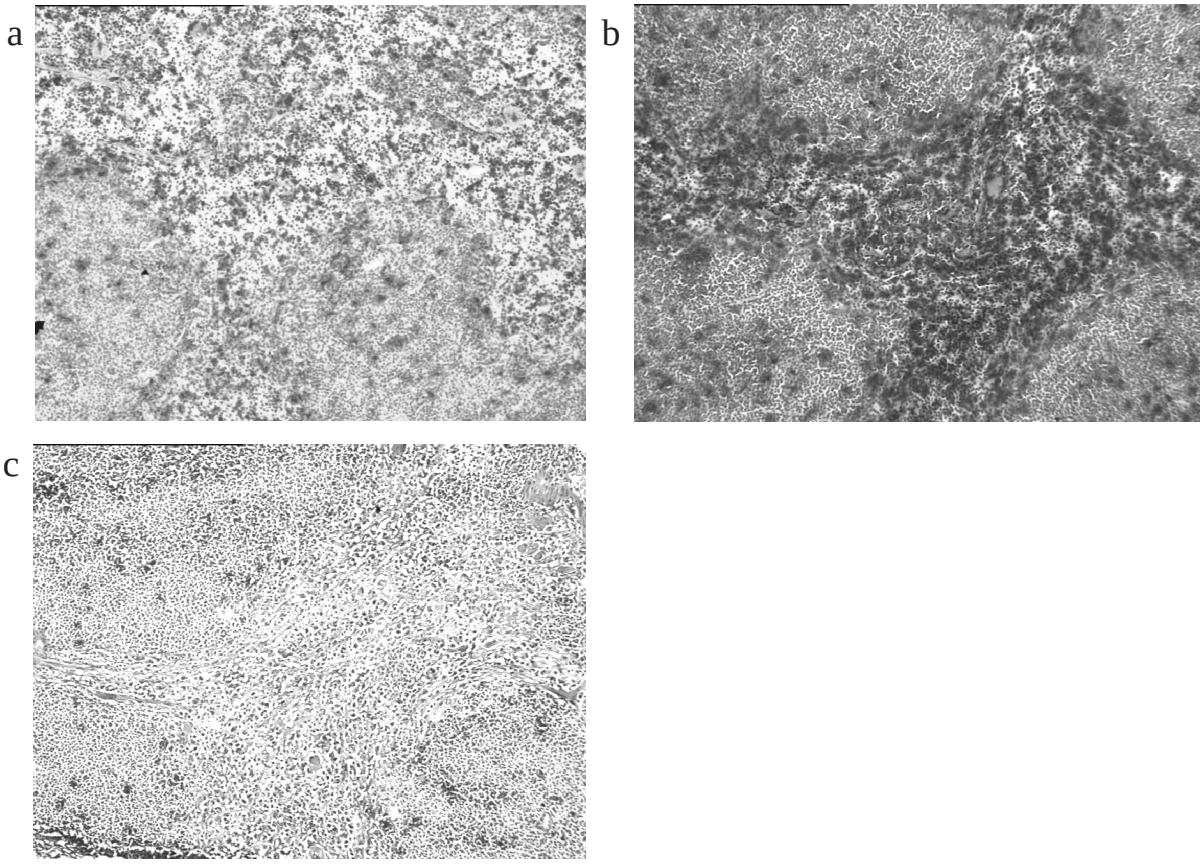

Figure 1. Effect of neutral (C) liposomes on the splenic macrophage density in mice. Mice were treated i.v. with 50 µl of either **a**: PBS, **b**: empty neutral (C) liposomes or **c**: clodronate-containing neutral (C) liposomes. Freeze sections of the spleen were made 24 h after treatment and stained for macrophages with acid phosphatase and counter stained with haematoxylin. The pictures are representative of 4 mice per group

Depletion of AMs in mice with neutral (C) liposomes

Having established the efficacy of an i.v. injection of neutral (C) liposomes, we used these liposomes to deplete AMs in mice via intratracheal instillation. Number and distribution of cells in the broncho-alveolar lavage fluid appeared normal 24 to 72 h after an intratracheal instillation of 50 µl empty neutral (C) liposomes (Fig. 2). More than 95% of the cells in the lavage fluid were AMs. Compared to the empty neutral (C) liposomes, the clodronate-containing neutral (C) liposomes significantly increased the total number of cells. Neutrophils largely accounted for the increase at all time points. Absolute AM numbers did not change significantly (Fig. 2). Relatively to the total cell numbers, AM numbers were decreased in the clodronate-containing neutral (C) liposome-treated animals (data not shown).

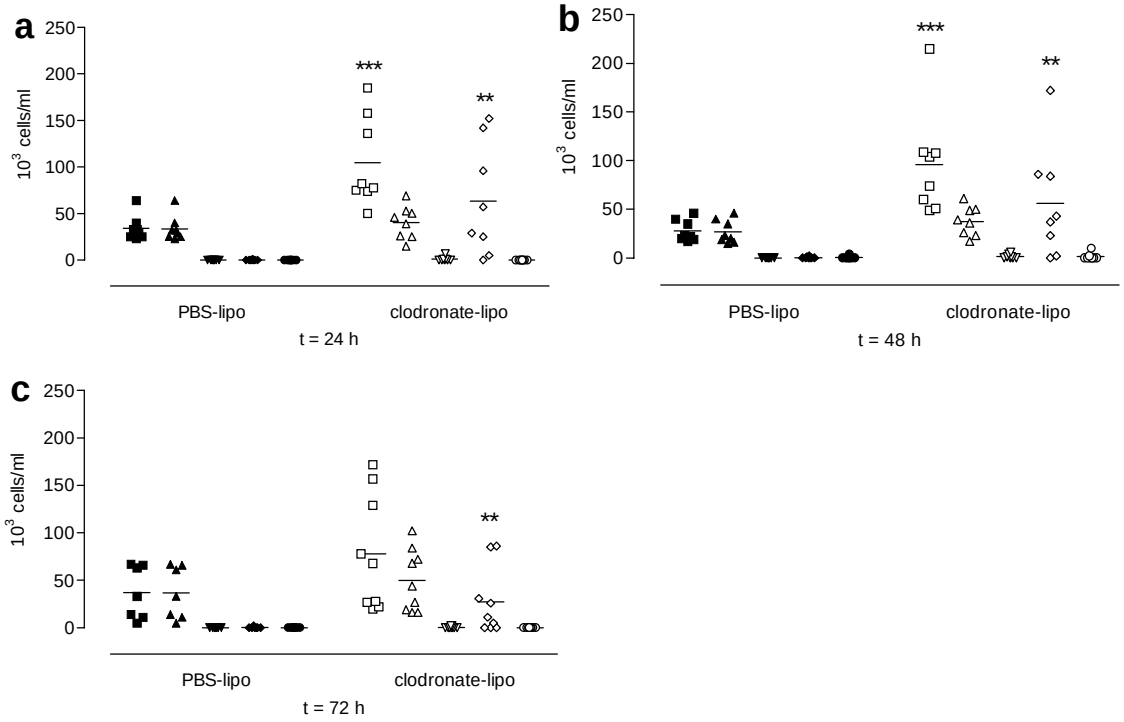

Figure 2. Total and differential cell numbers in the airway lumen of mice after neutral (C) liposome instillation. Lungs were lavaged 24 (**a**), 48 (**b**) and 72 h (**c**) after intratracheal instillation of 50 µl empty or clodronate-containing liposomes. Cells were differentiated into total number of cells (■), macrophages (▲), lymphocytes (▼) neutrophils (◆) and eosinophils (●). Results are expressed as individual data and mean (n=8). Significant differences are denoted by **: $p < 0.01$ or ***: $p < 0.001$ compared to treatment with empty liposomes.

Because a single instillation of clodronate-containing neutral (C) liposomes did not deplete AMs in mice, the effect of two instillations, given 24 h apart, was studied. Two instillations of empty neutral (C) liposomes had no effect on total and differential cell numbers, more than 95% of the cells consisting of AMs (Fig. 3). Similar treatment with clodronate-containing neutral (C) liposomes did not significantly change total cell numbers or deplete AMs and significantly increased the neutrophil numbers.

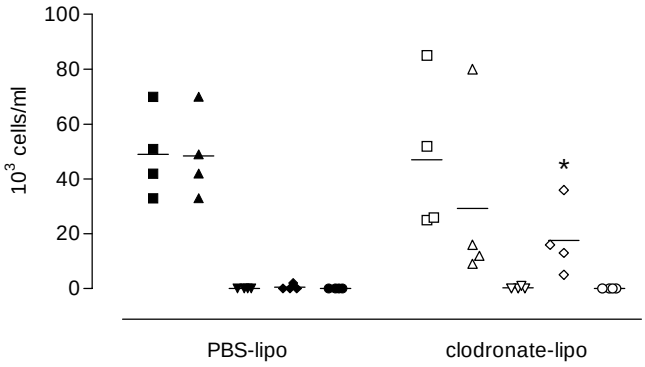

Figure 3. Total and differential cell numbers in the airway lumen of mice after repeated instillation of neutral (C) liposomes. Mice received 50 µl empty or clodronate-containing liposomes intratracheally on days 0 and 1 and lungs were lavaged 24 h thereafter. Cells were differentiated into total number of cells (■), macrophages (▲), lymphocytes (▼) neutrophils (◆) and eosinophils (●). Results are expressed as individual data and mean (n=4). Significant differences are denoted by *: $p < 0.05$ compared to treatment with empty liposomes.

Depletion of AMs in mice with neutral (AP) and negatively charged (AP) liposomes

Since increases in neutrophils might be explained by contamination of the liposome suspension with e.g. endotoxin, liposomes were prepared with pyrogen-free compounds under aseptic conditions. Two kinds of liposomes were prepared, liposomes with a neutral (AP) bilayer and liposomes with a negatively charged (AP) bilayer.

Mice instilled twice with 50 µl of the empty neutral (AP) liposomes, given 24 h apart, had total cell and AM numbers in the normal range (Fig. 4a), comparable to mice treated twice with empty neutral (C) liposomes (Fig. 3). Duplicate instillation of clodronate-containing neutral (AP)

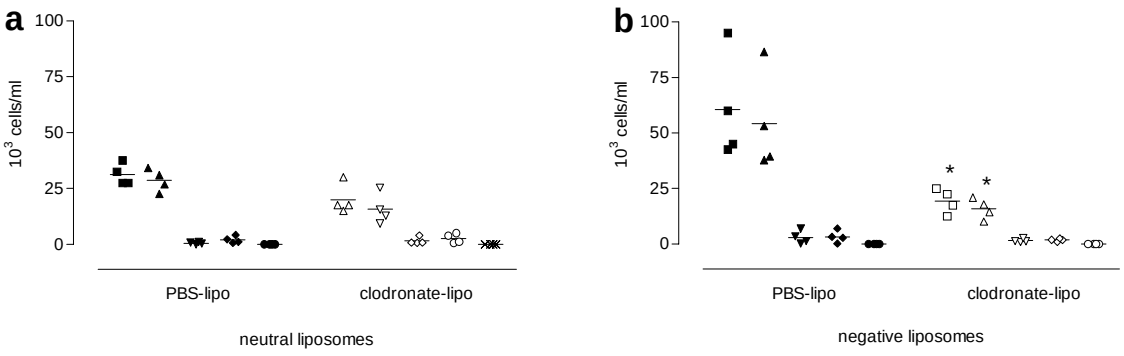

Figure 4. Total and differential cell numbers in the airway lumen of mice after repeated instillation of neutral (AP) and negatively charged (AP) liposomes. Mice received 50 µl of either empty or clodronate-containing neutral (AP) liposomes (**a**) or negatively charged liposomes (**b**) on days 0 and 1 and lungs were lavaged 24 hours thereafter. Cells were differentiated into total number of cells (■), macrophages (▲), lymphocytes (▼) neutrophils (◆) and eosinophils (●). Results are expressed as individual data and mean (n=4). Significant differences are denoted by *: $p < 0.05$ as compared to treatment with empty liposomes.

liposomes did not significantly deplete AMs from the airways 24 h after the last instillation compared to the empty neutral (AP) liposomes (Fig. 4a). In contrast, mice treated with the empty negatively charged (AP) liposomes had higher total cell and AM numbers as compared to the empty neutral (AP) liposomes, but clodronate-containing negatively charged (AP) liposomes depleted 70% of the AMs (Fig. 4b). Both neutral (AP) and negatively charged (AP) liposomes did not increase neutrophil numbers.

Depletion of AMs in rats with neutral (AP) and negatively charged (AP) liposomes

To study the efficacy of neutral (AP) and negatively charged (AP) liposomes in rats, these animals were instilled twice with 150 µl liposome suspension, given 24 h apart. Rats treated twice with either type of empty liposomes had normal total cell, AM and neutrophil numbers 24 h after the last treatment (Fig. 5). Duplicate instillation of clodronate-containing neutral (AP) liposomes depleted 40% of the AMs although this depletion was not significant compared to similar treatment with empty neutral (AP) liposomes (Fig. 5a). Rats treated with the clodronate-containing negatively charged (AP) liposomes showed a significant reduction in the number of AMs, being 70% less than that observed in rats treated with empty negatively charged (AP) liposomes (Fig. 5b). Therefore, it was decided to continue the experiments with the negatively charged (AP) liposomes.

In additional experiments, rats were instilled once with a higher dose (300 µl) of negatively charged (AP) liposomes and with a similar volume of PBS as a control for the liposome treatment (Fig. 6). PBS-treated rats had normal total cell and AM numbers in the lung lavage fluid. The empty negatively

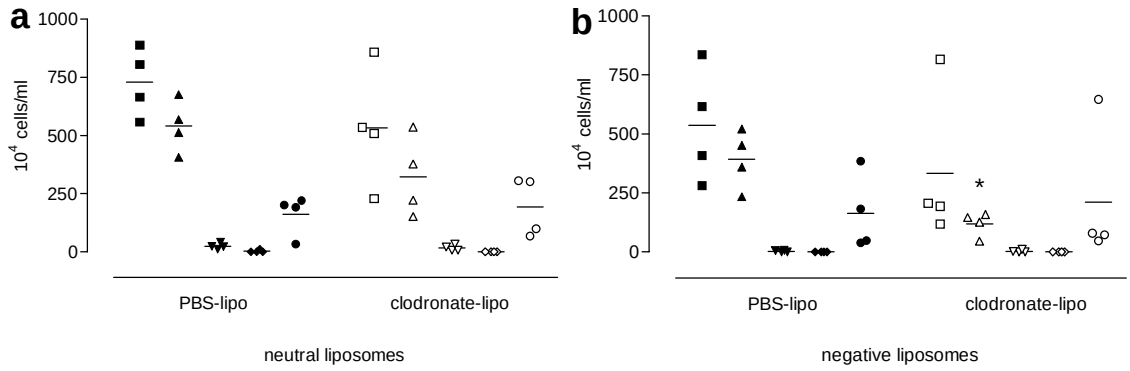

Figure 5. Total and differential cell numbers in the airway lumen of rats after repeated intratracheal instillation of neutral (AP) and negatively (AP) charged liposomes. Rats received 150 µl of either empty or clodronate containing neutral (AP) liposomes (**a**) or negatively charged (AP) liposomes (**b**) on days 0 and 1 and lungs were lavaged 24 h thereafter. Cells were differentiated into total number of cells (■), macrophages (▲), lymphocytes (▼) neutrophils (◆) and eosinophils (●). Results are expressed as individual data and mean (n=4). Significant differences are denoted by *: $p < 0.05$ as compared to treatment with empty liposomes.

charged (AP) liposome-treated rats showed a slight, but not significant, increase in total cell and AM numbers compared to the PBS-treated rats. The clodronate-containing negatively charged (AP) liposomes depleted AMs compared to both control treatments (79% depletion compared to PBS and 84% compared to empty negatively charged (AP) liposomes). There was no increase in neutrophil numbers in the lavage fluid obtained from all three groups of animals.

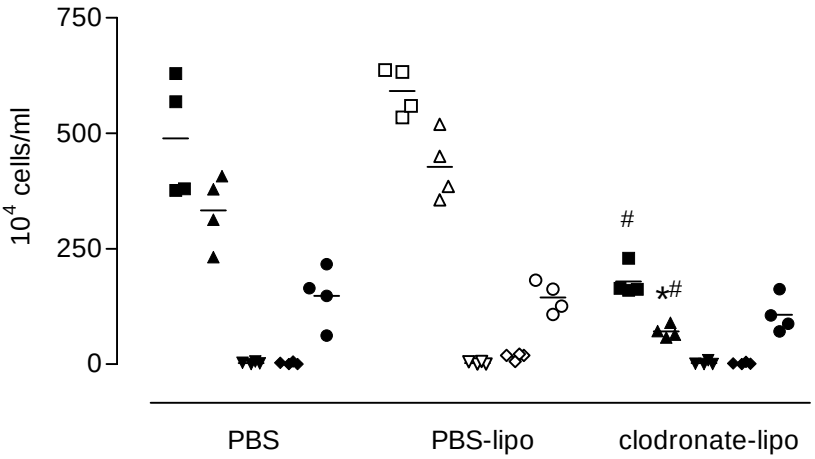

Figure 6. Total and differential cell numbers in the airways of rats after a single instillation of negatively charged (AP) liposomes. Rats received 300 µl PBS, empty or clodronate-containing liposomes on days 0 and 1 and lungs were lavaged 48 h thereafter. Cells were differentiated into total number of cells (■), macrophages (▲), lymphocytes (▼) neutrophils (◆) and eosinophils (●). Results are expressed as individual data and mean (n=4). Significant differences are denoted by *: $p < 0.05$ compared to PBS or #: $p < 0.05$ compared to treatment with empty liposomes.

Discussion

The present study shows that neutral (C) clodronate-containing liposomes depleted macrophages in the spleen in mice after intravenous injection, but failed to deplete AMs after intratracheal instillation. This failure is probably not due to the observed attraction of neutrophils to the airways, since neutral (AP) clodronate-containing liposomes that did not attract neutrophils, failed to significantly deplete AMs not only in mice but also in rats. Furthermore, negatively charged (AP) clodronate-containing liposomes efficiently depleted AMs in mice and rats without attracting neutrophils. The superiority of the latter liposomes in depleting AMs is probably related to a more efficient delivery of clodronate to the macrophages by the more efficient targeting to these cells (11, 15). Despite the superior activity of negatively charged (AP) clodronate-containing liposomes all published studies, so far as known, that aimed to deplete AMs by intratracheal instillation of clodronate used neutral liposomes (Table 1). Relative AM depletion reported by these studies varied

Table 1. Literature overview on types of clodronate-containing liposomes and their effects on AM depletion and influx of inflammatory cells after intratracheal instillation

Ref.	Type of liposome	Animal	Instillation volume	Reported AM depletion	Absolute cell numbers	Inflammatory cells
(22)	neutral*	mouse	50 µl	99% $	no	?
(4, 5)	neutral*	mouse	100 µl	80 - >95% $	no	?
(23)	neutral*	mouse	100 µl	76% $	yes	?
(24)	neutral*	mouse	120 µl	> 70% $	no	?
(25)	neutral*	rat	100 µl	74% #	yes	?
(26)	neutral*	rat	100 µl	50% $	yes	?
(7)	neutral*	rat	20 µl in 780 µl PBS	95% #	yes	no
(27)	neutral*	rat	100 µl in 200 µl PBS	74% $	no	?
(28)	neutral	mouse	30 µl	65% #	no	40-65% neutrophils
(29, 30)	neutral	mouse	100 µl	85 - 99% $	no	?
(31, 32)	neutral	mouse	100 µl	77 - 85% #	yes	no
(9)	neutral	mouse	100 µl	87% $	no	neutrophils
(20)	neutral	rat	80 µl in 1 ml HBSS	> 70% #	yes	minimal neutrophils
(33)	neutral	rat	200 µl	90% #	no	no
(34-37)	neutral	rat	200 µl	60 – 95% $	no	?
(38)	neutral	rat	300 µl	?	no	?
(9)	neutral	rat	1 ml	> 75% $	no	neutrophils

* Conventionally made liposomes

Effects reported as statistical significant compared to control

$ No statistics mentioned by authors

? No information given

considerably from 50-99%, but the degree of AM depletion obtained in most of these studies can not be assessed with certainty. Actually, the majority of the studies did not mention effects on absolute cell or AM numbers and/or on relative or absolute neutrophil numbers. Moreover, 15 of the 23 studies did not perform or report statistical analysis of the results. Anyhow, we did not find significant depletion of AMs after intratracheal administration of neutral clodronate-containing liposomes (Table 2). We, however, observed a significant influx of neutrophils after intratracheal treatment with neutral (C) clodronate-containing liposomes (Table 2). While 8 other studies using similar liposomes did not address effects on neutrophil numbers, only one study (7) reported the absence of such an effect (Table 1). However, various studies using other preparations of clodronate-containing neutral liposomes also found an influx of neutrophils. This influx might be explained by

contamination of the liposomes with pyrogens, like endotoxins. In our studies, these were probably present in the clodronate, because the empty liposomes did not attract neutrophils and were therefore not pyrogenic. Endotoxins are known to activate AMs to produce neutrophil chemoattractant substances, like macrophage inflammatory protein-2 (MIP-2), and the interleukin-8 homologues, murine KC and rat cytokine induced neutrophil chemoattractant (CINC) (16-19). Whereas these mediators may be derived from other cell types in the lung, the importance of the AM in the attraction of neutrophils following endotoxin instillation has been demonstrated in two independent studies (20, 21). Endotoxin instillation into the lungs of mice 24 h (20) or 72 h (21) after AM depletion resulted in less neutrophils in the lungs as compared to similar treatment of mice after sham-depletion.

Table 2. Effects of the three types of clodronate-containing liposomes on AM depletion and influx of inflammatory cells after intratracheal instillation

Figure	Type of liposome	Animal	Instillation volume	Relative AM depletion	Absolute cell/AM numbers	Inflammatory cells
2	neutral (C)	mouse	50 µl	0%	yes	neutrophils [#]
3	neutral (C)	mouse	2 x 50 µl	39%	yes	neutrophils [#]
4a	neutral (AP)	mouse	2 x 50 µl	45%	yes	no
5a	neutral (AP)	rat	2 x 150 µl	40%	yes	no
4b	negative (AP)	mouse	2 x 50 µl	71%	yes [#]	no
5b	negative (AP)	rat	2 x 150 µl	70%	yes [#]	no
6	negative (AP)	rat	300 µl	83%	yes [#]	no

[#] Statistically significant compared to control

The observation that neutral (C) clodronate-containing liposomes were effective in depleting splenic macrophages after i.v. injection but unable to significantly deplete AMs after intratracheal instillation is intriguing. This might be explained by differences in microenvironments in spleen tissue and the lung lumen. Furthermore, phenotypical and functional differences among different types of macrophages have been reported (1, 3), which might also be a reason for the variation in the effectiveness of clodronate-containing liposomes.

In general, we can conclude that negatively charged (AP) clodronate-containing liposomes are superior in depleting AMs compared to neutral clodronate-containing liposomes. Furthermore, to prevent the influx of inflammatory cells upon instillation of clodronate-containing liposomes for AM depletion of the lungs, aseptically made preparations are essential.

References

1. Laskin, D. L., B. Weinberger, and J. D. Laskin. 2001. Functional heterogeneity in liver and lung macrophages. *J Leukoc Biol 70:163.*
2. Van Rooijen, N., O. L. Wijburg, G. P. van den Dobbelsteen, and A. Sanders. 1996. Macrophages in host defense mechanisms. *Curr Top Microbiol Immunol 210:159.*
3. Dorger, M., S. Munzing, A. M. Allmeling, K. Messmer, and F. Krombach. 2001. Phenotypic and functional differences between rat alveolar, pleural, and peritoneal macrophages. *Exp Lung Res 27:65*.
4. Thepen, T., N. Van Rooijen, and G. Kraal. 1989. Alveolar macrophage elimination in vivo is associated with an increase in pulmonary immune response in mice. *J Exp Med 170:499*.
5. Zhang-Hoover, J., A. Sutton, N. Van Rooijen, and J. Stein-Streilein. 2000. A critical role for alveolar macrophages in elicitation of pulmonary immune fibrosis. *Immunology 101:501*.
6. Cheung, D. O., K. Halsey, and D. P. Speert. 2000. Role of pulmonary alveolar macrophages in defense of the lung against *Pseudomonas aeruginosa*. *Infect Immun 68:4585*.
7. Sekine, Y., L. K. Bowen, K. M. Heidler, N. Van Rooijen, J. W. Brown, O. W. Cummings, and D. S. Wilkes. 1997. Role of passenger leukocytes in allograft rejection: effect of depletion of donor alveolar macrophages on the local production of TNF-alpha, T helper 1/T helper 2 cytokines, IgG subclasses, and pathology in a rat model of lung transplantation. *J Immunol 159:4084*.
8. Tang, C., M. D. Inman, N. Van Rooijen, P. Yang, H. Shen, K. Matsumoto, and P. M. O'Byrne. 2001. Th type 1-stimulating activity of lung macrophages inhibits Th2- mediated allergic airway inflammation by an IFN-gamma-dependent mechanism. *J Immunol 166:1471*.
9. Hickman-Davis, J. M., S. M. Michalek, J. Gibbs-Erwin, and J. R. Lindsey. 1997. Depletion of alveolar macrophages exacerbates respiratory mycoplasmosis in mycoplasma-resistant C57BL mice but not mycoplasma-susceptible C3H mice. *Infect Immun 65:2278*.
10. Van Rooijen, N., and A. Sanders. 1994. Liposome mediated depletion of macrophages: mechanism of action, preparation of liposomes and applications. *J Immunol Methods 174:83*.
11. Ahsan, F., I. P. Rivas, M. A. Khan, and A. I. Torres Suarez. 2002. Targeting to macrophages: role of physicochemical properties of particulate carriers—liposomes and microspheres—on the phagocytosis by macrophages. *J Control Release 79:29*.
12. Metselaar, J. M., E. Mastrobattista, and G. Storm. 2002. Liposomes for intravenous drug targeting: design and applications. *Mini Rev Med Chem 2:319*.
13. Van Rijn, R. S., E. R. Simonetti, A. Hagenbeek, M. C. Hogenes, R. A. De Weger, M. R. Canninga-Van Dijk, K. Weijer, H. Spits, G. Storm, L. Van Bloois, G. Rijkers, A. C. Martens, and S. B. Ebeling. 2003. A new xenograft model for graft-versus-host disease by intravenous transfer of human peripheral blood mononuclear cells in RAG2-/-{gamma}c-/- double mutant mice. *Blood 102:2522*.
14. Fiske, C., and Y. Subbarov. 1926. The colorimetric determination of phosphorus. *J Biol Chem. 66:375*.

15. Raz, A., C. Bucana, W. E. Fogler, G. Poste, and I. J. Fidler. 1981. Biochemical, morphological, and ultrastructural studies on the uptake of liposomes by murine macrophages. *Cancer Res 41:487*.
16. Pauwels, R. A., J. C. Kips, R. A. Peleman, and M. E. Van Der Straeten. 1990. The effect of endotoxin inhalation on airway responsiveness and cellular influx in rats. *Am Rev Respir Dis 141:540*.
17. Palmberg, L., B. M. Larsson, P. Malmberg, and K. Larsson. 1998. Induction of IL-8 production in human alveolar macrophages and human bronchial epithelial cells in vitro by swine dust. *Thorax 53:260*.
18. Finck, C. M., M. G. Hodell, W. H. Marx, A. M. Paskanik, D. J. McGraw, C. J. Lutz, L. A. Gatto, A. L. Picone, and G. F. Nieman. 1998. Endotoxin-stimulated alveolar macrophage recruitment of neutrophils and modulation with exogenous surfactant. *Crit Care Med 26:1414*.
19. Mizgerd, J. P. 2002. Molecular mechanisms of neutrophil recruitment elicited by bacteria in the lungs. *Semin Immunol 14:123*.
20. Berg, J. T., S. T. Lee, T. Thepen, C. Y. Lee, and M. F. Tsan. 1993. Depletion of alveolar macrophages by liposome-encapsulated dichloromethylene diphosphonate. *J Appl Physiol 74:2812*.
21. Hashimoto, S., J. F. Pittet, K. Hong, H. Folkesson, G. Bagby, L. Kobzik, C. Frevert, K. Watanabe, S. Tsurufuji, and J. Wiener-Kronish. 1996. Depletion of alveolar macrophages decreases neutrophil chemotaxis to Pseudomonas airspace infections. *Am J Physiol 270:L819*.
22. Salez, L., V. Balloy, N. Van Rooijen, M. Lebastard, L. Touqui, F. X. McCormack, and M. Chignard. 2001. Surfactant protein A suppresses lipopolysaccharide-induced IL-10 production by murine macrophages. *J Immunol 166:6376*.
23. Nieuwenhuijzen, G. A., M. F. Knapen, T. Hendriks, N. Van Rooijen, and R. J. Goris. 1997. Elimination of various subpopulations of macrophages and the development of multiple-organ dysfunction syndrome in mice. *Arch Surg 132:533*.
24. Haeberle, H. A., R. Takizawa, A. Casola, A. R. Brasier, H. J. Dieterich, N. Van Rooijen, Z. Gatalica, and R. P. Garofalo. 2002. Respiratory syncytial virus-induced activation of nuclear factor-kappaB in the lung involves alveolar macrophages and toll-like receptor 4-dependent pathways. *J Infect Dis 186:1199*.
25. Lentsch, A. B., B. J. Czermak, N. M. Bless, N. Van Rooijen, and P. A. Ward. 1999. Essential role of alveolar macrophages in intrapulmonary activation of NF-kappaB. *Am J Respir Cell Mol Biol 20:692*.
26. Kradin, R. L., H. W. Liu, N. Van Rooijen, K. Springer, L. H. Zhao, and C. P. Leary. 1999. Pulmonary immunity to Listeria is enhanced by elimination of alveolar macrophages. *Am J Respir Crit Care Med 159:1967*.
27. Naidu, B. V., B. Krishnadasan, A. S. Farivar, S. M. Woolley, R. Thomas, N. Van Rooijen, E. D. Verrier, and M. S. Mulligan. 2003. Early activation of the alveolar macrophage is critical to the development of lung ischemia-reperfusion injury. *J Thorac Cardiovasc Surg 126:200*.

28. Broug-Holub, E., G. B. Toews, J. F. van Iwaarden, R. M. Strieter, S. L. Kunkel, R. Paine, 3rd, and T. J. Standiford. 1997. Alveolar macrophages are required for protective pulmonary defenses in murine *Klebsiella pneumonia*: elimination of alveolar macrophages increases neutrophil recruitment but decreases bacterial clearance and survival. *Infect Immun 65:1139*.

29. van Iwaarden, J. F., E. Claassen, S. H. Jeurissen, H. P. Haagsman, and G. Kraal. 2001. Alveolar macrophages, surfactant lipids, and surfactant protein B regulate the induction of immune responses via the airways. *Am J Respir Cell Mol Biol 24:452*.

30. Thepen, T., K. Hoeben, J. Breve, and G. Kraal. 1992. Alveolar macrophages down-regulate local pulmonary immune responses against intratracheally administered T-cell-dependent, but not T-cell-independent antigens. *Immunology 76:60*.

31. Maus, U. A., M. A. Koay, T. Delbeck, M. Mack, M. Ermert, L. Ermert, T. S. Blackwell, J. W. Christman, D. Schlondorff, W. Seeger, and J. Lohmeyer. 2002. Role of resident alveolar macrophages in leukocyte traffic into the alveolar air space of intact mice. *Am J Physiol Lung Cell Mol Physiol 282:L1245*.

32. Koay, M. A., X. Gao, M. K. Washington, K. S. Parman, R. T. Sadikot, T. S. Blackwell, and J. W. Christman. 2002. Macrophages are necessary for maximal nuclear factor-kappa B activation in response to endotoxin. *Am J Respir Cell Mol Biol 26:572*.

33. Limper, A. H., J. S. Hoyte, and J. E. Standing. 1997. The role of alveolar macrophages in *Pneumocystis carinii* degradation and clearance from the lung. *J Clin Invest 99:2110*.

34. Holt, P. G., J. Oliver, N. Bilyk, C. McMenamin, P. G. McMenamin, G. Kraal, and T. Thepen. 1993. Downregulation of the antigen presenting cell function(s) of pulmonary dendritic cells in vivo by resident alveolar macrophages. *J Exp Med 177:397*.

35. Thepen, T., C. McMenamin, J. Oliver, G. Kraal, and P. G. Holt. 1991. Regulation of immune response to inhaled antigen by alveolar macrophages: differential effects of in vivo alveolar macrophage elimination on the induction of tolerance vs. immunity. *Eur J Immunol 21:2845*.

36. Thepen, T., C. McMenamin, B. Girn, G. Kraal, and P. G. Holt. 1992. Regulation of IgE production in pre-sensitized animals: in vivo elimination of alveolar macrophages preferentially increases IgE responses to inhaled allergen. *Clin Exp Allergy 22:1107*.

37. Strickland, D. H., T. Thepen, U. R. Kees, G. Kraal, and P. G. Holt. 1993. Regulation of T-cell function in lung tissue by pulmonary alveolar macrophages. *Immunology 80:266*.

38. Madjdpour, C., U. R. Jewell, S. Kneller, U. Ziegler, R. Schwendener, C. Booy, L. Klausli, T. Pasch, R. C. Schimmer, and B. Beck-Schimmer. 2003. Decreased alveolar oxygen induces lung inflammation. *Am J Physiol Lung Cell Mol Physiol 284:L360*.

3

Effect of alveolar macrophage depletion on lung function and airway inflammation in a rat model for trimellitic anhydride-induced occupational asthma

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Abstract

Occupational exposure to low molecular weight chemicals, like trimellitic anhydride (TMA), can result in occupational asthma. Alveolar macrophages (AMs) are among the first cells to encounter these inhaled compounds. These cells can produce many different mediators that have a putative role in asthma. Therefore, in the present study we examined the role of AMs in lung function and airway inflammation of rats exposed to TMA. Female Brown Norway rats were sensitized by dermal application of TMA in vehicle (olive oil/acetone) or received vehicle alone on days 0 and 7. One day prior to challenge the rats were treated intratracheally with either empty liposomes or liposomes containing clodronate (dichloromethylene diphosphonate) to specifically deplete the lungs of AMs. On day 21 all groups of rats were challenged by inhalation of 30 mg/m^3 of TMA. Breathing frequency, tidal volume, and minute ventilation were measured before, during, within 1 h after, and 24 h after challenge and the gross respiratory rate score during challenge. Total and TMA-specific IgE levels were determined in serum and lung lavage fluid and parameters of inflammation and tissue damage were assessed in lung lavage fluid and/or lung tissue 24 h after challenge. Sensitization with TMA had no effect on the basal lung function before challenge, but led to decreased lung function parameters during and within 1 h after challenge as compared to the non-sensitized rats. AM depletion alleviated the TMA-induced drop in breathing frequency and minute ventilation and induced a faster recovery compared to sham-depleted TMA-sensitized rats. It also decreased the levels of serum IgE 24 h after challenge, but did not affect the sensitization-dependent increase in lung lavage fluid interleukin 6 and tissue tumor necrosis factor-alpha levels. AM depletion augmented the TMA-induced increases in non-specific tissue damage, as measured by lactate dehydrogenase, and increased inflammation 24 h after challenge, although the latter was not significant. In conclusion, AMs seem to have a dual role in asthma in our rat model for TMA-induced occupational asthma. Their presence deteriorated the immediate TMA-induced decrease in minute ventilation but tended to dampen the TMA-induced inflammatory reaction 24 h later.

Introduction

Alveolar macrophages (AMs) are phagocytic cells in the airways that are known to play an important role in the immune regulation of the airways to protein allergens. Notably, depletion of these cells in ovalbumin-sensitized rodents increased the serum IgE levels and airway inflammation after a local ovalbumin challenge indicating a role of the AM in decreasing pulmonary immune responses (1-3). AMs were found to do so by secreting mediators that inhibit allergen-induced T cell activation and by inducing T cell anergy by activating T cells in the absence of costimulatory signals (4-7).

Besides proteins, low molecular weight (LMW) chemicals are known to cause respiratory allergic diseases (8, 9). The development of LMW chemical-induced asthma requires sensitization, which is thought to be triggered by dermal or respiratory exposure to the compound, binding of the compound to self-proteins, and subsequent presentation of these conjugated proteins to T cells (10-12). In susceptible individuals exposure to LMW chemicals may result in the production of specific IgE antibodies and upon renewed contact with the LMW chemical secondary immune responses result in clinically manifest adverse health effects like cough, chest tightness, shortness of breath and wheezing (8). LMW chemicals, like diisocyanates, reactive dyes, and acid anhydrides, are a major cause of occupational respiratory allergies (13). Among the latter chemicals, trimellitic anhydride (TMA), used in the manufacture of paints, epoxy curing agents, printing inks and vinyl plasticizers, is most frequently implicated (11).

Since AMs were found to suppress immune responses to protein allergens, it is not unlikely that they play a similar role in LMW chemical-induced respiratory allergies. We, therefore, sought to determine the role of the AM in lung function and airway inflammation in a rat model for TMA-induced asthma (14) by depleting these cells by intratracheal instillation of clodronate-containing negatively charged liposomes one day before respiratory TMA challenge. This treatment was earlier shown to selectively deplete AMs without attracting inflammatory cells to the lung (Chapter 2).

Materials and Methods

Animals

Female, inbred Brown Norway/CrlBR rats (BN; 7-8 weeks of age) were purchased from Charles River (Sulzfeld, Germany). The animals were acclimatized for at least 5 days before the start of the study. They were kept under conventional laboratory conditions at the TNO Institute and received the Institute's grain-based open-formula diet and unfluoridated tap water ad libitum. The animals

were used at 8-9 weeks of age. All animal procedures were conducted in accordance with the Animal Ethics Committee of Utrecht University (Utrecht, The Netherlands).

Treatment schedule and groups

Studies were conducted according to the following scheme: day -1, blood withdrawal; days 0 and 7, dermal sensitization; day 19, blood withdrawal; day 20, assessment of basal lung function (breathing frequency and pattern) and liposome treatment; day 21, TMA challenge and assessment of lung function; day 22, assessment of lung function, blood sampling, broncho-alveolar lavage, and necropsy. The rats were divided into four groups as indicated in Table 1. All the results shown are representative for three separate experiments.

Table 1. Treatment schedule of BN rats.

	Sensitization		Liposomes	Challenge
	on flanks 300 µl	on ears 150 µl	intratracheal 300 µl	inhalation 30 mg/m^3; 15 min
Group designation	Day 0	Day 7	Day 20	Day 21
Veh/PBS	vehicle[1]	vehicle	PBS	TMA
Veh/clodro	vehicle	vehicle	clodronate	TMA
TMA/PBS	TMA	TMA	PBS	TMA
TMA/clodro	TMA	TMA	clodronate	TMA

[1]Vehicle was acetone/olive oil 4:1 (v/v).

Sensitization procedure

TMA (97% purity, Aldrich, Brussels, Belgium) was applied at a concentration of 50% (w/v) in a vehicle solution of 4:1 (v/v) acetone (HPLC grade; Merck, Darmstadt, Germany) and highly refined olive oil (Sigma, St. Louis, MO). Animals received 150 µl on each flank (approximately 12 cm^2 each), which had been shaved with an electrical razor at least 2-3 days earlier. Seven days after the first sensitization the animals received 75 µl of a 25% TMA solution on the dorsum of both ears. Control animals received vehicle solution.

Preparation of liposomes and depletion of alveolar macrophages

Negatively charged liposomes were made with pyrogen-free components under aseptic conditions at the Department of Pharmaceutics, Utrecht Institute for Pharmaceutical Sciences, Utrecht

University, Utrecht, the Netherlands, as described earlier (Chapter 2). Briefly, a mixture of egg-phosphatidylcholine, egg-phosphatidylglycerol (both from Lipoid GmbH, Ludwigshafen, Germany) and cholesterol (Sigma) were dissolved in ethanol in a molar ratio of 10:1:1.5 and evaporated to dryness by rotation under reduced pressure. The lipid film was hydrated in phosphate-buffered saline (PBS; pH 7.4) either or not containing 60 mg/ml of clodronate (dichloromethylene diphosphonate; Schering, Weesp, The Netherlands). Removal of unencapsulated clodronate was achieved by repeated washing with PBS and ultracentrifugation (Beckman Optima LE-80K, Palo Alto, CA) at 200,000 x *g* for 30 min. After the last washing step, the pellet was resuspended in PBS at a concentration of 90 mM phospholipid. The phospholipid concentration was determined according to Fiske and Subbarov as modified by King (15). The concentration of clodronate was determined spectrophotometrically at a wavelength of 238 nm after extraction and binding to Cu^{2+}. The final clodronate concentration of the liposome formulations was about 2.5 mg/ml.

Liposomes containing clodronate or PBS as a control (300 µl) were instilled intratracheally after light anesthesia using a mixture of midazolam (Roche Diagnostics, Basel, Switzerland), fluanisone, and phentanyl citrate (both from Janssen Pharmaceutica, Beerse, Belgium) (0.6 mg/kg, 1.2 mg/kg, and 40 µg/kg, respectively, i.m.).

TMA-atmosphere generation and analysis

An all glass nebulizer was used to generate the test atmosphere from a freshly prepared solution of TMA in acetone (16). The concentration of 30 mg/m^3 of TMA was based on previous studies performed in the rat (17). The acetone concentration was kept between 2000 and 5000 ppm (~5-12 g/m^3), being far below the level inducing sensory irritation (16). Atmospheric concentrations of TMA were determined gravimetrically by filter sampling and those of acetone by calculations based on the nominal concentration and its complete evaporation. The particle size distribution of TMA in the test atmosphere was determined using a 10-stage cascade impactor (Anderson, Atlanta, GA). The mass median aerodynamic diameter of the TMA aerosols was 1.5 µm with geometric standard deviation of 1.9.

Respiratory challenge and lung function measurements

For respiratory challenge and lung function measurements, rats were treated as described before (17). Briefly, lung function was assessed using a two-chamber whole-body plethysmograph. Per group one rat at a time was placed in one of the four nose-only tubes in the experimental setup and subsequently exposed to fresh air for 30 min (pre-challenge period), TMA atmosphere for exactly

15 min (challenge period), and fresh air for at least 1 h (post-challenge period). Breathing parameters (breathing frequency, tidal volume, and minute ventilation) were monitored by means of recording the pressure signal before, during and after challenge. Before challenge, breathing parameters were monitored approximately 20 s each minute, starting 10 min prior to the actual challenge. During the challenge, breathing parameters were monitored continuously, whereas after challenge, they were monitored approximately 20 s during each minute for the first 10 min followed by 20 s each 5 min for 50 min. Furthermore, breathing parameters were monitored approximately 20 s each minute for 15 min 24 h before AM depletion and 24 h after TMA challenge.

To determine the degree of respiratory distress, the gross respiratory response (GRR) was assessed according to Ritz et al (18) based on the number of normal breaths between retractions (irregularly lengthened pauses) relatively to normal breathing. The minimum score according to this classification is 0.0 (no retractions) and the maximum score is 7.0 (anaphylactic shock).

Serum collection, body and lung weights and necropsy

All animals were observed daily and weighed at the start of the experiment, at weekly intervals thereafter and just prior to necropsy. Individual serum samples were prepared from blood withdrawn 1 day before the start of the experiment and at day 19 via orbital puncture under light anesthesia. At day 22 animals received a lethal dose of sodium pentobarbitone (0.6 g/kg, i.p.; Cevasante Animale B.V., Maassluis, the Netherlands) and blood was withdrawn via cardiac puncture. Serum samples were stored at –20ºC until use. Animals were examined grossly for abnormalities. The trachea and lungs were excised *en bloc* and lungs were weighed.

Bronchoalveolar lavage

After lung weight determination the bronchus of the left lung was ligated. The right lung was lavaged, once with 2.5 ml of warmed PBS (37ºC) containing protease blockers (Roche Diagnostics) and then 3 times with 2.5 ml warm PBS keeping the recollected lavage fluid in tubes on ice. The bronchoalveolar cells were isolated by centrifugation for 10 min at 4ºC and pooled to determine total cell numbers using a Bürker Türk chamber. For differential cell counts, cytospin preparations were made and stained with Diff-Quick (Merz and Dade, Düdingen, Switzerland). The supernatant of the first 2.5 ml lavage fluid was analyzed for lactate dehydrogenase (LDH), a marker for general toxicity using an automatic analyzer (Hitachi 911, Hitachi Instruments Division, Japan), and for total and TMA-specific IgE, and cytokines as described below.

Lung homogenates

After total lung weight determination the left lung was separated, snap-frozen and stored at -20ºC. After assessment of the weight of the frozen lung it was homogenized in 1 ml ice cold PBS with protease blockers (Roche Diagnostics) in a round bottom vial at 4ºC using a politron (Ystral GmBH, Dottingen, Germany). After centrifugation at 390 x *g* the supernatants were collected and stored at -20ºC until determination of cytokines.

IgE and cytokine levels

Total and TMA-specific IgE were measured in serum and in lung lavage fluid by ELISA. In short, the total IgE determination was performed using 96-wells flat-bottomed plates (NUNC A/S, Roskilde, Denmark) coated with 5 µg/ml streptavidine (Sigma) in 0.1 M carbonate buffer (pH 9.6). Coated plates were incubated with a 1:800 dilution of biotin-conjugated mouse monoclonal anti-rat IgE (Serotec, Oxford, UK). Duplicate wells were then incubated with graded dilutions of test samples and a monoclonal rat-IgE standard (Serotec), and subsequently with a 1:200 dilution of peroxidase-conjugated mouse anti-rat IgE (Zymed Laboratories Inc., San Francisco, CA). After addition of the substrate TMB (3,3',5,5'-tetramethylbenzidine dehydrochloride dehydrate (ICN Biochemicals, Aurora, OH) and hydrogen peroxide) the reaction was terminated by addition of 0.9 M H_2SO_4. The absorbance was measured at 450 nm using a Microplate reader (Bio-Rad Laboratories, Hercules, CA). The concentration of IgE in the samples was calculated using a standard curve obtained with known quantities of monoclonal rat IgE and expressed as µg/ml serum. The TMA-specific IgE ELISA was performed using 96-wells flat-bottomed plates coated with TMA-BSA in 0.1 M carbonate buffer (pH 9.6), followed by a blocking step with 1% BSA. Duplicate wells were then incubated with graded dilutions of test samples and serum which had been scored positive for TMA-specific IgE as a standard, and subsequently with a 1:2000 dilution of a HRP-conjugated mouse anti-rat IgE (Serotec). Addition of the substrate and further steps were as described above. The amount of TMA-specific IgE in the samples was expressed as percentage (%) of the O.D. of the standard.

TNF-α and IL-6 levels in serum, lung homogenates, and lung lavage fluid were measured in duplicate using commercially available kits (R & D Systems Inc., Minneapolis, MN) with a detection limit of 10 pg/ml for IL-6 and 15 pg/ml for TNF-α. A microplate reader was used to measure absorbance at 450 nm.

Airway histopathology

Using separate groups of rats, the left lungs were inflated with Tissue Tec (Miles Inc., Elkhart, IN) diluted in PBS (1:1), snap-frozen in liquid nitrogen, and stored at –20ºC until used for histopathology. Frozen sections (5 μm) were prepared, fixed in acetone, and stained with haematoxylin and eosin to semi-quantitatively assess the degree of cellular infiltration around the terminal bronchioli as indicated in the legend of Table 2.

Data analysis

All data have been expressed as mean ± SEM. Cell numbers in the lung lavage fluid were statistically analyzed using the Mann-Whitney *U* test. Lung function parameters, organ weights, IgE concentrations, LDH, and cytokine levels were determined by one-way ANOVA followed by a Bonferroni multiple-comparison test. Inflammation scores around terminal bronchioli were determined by Kruskal-Wallis test followed by Dunn's multiple-comparison test. Differences were considered statistically significant if $p<0.05$. Analyses were performed by the usage of Graphpad Prism (version 3.0, San Diego, U.S.A.).

Results

Depletion of AM by clodronate liposomes

To confirm the macrophage depleting capacity of clodronate-containing negatively charged liposomes, naive BN rats received intratracheally 300 μl of PBS, empty liposomes, or a liposome suspension containing 0.83 mg clodronate. This dose of liposome-encapsulated clodronate depleted 83.1 ± 1.9 % of AMs relative to rats treated with control PBS-liposomes 24 h after administration, which was found to be the maximum depletion (Chapter 2).

Body and organ weights

TMA challenge of non-sensitized rats (veh/PBS and veh/clodro) did not change body and relative organ weights. TMA challenge of TMA-sensitized rats (TMA/PBS and TMA/clodro) did not change body, and relative liver and spleen weights as compared to the non-sensitized rats (data not shown), but, significantly increased relative lung weights 24 hours later (Fig. 1). AM depletion prior to challenge did not influence the effects of TMA challenge on body and organ weights in sensitized and control rats.

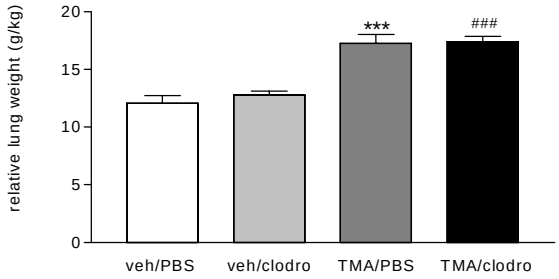

Figure 1. Effect of AM depletion prior to TMA challenge on relative lung weights 24 h after challenge. Groups of 6 rats received 150 µl vehicle or 50% TMA in vehicle on each shaved flank on day 0 and 75 µl vehicle or 25% TMA on the dorsum of both ears on day 7. On day 20 the animals received an intratracheal instillation of either empty or clodronate-liposomes followed 24 h later by an inhalation challenge with 30 mg/m^3 of TMA for 15 min. Lung weights were determined 24 h after challenge. Results are expressed relative to body weight and expressed as mean ± SEM. Significant differences are denoted by ***: p< 0.001 compared to veh/PBS and ###: p< 0.001 compared to veh/clodro.

Effect of AM depletion on IgE levels

Pretreatment serum levels of total IgE were below the baseline level of 1 µg/ml in all groups (data not shown). Total IgE and TMA-specific IgE levels remained low one day after TMA challenge in non-sensitized rats (veh/PBS and veh/clodro) irrespective of AM depletion, whereas these levels had significantly increased in the TMA-sensitized rats compared to the non-sensitized controls prior to AM depletion (day 19, data not shown). Surprisingly, AM depletion of TMA-sensitized rats (TMA/clodro) followed by TMA challenge resulted in significantly lower total and TMA-specific IgE levels in the serum than found in sham-depleted rats (TMA/PBS) (Fig. 2a and b).

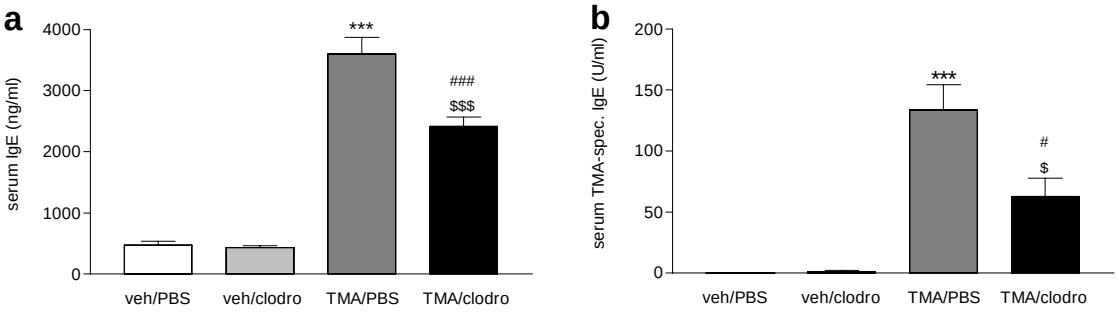

Figure 2. Effect of AM depletion prior to TMA challenge on serum IgE levels 24 h after challenge. Groups of 6 rats received 150 µl vehicle or 50% TMA in vehicle on each shaved flank on day 0 and 75 µl vehicle or 25% TMA on the dorsum of both ears on day 7. On day 20 the animals received an intratracheal instillation of either empty or clodronate-liposomes followed 24 h later by an inhalation challenge with 30 mg/m^3 of TMA for 15 min. Serum was collected 24 h after TMA challenge and levels of total (**a**) and TMA-specific IgE (**b**) were assessed. Results are expressed as mean ± SEM. Significant differences are denoted by ***: p< 0.001 compared to veh/PBS, ###: p< 0.001 compared to veh/clodro, $: p< 0.05, or $$$: p<0.001 compared to TMA/PBS.

Total and TMA-specific IgE levels in the lung lavage fluid of non-sensitized rats (veh/PBS and veh/clodro) were at baseline level. The levels were significantly higher in the TMA-challenged rats (TMA/PBS and TMA/clodro) as compared to the non-sensitized rats. AM depletion, however, had no effect on the IgE levels in the lung lavage fluid (Fig. 3a and b).

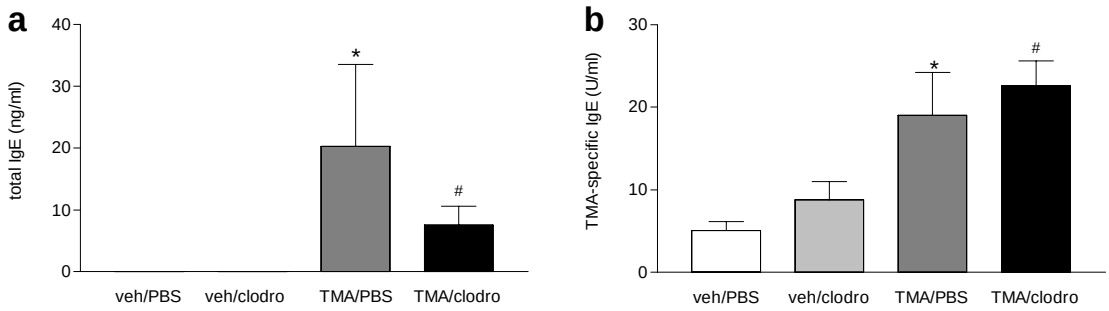

Figure 3. Effect of AM depletion prior to TMA challenge on IgE levels in the lung lavage fluid 24 h after challenge. Groups of 6 rats received 150 µl vehicle or 50% TMA in vehicle on each shaved flank on day 0 and 75 µl vehicle or 25% TMA on the dorsum of both ears on day 7. On day 20 the animals received an intratracheal instillation of either empty or clodronate-liposomes followed 24 h later by an inhalation challenge with 30 mg/m^3 of TMA for 15 min. Lungs were lavaged 24 h after TMA challenge and levels of total (**a**) and TMA-specific (**b**) IgE were assessed. Results are expressed as mean ± SEM. Significant differences are denoted by *: $p<0.05$, compared to veh/PBS, and #: $p<0.05$ compared to veh/clodro.

Effect of AM depletion on TMA-induced respiratory changes

Breathing frequencies were comparable for all groups prior to TMA challenge and did not change in the non-sensitized rats (veh/PBS and veh/clodro) at any time point measured during or after the challenge, irrespective of AM depletion (Fig. 4a). Compared to the non-sensitized rats, the breathing frequency of sensitized sham-depleted rats (TMA/PBS) significantly decreased during TMA challenge, was normal immediately thereafter, and significantly increased 24 h after challenge. Compared to the non-sensitized AM-depleted rats (veh/clodro), the breathing frequency of sensitized AM-depleted rats (TMA/clodro) decreased during the first 7 min of the challenge, was normal during the second 7 min of the challenge and within 1 h after challenge, and significantly increased 24 h after challenge. Compared to the sensitized sham-depleted rats (TMA/PBS), AM depletion of sensitized rats had no significant effects, but it tended to mitigate the TMA-induced decrease in breathing frequency during the TMA challenge.

The tidal volumes were comparable for all groups before challenge and did not change in the non-sensitized rats (veh/PBS and veh/clodro) at any time point measured during or after the challenge, irrespective of AM depletion (Fig. 4b). Compared to the non-sensitized rats, the tidal volume of the sensitized sham-depleted rats (TMA/PBS) was normal during the TMA challenge,

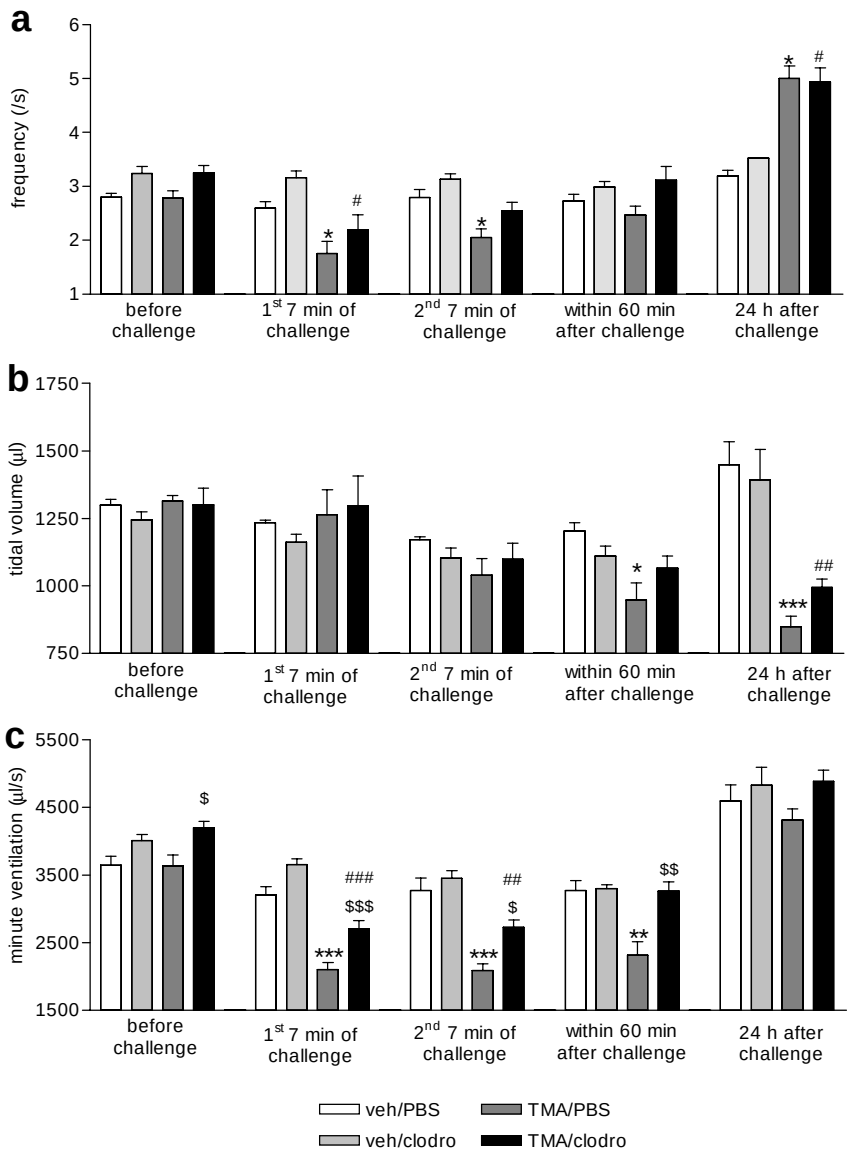

Figure 4. Effect of AM depletion on TMA-inhalation induced changes in lung function. Groups of 6 rats received 150 µl vehicle or 50% TMA in vehicle on each shaved flank on day 0 and 75 µl vehicle or 25% TMA on the dorsum of both ears on day 7. On day 20 the animals received an intratracheal instillation of either empty or clodronate-liposomes followed 24 h later by an inhalation challenge with 30 mg/m^3 of TMA for 15 min. Frequency (**a**), tidal volume (**b**) and minute ventilation (**c**) were measured before, during, within 1 h after, and 24 h after TMA challenge. Results are expressed as mean ± SEM. Significant differences are denoted by *: p<0.05, **: p<0.01 or *** p< 0.001 compared to veh/PBS, #: p<0.05, ##: p< 0.01, or ###; p<0.001 compared to veh/clodro, and $: p< 0.05, $$: p<0.01, or $$$: p<0.001 compared to TMA/PBS.

but decreased immediately after challenge and diminished even further 24 h after challenge. Compared to the non-sensitized AM-depleted rats (veh/clodro), the tidal volume of AM-depleted sensitized rats (TMA/clodro) was normal during the challenge and immediately after challenge, but significantly decreased 24 h after challenge. Compared to the sensitized sham-depleted rats (TMA/PBS), AM depletion of sensitized rats had no significant effects, but it tended to mitigate the TMA-induced decrease in tidal volume after TMA challenge.

The minute ventilation, a function of both frequency and tidal volume, was comparable for both non-sensitized rats (veh/PBS and veh/clodro) and did not change at any time point measured during or after the challenge, except for an increase 24 h after challenge, irrespective of AM depletion (Fig. 4c). Compared to the non-sensitized rats, the minute ventilation of the TMA-sensitized sham-depleted rats (TMA/PBS) was comparable before challenge, decreased immediately during challenge and stayed at the same level directly after challenge, but returned to the levels of non-sensitized rats 24 h after challenge. Compared to the non-sensitized AM-depleted rats (veh/clodro), the minute ventilation of the sensitized AM-depleted rats (TMA/clodro) was comparable before challenge, significantly decreased during TMA challenge, but was normal immediately and 24 h after challenge. Compared to the sensitized sham-depleted rats (TMA/PBS), AM depletion of sensitized rats (TMA/clodro) increased the minute ventilation before challenge but significantly diminished the decrease in minute ventilation during and directly after challenge.

The non-sensitized groups (veh/PBS and veh/clodro) did not show any signs of respiratory distress during TMA challenge and therefore were assigned a GRR-score of 0. The sensitized groups TMA/PBS and TMA/clodro had a GRR score of 3.4 ± 0.6 and 4.0 ± 0.0, respectively, corresponding to a mean of 6-15 respirations between each pause (18), indicating moderate respiratory distress irrespective of AM depletion.

Effect of AM depletion on TMA-induced airway inflammation

Lung lavage fluid of non-sensitized rats (veh/PBS) contained $148 \pm 16 \times 10^3$ cells/ml, the majority being AMs and eosinophils 24 h after TMA challenge (Fig. 5). AM depletion of non-sensitized rats (veh/clodro) prior to TMA challenge increased total cell number ($281 \pm 33 \times 10^3$ cells/ml) mainly because of an increase of eosinophils. Compared to the non-sensitized rats (veh/PBS), lung lavage fluid of sensitized sham-depleted rats (TMA/PBS) contained significantly more cells ($510 \pm 113 \times 10^3$ cells/ml) being due to increased neutrophil and particularly eosinophil numbers. AM depletion of sensitized rats (TMA/clodro) significantly increased the total cell number ($693 \pm 106 \times 10^3$) compared to the non-sensitized AM-depleted rats (veh/clodro) being due to increased neutrophil and eosinophil numbers. Compared to the sensitized sham-depleted rats (TMA/PBS), AM depletion had no effect on total cell number and neutrophil and eosinophil numbers, but increased mononuclear cell numbers, including AMs.

The inflammation around the terminal bronchioli was comparable for both groups of non-sensitized rats (veh/PBS and veh/clodro) (Table 2). Moderate numbers of inflammatory cells surrounded most of the terminal bronchioli. Compared to the non-sensitized sham-depleted rats (veh/PBS) the TMA-sensitized sham-depleted rats (TMA/PBS) had significantly more severe

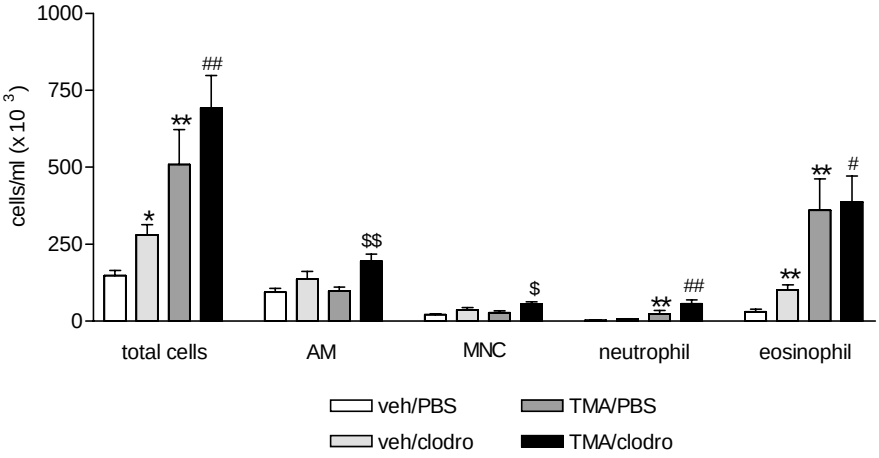

Figure 5. Effect of AM depletion prior to TMA-inhalation on the composition of lung lavage cells 24 h after challenge. Groups of 6 rats received 150 µl vehicle or 50% TMA in vehicle on each shaved flank on day 0 and 75 µl vehicle or 25% TMA on the dorsum of both ears on day 7. On day 20 the animals received an intratracheal instillation of either empty or clodronate-liposomes followed 24 h later by an inhalation challenge with 30 mg/ml of TMA for 15 min. Lungs were lavaged 24 h later. Cells were counted and differentiated into macrophages, MNC (lymphocytes and monocytes), eosinophils and neutrophils using Diff Quick. Results are expressed as mean ± SEM. Significant differences are denoted by *: $p<0.05$, or **: $p< 0.01$ compared to veh/PBS, #: $p<0.05$, or ##: $p< 0.01$ compared to veh/clodro, and $: $p< 0.05$, or $$: $p<0.01$ compared to TMA/PBS.

inflammation around the terminal bronchioli. AM depletion of sensitized rats (TMA/clodro) had no effect on the inflammation around the terminal bronchioli compared to the sensitized sham-depleted rats. In all groups, the inflammatory cells around the terminal bronchioli consisted mainly of eosinophils and some macrophages were found.

Table 2. Severity of inflammation around the terminal bronchioli 24 h after inhalation challenge with 30 mg/m^3 TMA.

	Severity of inflammation			
Treatment	No	Slight	Moderate	Severe
Veh/PBS	0 ± 0%	34 ± 5%	60 ± 4%	6 ± 2%
Veh/clodro	1 ± 1%	39 ± 4%	52 ± 3%	9 ± 3%
TMA/PBS	0 ± 0%	18 ± 4%	65 ± 3%	16 ± 2% *
TMA/clodro	1 ± 1%	22 ± 7%	58 ± 5%	19 ± 3%

Groups of 6 rats received 150 µl vehicle or 50% TMA in vehicle on each shaved flank on day 0 and 75 µl vehicle or 25% TMA on the dorsum of both ears on day 7. On day 20 the animals received an intratracheal instillation of either empty or clodronate-containing liposomes followed 24 h later by an inhalation challenge with 30 mg/m^3 of TMA for 15 min. On day 22 the left lungs were excised and inflated with Tissue Tec/PBS (1:1) to prepare HE stained sections for assessment of inflammation. The peribronchiolar inflammation of all terminal bronchioli per lung was scored; no: 0 cells; slight: ≤ 5 cells; moderate: ≤ 15 cells; severe: > 15 cells. Results are expressed as percentage of the total of terminal bronchioli per lung per group. Significant differences were denoted by *: $p< 0.05$ compared to veh/PBS.

Effect of AM depletion on damage markers following TMA-challenge

Low levels of LDH were measured in the lung lavage fluid of non-sensitized sham-depleted rats (veh/PBS) 24 h after TMA challenge. However, AM-depletion tended to increase the release of LDH (p=0.06) compared to the sham-depleted rats (Fig. 6). Compared to the non-sensitized rats, levels of LDH were increased in the sham-depleted TMA-sensitized rats (TMA/PBS) 24 h after TMA challenge. AM depletion of TMA-sensitized rat (TMA/clodro) increased the levels of LDH compared to the sham-depleted sensitized rats (TMA/PBS).

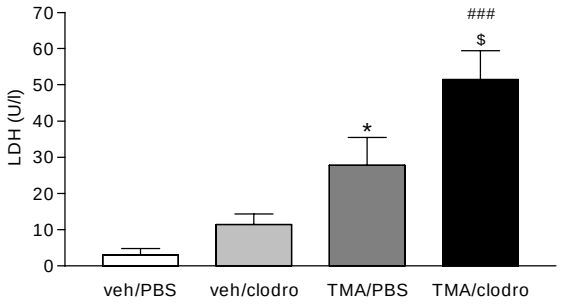

Figure 6. Effect of AM depletion 24 h before TMA challenge on LDH in the lung lavage fluid 24 h after challenge. Groups of 6 rats received 150 µl vehicle or 50% TMA in vehicle on each shaved flank on day 0 and 75 µl vehicle or 25% TMA on the dorsum of both ears on day 7. On day 20 the animals received an intratracheal instillation of either empty or clodronate-liposomes followed 24 h later by an inhalation challenge with 30 mg/m^3 of TMA for 15 min. The lungs were lavaged 24 h after TMA challenge and LDH was measured. Results are expressed as mean ± SEM. Significant differences are denoted by *: p< 0.05 compared to veh/PBS, ###: p< 0.001 compared to veh/clodro and $: p< 0.05 compared to TMA/PBS.

Effect of AM depletion on the production of cytokines following TMA challenge

IL-6 levels measured in lung homogenates of rats 24 h after TMA challenge were similar in non-sensitized and sensitized rats, irrespective of AM depletion (Fig. 7a). IL-6 levels measured in the lung lavage fluid 24 h after TMA challenge were very low in the non-sensitized rats (veh/PBS) and below the detection limit in the non-sensitized AM-depleted rats (veh/clodro) (Fig. 7b). Compared to the non-sensitized rats (veh/PBS and veh/clodro), IL-6 levels in the lung lavage fluid of sensitized rats (TMA/PBS and TMA/clodro) were increased by the TMA challenge irrespective of AM depletion. Serum IL-6 levels were below the detection limit of the assay in all groups.

TNF-α levels measured in lung homogenates of both groups of non-sensitized rats (veh/PBS and veh/clodro) 24 h after TMA inhalation were similar (Fig. 8). Compared to the non-sensitized rats (veh/PBS and veh/clodro), TNF-α levels in the lung homogenates of sensitized rats (TMA/PBS and TMA/clodro) were increased by the TMA challenge, irrespective of AM depletion. TNF-α levels both in lung lavage fluid and serum samples of all groups were below the detection limit of the assay.

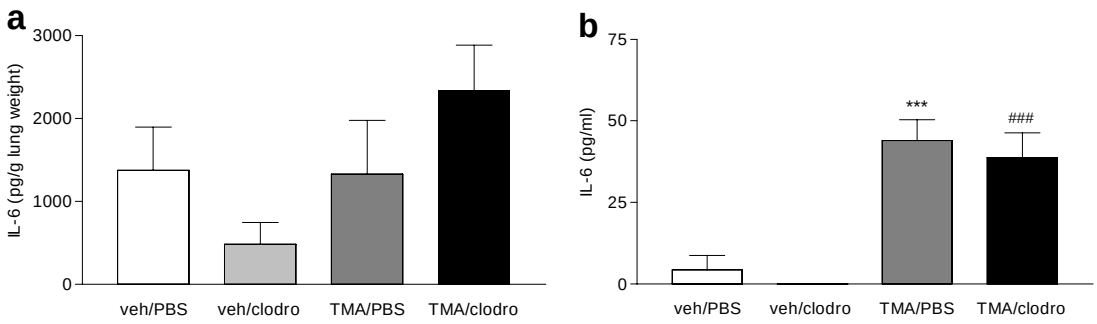

Figure 7. Effect of AM depletion 24 h before TMA challenge on IL-6 levels in lung homogenates and lung lavage fluid 24 h after challenge. Groups of 6 rats received 150 µl vehicle or 50% TMA in vehicle on each shaved flank on day 0 and 75 µl vehicle or 25% TMA on the dorsum of both ears on day 7. On day 20 the animals received an intratracheal instillation of either empty or clodronate-liposomes followed 24 h later by an inhalation challenge with 30 mg/m^3 of TMA for 15 min. The right lung was lavaged 24 h after challenge and the left lung was homogenized. IL-6 levels were assessed in homogenates (**a**) and in the lung lavage fluid (**b**). Results are expressed as mean ± SEM. Significant differences are denoted by ***: p< 0.001 compared to veh/PBS, and ###: p< 0.001 compared to veh/clodro.

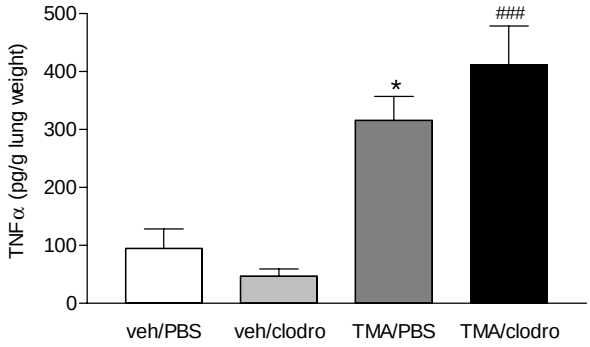

Figure 8. Effect of AM depletion 24 h before TMA challenge on TNF-α content in lung homogenates 24 h after challenge. Groups of 6 rats received 150 µl vehicle or 50% TMA in vehicle on each shaved flank on day 0 and 75 µl vehicle or 25% TMA on the dorsum of both ears on day 7. On day 20 the animals received an intratracheal instillation of either empty or clodronate-liposomes followed 24 h later by an inhalation challenge with 30 mg/m^3 of TMA for 15 min. The lungs dissected 24 h after challenge. Homogenates were made and TNF-α levels were assessed. Results are expressed as mean ± SEM. Significant differences are denoted by *: p< 0.05 compared to veh/PBS, and ###: p< 0.001 compared to veh/clodro.

Discussion

In this study we investigated the role of the AM in a model for TMA-induced occupational asthma in the BN rat. In this model, it was shown that TMA sensitization increased serum IgE levels and that subsequent respiratory TMA challenge resulted in immediate and late changes of lung function, increased inflammation as indicated by histology and leukocyte numbers in lung lavage fluid, and tissue damage as indicated by increased lung weights and raised levels of LDH in the lung lavage

fluid (this study, (14)). Furthermore, we also demonstrated increased inflammation around the terminal bronchioli and increased lung tissue levels of the proinflammatory cytokines, IL-6 and TNF-α (Table 3). AM depletion in this model for TMA-induced asthma had different effects on the diverse parameters measured. Regarding the respiratory symptoms, sensitized AM-depleted rats showed less bronchoconstriction as indicated by minute ventilation during and after TMA challenge than sensitized sham-depleted rats. This suggests that AMs contribute to the immediate bronchoconstrictive response. Since respiratory symptoms started almost instantaneously after TMA challenge of sensitized rats, irrespective of their AM status, AMs apparently do not initiate the immediate reaction. It is likely that they got activated by mast cell products that were probably released upon TMA challenge, since mast cells of mice (19) and rats (20) are known to become primed after TMA sensitization. Mast cell products, like histamine and TNF-α, may have been the mediators of the AM activation (21-23), which would result in the production of e.g. leukotrienes

Table 3. Summary of the influence of AM depletion of non-sensitized and TMA-sensitized BN rats on the different parameters during, within 1 h, and 24 h after TMA challenge

	TMA challenge								
	Sham-depletion			AM-depletion					
	TMA-sensitized * (TMA/PBS)			Non-sensitized * (veh/clodro)			TMA-sensitized # (TMA/clodro)		
Parameter[1]	during	t = 1h	t = 24h	during	t = 1h	t = 24h	during	t = 1h	t = 24h
Frequency	↓	=	↑	=	=	=	=	=	=
Tidal volume	=	↓	↓	=	=	=	=	=	=
Minute ventilation	↓	↓	=	=	=	=	↑	↑	=
GRR score	↑			=			=		
Serum IgE			↑			=			↓
BALF IgE			↑			=			=
BALF total leukocytes			↑			↑			↑
Peribronch. leukocytes			↑			=			=
Lung weight			↑			=			=
LDH			↑			= [2]			↑
BALF IL-6			↑			=			=
Tissue IL-6			=			=			=
Tissue TNF-α			↑			=			=

* Results compared to those of non-sensitized, sham-depleted rats (veh/PBS)

Results compared to those of TMA-sensitized, sham-depleted rats (TMA/PBS)

[1] Abbreviations used: BALF: lung lavage fluid, peribronch.: peribronchiolar

[2] Tends to be increased: p-value 0.06

and other mediators (23, 24). These mediators may be the cause of the more severe bronchoconstrictive response to TMA challenge in the presence of AMs. The protective effect of AM depletion appeared transient, since the bronchoconstriction in AM-depleted rats, as indicated by the decrease in tidal volume 24 h after TMA challenge, was similar to that of sham-depleted rats. At the latter time point, however, the AM-depleted rats demonstrated significantly more severe airway inflammation as indicated by total cell numbers in the lung lavage fluid than the sham-depleted rats. Furthermore, it has been shown that terminal bronchioli are more sensitive to the allergen ovalbumin than the larger airways (25). However, we did not find a correlation between the inflammation around the terminal bronchioli and the severity of bronchoconstriction. These results indicate that the airway inflammation is not clearly related to changes in lung function, although such a relation has often been suggested in protein allergen-induced asthma (26, 27). The reason why AM depletion of sensitized rats resulted in enhanced inflammation is not known, but the observation that AM depletion of non-sensitized rats also resulted in increased inflammation after TMA challenge suggests that part of the inflammatory reaction is antigen non-specific. The increased inflammation is probably due to irritation, since increased levels of LDH were measured in AM-depleted rats compared to sham-depleted rats, irrespective of sensitization. The irritant effect in its turn may have triggered the increased inflammation observed in AM-depleted rats 24 h after TMA challenge (14, 28, 29). Thus, AMs apparently protect against the irritant effect of TMA. Whereas the underlying mechanism is unknown, AMs derived from sensitized and naive BN rats appeared not affected by a range of concentrations of TMA *in vitro*, as judged by the failure of TMA to induce nitric oxide, TNF-α, and IL-6 (Chapter 5). The indifference of AMs to TMA and the well-known clearance function of AMs (30) may explain the attenuating effect of AMs on irritant inflammation. The attenuating effect, however, diverged as to cell type and immune status. The presence of AMs during TMA challenge prevented the sensitization-independent increase in eosinophils in the lung lavage fluid, but had no effect on the sensitization-dependent increase of these cells. For neutrophils, however, the presence of AMs during the TMA challenge only attenuated the sensitization-dependent increases in cells, while for AM and other mononuclear cells (monocytes/lymphocytes) the presence of AMs apparently prevented increases of these cells in sensitized animals. The observed increase in AMs is probably the combined result of chemotactic stimuli generated by the mere absence of AMs, the increased irritant effect of TMA in the absence of AMs, and mediators generated by the TMA-specific immune response in the absence of AMs. The latter mediators probably are different from IL-6 and TNF-α, because TMA challenge increased their levels in sensitized rats, but independently of the presence of AMs during challenge. Other resident cells than AMs, like mast cells or epithelial cells are probably the producers of these mediators.

The observed decrease in serum IgE levels 1 day after TMA challenge of sensitized AM-depleted rats as compared to sensitized sham-depleted rats is surprising. Since the serum IgE levels were comparable in both groups prior to liposome treatment (data not shown), the effect is related to the clodronate-liposome treatment, but difficult to explain, particularly because IgE levels in the lung were similar.

In summary, this study demonstrates that AMs have diverse effects on the reaction of non-sensitized and TMA-sensitized rats to an inhalation of TMA. On the one hand, AM depletion prior to TMA challenge had a protective effect on the lung function during challenge and reduced the levels of serum IgE. On the other hand, AM depletion increased the inflammatory response to TMA as indicated by the increase in tissue damage and the recruitment of inflammatory cells to the lungs.

Acknowledgements

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References

1. Tang, C., M. D. Inman, N. Van Rooijen, P. Yang, H. Shen, K. Matsumoto, and P. M. O'Byrne. 2001. Th type 1-stimulating activity of lung macrophages inhibits Th2- mediated allergic airway inflammation by an IFN-gamma-dependent mechanism. *J Immunol 166:1471*.
2. Thepen, T., N. Van Rooijen, and G. Kraal. 1989. Alveolar macrophage elimination in vivo is associated with an increase in pulmonary immune response in mice. *J Exp Med 170:499*.
3. Thepen, T., C. McMenamin, B. Girn, G. Kraal, and P. G. Holt. 1992. Regulation of IgE production in pre-sensitized animals: in vivo elimination of alveolar macrophages preferentially increases IgE responses to inhaled allergen. *Clin Exp Allergy 22:1107*.
4. Blumenthal, R. L., D. E. Campbell, P. Hwang, R. H. DeKruyff, L. R. Frankel, and D. T. Umetsu. 2001. Human alveolar macrophages induce functional inactivation in antigen-specific CD4 T cells. *J Allergy Clin Immunol 107:258*.
5. Strickland, D. H., T. Thepen, U. R. Kees, G. Kraal, and P. G. Holt. 1993. Regulation of T-cell function in lung tissue by pulmonary alveolar macrophages. *Immunology 80:266*.

6. Holt, P. G., J. Oliver, N. Bilyk, C. McMenamin, P. G. McMenamin, G. Kraal, and T. Thepen. 1993. Downregulation of the antigen presenting cell function(s) of pulmonary dendritic cells in vivo by resident alveolar macrophages. *J Exp Med 177:397*.
7. Kawabe, T., K. I. Isobe, Y. Hasegawa, I. Nakashima, and K. Shimokata. 1992. Immunosuppressive activity induced by nitric oxide in culture supernatant of activated rat alveolar macrophages. *Immunology 76:72*.
8. Lombardo, L. J., and J. R. Balmes. 2000. Occupational asthma: A review. *Environ Health Perspect 108 Suppl 4:697*.
9. Kirchner, D. B. 2002. The spectrum of allergic disease in the chemical industry. *Int Arch Occup Environ Health 75 Suppl:S107*.
10. Zhang, X. D., P. D. Siegel, and D. M. Lewis. 2002. Immunotoxicology of organic acid anhydrides (OAAs). *Int Immunopharmacol 2:239*.
11. Redlich, C. A., and M. H. Karol. 2002. Diisocyanate asthma: clinical aspects and immunopathogenesis. *Int Immunopharmacol 2:213*.
12. Kapsenberg, M. L. 1996. Chemicals and proteins as allergens and adjuvants. *Toxicol Lett 86:79*.
13. Mapp, C. E. 2001. Agents, old and new, causing occupational asthma. *Occup Environ Med 58:354*.
14. Arts, J. H., N. Bloksma, T. Leusink-Muis, and C. F. Kuper. 2003. Respiratory allergy and pulmonary irritation to trimellitic anhydride in Brown Norway rats. *Toxicol Appl Pharmacol 187:38*.
15. Fiske, C., and Y. Subbarov. 1926. The colorimetric determination of phosphorus. *J Biol Chem. 66:375*.
16. Schaper, M., and M. A. Brost. 1991. Respiratory effects of trimellitic anhydride aerosols in mice. *Arch Toxicol 65:671*.
17. Arts, J. H., C. F. Kuper, S. M. Spoor, and N. Bloksma. 1998. Airway morphology and function of rats following dermal sensitization and respiratory challenge with low molecular weight chemicals. *Toxicol Appl Pharmacol 152:66*.
18. Ritz, H. L., B. L. Evans, R. D. Bruce, E. R. Fletcher, G. L. Fisher, and K. Sarlo. 1993. Respiratory and immunological responses of guinea pigs to enzyme- containing detergents: a comparison of intratracheal and inhalation modes of exposure. *Fundam Appl Toxicol 21:31*.
19. Holliday, M. R., J. W. Coleman, R. J. Dearman, and I. Kimber. 1993. Induction of mast cell sensitization by chemical allergens: a comparative study. *J Appl Toxicol 13:137*.
20. Vento, K. L., R. J. Dearman, I. Kimber, D. A. Basketter, and J. W. Coleman. 1996. Selectivity of IgE responses, mast cell sensitization, and cytokine expression in the immune response of Brown Norway rats to chemical allergens. *Cell Immunol 172:246*.
21. Vignola, A. M., P. Chanez, P. Paul-Lacoste, N. Paul-Eugene, P. Godard, and J. Bousquet. 1994. Phenotypic and functional modulation of normal human alveolar macrophages by histamine. *Am J Respir Cell Mol Biol 11:456*.
22. Hart, P. H. 2001. Regulation of the inflammatory response in asthma by mast cell products. *Immunol Cell Biol 79:149*.

23. Hamid, Q., M. K. Tulic, M. C. Liu, and R. Moqbel. 2003. Inflammatory cells in asthma: mechanisms and implications for therapy. *J Allergy Clin Immunol 111:S5*.

24. Lee, T. H., and S. J. Lane. 1992. The role of macrophages in the mechanisms of airway inflammation in asthma. *Am Rev Respir Dis 145:S27*.

25. Wohlsen, A., S. Uhlig, and C. Martin. 2001. Immediate allergic response in small airways. *Am J Respir Crit Care Med 163:1462*.

26. Albertine, K. H., L. Wang, S. Watanabe, G. K. Marathe, G. A. Zimmerman, and T. M. McIntyre. 2002. Temporal correlation of measurements of airway hyperresponsiveness in ovalbumin-sensitized mice. *Am J Physiol Lung Cell Mol Physiol 283:L219*.

27. Tanaka, H., T. Masuda, S. Tokuoka, M. Komai, K. Nagao, Y. Takahashi, and H. Nagai. 2001. The effect of allergen-induced airway inflammation on airway remodeling in a murine model of allergic asthma. *Inflamm Res 50:616*.

28. Pauluhn, J., M. Emura, U. Mohr, A. Popp, and M. Rosenbruch. 1999. Two-week inhalation toxicity of polymeric diphenylmethane-4, 4'-diisocyanate (PMDI) in rats: analysis of biochemical and morphological markers of early pulmonary response. *Inhal Toxicol 11:1143*.

29. Swiercz, R., K. Rydzynski, J. Jajte, J. Stetkiewicz, and W. Majcherek. 2000. Studies on dermal, ocular and respiratory effects of 4-ethyltoluene in experimental animals. *Int J Occup Med Environ Health 13:307*.

30. Lohmann-Matthes, M. L., C. Steinmuller, and G. Franke-Ullmann. 1994. Pulmonary macrophages. *Eur Respir J 7:1678*.

4

Alveolar macrophages suppress non-specific inflammation caused by inhalation challenge with trimellitic anhydride conjugated to albumin

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Abstract

Occupational exposure to low molecular weight chemicals, like trimellitic anhydride (TMA), can result in occupational asthma. Alveolar macrophages (AMs) are among the first cells to encounter these inhaled compounds and were previously shown to affect TMA-induced asthma-like symptoms in the Brown Norway (BN) rat. TMA is a hapten that will bind to endogenous proteins upon entrance of the body. Therefore, in the present study we determined if TMA conjugated to serum albumin induced the same asthma-like symptoms as free TMA.Female BN rats were sensitized by dermal application of TMA in vehicle (olive oil/acetone) or received vehicle alone on days 0 and 7. One day prior to challenge the rats were treated intratracheally with either empty liposomes or liposomes containing clodronate (dichloromethylene diphosphonate) to specifically deplete the lungs of AMs. On day 21 all groups of rats were challenged by inhalation of 0.1% TMA-BSA. Breathing frequency, tidal volume, and minute ventilation were measured before, during, within 1 h, and 24 h after challenge and the gross respiratory rate score during challenge. Total and TMA-specific IgE levels were determined in serum and lung lavage fluid and parameters of inflammation and tissue damage were assessed in lung lavage fluid and/or lung tissue 24 h after challenge. Sensitization with TMA had no effect on the lung function before challenge. TMA-BSA challenge, however, resulted in an early asthmatic response as compared to the non-sensitized rats, irrespective of AM depletion. AM depletion had no effect on the sensitization-induced serum and lung lavage fluid IgE levels. TMA-BSA inhalation did not induce airway inflammation and tissue damage 24 h after challenge, irrespective of sensitization. This, however, was induced in both groups of AM-depleted rats, indicating a suppressive role of AMs on TMA BSA-induced immunologically non-specific damage and inflammatory cell influx into the lungs.

Introduction

Exposure both to high and low molecular weight compounds at the workplace can result in the development of respiratory allergic diseases. Among the low molecular weight (LMW) compounds, trimellitic anhydride (TMA), used in the manufacture of paints, epoxy curing agents, printing inks and vinyl plasticizers, is a known cause of occupational asthma (1, 2). TMA-induced occupational asthma shares characteristics of protein allergen-induced asthma, like airway hyperreactivity to specific and nonspecific (e.g. methacholine) stimuli, and airway inflammation (3, 4). However, in contrast to protein allergen-induced asthma, asthma symptoms due LMW chemical allergens can persist for months to years after cessation of exposure (5). The mechanism underlying induction of LMW chemical-induced occupational asthma has not been fully elucidated, but the development requires sensitization by dermal or respiratory exposure to the compound, binding of the compound to endogenous molecules, processing by antigen presenting cells, and subsequent presentation to specific T cells (4, 6, 7). In susceptible individuals exposure to LMW chemicals may result in the production of specific IgE antibodies and upon renewed contact with the compound secondary immune responses can result in clinically manifest adverse health effects (1).

Alveolar macrophages (AMs) are long-lived cells belonging to the family of mononuclear phagocytes and are located at the interface between air and lung tissue where they are among the first cells to encounter inhaled chemicals and small particles. It has been demonstrated that AMs are phenotypically and functionally activated in asthma (8-10) suggesting that they are involved in the immune response of the airways to allergens. Notably, depletion of these cells in ovalbumin-sensitized rodents caused an increase in airway inflammation, specific IgE, and airway reactivity upon local ovalbumin challenge indicating a role of the AM in decreasing pulmonary immune responses to protein allergens (11-13). In a previous study using TMA-sensitized Brown Norway (BN) rats, we found that depletion of AMs prior to inhalation challenge with TMA increased the airway inflammation, but diminished the respiratory symptoms, decreased the serum IgE levels 24 h after challenge and did not affect serum IgE levels 14 days after challenge (Chapter 2 and unpublished results). Apart from the increase in airway inflammation, the latter results do not correspond with the increased immune reactions after ovalbumin challenge of AM-depleted sensitized rodents as described above (11-13). This indicates that AMs of sensitized rodents react differently to respiratory challenge with protein allergens or LMW chemical allergens. Recently, it was shown that an inhalation challenge of TMA-sensitized rodents with TMA conjugated to albumin elicited airway inflammation and bronchoconstriction (14-16), resembling the effects seen in our studies with TMA-sensitized BN rats upon challenge with TMA in air (17, Chapter 3). Since respiratory

challenge with an aerosol of TMA-protein conjugates is more similar to airway challenge with protein allergens than challenge with TMA in air, we studied the effects of AM depletion of TMA-sensitized BN rats shortly before inhalation challenge with an aerosol of TMA-BSA conjugates.

Materials and Methods

Animals

Female, inbred Brown Norway/CrlBR rats (BN; 7-8 weeks of age) were purchased from Charles River (Sulzfeld, Germany). The animals were acclimatized for at least 5 days before the start of the study. They were kept under conventional laboratory conditions and received the TNO Institute's grain-based open-formula diet and unfluoridated tap water *ad libitum*. All animal procedures were conducted in accordance with the Animal Ethics Committee of Utrecht University (Utrecht, The Netherlands).

Treatment schedule and groups

Studies were conducted according to the following scheme: day -1, blood withdrawal; days 0 and 7, dermal sensitization; day 19, blood withdrawal; day 20, assessment of basal lung function (breathing frequency and pattern) and liposome treatment; day 21, TMA-BSA challenge and assessment of lung function; day 22, assessment of lung function, blood sampling, broncho-alveolar lavage, and necropsy. The rats were divided into four groups as indicated in Table 1.

Table 1. Treatment schedule of BN rats.

	Sensitization		Liposomes	Challenge
	On flanks 300 µl	On ears 150 µl	Intratracheal 300 µl	Inhalation 0.1%; 15 min
Group designation	Day 0	Day 7	Day 20	Day 21
Veh/PBS	vehicle[1]	vehicle	PBS	TMA-BSA
Veh/clodro	vehicle	vehicle	clodronate	TMA-BSA
TMA/PBS	TMA	TMA	PBS	TMA-BSA
TMA/clodro	TMA	TMA	clodronate	TMA-BSA

[1]Vehicle was acetone/olive oil 4:1 (v/v).

Sensitization procedure

TMA (97% purity, Aldrich, Brussels, Belgium) was applied at a concentration of 50% (w/v) in a vehicle solution of 4:1 (v/v) acetone (HPLC grade; Merck, Darmstadt, Germany) and highly refined olive oil (Sigma, St. Louis, MO). Animals received 150 µl on each flank (approximately 12 cm^2 each), which had been shaved with an electrical razor at least 2-3 days earlier. Seven days after the first sensitization the animals received 75 µl of a 25% TMA solution on the dorsum of both ears. Control animals received vehicle solution.

Preparation of liposomes and depletion of alveolar macrophages

Negatively charged liposomes were made under with pyrogen-free components under aseptic conditions at the department of Pharmaceutics (Utrecht Institute for Pharmaceutical Sciences, Utrecht University, Utrecht, the Netherlands) as described earlier (Chapter 2). Briefly, a mixture of egg-phosphatidylcholine, egg-phosphatidylglycerol (both from Lipoid GmbH, Ludwigshafen, Germany) and cholesterol (Sigma) were dissolved in ethanol in a molar ratio of 10:1:1.5 and evaporated to dryness by rotation under reduced pressure. The lipid film was hydrated in phosphate-buffered saline (PBS; pH 7.4) either or not containing 60 mg/ml clodronate (dichloromethylene diphosphonate; Schering, Weesp, The Netherlands). Removal of unencapsulated clodronate was achieved by repeated washing with PBS and ultracentrifugation (Beckman Optima LE-80K, Palo Alto, CA) at 200,000 x *g* for 30 min. After the last washing step, the pellet was resuspended in PBS at a concentration of 90 mM phospholipid. The phospholipid concentration was determined according to Fiske and Subbarov as modified by King (18). The concentration of clodronate was determined spectrophotometrically at a wavelength of 238 nm after extraction and binding to Cu^{2+}. The final clodronate concentration of the liposome formulations was about 2.5 mg/ml.

Liposomes containing clodronate or PBS as a control (300 µl) were instilled intratracheally after light anesthesia using a mixture of midazolam (Roche Diagnostics, Basel, Switzerland), fluanisone, and phentanyl citrate (both from Janssen Pharmaceutica, Beerse, Belgium) (0.6 mg/kg, 1.2 mg/kg, and 40 µg/kg, respectively, i.m.).

Preparation of TMA-BSA conjugates

The TMA-BSA conjugate was prepared by dissolving 10 mg/ml of BSA (cell culture tested; Sigma) in 0.1 M sodium borate buffer (pH 9.4). Approximately 1.5 mg TMA was added per ml BSA-solution and the mixture was stirred at room temperature. After 1 h the same amount of TMA was added and the mixture was stirred for 2 h at room temperature. After centrifugation at 390 x *g* for 5

min, the supernatant was dialyzed successively against PBS and distilled water for 24 h at 4ºC. The conjugate was lyophilized and stored at 4ºC until use. The degree of substitution of the TMA-BSA was assessed by determination of remaining free amino groups by reaction with 2,4,6-trinitrobenzene sulphonic acid (Sigma) as described previously (19). The conjugate substitution ratio was approximately 40 mol TMA to 1 mol of BSA.

Atmosphere generation and analysis

A humidified compressed air driven nebulizer (Schlick, Coburg, Germany, type 970/S) was used to generate the test atmosphere from a freshly prepared solution of TMA-BSA in aqua dest. The concentration of 0.1% TMA-BSA was based on a previous study performed in the rat (data not shown). Atmospheric concentrations of TMA-BSA were determined gravimetrically by filter sampling as described before (17). The mass median aerodynamic diameter of the TMA-BSA aerosols was approximately 3 μm.

Respiratory challenge and lung function measurements

For respiratory challenge and lung function measurements, rats were treated as described before (17). Briefly, lung function was assessed using a two-chamber whole-body plethysmograph. Per group one rat at a time was placed in one of the four nose-only tubes in the experimental setup and subsequently exposed to fresh air for 20-40 min (pre-challenge period), TMA-BSA atmosphere for exactly 15 min (challenge period), and fresh air for at least 1 h (post-challenge period). Breathing parameters (breathing frequency, tidal volume, and minute ventilation) were monitored by means of recording the pressure signal before, during and after challenge. Before challenge, breathing parameters were monitored approximately 20 s each minute, starting 10 min prior to the actual challenge. During the challenge, breathing parameters were monitored continuously, whereas after challenge, they were monitored approximately 20 s during each minute for the first 10 min followed by 20 s each 5 min for 50 min. Furthermore, breathing parameters were monitored approximately 20 s each minute for 15 min 24 h before AM depletion and 24 h after TMA challenge.

To determine the degree of respiratory distress, the gross respiratory response (GRR) was assessed according to Ritz et al (20) based on the number of normal breaths between retractions (irregularly lengthened pauses) relatively to normal breathing. The minimum score according to this classification is 0.0 (no retractions) and the maximum score is 7.0 (anaphylactic shock).

Serum collection, body and lung weights and necropsy

All animals were observed daily and weighed at the start of the experiment, at weekly intervals thereafter and just prior to necropsy. Individual serum samples were prepared from blood withdrawn 1 day before the start of the experiment and at day 19 via orbital puncture under light anesthesia. At day 22 animals received a lethal dose of sodium pentobarbitone (0.6 g/kg, i.p.; Cevasante Animale B.V., Maassluis, the Netherlands) and blood was withdrawn via cardiac puncture. Serum samples were stored at –20ºC until use. Animals were examined grossly for abnormalities. The trachea and lungs were excised *en bloc* and lungs were weighed.

Bronchoalveolar lavage

After lung weight determination the bronchus of the left lung was ligated. The right lung was lavaged, once with 2.5 ml of warmed PBS (37ºC) containing protease blockers (Roche Diagnostics) and then 3 times with 2.5 ml warm PBS keeping the recollected lavage fluid in tubes on ice. The broncho-alveolar cells were isolated by centrifugation for 10 min at 4ºC and pooled to determine total cell numbers using a Bürker Türk chamber. For differential cell counts, cytospin preparations were made and stained with Diff-Quick (Merz and Dade, Düdingen, Switzerland). The supernatant of the first 2.5 ml lavage fluid was concentrated 10 times using concentration tubes (10,000 mwco; Vivascience AG, Hanover, Germany). The lavage fluid concentrate was analyzed for lactate dehydrogenase (LDH), a marker for general toxicity, β-N-acetyl-glucosaminidase (β-NAG), a lysosomal enzyme released from activated or lysed phagocytes, and total protein using an automatic analyzer (Hitachi 911, Hitachi Instruments Division, Japan), and for total and TMA-specific IgE, and cytokines as described below.

IgE and cytokine levels in serum and lung lavage fluid

Total and TMA-specific IgE were measured in serum and in lung lavage fluid by ELISA. In short, the total IgE determination was performed using 96-wells flat-bottomed plates (NUNC A/S, Roskilde, Denmark) coated with 2 µg/ml mouse anti-rat IgE (BD Biosciences Pharmingen, San Diego, CA) in PBS. Duplicate wells were then incubated with graded dilutions of test samples and a monoclonal rat-IgE standard (Serotec), and subsequently with a 1:800 dilution of biotin-conjugated mouse anti-rat IgE (Serotec), followed by a 1:200 dilution of streptavidin-HRP (R & D Systems Inc., Minneapolis, MN). After addition of the substrate OPD (o-Phenylenediamine dihydrochloride (Sigma) and hydrogen peroxide) the reaction was terminated by addition of 4 M H_2SO_4. The absorbance was measured at 490 nm using a Microplate reader (Bio-Rad Laboratories, Hercules, CA). The

concentration of IgE in the samples was calculated using a standard curve obtained with known quantities of monoclonal rat IgE and expressed as μg/ml serum. The TMA-specific IgE ELISA was performed using 96-wells flat-bottomed plates coated with TMA-BSA in 0.1 M carbonate buffer (pH 9.6), followed by a blocking step with 1% BSA. Duplicate wells were then incubated with graded dilutions of test samples and serum which had been scored positive for TMA-specific IgE as a standard, and subsequently with a 1:2000 dilution of a HRP-conjugated mouse anti-rat IgE (Serotec). Addition of the substrate and further steps were as described above. The amount of TMA-specific IgE in the samples was expressed as percentage (%) of the O.D. of the standard.

TNF-α and IL-6 levels in the lavage fluid and serum were measured in duplicate using commercially available kits (R & D Systems) with a detection limit of 10 pg/ml for IL-6 and 15 pg/ml for TNF-α. A microplate reader was used to measure optical density at 450 nm.

Data analysis

Cell numbers in the lung lavage fluid were statistically analyzed using the Mann-Whitney *U* test. Lung function parameters, lung weights, IgE concentrations, biochemical parameters, and TNF-α and IL-6 levels were determined by one-way ANOVA followed by a Bonferroni multiple-comparison test. Differences were considered statistically significant if $p<0.05$. Analyses were performed by the usage of Graphpad Prism (version 3.0, San Diego, U.S.A.).

Results

Body and lung weights

TMA-BSA challenge did not cause any changes in body weights and lung weights of the non-sensitized rats and the TMA-sensitized rats, irrespective of AM depletion (data not shown).

Effect of AM depletion on respiratory changes due to TMA-BSA challenge

Breathing frequencies were comparable for all groups prior to liposome treatment (Fig. 1a). The breathing frequency of sham-depleted non-sensitized rats (veh/PBS) did not change at any time point measured but tended to decrease during and within 1 h after the challenge. Compared to the non-sensitized sham-depleted rats (veh/PBS), the breathing frequency of non-sensitized AM-depleted rats (veh/clodro) was increased before, during and within 1 h after challenge but was comparable to sham-depleted rats (veh/PBS) 24 h after challenge. Compared to the non-sensitized rats (veh/PBS

and veh/clodro), the breathing frequencies of sensitized rats (TMA/PBS and TMA/clodro) significantly decreased during the first 7 min of the challenge but were normal immediately thereafter, irrespective of AM depletion.

The tidal volumes were comparable for all groups before liposome treatment and did not change in the non-sensitized rats (veh/PBS and veh/clodro) at any time point measured prior, during, or after challenge, irrespective of AM depletion (Fig. 1b). Compared to the non-sensitized rats, the

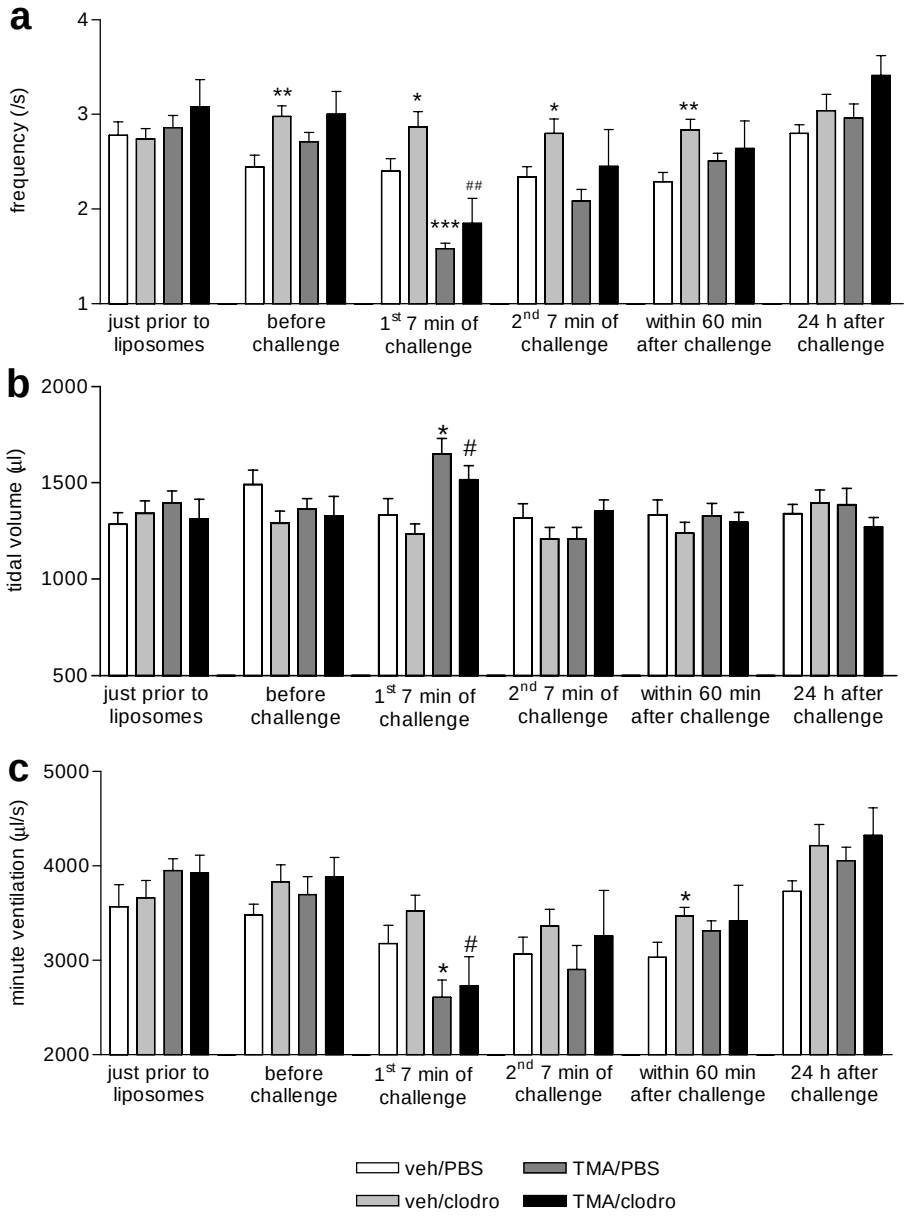

Figure 1. Effect of AM depletion on TMA-BSA inhalation induced changes in lung function. Groups of 6 rats received 150 µl vehicle or 50% TMA in vehicle on each shaved flank on day 0 and 75 µl vehicle or 25% TMA on the dorsum of both ears on day 7. On day 20 the animals received an intratracheal instillation of either empty or clodronate-liposomes followed 24 h later by an inhalation challenge with 0.1 % TMA-BSA for 15 min. Frequency (**a**), tidal volume (**b**) and minute ventilation (**c**) were measured prior to liposome treatment, before, during, directly after, and 24 h after challenge. Results are expressed as mean ± SEM. Significant differences are denoted by *: $p<0.05$, **: $p<0.01$ or *** $p< 0.001$ compared to veh/PBS and #: $p<0.05$, or ##: $p<0.01$ compared to veh/clodro.

tidal volumes of the sensitized rats (TMA/PBS and TMA/clodro) were normal just before challenge, significantly increased during the first 7 min of the challenge but were normal again during the second 7 min of the challenge, within 1 h after, and 24 h after the challenge, irrespective of AM depletion.

The minute ventilation, a function of both breathing frequency and tidal volume, was comparable for all groups before liposome treatment (Fig. 1c). The minute ventilation of sham-depleted non-sensitized rats (veh/PBS) did not change at any time point measured but tended to decrease during and directly after the challenge. The minute ventilation of AM-depleted non-sensitized rats (veh/clodro) did not differ from the sham-depleted non-sensitized rats (veh/PBS) except for an increase directly after challenge. Compared to the non-sensitized rats (veh/PBS and veh/clodro), the minute ventilation of sensitized rats (TMA/PBS and TMA/clodro) significantly decreased during the first 7 min of the challenge but returned to the levels of non-sensitized rats during the second 7 min of the challenge and stayed at the levels of non-sensitized rats the rest of the measurements, irrespective of AM depletion.

The non-sensitized groups (veh/PBS and veh/clodro) did not show any signs of respiratory distress during TMA-BSA challenge and therefore were assigned a GRR-score of 0. The sensitized groups TMA/PBS and TMA/clodro had a GRR score of 4.0 ± 0.0 and 4.0 ± 0.3, respectively, corresponding to a mean of 6-15 respirations between each pause (20), indicating moderate respiratory distress, irrespective of AM depletion.

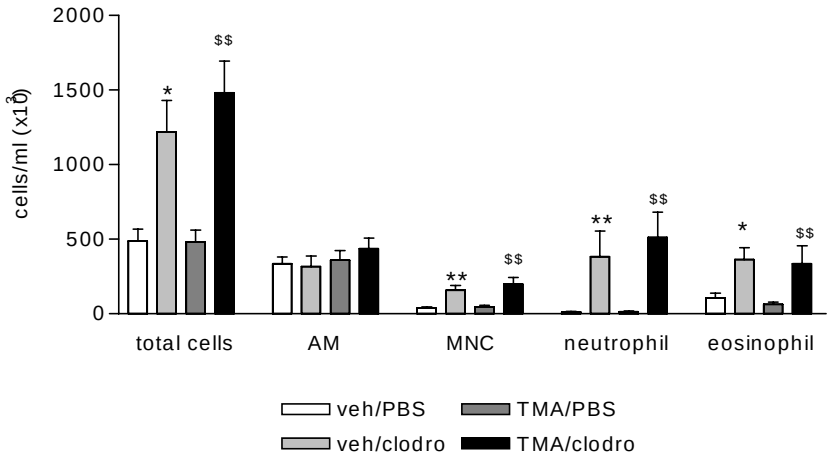

Figure 2. Effect of AM depletion prior to TMA-BSA inhalation on the composition of lung lavage cells 24 h after challenge. Groups of 6 rats received 150 µl vehicle or 50% TMA in vehicle on each shaved flank on day 0 and 75 µl vehicle or 25% TMA on the dorsum of both ears on day 7. On day 20 the animals received an intratracheal instillation of either empty or clodronate-liposomes followed 24 h later by an inhalation challenge with 0.1 % TMA-BSA for 15 min. Lungs were lavaged 24 h later. Cells were counted and differentiated into macrophages, MNC (lymphocytes and monocytes), neutrophils and eosinophils using Diff-Quick. Results are expressed as mean ± SEM. Significant differences are denoted by *: $p<0.05$, or **: $p<0.01$ compared to veh/PBS and $$: $p<0.01$ compared to TMA/PBS.

Effect of AM depletion on airway inflammation due to TMA-BSA inhalation

Lung lavage fluid of non-sensitized rats (veh/PBS) contained 488 ± 80 x 10^3 cells/ml, the majority being AMs and eosinophils, 24 h after TMA-BSA challenge (Fig. 2). AM depletion of non-sensitized rats (veh/clodro) prior to TMA-BSA challenge significantly increased the total cell number (1219 ± 212 x 10^3 cells/ml) mainly because of an increase in lymphocytes and monocytes (MNC), neutrophils, and eosinophils. Compared to the non-sensitized rats (veh/PBS), challenge of sensitized sham-depleted rats (TMA/PBS) led to similar total and differential numbers (482 ± 80 x 10^3 cells/ml). Likewise, AM depletion of sensitized rats (TMA/clodro) prior to challenge led to similar increases in total (1483 ± 211 x 10^3 cells/ml) and differential cell number as observed in non-sensitized AM-depleted rats (veh/clodro).

The inflammation around the terminal bronchioli was comparable for both groups of non-sensitized rats (veh/PBS and veh/clodro) (Table 2). Moderate numbers of inflammatory cells surrounded most of the terminal bronchioli. Compared to the non-sensitized rats (veh/PBS and veh/clodro) the inflammation around the terminal bronchioli was not significantly different in the TMA-sensitized rats (TMA/PBS and TMA/clodro), irrespective of AM depletion. In all groups, the inflammatory cells around the terminal bronchioli consisted mainly of eosinophils and some macrophages.

Table 2. Severity of inflammation around the terminal bronchioli 24 h after inhalation challenge with 0.1% TMA-BSA.

	Severity of inflammation			
Treatment	No	Slight	Moderate	Severe
Veh/PBS	0%	27 ± 4%	57 ± 4%	16 ± 5%
Veh/clodro	0%	19 ± 2%	64 ± 2%	17 ± 4%
TMA/PBS	0%	12 ± 4%	65 ± 3%	23 ± 5%
TMA/clodro	0%	12 ± 3%	56 ± 4%	33 ± 6%

Groups of 6 rats received 150 µl vehicle or 50% TMA in vehicle on each shaved flank on day 0 and 75 µl vehicle or 25% TMA on the dorsum of both ears on day 7. On day 20 the animals received an intratracheal instillation of either empty or clodronate-containing liposomes followed 24 h later by an inhalation challenge with 0.1% TMA-BSA for 15 min. On day 22 the left lungs were excised and inflated with Tissue Tec/PBS (1:1) to prepare HE stained sections for assessment of inflammation. The peribronchiolar inflammation of all terminal bronchioli per lung was scored; no: 0 cells; slight: ≤ 5 cells; moderate: ≤ 15 cells; severe: > 15 cells. Results are expressed as percentage of the total of terminal bronchioli per lung per group.

Effect of AM depletion on IgE levels

Pretreatment serum levels of total IgE were below the baseline level in all groups (data not shown). Total IgE and TMA-specific IgE levels remained low one day prior to liposome treatment (day 19) and one day after TMA-BSA challenge (day 22) in non-sensitized rats (veh/PBS and veh/clodro), irrespective of AM depletion. Compared to the non-sensitized controls (veh/PBS and veh/clodro), total IgE and TMA-specific IgE levels were significantly increased in the TMA-sensitized rats (TMA/PBS and TMA/clodro) one day prior to liposome treatment (day 19) and one day after TMA-BSA challenge (day 22), irrespective of AM depletion. However, inhalation challenge of TMA-sensitized rats (TMA/PBS and TMA/clodro) with TMA-BSA resulted in significantly lower TMA-specific IgE levels (day 22) compared to the levels measured at day 19 and significantly lower levels of total IgE in AM-depleted sensitized rats (TMA/clodro) (Fig. 3a and b).

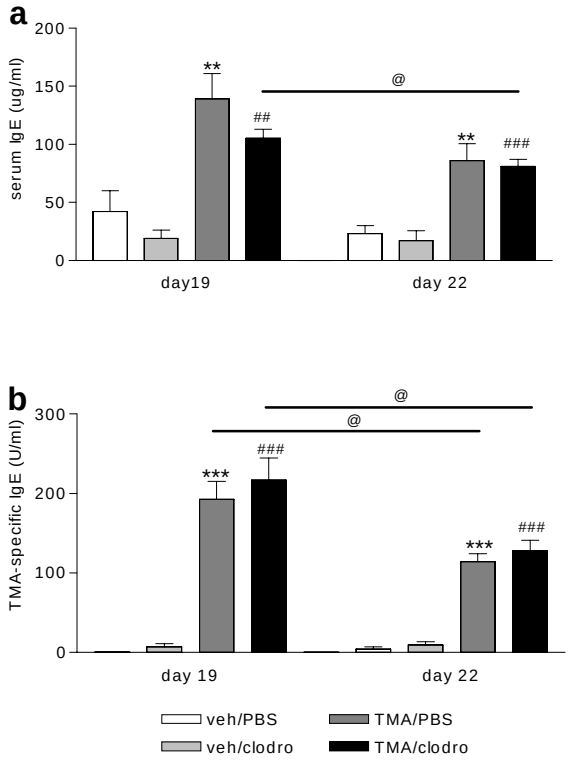

Figure 3. Effect of AM depletion prior to TMA-BSA inhalation on serum IgE levels. Groups of 6 rats received 150 µl vehicle or 50% TMA in vehicle on each shaved flank on day 0 and 75 µl vehicle or 25% TMA on the dorsum of both ears on day 7. On day 19 serum samples were drawn. On day 20 the animals received an intratracheal instillation of either empty or clodronate-liposomes followed 24 h later by an inhalation challenge with 0.1% TMA-BSA for 15 min. Serum was collected 24 h after challenge and levels of total (**a**) and TMA-specific IgE (**b**) were assessed. Results are expressed as mean ± SEM. Significant differences are denoted by *: $p<0.05$, **: $p<0.01$, or ***: $p< 0.001$ compared to veh/PBS, ##: $p<0.01$, or ###: $p< 0.001$ compared to veh/clodro, and @: $p<0.05$ compared to the levels at day 19.

Total and TMA-specific IgE levels in the lung lavage fluid of non-sensitized rats (veh/PBS and veh/clodro) were at baseline level. The levels were significantly higher in the TMA-BSA-challenged rats (TMA/PBS and TMA/clodro) as compared to the non-sensitized rats. AM depletion, however, had no effect on the IgE levels in the lung lavage fluid (Fig. 4a and b).

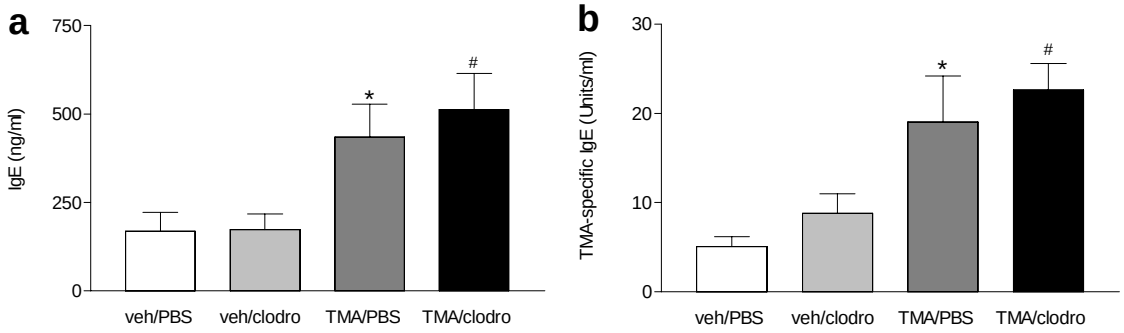

Figure 4. Effect of AM depletion prior to TMA-BSA inhalation on IgE levels in the lung lavage fluid 24 h after challenge. Groups of 6 rats received 150 µl vehicle or 50% TMA in vehicle on each shaved flank on day 0 and 75 µl vehicle or 25% TMA on the dorsum of both ears on day 7. On day 20 the animals received an intratracheal instillation of either empty or clodronate-liposomes followed 24 h later by an inhalation challenge with 0.1 % TMA-BSA for 15 min. Lungs were lavaged 24 h after challenge and levels of total (**a**) and TMA-specific (**b**) IgE were assessed. Results are expressed as mean ± SEM. Significant differences are denoted by *: $p<0.05$ compared to veh/PBS, and #: $p<0.05$ compared to veh/clodro.

Effect of AM depletion on the production of cytokines following TMA-BSA inhalation

TMA sensitization, with or without AM depletion, did not significantly change IL-6 serum levels 24 h after respiratory TMA-BSA challenge as compared to controls (Fig 5a). Serum TNF-α levels were low in non-sensitized rats, whether or not depleted of AMs, 24 h after TMA-BSA challenge (Fig 5b). Significantly increased serum TNF-α levels were found in TMA-BSA-challenged sensitized rats, but AM depletion completely prevented the increase.

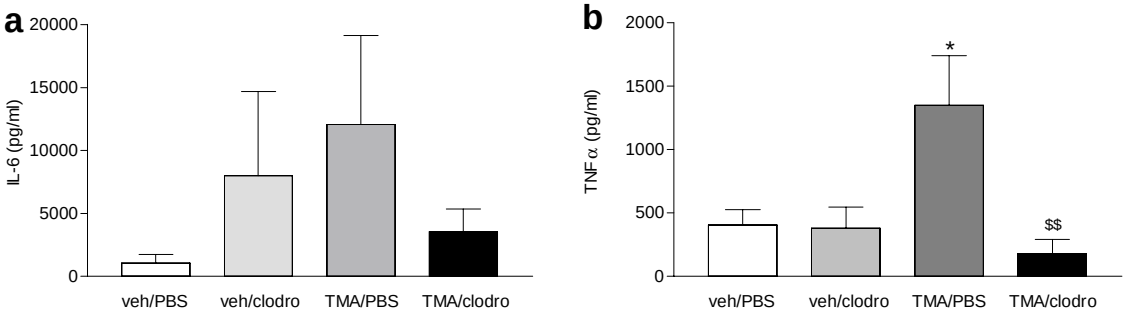

Figure 5. Effect of AM depletion 24 h before TMA-BSA inhalation on serum IL-6 and TNF-α levels 24 h after challenge. Groups of 6 rats received 150 µl vehicle or 50% TMA in vehicle on each shaved flank on day 0 and 75 µl vehicle or 25% TMA on the dorsum of both ears on day 7. On day 20 the animals received an intratracheal instillation of either empty or clodronate-liposomes followed 24 h later by an inhalation challenge with 0.1% TMA-BSA for 15 min. Serum was collected 24 h after challenge and IL-6 (**a**) and TNF-α (**b**) levels were assessed. Results are expressed as mean ± SEM. Significant differences are denoted by *: $p< 0.05$ compared to veh/PBS, and $: $p< 0.05$ compared to TMA/PBS.

Levels of IL-6 in lung homogenates were comparable for both non-sensitized groups (veh/PBS and veh/clodro) 24 h after TMA-BSA challenge, although the levels of the AM-depleted rats (veh/clodro) tended to be lower (Fig. 6a). Significantly decreased IL-6 levels were found in sensitized rats, irrespective of AM depletion, 24 h after TMA-BSA challenge. Lung homogenate TNF-α levels were decreased in non-sensitized AM-depleted rats (veh/clodro) and sensitized rats, with or without AMs (TMA/PBS and TMA/clodro), compared to non-sensitized sham-depleted controls (veh/PBS) 24 h after challenge (Fig 6b).

IL-6 and the TNF-α levels in the lung lavage fluid 24 h after TMA-BSA challenge of all groups were below the detection limit of the assays used.

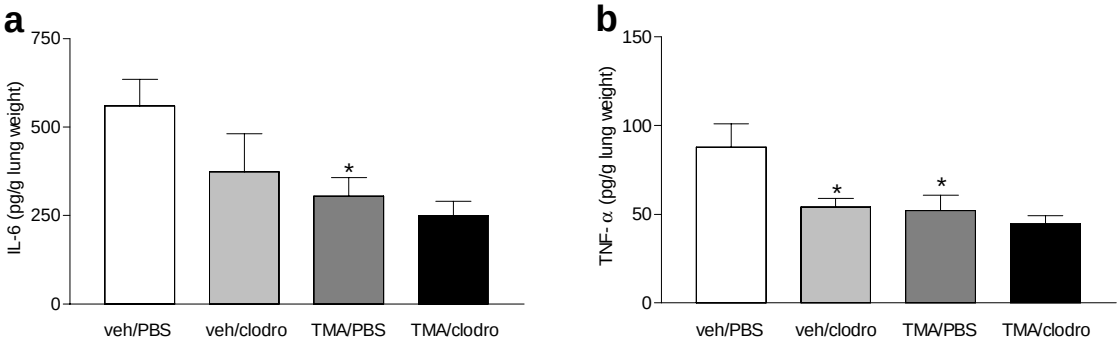

Figure 6. Effect of AM depletion 24 h before TMA-BSA inhalation on IL-6 and TNF-α levels in lung homogenates 24 h after challenge. Groups of 6 rats received 150 µl vehicle or 50% TMA in vehicle on each shaved flank on day 0 and 75 µl vehicle or 25% TMA on the dorsum of both ears on day 7. On day 20 the animals received an intratracheal instillation of either empty or clodronate-liposomes followed 24 h later by an inhalation challenge with 0.1% TMA-BSA for 15 min. Lung homogenates were made 24 h after challenge and IL-6 (**a**) and TNF-α (**b**) levels were assessed. Results are expressed as mean ± SEM. Significant differences are denoted by *: $p < 0.05$ compared to veh/PBS.

Effect of AM depletion on damage markers following TMA-BSA challenge

TMA-BSA challenge resulted in low levels of LDH in lung lavage fluids of all groups 24 h after challenge, except those of the non-sensitized AM-depleted rats (veh/clodro) that contained significantly increased levels (Fig 7a). Challenge with TMA-BSA induced low levels of β-NAG and total protein in lung lavage fluids of sham-depleted rats (veh/PBS and TMA/PBS) 24 h after challenge, but significantly increased these levels in lung lavage fluids of AM-depleted rats, irrespective of sensitization (veh/clodro and TMA/clodro) (Fig 7 b, c).

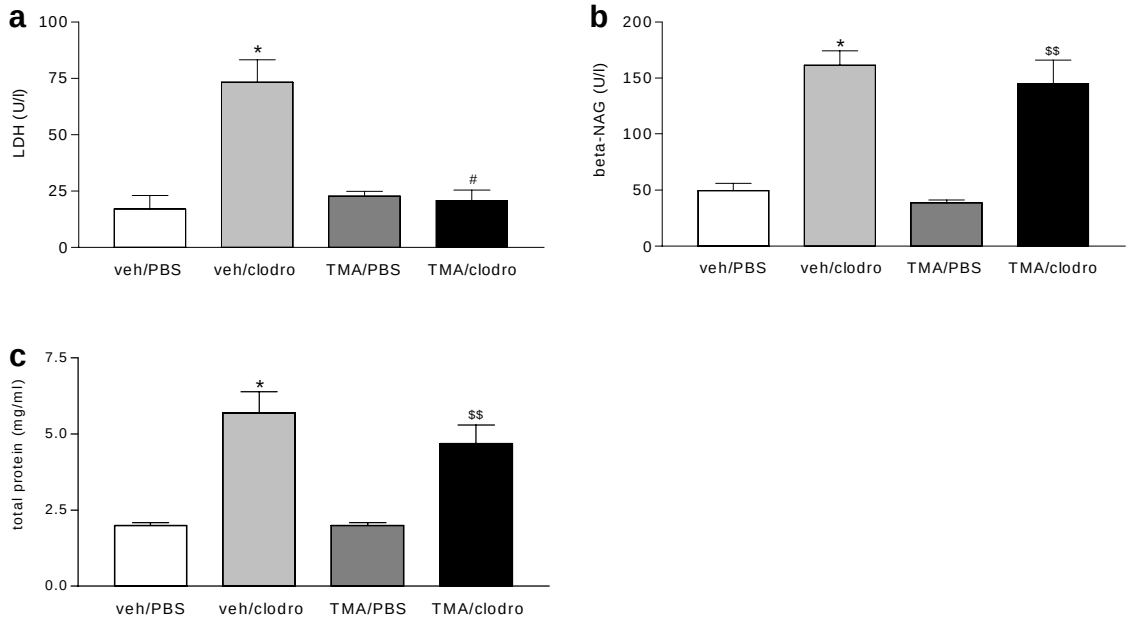

Figure 7. Effect of AM depletion 24 h before TMA-BSA challenge on LDH, β-NAG and total protein in the lung lavage fluid 24 h after challenge. Groups of 6 rats received 150 µl vehicle or 50% TMA in vehicle on each shaved flank on day 0 and 75 µl vehicle or 25% TMA on the dorsum of both ears on day 7. On day 20 the animals received an intratracheal instillation of either empty or clodronate-liposomes followed 24 h later by an inhalation challenge with 0.1% TMA-BSA for 15 min. The lungs were lavaged 24 h after TMA challenge and LDH (**a**), β-NAG (**b**), and total protein (**c**) were measured. Results are expressed as mean ± SEM. Significant differences are denoted by *: $p< 0.05$ compared to veh/PBS, #: $p<0.05$ and ##: $p< 0.01$ compared to veh/clodro and $: $p< 0.05$ and $$: $p<0.01$ compared to TMA/PBS.

Discussion

In this study the role of the AM in a rat model for TMA-induced occupational asthma was investigated. Since LMW allergens are known to conjugate to endogenous proteins upon entrance of the body (21), TMA conjugated to BSA was used as the challenge agent. It was found that TMA sensitization increased serum IgE levels and that subsequent respiratory challenge with TMA-BSA induced immediate changes in lung function reflecting an early asthmatic reaction. Decreased lung tissue levels of TNF-α and IL-6, and increased serum TNF-α levels were measured 24 h later (Table 3). TMA-BSA inhalation by sensitized rats, however, did not induce airway inflammation or increase relative lung weights. The inability of the TMA-BSA aerosol to induce airway inflammation was surprising, since inhalation challenge of similarly sensitized rats with 30 mg/m^3 of free TMA induced prominent airway inflammation (Chapter 3)(22). The absence of pulmonary inflammation might be related to the smaller amount of TMA in the TMA-BSA aerosol than in atmospheres of TMA in air. This is indicated by concentration-response studies with free TMA showing that 2 mg/m^3 of TMA

Table 3. Summary of the influence of AM depletion of non-sensitized and TMA-sensitized BN rats on the diverse parameters measured during, within 1 h, and 24 h after TMA-BSA challenge

	TMA-BSA challenge								
	Sham-depletion			AM-depletion					
	TMA-sensitized (TMA/PBS) *			Non-sensitized (veh/clodro) *			TMA-sensitized (TMA/clodro) #		
Parameter[1]	during	t = 1h	t = 24h	during	t = 1h	t = 24h	during	t = 1h	t = 24h
Frequency	↓	=	=	↑	↑	=	=	=	=
Tidal volume	↑	=	=	=	=	=	=	=	=
Minute ventilation	↓	=	=	=	↑	=	=	=	=
GRR score	↑			=			=		
Serum IgE			↑			=			=
BALF IgE			↑			=			=
BALF total leukocytes			=			↑			↑
Peribronch. leukocytes			=			=			=
Lung weight			=			=			=
Serum IL-6			=			=			=
Tissue IL-6			↓			=			=
Serum TNF-α			↑			=			↓
Tissue TNF-α			↓			↓			=
LDH			=			↑			=
β-NAG			=			↑			↑
Total protein			=			↑			↑

* Results compared to those of non-sensitized, sham-depleted rats
# Results compared to those of TMA-sensitized sham-depleted rats
[1] Abbreviations used: BALF: lung lavage fluid, peribronch.: peribronchiolar

in air was sufficient to elicit an early asthmatic response in sensitized BN rats, but failed to induce pulmonary inflammation and to increase relative lung weights, while 12 mg/m^3 induced both (23).

Depletion of AMs in TMA-sensitized rats prior to respiratory challenge with TMA-BSA aerosol did not change the early asthmatic response, suggesting that AMs are not at play in this response. By contrast, the early asthmatic response during challenge with TMA in air was previously shown to be mitigated by prior AM depletion of similarly sensitized BN rats (Chapter 3). This difference in responses in the absence of AMs is hard to explain, especially because TMA-BSA, but not free TMA, was found to induce the production of proinflammatory cytokines and NO by AMs derived from BN rats *in vitro* (Chapter 5). The observation that non-sensitized AM-depleted rats had higher breathing frequencies before and during TMA-BSA challenge compared to the non-sensitized sham-depleted rats seems to be an effect of the clodronate-liposome treatment. However,

no significant differences were measured between the breathing frequencies of the non-sensitized AM-depleted rats at different time points, which was also observed for the non-sensitized sham-depleted rats.

While inhalation challenge with TMA-BSA did not induce a local inflammatory response in sensitized BN rats, it did so in AM-depleted rats, irrespective of sensitization. Lung lavage fluid of the AM-depleted rats contained significantly increased numbers of neutrophils, eosinophils, and mononuclear cells. Apparently, TMA-BSA conjugates are potent inducers of immunologically non-specific pulmonary inflammation that is vigorously prevented in the presence of AMs. The underlying mechanisms are not known, but are not related to AM depletion as such, since depletion induced according to the same protocol as used in the present study did not evoke airway inflammation in BN rats (Chapter 2). The inflammation, therefore, is probably the result of a combined effect of AM depletion and TMA-BSA inhalation, and may be related to irritation and cell damage. The latter is indicated by the observation that the inflammation was paralleled by increases in the cytotoxicity markers, β-NAG, proteins, and, except for the sensitized AM-depleted group (TMA/clodro), LDH in the lung lavage fluid of AM-depleted rats. Thus, it is not unlikely that AM depletion makes the airways more vulnerable to the immunologically non-specific inflammatory and/or toxic effects of TMA-BSA. The reason why LDH levels were not increased in the sensitized AM-depleted rats is not known. However, it may involve inactivation of TMA-BSA by specific antibodies that were induced by sensitization thereby specifically decreasing the induction of LDH. The nature of the non-specific inflammatory effects of TMA-BSA in AM-depleted rats is not known. The inflammation may be the result of cytotoxic effects of the conjugate on lung cells in the absence of AMs. Another, not mutually exclusive, explanation may be that TMA-BSA induced cytokine production by lung cells, other than AMs. This is not unlikely, because TMA-BSA, like LPS from gram-negative bacteria, was found to induce TNF-α production by freshly isolated AMs of BN rats *in vitro* (Chapter 5). Other cell types may react similarly, but this explanation in its turn does not clarify why TMA-BSA did not induce inflammation in AM-replete BN rats. The observation that TNF-α and IL-6 levels were absent in the lung lavage fluid and that their levels were decreased in lung tissue of all treatment groups compared to controls (veh/PBS) 24 h after TMA-BSA challenge suggests that the time of measurement was too late to detect increased levels of these cytokines, particularly because of their short half-life (24).

The fact that TMA-BSA and TMA only partly induced the same effects in our rat model for occupational asthma bears relevance to toxicological hazard identification. Protein conjugates are frequently used, because respiratory challenge with the free, usually highly reactive, LMW chemicals requires specific and expensive equipment and many safety precautions. However, in order to make

the right assumptions of the effects of LMW chemicals on the airways, challenge with the free compound is to be preferred.

In conclusion, an early asthmatic response to TMA-BSA is induced in the absence of airway inflammation and depletion of AMs before challenge had no influence on the asthmatic reaction. AM depletion, however, resulted in immunologically non-specific airway inflammation and damage 24 h after challenge, indicating that AMs suppress non-specific inflammation caused by TMA-BSA challenge.

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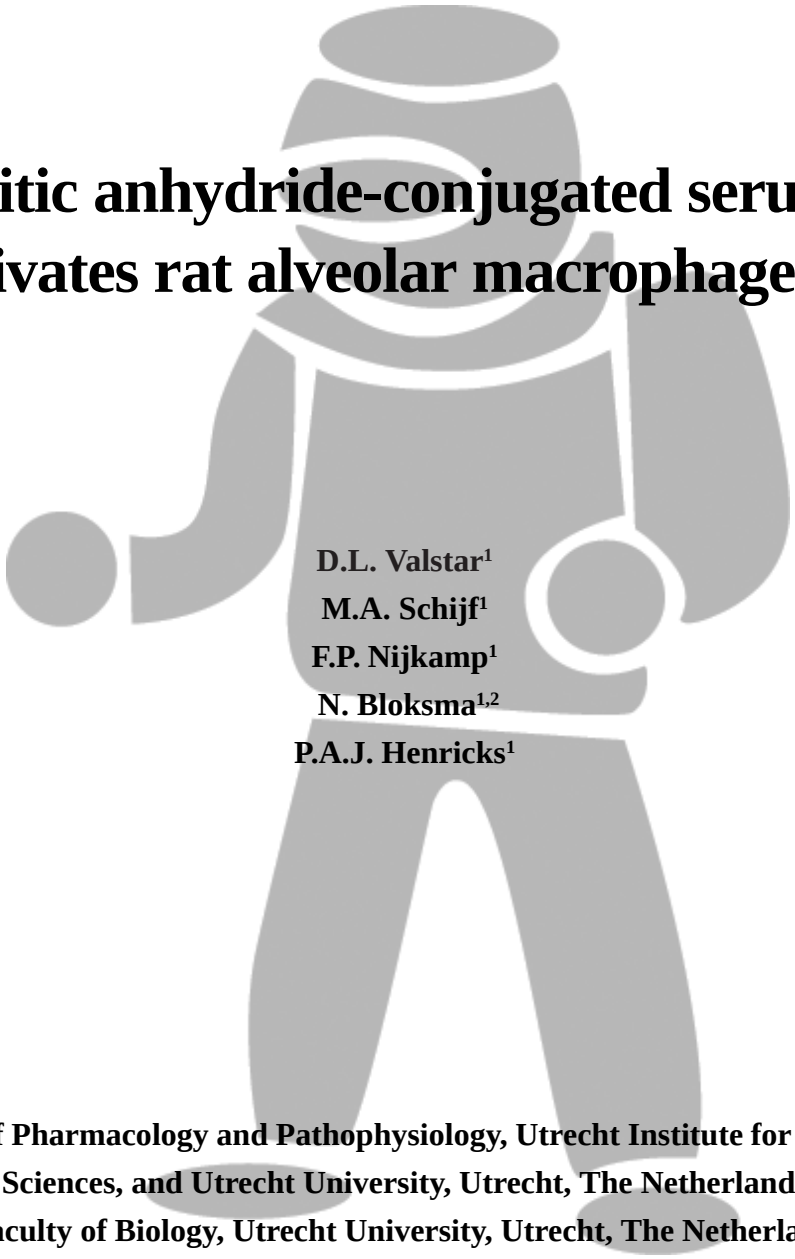
References

1. Lombardo, L. J., and J. R. Balmes. 2000. Occupational asthma: A review. *Environ Health Perspect 108 Suppl 4:697*.
2. Kirchner, D. B. 2002. The spectrum of allergic disease in the chemical industry. *Int Arch Occup Environ Health 75 Suppl:S107*.
3. Hamid, Q., M. K. Tulic, M. C. Liu, and R. Moqbel. 2003. Inflammatory cells in asthma: mechanisms and implications for therapy. *J Allergy Clin Immunol 111:S5*.
4. Mapp, C. E., M. Saetta, P. Maestrelli, A. Di Stefano, P. Chitano, P. Boschetto, A. Ciaccia, and L. M. Fabbri. 1994. Mechanisms and pathology of occupational asthma. *Eur Respir J 7:544*.
5. Vandenplas, O., K. Toren, and P. D. Blanc. 2003. Health and socioeconomic impact of work-related asthma. *Eur Respir J 22:689*.
6. Zhang, X. D., P. D. Siegel, and D. M. Lewis. 2002. Immunotoxicology of organic acid anhydrides (OAAs). *Int Immunopharmacol 2:239*.
7. Redlich, C. A., and M. H. Karol. 2002. Diisocyanate asthma: clinical aspects and immunopathogenesis. *Int Immunopharmacol 2:213*.
8. Lane, S. J., A. R. Sousa, and T. H. Lee. 1994. The role of the macrophage in asthma. *Allergy 49:201*.
9. Lensmar, C., J. Prieto, B. Dahlen, A. Eklund, J. Grunewald, and A. Roquet. 1999. Airway inflammation and altered alveolar macrophage phenotype pattern after repeated low-dose allergen exposure of atopic asthmatic subjects. *Clin Exp Allergy 29:1632*.

10. Viksman, M. Y., B. S. Bochner, R. S. Peebles, R. P. Schleimer, and M. C. Liu. 2002. Expression of activation markers on alveolar macrophages in allergic asthmatics after endobronchial or whole-lung allergen challenge. *Clin Immunol 104:77*.
11. Thepen, T., C. McMenamin, J. Oliver, G. Kraal, and P. G. Holt. 1991. Regulation of immune response to inhaled antigen by alveolar macrophages: differential effects of in vivo alveolar macrophage elimination on the induction of tolerance vs. immunity. *Eur J Immunol 21:2845*.
12. Thepen, T., C. McMenamin, B. Girn, G. Kraal, and P. G. Holt. 1992. Regulation of IgE production in pre-sensitized animals: in vivo elimination of alveolar macrophages preferentially increases IgE responses to inhaled allergen. *Clin Exp Allergy 22:1107*.
13. Tang, C., M. D. Inman, N. Van Rooijen, P. Yang, H. Shen, K. Matsumoto, and P. M. O'Byrne. 2001. Th type 1-stimulating activity of lung macrophages inhibits Th2- mediated allergic airway inflammation by an IFN-gamma-dependent mechanism. *J Immunol 166:1471*.
14. Cui, Z. H., M. Sjostrand, T. Pullerits, P. Andius, B. E. Skoogh, and J. Lotvall. 1997. Bronchial hyperresponsiveness, epithelial damage, and airway eosinophilia after single and repeated allergen exposure in a rat model of anhydride-induced asthma. *Allergy 52:739*.
15. Regal, J. F., M. E. Mohrman, and D. M. Sailstad. 2001. Trimellitic anhydride-induced eosinophilia in a mouse model of occupational asthma. *Toxicol Appl Pharmacol 175:234*.
16. Larsen, C. P., and J. F. Regal. 2002. Trimellitic anhydride (TMA) dust induces airway obstruction and eosinophilia in non-sensitized guinea pigs. *Toxicology 178:89*.
17. Arts, J. H., C. F. Kuper, S. M. Spoor, and N. Bloksma. 1998. Airway morphology and function of rats following dermal sensitization and respiratory challenge with low molecular weight chemicals. *Toxicol Appl Pharmacol 152:66*.
18. Fiske, C., and Y. Subbarov. 1926. The colorimetric determination of phosphorus. *J Biol Chem. 66:375*.
19. Sashidhar, R. B., A. K. Capoor, and D. Ramana. 1994. Quantitation of epsilon-amino group using amino acids as reference standards by trinitrobenzene sulfonic acid. A simple spectrophotometric method for the estimation of hapten to carrier protein ratio. *J Immunol Methods 167:121*.
20. Ritz, H. L., B. L. Evans, R. D. Bruce, E. R. Fletcher, G. L. Fisher, and K. Sarlo. 1993. Respiratory and immunological responses of guinea pigs to enzyme- containing detergents: a comparison of intratracheal and inhalation modes of exposure. *Fundam Appl Toxicol 21:31*.
21. Karol, M. H., C. Graham, R. Gealy, O. T. Macina, N. Sussman, and H. S. Rosenkranz. 1996. Structure-activity relationships and computer-assisted analysis of respiratory sensitization potential. *Toxicol Lett 86:187*.
22. Arts, J. H., N. Bloksma, T. Leusink-Muis, and C. F. Kuper. 2003. Respiratory allergy and pulmonary irritation to trimellitic anhydride in Brown Norway rats. *Toxicol Appl Pharmacol 187:38*.
23. Arts, J. H., M. W. de Koning, N. Bloksma, and C. F. Kuper. 2004. Respiratory allergy to trimellitic anhydride in rats: concentration-response relationships during elicitation. *Inhal Toxicol 16:259*.
24. Beutler, B. A., I. W. Milsark, and A. Cerami. 1985. Cachectin/tumor necrosis factor: production, distribution, and metabolic fate in vivo. *J Immunol 135:3972*.

5

Trimellitic anhydride-conjugated serum albumin activates rat alveolar macrophages *in vitro*



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Abstract

Occupational exposure to airborne low molecular weight chemicals, like trimellitic anhydride (TMA), can result in occupational asthma. Alveolar macrophages (AMs) are among the first cells to encounter these inhaled compounds and were previously shown to affect TMA-induced asthma-like symptoms in the BN rat. TMA is a hapten that will bind to endogenous proteins upon entrance of the body. Therefore, in the present study we determined if TMA and TMA conjugated to serum albumin induced the production of the macrophage mediators nitric oxide (NO), tumor necrosis factor-alpha (TNF-α), and interleukin 6 (IL-6) *in vitro* using the rat AM cell line NR8383 and primary AMs derived from TMA-sensitized and control BN rats. Cells were incubated with different concentrations of TMA, TMA conjugated to bovine serum albumin (BSA), and BSA as a control for 24 h and the culture supernatant was analyzed for mediator content. TMA alone was not able to induce the production of mediators by NR8383 cells and primary AMs from sham-sensitized and control rats. TMA-BSA, on the contrary, dose-dependently stimulated the production of NO, TNF-α, and IL-6 by NR8383 cells and of NO and TNF-α, but not IL-6, by primary AMs independent of sensitization. These results suggest that although TMA is a highly reactive compound, conjugation to a suitable protein is necessary to induce mediator production by AMs, since TMA conjugated to BSA very potently stimulated AMs. Furthermore, the effects of TMA-BSA were not sensitization-dependent which is suggestive of the involvement of an immunologically non-specific receptor. In the discussion it is argued that a macrophage scavenger receptor is a likely candidate.

Introduction

Trimellitic anhydride (TMA) is a reactive low molecular weight (LMW) chemical used in the manufacture of paints, epoxy curing agents, printing inks and vinyl plasticizers and is known to cause occupational asthma characterized by airflow obstruction, airway inflammation and non-specific bronchial hyperreactivity (1, 2). The development of this allergic disease requires sensitization triggered by dermal or respiratory exposure to TMA followed by its binding to proteins (3). These so formed TMA-protein conjugates will then be taken up by antigen-presenting cells, transported to the regional lymph node, and presented to TMA-specific T cells resulting in T cell memory and the production of TMA-specific IgE antibodies. These antibodies will then bind to the high-affinity IgE receptor on mast cells (4, 5) and upon renewed contact with TMA mediate cross-linking of the IgE-receptors with subsequent release of mediators, that in their turn cause bronchoconstriction and attraction of inflammatory cells (6-8).

Alveolar macrophages (AMs) are among the first cells that encounter inhaled small particles and chemicals in the airways, since these cells are located at the interface between air and lung tissue. AMs are long-lived cells belonging to the family of mononuclear phagocytes. They represent a non-specific cellular host defense mechanism and can do so by binding and uptake of microorganisms and macromolecules via pattern recognition receptors, and the secretion of a broad repertoire of mediators that regulate inflammatory and immune reactions in the lung (9-12). Previously, it has been shown that depletion of AMs in TMA-sensitized Brown Norway (BN) rats prior to inhalation challenge with TMA resulted in ameliorated lung function during the challenge. Furthermore, an increased influx of inflammatory cells into the lung lumen was observed 24 h after challenge with TMA and TMA-BSA in AM-depleted rats compared to non-depleted control rats (TMA and TMA-BSA chapters). Therefore, we investigated the direct effects of TMA and TMA-BSA on the production of nitric oxide (NO), tumor necrosis factor-alpha (TNF-α), and interleukin 6 (IL-6) by AMs using the rat AM cell line NR8383 and AMs derived from TMA-sensitized and control BN rats.

Materials and Methods

Materials

TMA (97% purity) was obtained from Aldrich (Brussels, Belgium) and acetone (HPLC grade) from Merck (Darmstadt, Germany). Highly refined olive oil, 2,4,6-trinitrobenzene sulphonic acid, bovine serum albumine (BSA; cell culture tested), sulphanilamide, and naphthyl-ethylenediamide were purchased from Sigma (St. Louis, MO). Sodium pentobarbitone was obtained from Cevasante Animale B.V. (Maassluis, the Netherlands). K-medium contained Dulbecco's Modified Eagle Medium (DMEM; Cambrex BioScience, Verviers, Belgium) supplemented with 10% fetal calf serum (FCS; Invitrogen BV, Breda, The Netherlands), 10 mM HEPES (Merck), 4 mM L-glutamine, 1 mM sodium pyruvate, 100 U/ml of penicillin, 100 mg/ml of streptomycin, 0.05 mM β-mercapto-ethanol (all from Sigma) and 100 mg/ml gentamycin (Invitrogen). Ham's F12 medium was obtained from Invitrogen, lipopolysaccharide (LPS; *E. coli* O111:B4) from Sigma, and IFN-γ from Genentech Inc. (San Francisco, CA). The TNF-α and IL-6 ELISA kits were purchased from R&D Systems Inc. (Minneapolis, MN).

Animals

Female, inbred Brown Norway/CrlBR rats (BN; 7-8 weeks of age) were purchased from Charles River (Maastricht, The Netherlands). The animals were acclimatized at least 5 days before the start of the study. They were kept under conventional laboratory conditions and received food (Tecnilab BMI, Helmond, The Netherlands) and tap water *ad libitum.* All animal procedures were conducted in accordance with the Animal Ethics Committee of Utrecht University (Utrecht, The Netherlands).

Sensitization procedure

TMA was applied at a concentration of 50% (w/v) in a vehicle solution of 4:1 (v/v) acetone and olive oil. Animals received 150 µl on each flank (approximately 12 cm^2 each), which had been shaved with an electrical razor 2-3 days earlier. Seven days after the first sensitization the animals received 75 µl of a 25% TMA solution on the dorsum of both ears. Control animals received vehicle solution. Increased TMA-specific IgE serum levels verified the sensitization status of the TMA-sensitized rats (chapters TMA and TMA-BSA).

Preparation of TMA-BSA conjugate

The TMA-BSA conjugate was prepared under aseptic conditions by dissolving 10 mg/ml of BSA in 0.1 M sodium borate buffer (pH 9.4), adding approximately 1.5 mg TMA per ml BSA-solution and stirring at room temperature. After 1 h the same amount of TMA was added and the mixture was stirred for 2 h at room temperature. After centrifugation at 390 x *g* for 5 min, the supernatant was dialyzed successively against PBS and distilled water for 24 h at 4ºC. The conjugate was lyophilized and stored at 4ºC until use. The degree of substitution of the TMA-BSA was assessed by determination of remaining free amino groups by reaction with 2,4,6-trinitrobenzene sulphonic acid as described previously (13). The conjugate substitution ratio was approximately 40 mol TMA to 1 mol of BSA.

Cell culture and stimulation

AMs from TMA-sensitized and control rats were obtained by lung lavage at day 20 after treatment. Rats were killed with an overdose of sodium pentobarbitone (0.6 g/kg, i.p.). A cannula was inserted into the trachea, the lungs were lavaged 4 times with 8 ml aliquots of PBS warmed to 37°C and the lavage fluid was immediately thereafter put on ice. The cells were collected by centrifugation for 10 min at 390 x *g* (4ºC). After washing 3 times with PBS, the cells were resuspended in K-medium and incubated at 37°C for 2 h in 100 ml culture flasks (Greiner Bio-One, Alphen a/d Rijn, The Netherlands). After washing away non-adherent cells, the adherent cells (AMs) were scraped off in fresh K-medium supplemented with 1% FCS, adjusted to 1 x 10^6 cells/ml, and seeded in a total volume of 100 µl into sterile flat-bottom 96-wells plates (Costar, Cambridge, MA). After 1 h, 25 µl medium, stimulants (2 µg LPS admixed with 10 U IFN-γ per ml, 0.1-3 mg/ml of TMA-BSA, 0.1-3 mg/ml of BSA) in medium, or 10 and 100 µM TMA in 0.01 and 0.1% ethanol were added. All stimulants were filtered through a 0.22 µm syringe-filter (TPP, Trasadingen, Switzerland) before use. After 24 h the supernatants were collected and kept at –20ºC until use. Control stimulation of cells with 0.01 and 0.1% ethanol in medium did not affect cells (data not shown).

The AM cell line, NR8383, derived from Sprague Dawley rats, was purchased from the ATCC (Manassas, VA, USA) and maintained in Ham's F12 medium supplemented with 15% FCS, 100 U/ml of penicillin and 100 µg/ml of streptomycin. Cells were subcultured once per week. For that purpose, floating and scraped-off adherent cells were collected by centrifugation, resuspended in fresh medium, and seeded into new culture flasks. For stimulation experiments, both adherent and floating cells were harvested, resuspended in Ham's F12 medium supplemented with 1% FCS, penicillin, and streptomycin, and seeded into flat-bottom 96-wells plates at a density of 1 x 10^6

cells/ml. After 1 h, test compounds were added and the cells were further incubated for 24 h as described above. All cells were cultured at 37°C in a humidified atmosphere containing 5% CO_2.

NO measurements

The amount of NO secreted into the culture supernatants was assessed by determination of the concentration of its reaction product, nitrite, using the Griess reaction (14). Griess reagent (100 µl of 1% sulphanilamide and 0.1% naphthyl-ethylenediamide in 5% phosphoric acid) was added to 100 µl of sample medium. After incubation at room temperature for 10 min the optical density was measured at 550 nm using a microplate reader (Bio-Rad Laboratories, Hercules, CA). Calibration curves were made with $NaNO_2$ dissolved in the culture medium.

Cytokine assays

Levels of TNF-α and IL-6 in the culture supernatant of control and stimulated cell cultures were measured using commercial ELISA kits according to the manufacturer instructions. The IL-6 and TNF-α ELISA kits had detection limits of 10 pg/ml and 15 pg/ml, respectively. A microplate reader was used to measure the optical density at 450 nm.

Statistical analysis

All data are expressed as mean ± SEM. NO, TNF-α, and IL-6 levels were statistically analyzed using an unpaired t-test. Differences were considered statistically significant if $p<0.05$. Analyses were performed by the usage of Graphpad Prism (version 3.0, San Diego, U.S.A.).

Results

Effects of different stimuli on the production of NO, TNF-α, and IL-6 by NR8383 cells

NR8383 cells produced very low levels of NO, TNF-α, and IL-6 when cultured for 24 h in medium (Fig. 1). Incubation with LPS/IFN-γ induced the production of TNF-α and IL-6 already after 6 h (data not shown). After 24 h of incubation, LPS/IFN-γ induced the production of NO and further increased the production of TNF-α and IL-6. TMA was not able to stimulate the production of any of the mediators by NR8383 cells at any time, but TMA-BSA induced TNF-α and IL-6 production in a concentration-dependent manner after 6 h (data not shown). After 24 h of incubation, TMA-BSA induced the production of NO and further increased the production of TNF-α and IL-6 pro-

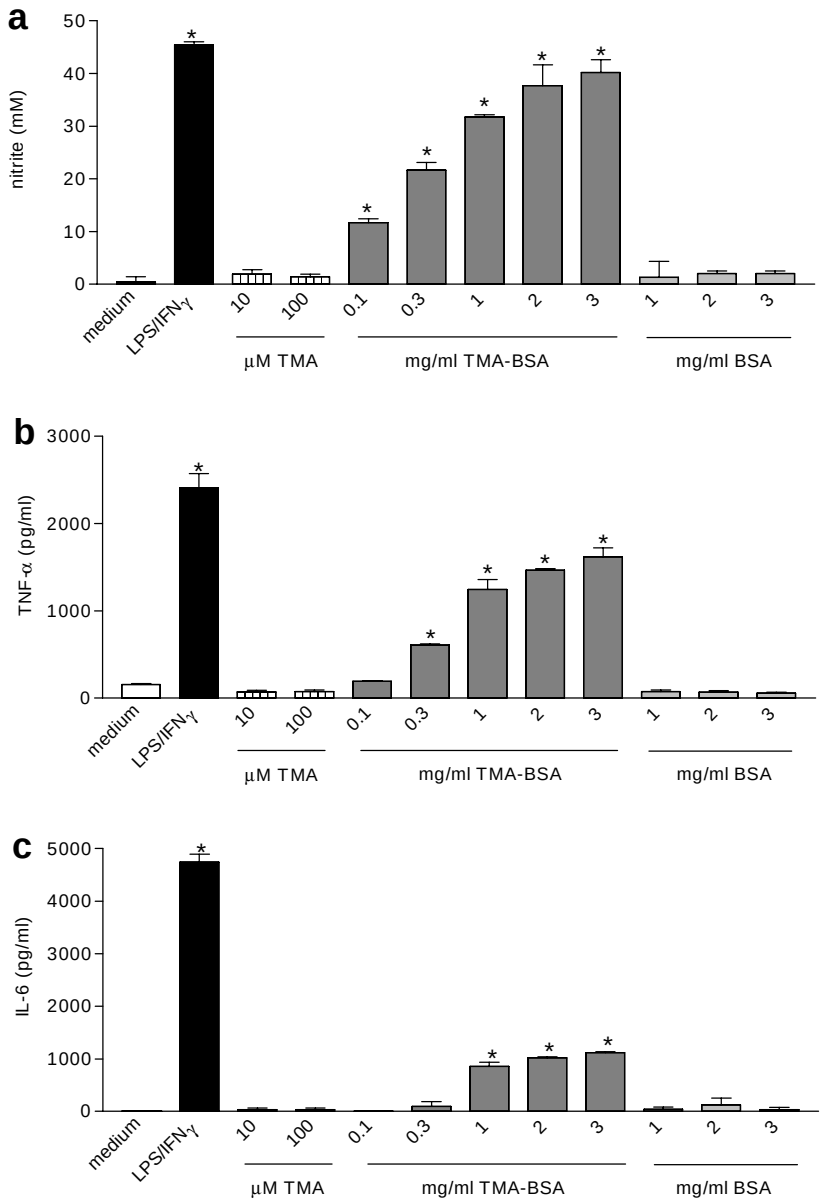

Figure 1. Effect of different stimuli on the production of NO, TNF-α, and IL-6 by NR8383 cells. Cells were incubated with medium (white bars), LPS/IFN-γ (black bars), TMA (striped bars) TMA-BSA (dark gray bars) and BSA (light gray bars) for 24 h. The culture supernatants were analyzed for NO (**a**), TNF-α (**b**) and IL-6 (**c**). Results are expressed as mean ± SEM. Significant differences are denoted by *: $p<0.05$ compared to medium incubation.

duction by NR8383 cells. However, the lowest concentrations of TMA-BSA to induce significant production of the separate mediators, and the amounts produced relative to those induced by LPS/IFN-γ diverged. NO was already induced by the lowest concentration of TMA-BSA (0.1 mg/ml) and the NO levels induced by the two highest concentrations were similar to the level induced by LPS/IFN-γ. TNF-α was induced at TMA/BSA concentrations of 0.3 mg/ml or higher and the maximum TNF-α level that was induced by 3 mg/ml was approximately 65% of that induced by LPS/IFN-γ. IL-6 was induced at TMA/BSA concentrations of 1 mg/ml or higher and the maximum IL-

6 level that was induced by 3 mg/ml was approximately 25% of that induced by LPS/IFN-γ.

Preincubation of NR8383 cells with 5, 10, and 15% serum derived from either TMA-sensitized or control rats for 1 h did not affect the capacity of LPS/IFN-γ, TMA, TMA-BSA, or BSA to induce the production of NO, TNF-α, and IL-6 (data not shown).

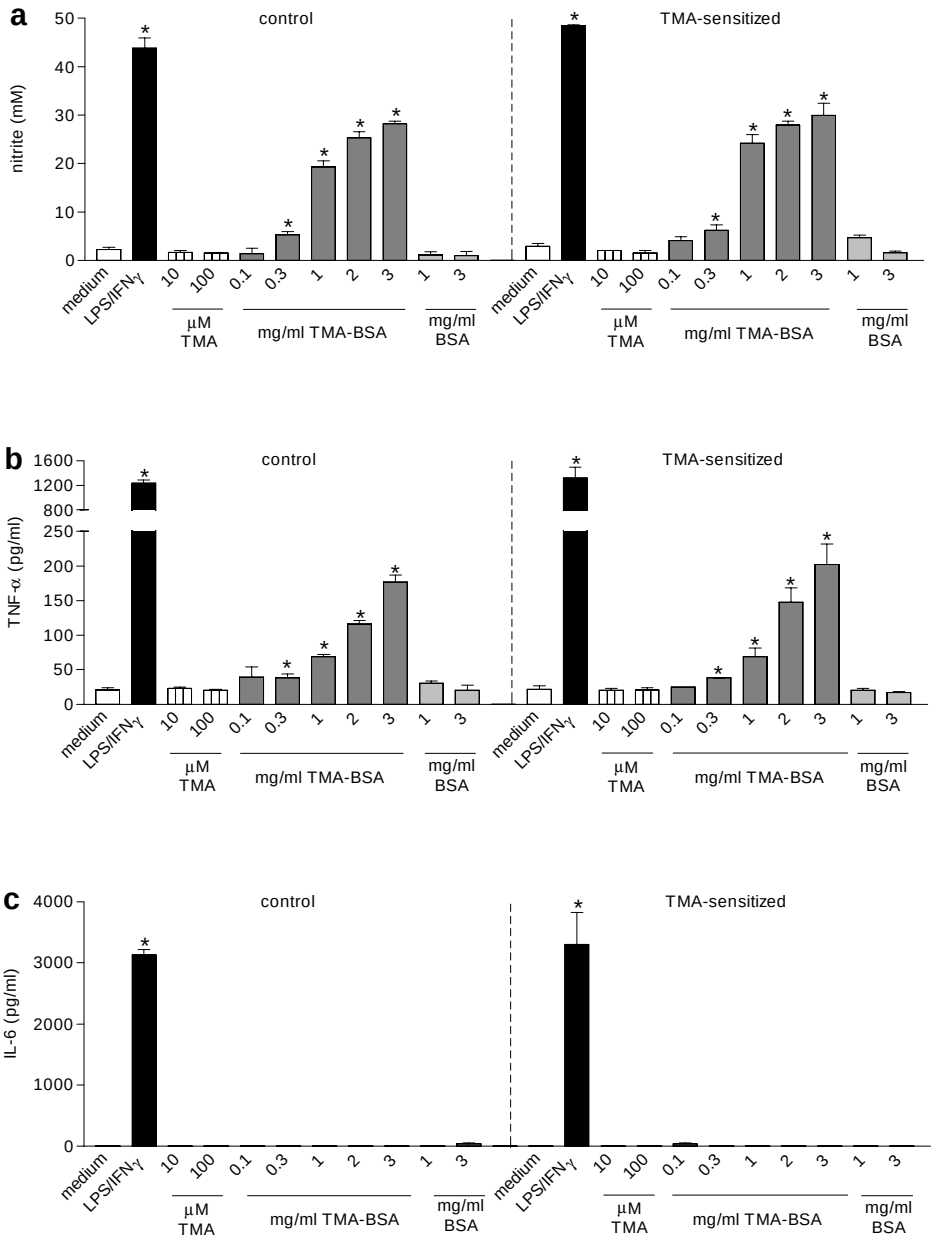

Figure 2. Effect of different stimuli on the production of NO, TNF-α, and IL-6 by AMs derived from either control or TMA-sensitized BN rats. Rats received 150 µl vehicle or 50% TMA in vehicle on each shaved flank on day 0 and 75 µl vehicle or 25% TMA on the dorsum of both ears on day 7. On day 21 the animals were sacrificed and the lungs were lavaged. AMs obtained from the lung lavage fluid were incubated with medium (white bar), LPS/IFN-γ (black bar), TMA (striped bars) TMA-BSA (dark gray bars) and BSA (light gray bars) for 24 h. The culture supernatants were analyzed for NO (**a**), TNF-α (**b**) and IL-6 (**c**). Results are expressed as mean ± SEM. Significant differences are denoted by *: $p<0.05$ compared to medium incubation.

Effect of different stimuli on the production of NO, TNF-α, and IL-6 by AMs from TMA-sensitized and control rats

AMs from TMA-sensitized and control rats produced very low levels of NO, TNF-α and IL-6 when cultured for 24 h in medium. Incubation with LPS/IFN-γ induced equal productions of mediators by AMs from both TMA-sensitized and control rats (Fig. 2). The LPS/IFN-γ induced NO production by AMs was comparable to that by NR8383 cells after incubation. The LPS/IFN-γ induced TNF-α and IL-6 production by AMs, however, was lower than by NR8383 cells. TMA did not induce the production of mediators by AMs after 24 h. TMA-BSA, however, induced a concentration-dependent increase in NO and TNF-α production by AMs, but did not induce the production of IL-6. Significant levels of both NO and TNF-α were induced by 0.3 mg/ml TMA-BSA or higher. The maximum NO level that was induced by TMA-BSA was approximately 60% of that induced by LPS/IFN-γ (Fig. 2a) and the maximum TNF-α level induced by TMA-BSA was approximately 15% of that induced by LPS/IFN-γ (Fig. 2b).

Discussion

The present study showed that TMA-BSA conjugates, but not free TMA or BSA, were able to induce the production of the mediators NO, TNF-α, and IL-6 by the cell line, NR8383, and, IL-6 excepted, by primary AMs *in vitro*. This stimulation is probably not immunologically specific for two reasons. Firstly, preincubation with serum containing TMA-specific IgE did not affect the capacity of TMA-BSA to stimulate mediator production by NR8383 cells. Secondly, primary AMs from TMA-sensitized and control rats reacted similarly to TMA-BSA. The lack of effect of TMA-specific IgE was not expected, since AMs express the IgE receptors, FcεRI and CD23. Moreover, their expression is increased in the presence of IgE (15) and upon *in vivo* sensitization (16, 17). Apparently, there is no cross-linking of IgE at the surface of AMs by TMA-BSA or cross-linking does not trigger mediator production in response to TMA-BSA, although similarly prepared conjugates have been reported to trigger degranulation of IgE-primed mast cells (5).

Regarding the nature of the immunologically non-specific AM stimulation, structural similarities between TMA-conjugated BSA and maleylated-BSA may point at the involvement of a member of the family of scavenger receptors. These receptors belong to the large family of pattern recognition receptors that exhibit binding specificity for structural patterns typically displayed by cell surface molecules of many microorganisms that are not normally found on the surface of eukaryotic cells (18). Macrophages are known to express multiple scavenger receptors (19, 20) and

a variety of ligands, including maleylated-BSA and LPS, have been shown to induce the production of NO, TNF-α, and IL-6 via these scavenger receptors (21-23). Since these mediators were also induced after stimulation of AMs with TMA-BSA, but not free BSA, combined with the structural similarities of TMA-BSA with maleylated-BSA, it is likely that the observed effects were regulated via these receptors.

In the present study, stimulation with LPS/IFN-γ of NR8383 cells induced higher TNF-α and IL-6 levels compared to the primary AMs. Since both NR8383 cells and primary cells were AMs, differences in genetic background may explain the variation in mediator production, given the fact that the NR8383 cells were derived from Sprague Dawley rats (24), while the primary AMs were derived from BN rats. Furthermore, Rao et al. (25) demonstrated that stimulation of NR8383 cells with LPS, with or without IFN-γ, activated all three families of mitogen-activated protein kinases while only one of the three families was activated in primary AMs derived from Sprague Dawley rats, indicating differences between primary cells and immortalized cells.

The observation that TMA-BSA induced equal amounts of NO in NR8383 cells as LPS/IFN-γ and only 40% less in primary AMs indicates that TMA-BSA is a powerful macrophage activating agent. It is probably more potent than LPS as such, since the amount of NO produced by AMs in response to LPS was reported to be only 0-35 % of the response after LPS/IFN-γ incubation of primary AMs derived from Sprague Dawley and BN rats (26). Despite the potent *in vitro* AM-activating capacity of TMA-BSA and the lack of effect of TMA in this respect, inhalation challenge of TMA-sensitized BN rats with either TMA or TMA-BSA induced similar immediate reduction in minute ventilation (Chapters 3 and 4), although TMA-BSA, in contrast to TMA, did not induce an influx of inflammatory cells into the airway lumen. This, however, is likely to be due to the lower challenge concentration of TMA-BSA. The substantial differences between TMA and TMA-BSA in their *in vitro* AM-activating capacity are apparently not at play upon inhalation challenge with these compounds. A possible explanation for this controversy might be that inhalation of TMA leads to rapid conjugation with endogenous proteins. The observation that TMA challenge of BN rats caused immediate bronchoconstriction is indicative of rapid conjugation, since the immediate bronchoconstriction is likely to be due to mast cell degranulation triggered by IgE receptor cross-linking with a multivalent TMA ligand as provided by self-protein conjugated with multiple TMA molecules. Formation of such conjugates, however, is probably not feasible *in vitro* due to the static culture conditions.

Formation of conjugates of TMA with endogenous proteins is considered to be required for sensitization (27). When such protein-conjugates, like TMA-BSA *in vitro*, induce the production

of NO and proinflammatory cytokines *in vivo*, then TMA can be considered as an inducer of danger signals. Thus, TMA-protein-conjugates, like the LPS-induced danger signal in the case of bacteria, can act as an adjuvant for TMA sensitization. An interesting question in this respect is, whether the most potent inducers of LMW chemical-induced occupational respiratory allergic disease share this intrinsic adjuvant activity. If so, toxicological hazard identification may benefit from screening for macrophage-activating activity of proteins conjugated with reactive LMW compounds.

In summary, the results of the present study demonstrate that although TMA is a highly reactive chemical, it needs to be conjugated with a suitable protein to exert an effect on AMs, as was observed for the TMA-BSA conjugate. The effects of TMA-BSA on AMs were not dependent on sensitization indicating that the interaction of TMA-BSA with AMs is probably mediated via an immunologically non-specific scavenger receptor.

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References

1. Lombardo, L. J., and J. R. Balmes. 2000. Occupational asthma: A review. *Environ Health Perspect 108 Suppl 4:697*.
2. Kirchner, D. B. 2002. The spectrum of allergic disease in the chemical industry. *Int Arch Occup Environ Health 75 Suppl:S107*.
3. Karol, M. H. 2001. Bonding and transfer: do epithelial conjugates have a role in chemical asthma? *Clin Exp Allergy 31:357*.
4. Holliday, M. R., J. W. Coleman, R. J. Dearman, and I. Kimber. 1993. Induction of mast cell sensitization by chemical allergens: a comparative study. *J Appl Toxicol 13:137*.
5. Vento, K. L., R. J. Dearman, I. Kimber, D. A. Basketter, and J. W. Coleman. 1996. Selectivity of IgE responses, mast cell sensitization, and cytokine expression in the immune response of Brown Norway rats to chemical allergens. *Cell Immunol 172:246*.
6. Prussin, C., and D. D. Metcalfe. 2003. 4. IgE, mast cells, basophils, and eosinophils. *J Allergy Clin Immunol 111:S486*.
7. Hart, P. H. 2001. Regulation of the inflammatory response in asthma by mast cell products. *Immunol Cell Biol 79:149*.
8. Hamid, Q., M. K. Tulic, M. C. Liu, and R. Moqbel. 2003. Inflammatory cells in asthma: mechanisms and implications for therapy. *J Allergy Clin Immunol 111:S5*.
9. Laskin, D. L., B. Weinberger, and J. D. Laskin. 2001. Functional heterogeneity in liver and lung macrophages. *J Leukoc Biol 70:163*.

10. Van Rooijen, N., O. L. Wijburg, G. P. van den Dobbelsteen, and A. Sanders. 1996. Macrophages in host defense mechanisms. *Curr Top Microbiol Immunol 210:159.*
11. Dorger, M., S. Munzing, A. M. Allmeling, K. Messmer, and F. Krombach. 2001. Phenotypic and functional differences between rat alveolar, pleural, and peritoneal macrophages. *Exp Lung Res 27:65.*
12. Peiser, L., S. Mukhopadhyay, and S. Gordon. 2002. Scavenger receptors in innate immunity. *Curr Opin Immunol 14:123.*
13. Sashidhar, R. B., A. K. Capoor, and D. Ramana. 1994. Quantitation of epsilon-amino group using amino acids as reference standards by trinitrobenzene sulfonic acid. A simple spectrophotometric method for the estimation of hapten to carrier protein ratio. *J Immunol Methods 167:121.*
14. Green, L. C., D. A. Wagner, J. Glogowski, P. L. Skipper, J. S. Wishnok, and S. R. Tannenbaum. 1982. Analysis of nitrate, nitrite, and [15N]nitrate in biological fluids. *Anal Biochem 126:131.*
15. Boltz-Nitulescu, G., J. M. Plummer, and H. L. Spiegelberg. 1984. Increased expression of the IgE Fc receptors on rat macrophages induced by elevated serum IgE levels. *Immunology 53:9.*
16. Mencia-Huerta, J. M., B. Dugas, E. Boichot, C. Petit-Frere, N. Paul-Eugene, V. Lagente, M. Capron, F. T. Liu, and P. Braquet. 1991. Pharmacological modulation of the antigen-induced expression of the low-affinity IgE receptor (Fc epsilon RII/CD23) on rat alveolar macrophages. *Int Arch Allergy Appl Immunol 94:295.*
17. Humbert, M., J. A. Grant, L. Taborda-Barata, S. R. Durham, R. Pfister, G. Menz, J. Barkans, S. Ying, and A. B. Kay. 1996. High-affinity IgE receptor (FcepsilonRI)-bearing cells in bronchial biopsies from atopic and nonatopic asthma. *Am J Respir Crit Care Med 153:1931.*
18. Gordon, S. 2002. Pattern recognition receptors: doubling up for the innate immune response. *Cell 111:927.*
19. Peiser, L., and S. Gordon. 2001. The function of scavenger receptors expressed by macrophages and their role in the regulation of inflammation. *Microbes Infect 3:149.*
20. Gough, P. J., and S. Gordon. 2000. The role of scavenger receptors in the innate immune system. *Microbes Infect 2:305.*
21. Alford, P. B., Y. Xue, S. F. Thai, and R. E. Shackelford. 1998. Maleylated-BSA enhances production of nitric oxide from macrophages. *Biochem Biophys Res Commun 245:185.*
22. Coller, S. P., and D. M. Paulnock. 2001. Signaling pathways initiated in macrophages after engagement of type A scavenger receptors. *J Leukoc Biol 70:142.*
23. Paulnock, D. M., K. P. Demick, and S. P. Coller. 2000. Analysis of interferon-gamma-dependent and -independent pathways of macrophage activation. *J Leukoc Biol 67:677.*
24. Helmke, R. J., R. L. Boyd, V. F. German, and J. A. Mangos. 1987. From growth factor dependence to growth factor responsiveness: the genesis of an alveolar macrophage cell line. *In Vitro Cell Dev Biol 23:567.*
25. Rao, K. M., T. Meighan, and L. Bowman. 2002. Role of mitogen-activated protein kinase activation in the production of inflammatory mediators: differences between primary rat alveolar macrophages and macrophage cell lines. *J Toxicol Environ Health A 65:757.*

26. Jesch, N. K., M. Dorger, K. Messmer, and F. Krombach. 1998. Formation of nitric oxide by rat and hamster alveolar macrophages: an interstrain and interspecies comparison. *Toxicol Lett 96-97:47*.

27. Karol, M. H., O. T. Macina, and A. Cunningham. 2001. Cell and molecular biology of chemical allergy. *Ann Allergy Asthma Immunol 87:28*.

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Glutathione-conjugated toluene diisocyanate causes airway inflammation in sensitized mice

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Abstract

Toluene diisocyanate (TDI) is a highly volatile compound that reacts readily with nucleophilic compounds, sulfhydryl groups in particular. Since the epithelial lining fluid of the airways contains high levels of the sulfhydryl, glutathione (GSH), inhalation of TDI is likely to result in the formation of GS-TDI conjugates. We, therefore, investigated whether GS-TDI is capable of provoking irritant and/or allergic reactions.Irritant effects of GS-TDI were studied after intratracheal administration of a range of doses of GS-TDI in saline to naive BALB/c mice. GS-TDI caused a dose-dependent increase in neutrophils in the lungs 24 h after instillation. A dose equivalent to 150 μg of TDI or lower had no effect. For provocation of allergic reactions, mice were sensitized by application of 1 % TDI onto the skin on days 0 and 1, and challenged intratracheally with a sub-irritant dose of GS-TDI on day 8. GS-TDI did not induce non-specific tracheal hyperreactivity to carbachol 24 and 48 h after challenge in TDI-sensitized mice. However, it increased the numbers of neutrophils in the lungs as compared to the control mice.These findings suggest that GSH conjugation does not diminish the capacity of TDI to elicit irritant-induced inflammation in the lungs of mice at doses above 150 μg of TDI in the conjugate. Moreover, the capacity to induce allergic-specific inflammation was retained at concentrations of GS-TDI being devoid of irritant activity. However, the GS-TDI conjugate failed to induce non-specific tracheal hyperreactivity. This may be the consequence of the deposition of excess of GSH upon local dissociation of the conjugate.

Introduction

Diisocyanates, like toluene diisocyanate (TDI), are highly reactive low molecular weight compounds used in paint and plastic industries. They can cause occupational asthma characterized by symptoms like cough, chest tightness, shortness of breath, and wheezing (1). The pathogenic mechanism of TDI-induced asthma is far from clear but involves specific sensitization to the compound. This sensitization is supposed to require binding of TDI to carrier molecules in the body (2, 3). Because TDI is a typical electrophile, it is capable of reacting with hydroxyl, amino, and thiol groups on proteins, thereby forming hapten-carrier-complexes. The latter can be recognized as a foreign antigen and may cause sensitization (1, 3, 4). In sensitized subjects renewed contact with the antigen can provoke a more vigorous and accelerated secondary immune responses that may result in clinically manifest adverse health effects. Therefore, the identification of carrier molecules that permit sensitization and secondary immune responses upon binding of TDI is central in understanding diisocyanate-induced asthma.

TDI has been found to bind to diverse molecules. Following inhalation exposure of guinea pigs, TDI has been shown to bind to the tubulin of cilia of respiratory epithelium (5, 6). In broncho-alveolar lavage fluid of guinea pigs that had been exposed to TDI vapor, TDI was found to bind to at least five different proteins, of which serum albumin was the most prominent carrier (7, 8). In rat studies using ^{14}C-labeled TDI for inhalation exposure, the majority of radioactivity in the blood was associated with albumin or albumin-like proteins, while the remainder was located in erythrocytes bound to hemoglobin (9). Hemoglobin adducts were also found in blood of guinea pigs after TDI inhalation (8) indicating that TDI, though highly reactive, can cross cells and cell membranes to ultimately bind to intracellular structures.

Inhalation of highly reactive TDI vapor by animals requires specific and expensive equipment. Since TDI has been found to bind rapidly to diverse molecules, water-soluble TDI conjugates, like TDI-serum albumin conjugates, are frequently used for respiratory challenge. Such conjugates appeared capable of eliciting an asthmatic response in sensitized animals (10, 11). Serum albumin, however, is normally not present at high concentration in the airways. Therefore, a prominent role of TDI conjugates of this protein is less likely. The non-protein tripeptide, glutathione (L,γ-glutamyl-L-cysteinglycine), is probably a more relevant target for TDI. This small molecule is present in all mammalian tissues where it is the most abundant thiol. It participates in functions, like free radical scavenging, redox reactions, xenobiotic metabolism, and amino acid transport. In the airway epithelial lining fluid the glutathione concentration is more than a 100-fold higher than in plasma and more

than 95 % of the molecule is in the reduced form (GSH) (12-14). Because of the high GSH concentration in the airway lining fluid and the high reactivity of TDI with thiol groups, inhalation of TDI is likely to result in the formation of GS-TDI conjugates. This has been demonstrated in mice where inhalation of TDI vapor caused a 30-50% reduction in total lung thiol content, mainly because of the formation of GS-TDI conjugates (15). *In vitro* studies with human bronchial epithelial cells gave similar results (15, 16).

Generally, GSH conjugation to electrophilic compounds results in their detoxification and elimination, but since GSH conjugation to TDI is reversible (8, 17, 18), detoxification may not be the case for TDI. Therefore, the aim of the present study was to investigate whether TDI coupled to GSH is able to cause airway inflammation and non-specific airway hyperreactivity in a mouse model for TDI-induced occupational asthma.

Materials and methods

Materials

Reduced glutathione, 2,4-toluene diisocyanate (97% purity), highly refined olive oil, and carbachol were purchased from Sigma Chemical Company (St. Louis, MO). Acetone, HPLC grade, was obtained from Merck (Darmstadt, Germany). Sodium pentobarbitone was purchased from Sanofi BV (Maassluis, the Netherlands), ketamine from Cassot (Hasselt, Belgium) and xylazine from Eurovet (Bladel, The Netherlands). Diff-Quick was obtained from Merz and Dade (Düdingen, Switzerland).

Synthesis of GS-TDI conjugates

TDI was coupled to glutathione as described by Day et al. (1997). Briefly, 1 mmol GSH was dissolved in 10 ml 0.1 M NH_4HCO_3 (pH 7.73). Next, 1.2 mmol of 2,4-TDI was added drop-wise under constant shaking at ambient temperature for 10 minutes. The solution was centrifuged at 390 x g for 10 minutes. Residual unreacted TDI, visible as droplets at the surface, was removed and the remainder subsequently lyophilized in a speedvac (New Brunswick Scientific B.V., Nijmegen, The Netherlands), yielding a white powder that was stored at -70ºC.

Products resulting from the admixture of TDI and GSH were tested with ESI-mass spectrometry and NMR analysis as described by Day et al. (1997).

Animals

Male BALB/c mice (6-8 weeks of age) were obtained from the Utrecht University Central Animal Laboratory (Utrecht, The Netherlands). They were housed in macrolon cages under filter tops (six animals per cage). Tap water and food (Hope Farms, Woerden, The Netherlands) were allowed ad libitum. All animal procedures were conducted in accordance with the Animal Ethics Committee of Utrecht University (Utrecht, The Netherlands).

Inflammatory responses of the lungs to GS-TDI conjugates

Groups of naive mice received different concentrations of GS-TDI containing 138 μg, 275 μg and 550 μg of TDI in 50 μl saline by instillation into the trachea under light anesthesia (ketamine 30 mg/kg; xylazine 5 mg/kg, i.m.). After 24 h, the mice were killed with an overdose of sodium pentobarbitone (0.6 g/kg, i.p.) and the lungs were lavaged for determination of leukocytes. A cannula was inserted into the trachea, and the lungs were lavaged 5 times with 1 ml aliquots of saline warmed to 37°C. The cells were collected by centrifugation (390 x g, 10 minutes, 4ºC), and resuspended in 150 μl cold PBS. A Bürker-Türk chamber was used to count the total number of lavage cells. For differential cell counts cytospin preparations were made and stained with Diff-Quick. Per cytospin 200 cells were counted and differentiated into macrophages, monocytes/ lymphocytes and neutrophils by standard morphology.

Sensitization and challenge procedure

Groups of mice received vehicle solution (acetone:olive oil, 4:1) or TDI (1%, v/v) in vehicle twice daily on the shaved abdomen (100 μl) on days 0 and 1 and onto the four paws (100 μl) on day 0. On day 8, both vehicle- and TDI-treated mice were challenged intratracheally with 50 μl of indicated concentrations GS-TDI in saline under light anesthesia. A positive ear swelling 24 h after local challenge with TDI in vehicle solution verified the sensitization procedure (data not shown).

Airway reactivity in vitro

Groups of sensitized or vehicle-treated control mice were killed with an overdose of sodium pentobarbitone 24 or 48 h after intratracheal challenge with GS-TDI. The trachea was resected *in toto* and carefully cleaned of connective tissue using a binocular microscope. A nine ring piece of trachea was then transferred to a 10 ml organ bath containing a modified, aerated (95% O_2, 5% CO_2) Krebs solution (118 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 0.5 mM $MgCl_2$, 25 mM $NaHCO_3$, 1 mM $NaHPO_4$ and 11.1 mM glucose) and maintained at 37ºC. The trachea was directly slipped

onto two supports, one coupled to the organ bath and the other to an isometric transducer (Harvard Bioscience, Kent, UK). The tracheal tension was set at an optimal counterweight of 1000 mg and isometric measurements were made using a force displacement transducer and a two-channel recorder and measurements were expressed as changes in mg force. The trachea was allowed to equilibrate for at least one hour before effects of carbachol were determined. During the equilibrium phase the bath fluid was exchanged every 15 min. To assess airway reactivity, a cumulative concentration response-curve to carbachol (10^{-8} to 10^{-4} M) was determined.

Data analysis

Data are expressed as mean and standard error of the mean (SEM). Data on the cellular accumulation were statistically analyzed using the Mann-Whitney *U* test. EC_{50} and E_{max} values for the carbachol-induced tracheal contractions were calculated by non-linear least-squares regression analysis of the measured contractions versus carbachol concentrations using the sigmoid concentration-response relationship and were analyzed by using the two-tailed Student's t test. Differences were considered statistically significant if $p<0.05$. Analyses were performed by the usage of Graphpad Prism (version 3.0, San Diego, U.S.A.).

Results

Analysis of GS-TDI conjugates

Analysis by ESI-mass spectrometry and by NMR analysis showed that approximately 95% of the reaction product of TDI and GSH was the bisGS-TDI adduct, the remaining 5% consisted of unreacted GSH, GSSG and the mono-adduct (Fig. 1).

Airway inflammation after GS-TDI treatment of naive mice

Intratracheal instillation of different doses of GS-TDI caused a dose-dependent increase in neutrophils in the airways compared to the vehicle-treated mice (Fig. 2). The lowest concentration of the conjugate containing 138 μg of TDI did not induce an increase in cells into the lungs 24 h after administration. The doses of 275 μg and 550 μg TDI induced significant increases of neutrophils into the airways. Therefore, it was decided to continue the experiments with 150 μg TDI.

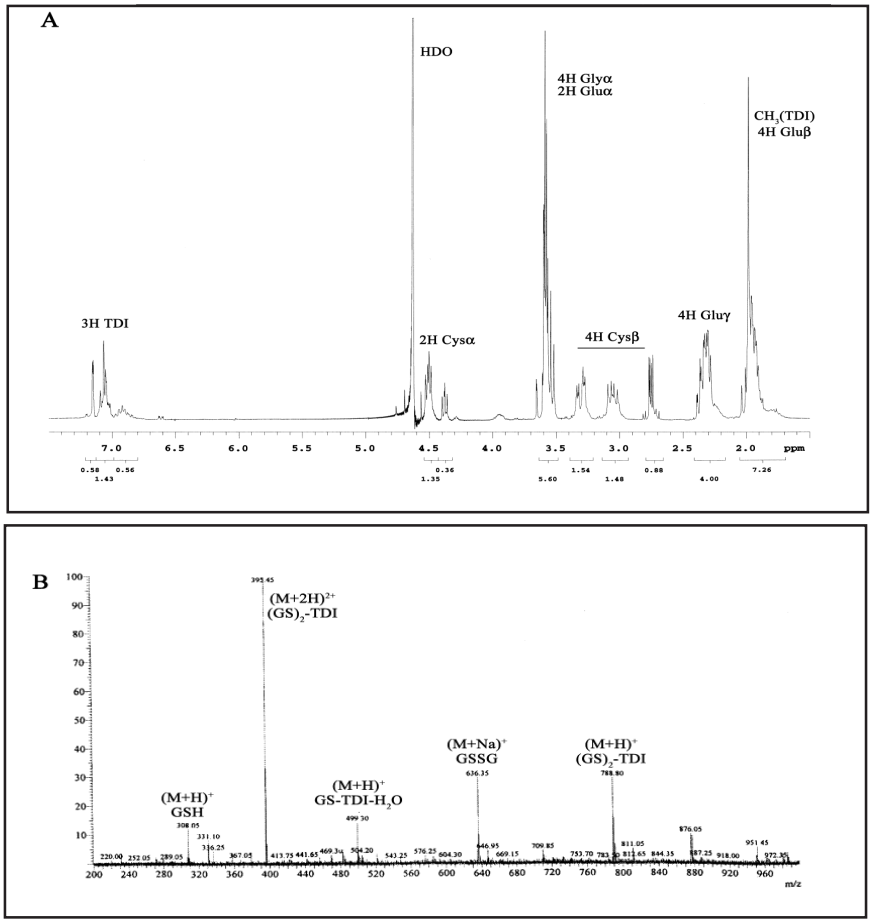

Figure 1. NMR spectrum (**A**) and ESI-mass spectrum (**B**) of the admixture of TDI and GSH. The NMR spectrum was recorded at ambient temperature at 300 MHz in D_2O. The y-axis of B is in arbitrary percentage units.

Sensitization-dependent airway inflammation

After establishing the non-inflammatory dose of GS-TDI, an equivalent of 150 µg TDI was used to challenge vehicle-treated and TDI-sensitized mice via intratracheal instillation. GS-TDI challenge of the vehicle-treated mice did not result in an influx of inflammatory cells into the airways 24 h later (Fig. 3). In contrast, a similar challenge of TDI-sensitized mice resulted in a significant increase in neutrophils in the lavage fluid compared to vehicle-treated control mice 24 h after GS-TDI challenge. The numbers of macrophages and lymphocytes were not significantly different between both groups.

In vitro tracheal reactivity

To investigate if the pulmonary inflammation coincided with non-specific airway hyperreactivity we measured the *in vitro* tracheal reactivity to carbachol 24 and 48 h after intratracheal challenge. The vehicle-treated, GS-TDI challenged control group showed a normal concentration-dependent

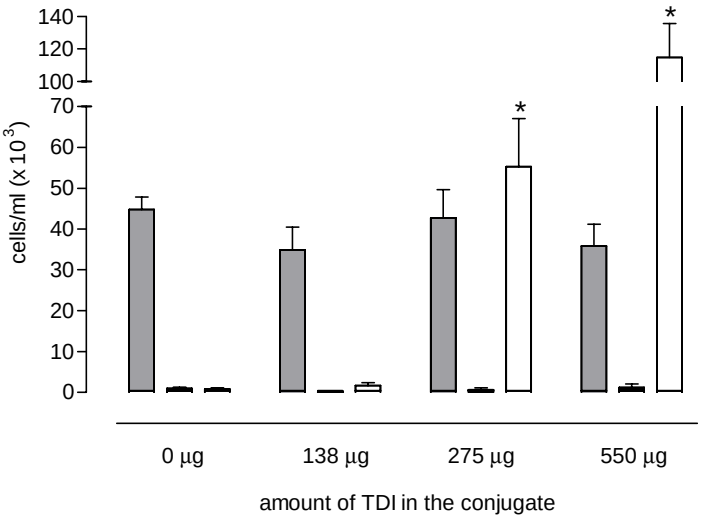

Figure 2. Leukocyte numbers in lung lavage fluid of naive BALB/c mice 24 h after intratracheal treatment with different doses of GS-TDI in 50 µl saline. The x-axis indicates the amount of 2,4-TDI present in the instilled conjugate. Cells were differentiated into macrophages (black bars), monocytes/lymphocytes (gray bars) and neutrophils (white bars). Results are expressed as mean +/- SEM (n=4). Significant differences compared to 0 µg are denoted by * ($p < 0.05$).

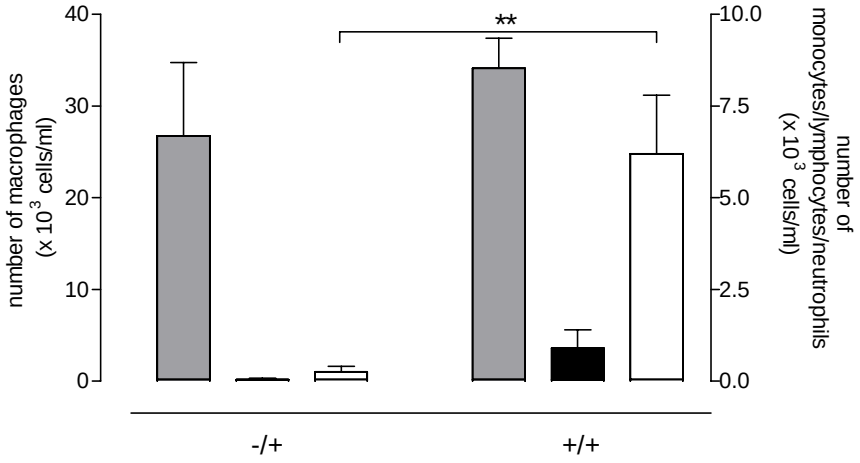

Figure 3. Cells in lung lavage fluid of TDI-sensitized (+/+) and vehicle-treated (-/+) BALB/c mice 24 h after intratracheal challenge with GS-TDI in saline. Groups of 5 mice were treated epicutaneously twice daily with vehicle solution (acetone:olive oil, 4:1) or 2,4-TDI in vehicle (1%, v/v). It was applied to the shaved abdomen (100 µl) on days 0 and 1 and to the four paws (100 µl) on day 0. On day 8, both vehicle- and TDI-treated mice were challenged intratracheally with GS-TDI in 50 µl saline containing a dose-equivalent of 150 µg TDI. Cells were differentiated into macrophages (black bars), monocytes/lymphocytes cells (gray bars) and neutrophils (white bars). Results are expressed as mean ± SEM. Significant differences are denoted by ** ($p < 0.01$).

increase in tracheal reactivity. Challenge with the GS-TDI conjugate did not cause a significant change in tracheal reactivity to carbachol 24 (Fig. 4) and 48 h (data not shown) later in the TDI-sensitized mice compared to the vehicle-treated control mice.

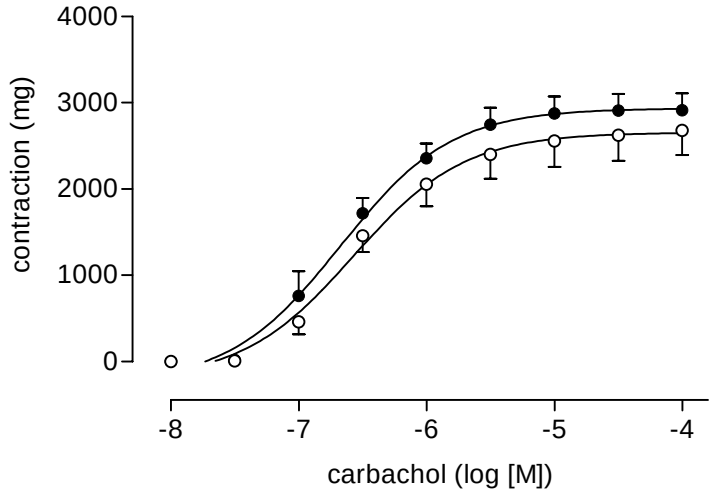

Figure 4. *In vitro* non-specific tracheal reactivity 24 h after challenge with GS-TDI in saline. Groups of 5 mice were treated epicutaneously twice daily with 2,4-TDI (1%, v/v) or vehicle solution (acetone:olive oil, 4:1). It was applied to the shaved abdomen (100 µl) on days 0 and 1 and to the four paws (100 µl) on day 0. On day 8, both vehicle- (open circles) and TDI-treated (closed circles) mice were challenged intratracheally with GS-TDI in 50 µl saline containing a dose-equivalent of 150 µg TDI. Concentration-response curves to carbachol were measured 24 h after challenge. Results are expressed as mean +/- SEM.

Discussion

In the present study we have found that intratracheal instillation of TDI conjugated to GSH induced a dose-dependent influx of inflammatory cells into the airways of naive mice. This increase in cells was most prominent for neutrophils. Since the inflammatory reaction was induced in non-sensitized mice, it can be concluded that the inflammation was the result of an irritant reaction to the GS-TDI conjugate. Apparently, GSH conjugation is not capable of neutralizing the irritant effects of TDI. Furthermore, instillation of a sub-irritant dose of the GS-TDI conjugate caused a significant influx of neutrophils into the airway lumen of TDI-sensitized but not in vehicle-treated control mice. This sensitization-dependent neutrophilic airway inflammation is probably a typical manifestation of TDI-induced asthma since bronchoalveolar neutrophilia has been reported to be associated with asthma in TDI-exposed workers (3, 19, 20). Our animal data are in agreement with the reported influx of neutrophils into the airways of sensitized C57BL/6J mice 24 h after repeated inhalation challenge with aerosols containing 100 ppm TDI as compared with vehicle aerosols (21). Our data are also in agreement with the neutrophil influx into the airways observed in sensitized BALB/c mice 6 to 72 h after inhalation challenge with an aerosol of 1% TDI in ethyl acetate:olive oil that was not seen in vehicle-treated mice (22). However, no inflammatory cell influx was found in sensitized BALB/c mice after an intranasal challenge with 1% TDI in ethyl acetate:olive oil (23).

Since the sensitization protocol and the time of challenge of the latter study were similar to those used in our study, the intranasal challenge protocol probably has been responsible for the lack of bronchoalveolar inflammation. We administered a GS-TDI conjugate containing 150 µg TDI in saline intratracheally instead of 243 µg of "free" TDI in ethyl acetate/olive oil intranasally. Interestingly, Ebino et al. (1999) demonstrated that intranasal application of TDI in ethyl acetate/ olive oil did not reach the lower airways. Therefore, the applied oily vehicle used by Scheerens et al. (1996) may explain the observed lack of lower airway inflammation. This problem is not encountered after intratracheal challenge with GS-TDI in saline as used in our study.

In contrast to our study, the neutrophilic inflammation into the airways of sensitized C57BL/ 6J and BALB/c mice after inhalation challenge with TDI vapor (21, 22) was attended by bronchial hyperreactivity, another hallmark of asthma. The discrepancy in bronchial hyperreactivity may be related to our use of TDI as a GSH conjugate. The sulfhydryl moiety of GSH is carbamoylated to each of both isocyanate groups in the GS-TDI conjugate. This binding was found to be reversible (8, 18, 24) thereby releasing one TDI molecule and two GSH molecules per conjugate. Thus decomposition of the conjugate will lead to an increase of the GSH concentration in the epithelial lining fluid. Complete dissociation of the applied dose of conjugate may have resulted in the release of 150 µg TDI and 532 µg GSH. It has been demonstrated that only 75 µg GSH is present in the normal mouse lung (25). Therefore, the increase in GSH might provide an explanation for the absence of airway hyperreactivity, since it is known that GSH relaxes tracheal smooth muscles as tested with epithelium denuded guinea pig tracheas in perfused organ baths (26). Also, airway reactivity is increased when GSH is depleted from the airways by substances like BSO (D,R-buthionine-L-sulfoximine; a GSH synthesis inhibitor) (17, 27). Furthermore, inhalation of nebulized GSH decreased the non-specific airway hyperreactivity in asthmatic patients (28) and decreased the airway obstruction in patients with COPD (29).

In summary, conjugation with GSH does not detoxify TDI since GS-TDI is able to cause inflammatory reactions in naive mice. Moreover, a sub-irritant concentration of the conjugate was able to elicit a sensitization-dependent reaction in TDI-sensitized mice. This immune reaction consisted of an influx of inflammatory cells into the airways but non-specific tracheal hyperreactivity could not be measured. This failure may well be the result of the increase of GSH that is present after dissociation of the conjugate.

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References

1. Redlich, C. A., and M. H. Karol. 2002. Diisocyanate asthma: clinical aspects and immunopathogenesis. *Int Immunopharmacol 2:213*.
2. Raulf-Heimsoth, M., and X. Baur. 1998. Pathomechanisms and pathophysiology of isocyanate-induced diseases— summary of present knowledge. *Am J Ind Med 34:137*.
3. Wisnewski, A. V., and C. A. Redlich. 2001. Recent developments in diisocyanate asthma. *Curr Opin Allergy Clin Immunol 1:169*.
4. Brown, W. E., and A. L. Burkert. 2002. Biomarkers of toluene diisocyanate exposure. *Appl Occup Environ Hyg 17:840*.
5. Karol, M. H., R. Jin, and R. C. Lantz. 1997. Immunohistochemical detection of toluene diisocyanate (TDI) adducts in pulmonary tissue of guinea pigs following inhalation exposure. *Inhalation Toxicology 9:63*.
6. Lange, R. W., R. C. Lantz, D. B. Stolz, S. C. Watkins, P. Sundareshan, R. Lemus, and M. H. Karol. 1999. Toluene diisocyanate colocalizes with tubulin on cilia of differentiated human airway epithelial cells. *Toxicol Sci 50:64*.
7. Jin, R., B. W. Day, and M. H. Karol. 1993. Toluene diisocyanate protein adducts in the bronchoalveolar lavage of guinea pigs exposed to vapors of the chemical. *Chem Res Toxicol 6:906*.
8. Day, B. W., R. Jin, and M. H. Karol. 1996. In vivo and in vitro reactions of toluene diisocyanate isomers with guinea pig hemoglobin. *Chem Res Toxicol 9:568*.
9. Kennedy, A. L., T. R. Wilson, M. F. Stock, Y. Alarie, and W. E. Brown. 1994. Distribution and reactivity of inhaled 14C-labeled toluene diisocyanate (TDI) in rats. *Arch Toxicol 68:434*.
10. Huang, J., L. L. Millecchia, D. G. Frazer, and J. S. Fedan. 1998. Airway hyperreactivity elicited by toluene diisocyanate (TDI)-albumin conjugate is not accompanied by airway eosinophilic infiltration in guinea pigs. *Arch Toxicol 72:141*.
11. Shiotsuka, R. N., D. L. Warren, A. T. Halliburton, and D. W. Sturdivant. 2000. A Comparative Respiratory Sensitization Study of 2,4- and 2,6-Toluene Diisocyanate Using Guinea Pigs. *Inhal Toxicol 12:605*.
12. Cantin, A. M., S. L. North, R. C. Hubbard, and R. G. Crystal. 1987. Normal alveolar epithelial lining fluid contains high levels of glutathione. *J Appl Physiol 63:152*.

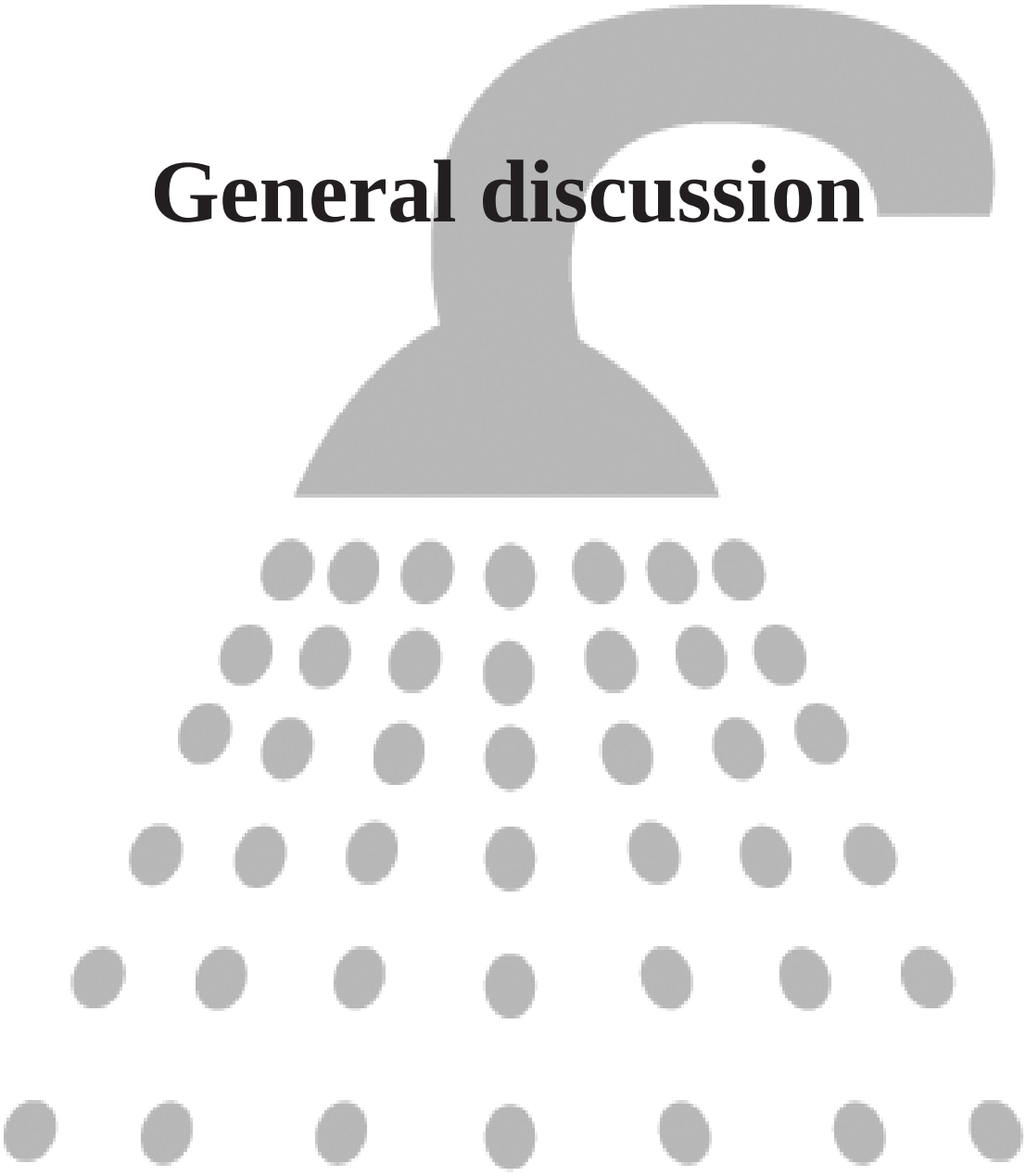
13. Hatch, G. E. 1992. Comparative biochemistry of airway lining fluid. In *Comparative biology of the normal lung*, Vol. 1. R. A. Parent, ed. CRC Press, Boca Raton, p. 617.
14. Kelly, F. J. 1999. Gluthathione: in defence of the lung. *Food Chem Toxicol 37:963*.
15. Lange, R. W., B. W. Day, R. Lemus, V. A. Tyurin, V. E. Kagan, and M. H. Karol. 1999. Intracellular S-glutathionyl adducts in murine lung and human bronchoepithelial cells after exposure to diisocyanatotoluene. *Chem Res Toxicol 12:931*.
16. Lantz, R. C., R. Lemus, R. W. Lange, and M. H. Karol. 2001. Rapid reduction of intracellular glutathione in human bronchial epithelial cells exposed to occupational levels of toluene diisocyanate. *Toxicol Sci 60:348*.
17. Kloek, J., G. Folkerts, N. Bloksma, C. M. Lilly, F. De Clerck, J. M. Drazen, and F. P. Nijkamp. 2001. Glutathione modulates non-specific and allergen-induced airway responsiveness in the guinea pig. In *ATS*, Vol. 163. Am J Respir Crit Care Med, San Francisco, California, p. A925.
18. Day, B. W., R. Jin, D. M. Basalyga, J. A. Kramarik, and M. H. Karol. 1997. Formation, solvolysis, and transcarbamoylation reactions of bis(S- glutathionyl) adducts of 2,4- and 2,6-diisocyanatotoluene. *Chem Res Toxicol 10:424*.
19. Fabbri, L. M., P. Boschetto, E. Zocca, G. Milani, F. Pivirotto, M. Plebani, A. Burlina, B. Licata, and C. E. Mapp. 1987. Bronchoalveolar neutrophilia during late asthmatic reactions induced by toluene diisocyanate. *Am Rev Respir Dis 136:36*.
20. Park, H., K. Jung, H. Kim, D. Nahm, and K. Kang. 1999. Neutrophil activation following TDI bronchial challenges to the airway secretion from subjects with TDI-induced asthma. *Clin Exp Allergy 29:1395*.
21. Redlich, C. A., A. V. Wisnewski, and T. Gordon. 2002. Mouse models of diisocyanate asthma. *Am J Respir Cell Mol Biol 27:385*.
22. Lee, Y. C., Y. G. Kwak, and C. H. Song. 2002. Contribution of vascular endothelial growth factor to airway hyperresponsiveness and inflammation in a murine model of toluene diisocyanate-induced asthma. *J Immunol 168:3595*.
23. Scheerens, H., T. L. Buckley, E. M. Davidse, J. Garssen, F. P. Nijkamp, and H. Van Loveren. 1996. Toluene diisocyanate-induced in vitro tracheal hyperreactivity in the mouse. *Am J Respir Crit Care Med 154:858*.
24. Karol, M. H. 2001. Bonding and transfer: do epithelial conjugates have a role in chemical asthma? *Clin Exp Allergy 31:357*.
25. Smith, L. J., J. Anderson, and M. Shamsuddin. 1992. Glutathione localization and distribution after intratracheal instillation. Implications for treatment. *Am Rev Respir Dis 145:153*.
26. Kloek, J., I. van Ark, N. Bloksma, F. De Clerck, F. P. Nijkamp, and G. Folkerts. 2002. Glutathione and other low-molecular-weight thiols relax guinea pig trachea ex vivo: interactions with nitric oxide? *Am J Physiol Lung Cell Mol Physiol 283:L403*.
27. Kloek, J. 2001. Acute glutathione depletion underlies the early asthmatic response in a guinea pig model of allergic asthma. In *Faculty of Pharmaceutical Sciences*. Utrecht University, Utrecht, p. 47.

28. Bagnato, G. F., S. Gulli, R. De Pasquale, O. Giacobbe, G. Spatari, and F. Purello D'Ambrosio. 1999. Effect of inhaled glutathione on airway response to 'Fog' challenge in asthmatic patients. *Respiration 66:518*.

29. Lamson, D. W., and M. S. Brignall. 2000. The use of nebulized glutathione in the treatment of emphysema: a case report. *Altern Med Rev 5:429*.

7

General discussion



Asthma is a very common disorder and its prevalence has increased over the past two to three decades. The proportion of cases attributable to occupational exposure at the workplace is estimated at ~10% of adult-onset asthma. The number of cases is thought to increase in the future, mainly due to new compounds that are introduced into the workplace. Occupational asthma can be caused by HMW compounds that induce occupational asthma via an IgE-mediated mechanism, but most cases of occupational asthma are caused by LMW compounds. These compounds are too small to be immunogenic by themselves and need to bind to endogenous proteins upon entrance of the body to do so. Although in only a minority of cases specific IgE antibodies could be demonstrated, LMW compound-induced occupational asthma, developed upon prolonged exposure to low concentrations, is thought to be immunologically mediated. In many studies, using both human subjects and animal models, investigators have tried to unravel the mechanisms underlying LMW compound-induced occupational asthma. However, the mechanisms have not been clearly identified. Most of the studies have focussed on the induction of specific antibodies, cytokines, and the presence and activity of inflammatory cells, like T cells, eosinophils, and neutrophils. Relatively little is known about the role of the alveolar macrophage (AM) in LMW compound-induced occupational asthma. This is rather surprising, since AMs are one of the first cells to encounter these LMW compounds upon inhalation due to their location at the interface between air and lung tissue. For that reason, it was tried in the present thesis to unravel some of the functions of AMs in LMW compound-induced occupational asthma.

Depletion of AMs to study their role in LMW compound-induced occupational asthma

Depletion of AMs from the airways is a valid approach to study the role of AMs in asthma and other airway diseases. One of the most used methods to achieve this depletion is intratracheal instillation of clodronate-containing liposomes. In most studies, however, effects of the treatment with liposomes are not properly reported (Chapter 2, table 1). It has been shown that AM depletion by intratracheal instillation of particular preparations of clodronate-containing liposomes could be paralleled by neutrophilic inflammation (Chapter 2). Therefore, it was recognized that liposome preparations, to be used for intratracheal instillation, should be made under aseptic conditions with pyrogen-free components. Contamination with pyrogens of liposomes made under conventional conditions most probably caused the observed inflammatory cell influx into the lung lumen (Chapter 2). Although most studies addressing the effects of AM depletion in lung diseases used liposomes with a neutral bilayer, negatively charged liposomes were shown to be superior (Chapter 2). This is likely due to the intrinsic capacity of negatively charged liposomes to associate more effectively with, and deliver their content more efficiently to macrophages than neutral liposomes (1, 2).

Role of AMs in the early asthmatic response to challenge with LMW compounds

AMs are the predominant cell type in the airway lumen and have the capacity to release both pro- and anti-inflammatory mediators during airway inflammation (3, 4), indicating that these cells may have the capacity of affecting the asthmatic response both in a positive and negative way. Furthermore, it has been demonstrated that AMs from asthmatic patients have different phenotypes compared to healthy individuals (5-7). A positive effect of AM depletion was observed in TMA-sensitized BN rats when carried out prior to inhalation challenge with TMA, since it partly protected against the early asthmatic response (Chapter 3). However, when such animals were challenged with TMA-BSA, instead of TMA, no protection was observed (Chapter 4). The protein nature of TMA-BSA was probably not responsible for the lack of protection. This is indicated by the observation that the early asthmatic response to an ovalbumin aerosol in ovalbumin-sensitized BN rats was prevented when their AM population was replaced by naive AMs before challenge (8). Moreover, the differential effect of AM depletion can not be explained by the differential effects of TMA and TMA-BSA on AMs *in vitro*, since TMA lacked any effect, while TMA-BSA stimulated the production of mediators by primary BN AMs and NR8383 cells (Chapter 5). This suggests that AMs possibly modulate the reaction of other cell types in the airways to TMA, but not to TMA-BSA. However, since the *in vitro* study did not measure acute AM responses to TMA and TMA-BSA (Chapter 5), while AMs clearly affected the early asthmatic response to TMA (Chapter 3), the *in vitro* observations probably can not aid understanding of the role of AMs in the early asthmatic response. More research into the direct effects of TMA and TMA-BSA on AMs is necessary to gain information about their role in the early asthmatic response.

Role of AMs in allergic airway inflammation due to challenge with LMW compounds

Although AMs apparently are not major players in the early asthmatic response of the airways after inhalation of both TMA and TMA-BSA, these cells do play a role in the inflammatory response to these compounds (Chapters 3 and 4). Their function in the rat model for TMA-induced occupational asthma was found to be suppressive, since in the absence of AMs the inflammatory reaction both to TMA and TMA-BSA was increased. The increased inflammatory response in the absence of AMs in both sensitized and control rats indicates that the role of the AM in the inflammatory response to TMA and TMA-BSA is mainly immunologically non-specific. This immunologically non-specific reaction of AMs was corroborated *in vitro*, since TMA-BSA induced similar mediator production in primary AMs derived from sensitized and control rats (Chapter 5). The observation that TMA did not stimulate mediator production by AMs *in vitro* was likely to be due to its inability to form

appropriate conjugates because of the static culture conditions (Chapter 5). Irritant properties of both TMA and TMA-BSA are likely to be responsible for the non-specific inflammatory reaction in the absence of AMs, since irritation-induced increases in inflammatory parameters due to LMW compounds have been observed before (9-11). Furthermore, the influx of neutrophils into the airways of AM-depleted sensitized and control rats induced by TMA-BSA, which was not seen in sham-depleted rats supports this opinion (Chapter 4). Thus, AMs apparently are protective against irritation by TMA and TMA-BSA.

Since maleylated-BSA has structural similarities with TMA-BSA and induces the production of mediators by macrophages via scavenger receptors (12-14), it is likely that the immunologically non-specific activation of AMs by TMA-BSA *in vitro* is regulated via these scavenger receptors. The fact that these receptors play a role in innate immunity due to their ability to bind to and subsequently remove pathogens and apoptotic cells, thereby suppressing a local inflammatory response (15, 16), suggests that these receptors are also of importance in the regulation of the inflammatory reaction due to TMA-BSA and TMA *in vivo.*

The effects of LMW compound-carrier conjugates in animal models for LMW compound-induced occupational asthma

In vitro preformed conjugates of LMW compounds with proteins, e.g. albumin, are frequently used for airway challenge to study airway responses in animal models of LMW compound-induced occupational asthma, since challenge with free LMW compounds often requires specific and expensive equipment and many safety precautions. Moreover, conjugation of LMW compounds to endogenous proteins is considered necessary to enable cross-linking of mast cell-bound IgE in order to induce mast cell degranulation (17). The fact that mediator production was induced *in vitro* only by TMA-BSA, but not by TMA, indicates that conjugation is probably also necessary for AM activation *in vitro* (Chapter 5). Conjugates of LMW compounds with endogenous structures, like albumin and hemoglobin, have been observed in lung tissue and blood after exposure to these compounds (18-23). However, it is not known which LMW compound-protein conjugates mediate the effects observed in asthmatic patients (24). The conjugates used to study the effects of LMW compounds in animal models do not necessarily induce similar asthma-like symptoms as observed in patients with LMW compound-induced occupational asthma. TDI conjugated to reduced glutathione, a compound that is normally abundantly present in the airway lining fluid, did not induce tracheal hyperreactivity but induced immunologically specific airway inflammation in TDI-sensitized mice after intratracheal instillation (Chapter 6). Although it was observed that both TMA

and TMA-BSA induced a comparable early asthmatic response in sensitized BN rats following respiratory challenge, their effects diverged for the inflammatory reaction (Chapters 3 and 4). Thus, the fact that TMA and the conjugates TMA-BSA and GS-TDI only partly induced the same effects in our rodent models for occupational asthma as induced in human patients, is of importance for estimation of the usefulness these animal models; not only for studies into the mechanisms that underlie LMW compound-induced occupational asthma but also for toxicological hazard identification. Moreover, since TMA challenge partly induced different effects compared to challenge with TMA-BSA, inhalation challenge with the free compound is preferred in order to make the right assumptions of the effects of LMW compounds on the airways of patients with LMW-induced occupational asthma.

Final conclusion

The experiments described in this thesis show that the role of AMs in a model for TMA-induced occupational asthma is not straightforward. These cells intensify the early asthmatic response but only in reaction to TMA. Their role in allergic airway inflammation is mainly immunologically non-specific. Due to their capacity to reduce irritant properties of TMA and TMA-BSA they probably have an important function in the clearance of these compounds from the airways.

References

1. Van Rooijen, N., and A. Sanders. 1994. Liposome mediated depletion of macrophages: mechanism of action, preparation of liposomes and applications. *J Immunol Methods 174:83*.
2. Ahsan, F., I. P. Rivas, M. A. Khan, and A. I. Torres Suarez. 2002. Targeting to macrophages: role of physicochemical properties of particulate carriers—liposomes and microspheres—on the phagocytosis by macrophages. *J Control Release 79:29*.
3. Lee, T. H., and S. J. Lane. 1992. The role of macrophages in the mechanisms of airway inflammation in asthma. *Am Rev Respir Dis 145:S27*.
4. Sirois, J., G. Menard, A. S. Moses, and E. Y. Bissonnette. 2000. Importance of histamine in the cytokine network in the lung through H2 and H3 receptors: stimulation of IL-10 production. *J Immunol 164:2964*.
5. Godard, P., J. Chaintreuil, M. Damon, M. Coupe, O. Flandre, A. Crastes de Paulet, and F. B. Michel. 1982. Functional assessment of alveolar macrophages: comparison of cells from asthmatics and normal subjects. *J Allergy Clin Immunol 70:88*.
6. Lensmar, C., J. Prieto, B. Dahlen, A. Eklund, J. Grunewald, and A. Roquet. 1999. Airway inflammation and altered alveolar macrophage phenotype pattern after repeated low-dose allergen exposure of atopic asthmatic subjects. *Clin Exp Allergy 29:1632*.

7. Alexis, N. E., J. Soukup, S. Nierkens, and S. Becker. 2001. Association between airway hyperreactivity and bronchial macrophage dysfunction in individuals with mild asthma. *Am J Physiol Lung Cell Mol Physiol 280:L369*.
8. Careau, E., and E. Y. Bissonnette. 2004. Adoptive Transfer of Alveolar Macrophages Abrogates Bronchial Hyperresponsiveness. *Am J Respir Cell Mol Biol 31:22*.
9. Arts, J. H., N. Bloksma, T. Leusink-Muis, and C. F. Kuper. 2003. Respiratory allergy and pulmonary irritation to trimellitic anhydride in Brown Norway rats. *Toxicol Appl Pharmacol 187:38*.
10. Pauluhn, J., M. Emura, U. Mohr, A. Popp, and M. Rosenbruch. 1999. Two-week inhalation toxicity of polymeric diphenylmethane-4, 4'-diisocyanate (PMDI) in rats: analysis of biochemical and morphological markers of early pulmonary response. *Inhal Toxicol 11:1143*.
11. Swiercz, R., K. Rydzynski, J. Jajte, J. Stetkiewicz, and W. Majcherek. 2000. Studies on dermal, ocular and respiratory effects of 4-ethyltoluene in experimental animals. *Int J Occup Med Environ Health 13:307*.
12. Alford, P. B., Y. Xue, S. F. Thai, and R. E. Shackelford. 1998. Maleylated-BSA enhances production of nitric oxide from macrophages. *Biochem Biophys Res Commun 245:185*.
13. Paulnock, D. M., K. P. Demick, and S. P. Coller. 2000. Analysis of interferon-gamma-dependent and -independent pathways of macrophage activation. *J Leukoc Biol 67:677*.
14. Coller, S. P., and D. M. Paulnock. 2001. Signaling pathways initiated in macrophages after engagement of type A scavenger receptors. *J Leukoc Biol 70:142*.
15. Platt, N., and S. Gordon. 2001. Is the class A macrophage scavenger receptor (SR-A) multifunctional? - The mouse's tale. *J Clin Invest 108:649*.
16. Peiser, L., and S. Gordon. 2001. The function of scavenger receptors expressed by macrophages and their role in the regulation of inflammation. *Microbes Infect 3:149*.
17. Holliday, M. R., J. W. Coleman, R. J. Dearman, and I. Kimber. 1993. Induction of mast cell sensitization by chemical allergens: a comparative study. *J Appl Toxicol 13:137*.
18. Griffin, P., L. Allan, P. Beckett, J. Elms, and A. D. Curran. 2001. The development of an antibody to trimellitic anhydride. *Clin Exp Allergy 31:453*.
19. Karol, M. H. 2001. Bonding and transfer: do epithelial conjugates have a role in chemical asthma? *Clin Exp Allergy 31:357*.
20. Lange, R. W., R. C. Lantz, D. B. Stolz, S. C. Watkins, P. Sundareshan, R. Lemus, and M. H. Karol. 1999. Toluene diisocyanate colocalizes with tubulin on cilia of differentiated human airway epithelial cells. *Toxicol Sci 50:64*.
21. Jin, R., B. W. Day, and M. H. Karol. 1993. Toluene diisocyanate protein adducts in the bronchoalveolar lavage of guinea pigs exposed to vapors of the chemical. *Chem Res Toxicol 6:906*.
22. Day, B. W., R. Jin, and M. H. Karol. 1996. In vivo and in vitro reactions of toluene diisocyanate isomers with guinea pig hemoglobin. *Chem Res Toxicol 9:568*.
23. Kennedy, A. L., T. R. Wilson, M. F. Stock, Y. Alarie, and W. E. Brown. 1994. Distribution and reactivity of inhaled ^{14}C-labeled toluene diisocyanate (TDI) in rats. *Arch Toxicol 68:434*.
24. Karol, M. H., O. T. Macina, and A. Cunningham. 2001. Cell and molecular biology of chemical allergy. *Ann Allergy Asthma Immunol 87:28*.

Samenvatting

Astma is een chronische aandoening van de luchtwegen. Het is geschat dat wereldwijd ongeveer 300 miljoen mensen deze aandoening hebben. De belangrijkste symptomen zijn piepen, hoesten, benauwdheid en het hebben van een beklemmend gevoel op de borst. De symptomen kunnen variëren van mild tot levensbedreigend. Astma kan zich ontwikkelen na langdurige blootstelling aan stoffen in de omgeving, de zogenaamde allergenen, zoals bijvoorbeeld huisstofmijt en pollen. In de daarvoor gevoelige personen zullen na contact met het allergeen specifieke antistoffen worden gemaakt, de zogenoemde IgE antilichamen. Deze IgE antilichamen binden zich aan mestcellen die in de luchtwegen aanwezig zijn, dit proces heet sensibilizatie. Bij een tweede contact met het allergeen kan dit binden aan de IgE antilichamen op de mestcellen. Deze binding zorgt voor mestcelactivatie en de uitscheiding van mestcelmediatoren. Deze stoffen zijn verantwoordelijk voor de vernauwing van de luchtwegen waardoor patiënten zich benauwd voelen. Tevens stimuleren de mestcelmediatoren de slijmproductie en zorgen ze voor een verhoogde doorlaatbaarheid van de bloedvaten, waardoor er vocht in het longweefsel kan komen. Door de mestcelmediatoren worden ook ontstekingscellen, zoals T cellen, eosinofiele granulocyten en macrofagen, aangetrokken, resulterend in ontsteking van de luchtwegen. De ontstekingscellen scheiden samen met lokale cellen, zoals epitheel- en spiercellen, ook ontstekingsmediatoren uit. Verder zijn de luchtwegen van astmapatiënten gevoeliger voor specifieke stimuli (allergenen) maar ook aspecifieke stimuli (o.a. koude lucht en sigarettenrook) dan gezonde personen. Dit wordt hyperreactiviteit van de luchtwegen genoemd. Doordat men vaak langdurig is blootgesteld aan allergenen, zal de ontsteking van de luchtwegen een chronisch karakter krijgen. Als gevolg van deze chronische onsteking verandert de structuur van het longweefsel wat weer verantwoordelijk is voor een afnemende longfunctie die na verloop van jaren wordt gemeten in astmapatiënten.

In ongeveer 10% van de volwassenen die astma ontwikkelen, wordt de ziekte veroorzaakt door blootstelling aan stoffen op het werk en wordt beroepsastma genoemd. Van ongeveer 250 stoffen is het bekend dat ze beroepsastma kunnen veroorzaken. Het aantal gevallen van beroepsastma zal in de toekomst waarschijnlijk toenemen doordat steeds nieuwe stoffen worden geïntroduceerd in de industrie. Beroepsastma kan veroorzaakt worden door stoffen met een hoog molecuulgewicht. Dit zijn meestal eiwitten die voorkomen in meel, latex, in urine en huidschilfers van dieren. De grootste groep stoffen die beroepsastma veroorzaken zijn echter stoffen met een laag molecuulgewicht. Dit zijn meestal chemicaliën zoals isocyanaten, zure anhydriden en reactieve kleurstoffen. Deze stoffen zijn op zich zelf te klein om door het lichaam opgemerkt te worden maar door binding aan lichaamseigen stoffen veroorzaken zij ook een reactie. Ondanks het feit dat in

patiënten met beroepsastma door blootstelling aan deze chemicaliën de IgE-specifieke antilichamen tegen deze stoffen vaak niet zijn aan te tonen, veronderstelt men dat een immunologisch mechanisme ten grondslag ligt aan deze aandoening.

Er zijn al veel studies gedaan naar de onderliggende mechanismen van beroepsastma veroorzaakt door stoffen met een laag molecuulgewicht in zowel patiënten als in proefdiermodellen. In het merendeel van deze studies was de focus gericht op de inductie van specifieke antilichamen, de productie van ontstekingsmediatoren en de aanwezigheid van ontstekingscellen, zoals T cellen en eosinofiele granulocyten. Er is echter erg weinig bekend over de rol van alveolaire macrofagen (AMs) in beroepsastma veroorzaakt door stoffen met een laag molecuulgewicht. Dit is enigszins vreemd, aangezien AMs, door hun unieke locatie op de scheiding tussen lucht en longweefsel, één van de eerste cellen zijn die in contact komen met allergenen. AMs zijn de meest voorkomende cellen in het lumen van de luchtwegen, ~ 95% van de cellen in de longspoelvloeistof van gezonde personen zijn AMs. Het zijn belangrijke cellen in de afweer tegen en het opruimen van ingeademde deeltjes en micro-organismen. AMs kunnen een breed scala aan mediatoren uitscheiden die ontstekings- en immuunreacties in de longen reguleren. Tevens is het aangetoond dat ze een belangrijke rol spelen in het verminderen van de locale immuunrespons tegen ingeademde allergenen.

In dit proefschrift is de rol van AMs bestudeerd in 2 diermodellen voor beroepsastma geïnduceerd door stoffen met een laag molecuulgewicht, namelijk een rattenmodel voor beroepsastma geïnduceerd door trimellietzuuranhydride (TMA) en een muizenmodel voor beroepsastma geïnduceerd door toluene diisocyanaat (TDI). Een goede manier om de rol van AMs te bestuderen is door de cellen te depleteren uit de luchtwegen. Dit kan gedaan worden met behulp van liposomen (inerte balletjes), die gevuld zijn met clodronaat. Nadat de met clodronaat gevulde liposomen in de longen via de luchtpijp zijn ingebracht, zullen ze specifiek worden opgenomen door AMs waarna het clodronaat in de cel vrijkomt. Dit zorgt ervoor dat deze cellen in apoptose gaan, een vorm van celdood waarbij cellen geen ontstekingsreactie opwekken in het omliggende weefsel. In **hoofdstuk 2** is aangetoond dat de oppervlaktelading van de liposomen belangrijk is in het effect dat ze hebben. Liposomen met een negatieve lading zorgen voor een betere depletie van AMs in vergelijking met liposomen zonder lading. Verder is ook aangetoond dat het belangrijk is om met liposomen te werken die gemaakt zijn onder steriele omstandigheden met schone componenten. Is dit niet het geval dan wordt een ontstekingsreactie opgewekt in de luchtwegen die gepaard gaat met het aantrekken van ontstekingscellen in plaats van het depleteren van AMs.

In **hoofdstuk 3** is de rol van AMs bestudeerd in een ratmodel voor beroepsastma geïnduceerd door de chemische stof TMA. In dit model zijn ratten gesensibiliseerd met TMA op dag 0 en 7. De AMs zijn op dag 20 gedepleteerd, dit is 1 dag voor de luchtwegblootstelling (challenge) met TMA.

Tijdens de challenge met TMA werd een vroege astmatische reactie gemeten in de gesensibiliseerde dieren maar niet in de controle dieren. In de gesensibiliseerde dieren zonder AMs was de vroege astmatische reactie minder ernstig en was het herstel van de luchtwegfuncties na de challenge sneller. Sensibilisatie met TMA resulteerde in een verhoging van zowel totaal als specifiek IgE. De waarden van IgE waren lager 24 uur na challenge in de gesensensibiliseerde ratten zonder AMs dan in de gesensibiliseerde ratten met AMs. De depletie van AMs voor de challenge had geen effect op de sensibilisatie-afhankelijke inductie van de ontstekingsmediatoren interleukine 6 (IL-6) en tumor necrosis factor alpha (TNF-α) 24 uur na challenge. AM depletie had echter wel een effect op de niet-specifieke weefselschade, zoals aangetoond door een toename in de schademarker lactaat dehydrogenase (LDH). De influx van ontstekingscellen leek versterkt te worden in afwezigheid van AMs, hoewel deze gegevens niet statistisch significant waren. Deze resultaten tonen dat AMs een dubbele rol spelen in het model voor TMA-geïnduceerd beroepsastma. Het lijkt erop dat de aanwezigheid van AMs de vroege astmatische reactie op TMA verergert terwijl 24 uur later deze cellen juist de ontstekingsreactie dempen.

In **hoofdstuk 4** de rol van AMs onderzocht na challenge met TMA gekoppeld aan albumine (TMA-BSA) in hetzelfde model als beschreven in hoofdstuk 3. TMA is een klein reactief molecuul dat bindt aan lichaamseigen stoffen. Conjugaten van TMA met het eiwit albumine zijn aangetoond in bloed van patiënten met beroepsastma geïnduceerd door TMA. Challenge met TMA-BSA zorgde voor een vroege astmatische reactie van de luchtwegen in de gesensibiliseerde ratten. De depletie van AMs 1 dag voor challenge had geen effect op de vroege astmatische reactie. Inhalatie van TMA-BSA resulteerde 24 uur na challenge niet in schade en influx van ontstekingscellen in de luchtwegen van de gesensibiliseerde en controle ratten met AMs. In tegenstelling tot beide groepen ratten met AMs werd in de ratten zonder AMs 24 uur na TMA-BSA inhalatie juist wel schade en onstekingscellen in de luchtwegen gemeten, onafhankelijk van sensibilisatie. Dit suggereert een dempende rol van AMs op de immunologisch niet-specifieke schade en influx van ontstekingscellen in de luchtwegen.

In **hoofdstuk 5** zijn de effecten beschreven van TMA en TMA-BSA op de productie van de macrofaagmediatoren stikstof oxide (NO), TNF-α en IL-6. Deze mediatoren zijn gemeten in celkweken met de ratten AM cellijn NR8383 en primaire AMs die geïsoleerd zijn uit gesensibiliseerde en controle ratten. De cellen zijn 24 uur geïncubeerd met verschillende concentraties van TMA, TMA-BSA en als controle BSA, waarna de celvrije kweekvloeistof geanalyseerd is voor de verschillende mediatoren. TMA en BSA alleen waren niet in staat om de productie van de mediatoren te induceren in zowel de cellijn als in de primaire cellen. Dit was echter wel het geval voor TMA-BSA, dat een concentratie-afhankelijke productie stimuleerde van NO, TNF-α en IL-6 in de NR8383

cellen en van NO en TNF-α, maar niet van IL-6, in de primaire cellen. Deze resultaten laten zien dat, ondanks het feit dat TMA een zeer reactieve stof is, de binding aan een geschikt eiwit nodig is om de bovengenoemde mediatoren te induceren. De effecten van TMA-BSA op de cellen waren niet afhankelijk van sensibilisatie, wat suggereert dat een immunologisch niet-specifieke receptor betrokken kan zijn bij de stimulatie van de cellen. Zo'n immunologisch niet-specifieke receptor zou bijvoorbeeld een scavenger-receptor kunnen zijn. Deze receptoren zijn aanwezig op macrofagen en zijn betrokken bij het verwerken van bacteriën en gemodificeerde eiwitten.

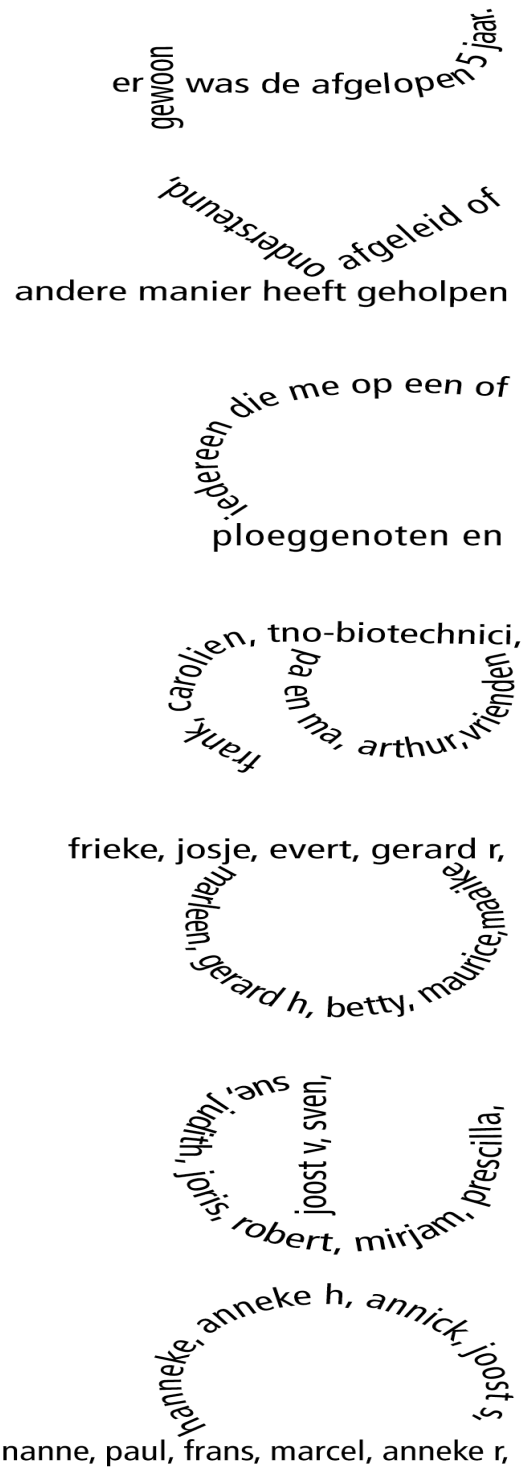
TDI is een klein reactief molecuul dat net als TMA beroepsastma kan veroorzaken. In **hoofdstuk 6** is onderzocht of TDI gekoppeld aan glutathion een irriterende of allergische reactie kan veroorzaken in de muis. Glutathion is een molecuul dat in hoge concentratie aanwezig is in de luchtwegen. Het heeft een beschermende functie en het is aangetoond dat na inademen van TDI de glutathionspiegels zijn verlaagd. De irriterende effecten van verschillende doses van het gekoppelde TDI (GS-TDI) zijn bestudeerd in naïeve muizen 24 uur na toediening in de longen via de luchtpijp. Het GS-TDI is in staat om 24 uur na toediening een dosisafhankelijke irritatie te veroorzaken, die gekenmerkt wordt door de influx van neutrofiele granulocyten in de luchtwegen. Een dosisequivalent van 150 μg TDI of minder veroorzaakte geen influx van ontstekingscellen. Deze dosis is daarom gebruikt om een allergische reactie op te wekken in gesensibiliseerde muizen. Deze muizen zijn gesensibiliseerd op dag 0 en 1 met TDI en op dag 8 hebben ze via de luchtpijp een toediening met GS-TDI gekregen. Het conjugaat was niet in staat om 24 en 48 uur na toediening hyperreactiviteit van de luchtwegen te induceren. Het veroorzaakte echter wel een influx van ontstekingscellen in de luchtwegen. Deze resultaten geven aan dat de koppeling van glutathion met TDI de reactiviteit van TDI niet vermindert, aangezien het een irritatiereactie induceerde in naïeve muizen. Een mogelijke verklaring voor de observatie dat de niet-irriterende dosis GS-TDI wel een allergische ontsteking maar geen hyperreactiviteit van de luchtwegen veroorzaakte in gesensibiliseerde muizen kan zijn dat na toediening het glutathion vrijkomt uit het conjugaat. Deze stof is normaal belangrijk in de bescherming van de luchtwegen tegen schadelijke stoffen en zou kunnen zorgen voor relaxatie van de spieren in de luchtwegen.

De experimenten beschreven in dit proefschrift tonen aan dat de rol van de AMs niet eenzijdig is in het rattenmodel voor TMA-geïnduceerde beroepsastma. De cellen verergeren de vroege astmatische reactie, maar alleen als reactie op TMA en niet op TMA-BSA. De rol van AMs in de allergische luchtwegontsteking is voor een groot deel immunologisch niet-specifiek. AMs spelen waarschijnlijk een belangrijke rol in het opruimen van deze stoffen, met name door de capaciteit van de AMs om de irritatie als gevolg van inhalatie van TMA en TMA-BSA te verminderen. Een belangrijke observatie is dat TMA en de conjugaten TMA-BSA en GS-TDI maar gedeeltelijk dezelfde

effecten induceren in de proefdiermodellen. Dit is belangrijk voor het schatten van de waarde van deze proefdiermodellen. Het is niet alleen belangrijk voor studies waarin de onderliggende mechanismen bestudeerd worden maar ook voor de studies naar toxicologische risicoschattingen van reactieve chemicaliën. Het feit dat inhalatie van TMA maar gedeeltelijk dezelfde effecten induceerde in vergelijking met TMA-BSA, geeft aan dat inhalatieblootstelling van proefdieren met de ongebonden stof de voorkeur geniet boven de gebonden vorm. Dit is nodig om de juiste conclusies te trekken over de effecten van stoffen met een laag molecuulgewicht op de luchtwegen van patiënten met beroepsastma geïnduceerd door deze stoffen.

Dankwoord

Het is dan eindelijk zo ver, het proefschrift is af.
Dus de tijd is gekomen om te zeggen:



Ik had het niet zonder jullie kunnen doen!

Inge

Curriculum vitae

De auteur van dit proefschrift werd geboren op 6 juni 1973 te ‘s-Gravenhage. Na het behalen van het HAVO diploma in juni 1990 en het Atheneum diploma in juni 1992, beiden aan de Dalton Scholengemeenschap te ‘s-Gravenhage, begon zij in augustus 1992 aan de opleiding Biomedische Wetenschappen aan de Faculteit der Geneeskunde van de Rijks Universiteit Leiden. Tijdens de doctoraalfase van de studie werden drie onderzoeksstages voltooid in het Academisch Ziekenhuis te Leiden. Onder begeleiding van Prof. M. Daha en ing. Y. Muizert van de afdeling Nierziekten deed zij 3 maanden onderzoek naar een nieuwe methode om het complement CH50 en AP50 te analyseren in serum van patiënten. Vervolgens deed ze 6 maanden onderzoek naar de rol van basisch fibroblast groeifactor in de proliferatie en differentiatie van keratinocyten onder begeleiding van dr. S. Gibbs en dr. M. Ponec van de afdeling Dermatologie. De studie werd afgesloten met een 9-maandse afstudeerstage op de afdeling Infectieziekten onder begeleiding van dr. P. Nibbering waarin werd onderzocht wat de rol was van antimicrobiële eiwitten geproduceerd door bronchiaal epitheelcellen in de afweer tegen luchtweginfecties. Na het behalen van haar doctoraalexamen in augustus 1997 heeft ze 10 maanden gewerkt in een kibboetz in Israël. In augustus 1999 is ze begonnen met een promotieonderzoek bij de disciplinegroep Farmacologie en Pathofysiology van de Faculteit Farmaceutische Wetenschappen aan de Universiteit Utrecht. Dit onderzoek heeft ze uitgevoerd in samenwerking met ing. M. Schijf onder leiding van dr. N. Bloksma, dr. P. Henricks en prof. dr. F. Nijkamp. De resultaten van het onderzoek staan beschreven in dit proefschrift.

List of publications and abstracts

Articles

Valstar D.L, Schijf M.A., Nijkamp F.P., Storm G., Arts J.E.H., Kuper C.F., Bloksma N., Henricks P.A.J. Effect of alveolar macrophage depletion on lung function and airway inflammation in a rat model for trimellitic anhydride-induced occupational asthma. (*submitted*)

Valstar D.L, Schijf M.A., Nijkamp F.P., Bloksma N., Henricks P.A.J. (2004). Glutathione-conjugated toluene diisocyanate causes airway inflammation in sensitised mice. Archives of Toxicology (*in press*).

Gibbs S., Vicanó J., Bouwstra J., Valstar D., Kempenaar J., Ponec M. (1997). Culture of reconstructed epidermis in a defined medium at 33°C shows a delayed epidermal maturation, prolonged lifespan and improved stratum corneum. Archives of Dermatological Research 289:585-595.

Abstracts

Valstar D.L, Schijf M.A., Henricks P.A.J., Nijkamp F.P., Arts J.H.E., Kuper C.F., Bloksma N (2004). Effect of macrophage depletion on lung function and airway inflammation in a rat model for TMA-induced occupational asthma. Naunyn-Schmiedebergs Archives of Pharmacology 369, suppl.1: R177.

Valstar D.L, Schijf M.A., Henricks P.A.J., Nijkamp F.P., Arts J.H.E., Kuper C.F., Bloksma N (2003). Effect of macrophage depletion on lung function and airway inflammation in a rat model for TMA-induced occupational asthma. FASEB Journal 17(7): C250.

Valstar D.L., M.A. Schijf, P.A.J. Henricks, N. Bloksma, F.P. Nijkamp (2002). Airway challenge of sensitized mice with glutathione-conjugated toluene diisocyanate (TDI) gives an influx of inflammatory cells into the lungs. American Journal of Respiratory and Critical Care Medicine 165(8): A523.

Valstar D.L., Henricks P.A.J., Kraneveld A.D., Thepen T., van Rooijen N., Nijkamp F.P. (2001). Alveolar macrophages are important in the development of tracheal hyperreactivity during pulmonary DTH reactions. *Keystone Symposium* "Macrophage activation and deactivation: links between innate and acquired immunity".