De lokkende roep van het sneeuwhoen…

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# Adjuvant activity of particulate air pollutants

Adjuvant activiteit van deeltjesvormige luchtverontreiniging

(met een samenvatting in het Nederlands)

Proefschrift

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door

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**Abbreviations**

| | |
|---|---|
| ANOVA | analysis of variance |
| AP | alkaline phosphatase |
| APC | antigen presenting cell |
| BAL | bronchial alveolar lavage |
| BSA | bovine serum albumin |
| CBP | carbon black particles |
| CD | cluster of differentiation |
| DC | dendritic cell |
| DEP | diesel exhaust particles |
| DEP1 | derived from J. L. Mauderly |
| DEP2 | derived from prof dr P. Scheepers |
| DEP3 | derived from dr F. Cassee |
| EAE | experimental autoimmune encephalomyelitis |
| ELISA | enzyme-linked immunosorbent assay |
| ELISPOT | enzyme-linked immunosorbent spot assay |
| EO | eosinophil |
| FITC | fluorescen isothiocyanate |
| FCS | foetal calf serum |
| GM-CSF | granulocyte/macrophage-colony stimulating factor |
| IFN | interferon |
| Ig | immunoglobulin |
| IL | interleukin |
| KLH | keyhole limpet hemocyanin |
| MBP | major basic protein |
| MHC | major histocompatability complex |
| min | minute(s) |
| n | number |
| NK | natural killer |
| NO | nitrogen oxide |
| $NO_2$ | nitrogen dioxide |
| $O_3$ | ozone |
| OVA | ovalbumin |
| PBLN | peribronchial lymph nodes |
| PBS | phosphate-buffered saline |
| PBS-T | PBS supplemented with 0.05% Tween20 |
| PE | phycoerythrin |
| $PGE_2$ | prostaglandin E2 |
| PLN | popliteal lymph node |
| PLNA | popliteal lymph node assay |
| PM | particulate matter |
| PM10 | PM, mean aerodynamic diameter <10 µm |
| PUFA | polyunsaturated fatty acid |
| ROFA | residual oil fly ash |
| RT | room temperature |
| SI | stimulation index |
| sc | subcutaneously |
| SD | standard deviation |
| SIP | amorphous silica particles |
| $SO_2$ | sulfur dioxide |
| TCR | T cell receptor |
| Tc | T cytotoxic |
| TGF | transforming growth factor |
| Th | T helper |
| TNF | tumor necrosis factor |
| TNP | 2,4,6-trinitrophenyl |
| TNP-OVA | trinitrophenyl-ovalbumin |

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# CHAPTER 1

# GENERAL INTRODUCTION

The prevalence of respiratory allergy has increased during the last few decades. Air pollution is one of the factors that may contribute to this process by stimulating immune responses to common allergens. Particulate matter (PM), a heterogeneous air pollutant, is involved in both acute and long-term respiratory effects. One of its main components, diesel exhaust particles (DEP), is known to have immunomodulating properties. The introduction to this thesis gives an overview of the present knowledge with respect to the role of air pollution in respiratory allergy.

**Basics of the adaptive immune response**

The immune system consists of two closely connected defense layers, the innate and the adaptive (also called specific) immune system. The first is evolutionary older and consists of barriers such as skin and mucosal surfaces and of broad pattern recognition leading to phagocytosis, complement activation, and extracellular killing. The specific immune system consists of B and T lymphocytes, which bear receptors that specifically bind to unique structures (antigens). The adaptive immune response has different effector mechanisms at its disposal, and forms a memory system to enable a faster and greater response at a subsequent exposure (Roitt 1988, Janeway and Travers 1994). T and B cells need 2 signals for activation (Bretscher 1992). Signal 1 is provided by antigen recognition, while signal 2 consists of non-specific costimulation. Signal 1 in the absence of signal 2 leads to inactivation by means of induction of unresponsiveness or apoptosis.

**T cell activation**

Two T cell subsets can be distinguished based on the expression of the accessory molecules cluster of differentiation (CD) CD4 and CD8. T helper (Th) cells express CD4 and use this molecule as a coreceptor in association with their antigen-specific T cell receptor (TCR) to recognize antigen. TCRs are generated by means of gene-segment recombination, leading to TCR polymorphism. The antigen is bound to major histocompatability complex class II (MHCII) present on antigen presenting cells (APC) and B cells and consists of enzymatically cleaved peptide fragments (8-10 amino acids) of protein antigen. A first signal for activation of the Th cell with the correct specificity is provided by the recognition of this MHCII-peptide complex via the TCR-CD3 complex. Recognition of a specific epitope by an effector Th cell induces the expression of CD40-ligand and CD28 on the T cell surface (Noelle *et al.,* 1992). Binding of this CD28 molecule to either B7-1 (CD80) or B7-2 (CD86) present on the APC generates signal 2 for T cell

activation (Clark and Ledbetter 1994). The activated Th cell on its turn can provide signal 2 for T cytotoxic (Tc) cells and B cells, and produces immunomodulating cytokines such as interleukin (IL)-2.
Cytotoxic T cells express CD8 in combination with their TCR, which recognizes antigen presented in context of MHC class-I molecules. The latter are expressed on all nucleated cells, and in general display peptide fragments of proteins present within the cell cytoplasm. However, dendritic cells (DC) are also able to present exogenous antigen on MHCI (Machy *et al.,* 2000). In case of presented non-self peptides and in the presence of signal 2, Tc cells can become effector cells that produce cytokines and lyse target cells. Signal 2 can be provided either by costimulation from the APC or by a Th cell specific for the same antigen.

**B cell activation**

B cells possess membrane-inserted immunoglobulins (Ig) by which they specifically recognize native protein, binding of which provides signal 1. Requirement for signal 2 depends on the nature of the antigen. The majority of antigens are so called thymus-dependent antigens, and B cells need costimulation from activated Th cells to respond to them (Clark and Ledbetter 1994). After binding, internalization and proteolytical cleavage, their epitopes are presented on MHCII molecules on the B cell surface. Ligation of CD40-ligand on the activated T cell with its receptor CD40 on the B cell delivers signal 2 leading to the activation of the B cell (Noelle *et al.,* 1992). Activated B cells that express costimulatory molecule B7-1 or B7-2 (CD80/CD86) are able to function as APC, and are receptive for cytokines augmenting proliferation and differentiation. Some of the daughter cells become long-lived memory cells, while most of them become antibody-producing plasma cells, initially secreting IgM. In the course of a response, B cells are able to switch to the production of other isotypes like IgG and IgE by means of gene-segment rearrangement of the constant part of the Ig molecule. The different isotypes activate distinct effector mechanisms after binding to antigen. In case of respiratory allergy the antibody switch to IgE is a crucial event, as is described in paragraph “IgE-mediated respiratory allergy”.

**T helper cell subsets**

Th cells in mouse (Mosmann *et al.,* 1986, Mosmann and Coffman 1989) and man (Wierenga *et al.,* 1990, Romagnani 1991) can be divided in the functional subsets Th1 and

Th2 cells, according to the pattern of cytokines they produce. Both arise from Th0 cells and direct effector mechanisms against intra- and extracellular antigens, respectively. While Th0 cells stimulate both type 1 and type 2 responses by producing a mixture of cytokines such as IL-2, IL-4 and interferon (IFN)γ, Th1 cells produce IFNγ and IL-2, and Th2 cells produce IL-4, IL-5, IL-6, IL-10 and IL-13 (Fig. 1) (Openshaw *et al.,* 1995). Type 1 responses are cellular responses characterized by activation of Tc cells and macrophages and production of IFNγ and IgG2a in mouse. The Th2 subset, on the other hand, provides support for humoral responses, characterized by IgG1 and IgE production, sensitization of mast cells, and eosinophil maturation (Kapsenberg *et al.,* 1991). Tc cells can likewise be divided into Tc1 and Tc2.

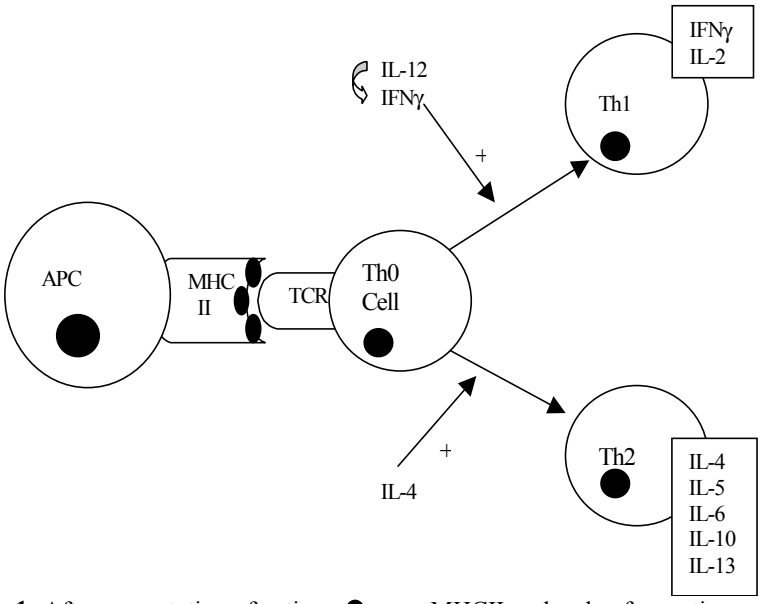


**Figure 1:** After presentation of antigen ● on a MHCII molecule of an antigen presenting cell (APC), the Th0 cell differentiates into either a Th1 or a Th2 cell, stimulated by respectively IFNγ or IL-4.

In addition to Th1 and Th2 cells, also Th3 cells, producing high amounts of transforming growth factor (TGF)β, have been defined (Mosmann and Sad, 1996). Initially, Th1 and Th2 cells were regarded as complete opposites, inhibiting each other's actions (Maggi *et al.,* 1992), and their balance determining which effector mechanism was activated (Romagnani *et al.,* 1997). However, in practice the complete distinction between Th1 and Th2 appears not always appropriate. Immune responses often comprise a mixture of Th1 (type 1) and Th2 (type 2) effector mechanisms, and Th1 and Th2 responses can be predominant at

different stages of the immune response (Mosmann and Sad 1996). Moreover, single Th cells are able to display great diversity in their cytokine profile (Kelso *et al.,* 1999), and instead of counteracting, Th1 and Th2 cells may even reinforce each other's actions (Randolph *et al.,* 1999, Hansen *et al.,* 1999). Therefore a model in which there are no discrete subsets but rather a continuum of different combinations of cytokine secretion may be more appropriate.

## IgE-mediated respiratory allergy

### *Sensitization and challenge*

Allergy can be defined as an inappropriate response to an innocuous foreign substance (Janeway and Travers 1994). The inherited tendency to develop high IgE responses to an antigen is called atopy. Several genes have been suggested to be involved in this tendency and to increase the risk and severity of allergy (Howard *et al.,* 2000). The definition of atopy is not clear, since several indicators such as total IgE, specific IgE, positive skin prick test and clinical manifestations have been used. Respiratory allergy to common protein antigens like pollen is, in contrast to low molecular weight allergens (Scheerens *et al.,* 1999), a Th2-driven, IgE-mediated, immediate reaction. However, in asthmatics possibly also Th1 cells play a role (Krug *et al.,* 1996, Krug and Frew 1997).

The allergic response can be divided in two temporally distinct phases, the sensitization and the challenge (Fig. 2). During sensitization, the antigen is presented to specific T cells, which differentiate into different subsets and memory cells. In IgE-mediated allergy the formation of a Th2 subset is pivotal for the production of specific IgE which binds to the FcεRI receptor on mast cells and basophils, thereby "sensitizing" them. During a second contact with the antigen, which is called the challenge, the antigen crosslinks the surface-bound IgE of the basophil/mastcell. This causes the cell to degranulate and release several mediators, such as histamines, leukotrienes, prostaglandins and cytokines, producing symptoms characteristic for respiratory allergy like rhinitis, conjunctivitis and airway hyperresponsiveness. While some conclude IgE is indispensable for hyperresponsiveness (Hamelmann *et al.,* 1997) and for eosinophil infiltration (Coyle *et al.,* 1996), others claim that alternative, IgE-independent pathways exist (Mehlop *et al.,* 1997). In addition to the key role of Th2 cells in the initiation of disease by inducing IgE production, their cytokines also play an important role in the effector phase. IL-4 (Corry *et al.,* 1996) and IL-5 (Foster *et al.,* 1996) appear to be crucial in processes like respectively hyperreactivity and eosinophilia, although differences between mice strains and sensitization protocols are observed (Hogan *et al.,* 1998).

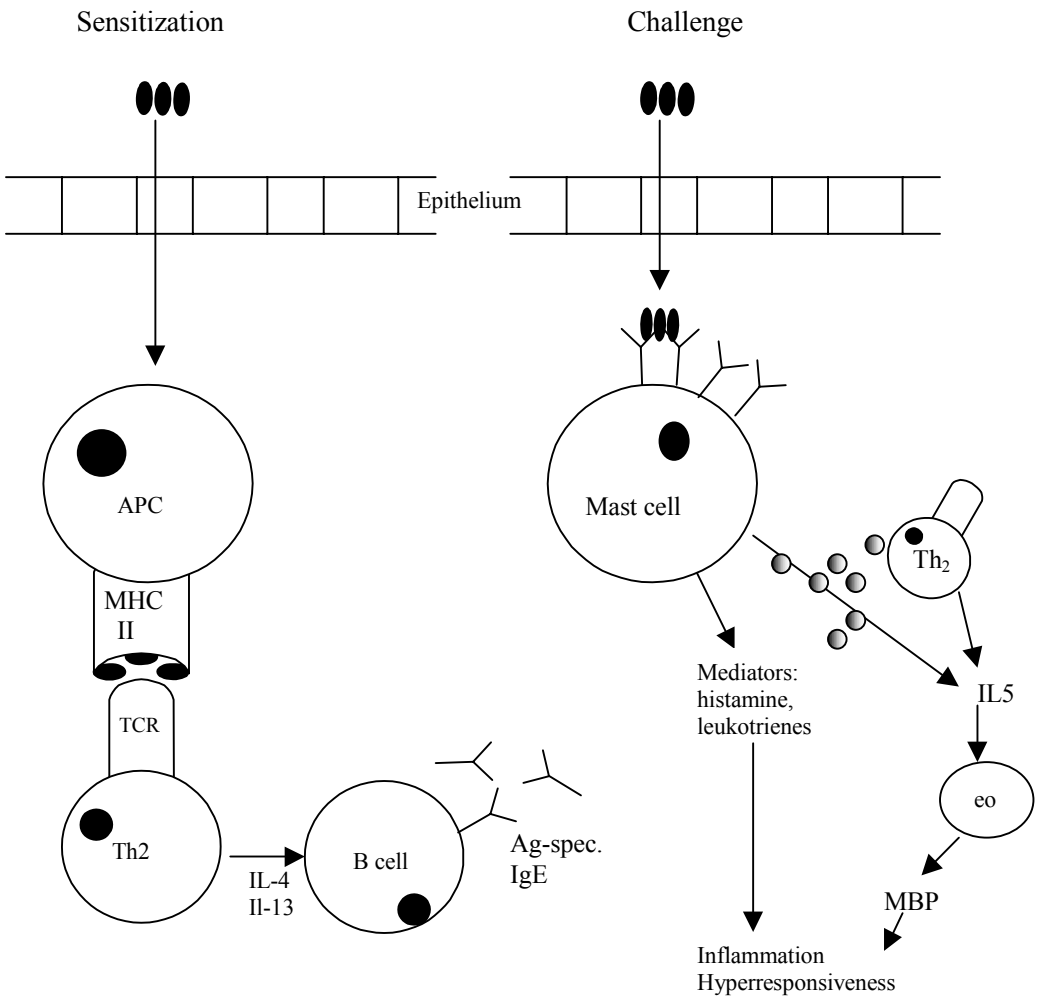


**Figure 2:** The allergic response can be divided into two temporally distinct phases: the sensitization phase (left) and the challenge phase (right). eo = eosinophil, MBP = major basic protein. Antigen is depicted as ⬮

Tumor necrosis factor (TNF) α (Kips *et al.,* 1993) and IFNγ (Hessel *et al.,* 1997) may also contribute to hyperresponsiveness. Other Th2 cytokines, such as GM-CSF, IL-3 and IL-5 act on eosinophils by chemoattracting them, supporting their effector function, and prolonging their survival (Weller *et al.,* 1996). Eosinophils are clearly participants in promoting the pathogenesis of allergy by releasing mediators such as leukotriene C4,

oxygen radicals and cationic proteins such as major basic protein (MBP). These may cause inflammation and destruction of other cells like the epithelium. This may lead to a higher susceptibility of underlying smooth muscle for contractile mediators (Corry *et al.,* 1996) or exposure of sensory nerve endings (Motojima *et al.,* 1989), both contributing to airway hyperresponsiveness.

*Determining T helper subset balance in the lung*

The crucial factor causing a Th2 subset to be formed is unknown, but several factors are involved during the innate and the adaptive immune response (Kirman and Le Gros 1998). The innate immune system is the first line of defense an antigen encounters when entering the body. But it probably serves a more important function than just bridging time until the more sophisticated adaptive immune response develops (Medzhitov and Janeway Jr., 1997), for it has the ability to discriminate between potential pathogens and innocuous antigens (Fearon and Locksley 1996, Matzinger 1994). Innate compounds that select proteins for endocytosis by APC, like mannose or scavenger receptors on DC and macrophages or complement factor CD3 on antigen, determine which antigens activate Th cells. Furthermore, cellular and soluble components of the innate immune system provide instruction that enables the adaptive immune system to develop appropriate strategies for elimination. For example, activation of macrophages through cell surface pattern recognition receptors causes the production of cytokines like IL-12, which is involved in regulation of Th1/Th2 balance (Fearon and Locksley 1996). IL-12 induces IFNγ production by T cells, which suppresses Th2 development. In atopic subjects, the frequency of Th cells that are unresponsive to IL-12 is high (Hilkens *et al.,* 1996). However, alveolar macrophages of asthmatics display enhanced IL-12 production (Magnan *et al.,* 1998), and IL-12 deficient mice do not show generalized enhancement of type 2 cytokines or antibodies (Rempel *et al.,* 2000). IL-4 also plays an important role by stimulating Th2 responses and simultaneously inhibiting Th1 development (Trinchieri 1995). In the early phase of the response the cellular source of IL-4 are possibly natural killer (NK) $1.1^+$ T cells (Vikari and Zlotnik, 1996), while in a later phase of the Th2 response mast cells (Bradding *et al.,* 1992) or basophils (Aoki *et al.,* 1995) are the main source in addition to Th2 cells themselves. Except for the latter these are all cells belonging to the innate immune system. Another possibility is a role for IL-6, produced by APC or one of the many other cell types able to produce IL-6 like macrophages, in T cell differentiation. IL-6 directly stimulates T cells to generate small amounts of IL-4, sufficient to induce autocrine upregulation of IL-4 and its receptor (Rincon *et al.,* 1997).

In this microenvironment with different cells and soluble mediators, the APC takes up antigen in order to present it to T cells. The most efficient APC is the dendritic cell (DC), which resides mainly in peripheral non-lymphoid tissue where many antigens are encountered, such as the airways. In the lung, resting DCs preferentially stimulate Th2 responses, while Th1 immunity relies completely on the provision of appropriate microenvironmental stimuli such as TNFα (Stumbles et al., 1998). In general, however, the most important stimuli appear to be IFNγ and $PGE_2$, inducing respectively maturation of DC that produce high levels of IL-12 (stimulating Th1 responses) and maturation of DC that produce $PGE_2$ (stimulating Th2 responses) (Kapsenberg et al., 1999). After antigen uptake by means of macropinocytosis or via mannose or Fc receptors, the DC migrates to the draining lymph node to present antigen to T cells. During migration the DC loses its capacity to take up antigen, but it upregulates the expression of MHCII and various adhesion and costimulatory molecules, and increases its unique ability to stimulate naive T cells. Of the latter, B7-1 (CD80) and B7-2 (CD86) interact with CD28 on the T cell, and have been proposed to be involved in the course (Morikawa and Nagashima 2000, Larche et al., 1998) of Th1 and Th2 reactions respectively. The definite role of B7-1 and B7-2 in the development of Th1 and Th2-mediated responses respectively (Kuchroo et al. (1995)) remains unclear.

While present in the airways, however, the antigen-presenting functions of the DC are suppressed, mainly by signals from adjacent cell populations like (subpopulations of) alveolar macrophages. Alveolar macrophages suppress T-cell-dependent immunity by means of cytokines which inhibit proliferation of T cells and APC-activity of DC (Holt *et al.,* 1993, Holt 1996, Poulter *et al.,* 1994, Strickland *et al.,* 1996, Thepen *et al.,* 1992). Suppressing activity of alveolar macrophages may be partly blocked in asthmatics by the presence of high levels of GM-CSF (Bilyk and Holt 1993, Holt *et al.,* 1996). Moreover, airway macrophages from asthmatic subjects show higher APC activity (Larche *et al.,* 1998).

Nature and number of doses of the antigen also play a role in Th differentiation. Intrinsic characteristics of the allergen may stimulate either Th1 or Th2 differentiation, as may for example be concluded from the fact that modification of allergen structure can cause a change in Th cell differentiation (Gieni *et al.,* 1993). Also dose is important in determining Th differentiation, as moderate and/or high doses in general induce Th2 differentiation, whereas low or very high doses stimulate Th1 (Rogers and Croft 2000).

Finally age also influences Th balances. The intrauterine environment is biased toward a Th2-response, and all newborns are biased toward Th2 responses (Warner *et al.,* 2000). While normally the Th1 branch develops within few years, this process is disturbed

in children who develop allergy (Holt and Macaubas 1997, Prescott *et al.,* 1998). Although respiratory allergy is a Th2-mediated process, immune deviation to a Th1 response does not necessarily ameliorate the disease. The same goes for the Th1-mediated process leading to experimental autoimmune encephalomyelitis, which can, in a slightly different form, also be induced by Th2-cells (Lafaille *et al.,* 1997). In a murine model of eosinophilic airway inflammation Th1 cells fail to counterbalance the effects of Th2 cells (Hansen *et al.,* 1999, Li *et al.,* 1998), and they may actually cooperate with Th2 cells (Randolph *et al.,* 1999). The inflammatory reaction initiated by Th1 cells plays a crucial role in this process. This supports the importance of the Th2-default in the lungs (Constant *et al.,* 2000).

Overall, the final character of the immune response is determined by various factors such as the APC, cytokines produced by the APC and other cells, nature and dose of the antigen, age, genetic background, route of administration and number of administrations.

**Increasing prevalence of respiratory allergy**

The prevalence of allergic airway diseases has increased during the last few decades in western and westernized societies (Schäfer and Ring, 1997, Lundbäck 1998, Beasley *et al.,* 2000). Since the genetic make up of a diverse population cannot change so rapidly, the cause(s) will most likely be found in our changing lifestyle and environment (Cookson *et al.,* 1997, von Mutius *et al.,* 1998, ISAAC 1998, Alm *et al.,* 1999). While some argue that these factors should act in a certain "critical period" during childhood (first 1-2 years after birth) to permanently retain the Th2-biased response after birth (Prescott *et al.,* 1999), studies from East/West Germany show that children older than 3 years are still influenced (von Mutius *et al.,* 1998).

*Factors in Western lifestyle*

Different factors (Hopkin 1997) associated with western lifestyle, such as smaller family size (Matricardi *et al.,* 1998), vaccinations and childhood infections (Cookson 1997, Brehler and Luger 1999, Kramer *et al.,* 1998), intestinal microflora (Björksten *et al.,* 1999), Western diet (Black and Sharpe 1997) and lifestyle-associated environmental changes such as in- and outdoor air pollution (Frew and Salvi, 1997, Steerenberg *et al.,* 1999b) have been proposed to explain the increasing prevalence (Fig. 3). The first three all support the idea that diminished exposure to infection, especially at a young age, might promote atopy. The Th1 environment created during infections like tuberculosis and measles may modify the developing immune system by inhibiting Th2 reactions, whereas the absence of such infections may give Th2 mechanisms space to act (Shirakawa *et al.,* 1997). In apparent

contrast seems to be the fact that Th1 cells cooperate with Th2 cells as mentioned before (Hansen *et al.,* 1999, Randolph *et al.,* 1999), and reports on measles and other infections increasing the risk for allergy (Paunio *et al.,* 2000, Bodner *et al.,* 1998). It is evident that different types of infections have distinct effects on the development of allergy. Matricardi *et al.* (2000) demonstrated that respiratory allergy was less frequent in people heavily exposed to orofecal and foodborne microbes, while this was not the case for highly infectious airborne viruses such as mumps and measles. Whereas some helminthic infections may inhibit atopic immune disorder, other members of this heterogeneous group do not (Mao *et al.* 2000).

The fact that our contemporary diet contains increased amounts of ω–6 polyunsaturated fatty acids (PUFAs) and less ω–3 PUFAs may cause Th2-mediated allergy. Ω–6 PUFAs such as linoleic acid are precursors of arachidonic acid, which can be converted to prostaglandin (PG) E2. Ω–3 PUFAs as present in oily fish inhibit this formation (Black and Sharpe, 1997). $PGE_2$ on its turn reduces IFNγ production by T lymphocytes and thus stimulates Th2 responses (Black and Sharpe, 1997, Kalinski *et al.,* 1999).

A plausible but unproven case can be made for increasing levels of allergens like house dust mite in modern heated, well isolated houses (Leung *et al.,* 1998, Garrett *et al.,* 1998). For several allergens, differences in allergy-prevalence could not be explained by differences in exposure (Jogi *et al.,* 1998).

*Air pollution*

Indoor (von Mutius *et al.,* 1996, Maier *et al.,* 1997) and outdoor (Wjst *et al.,* 1993, Lipsett *et al.,* 1997, Duhme *et al.,* 1996, Rusznak *et al.,* 1994, Popp *et al.,* 1989, Ulvestad *et al.,* 2000) air pollution appear to play an immunomodulatory role in the lungs. It is important to distinguish that air pollutants may cause sensitization, thereby increasing the prevalence of allergic disease, and/or exacerbate already existing allergy, hence increase its severity (Wardlaw 1992). Although the increase in severity is undoubtedly clear (Korrick *et al.,* 1998, Lipsett *et al.,* 1997, Jacobs *et al.,* 1997, Pope and Dockery 1992, Duhme *et al.,* 1996), the role of air pollution in sensitization is controversial (van Vliet *et al.,* 1997, Ishizaki *et al.,* 1987, von Mutius *et al.,* 1998, ISAAC 1998).

The fact that humans are exposed to a mixture of pollutants makes discrimination between the roles of the different components virtually impossible (Lipsett *et al.,* 1997). However different pollutants appear to have distinct effects, therefore a distinction has to be made between different types of air pollution.

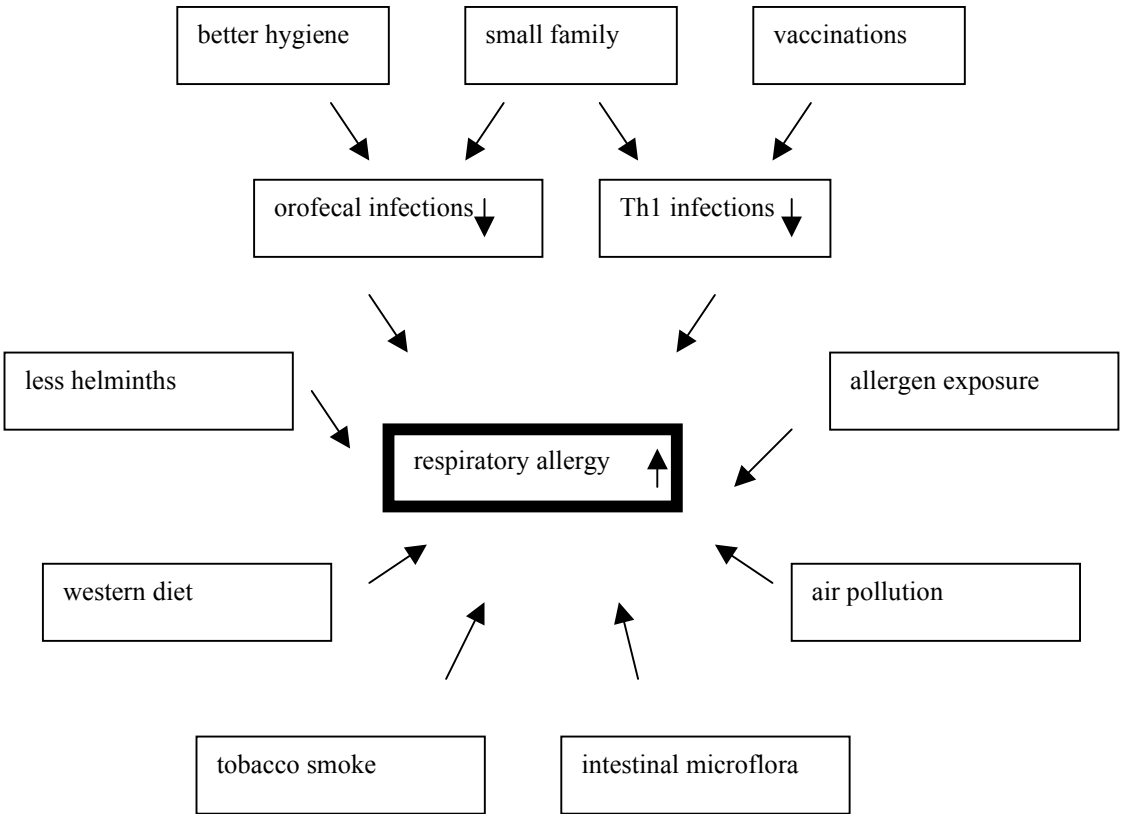


**Figure 3:** Factors in Western lifestyle that possibly influence the prevalence of respiratory allergy.

Pollutants like (ultra) fine particles and oxidant gases such as nitrogen dioxide ($NO_2$) and ozone ($O_3$) appear to influence the functioning of the immune system, thereby possibly playing a role in airway allergy (Devalia *et al.,* 1996, Behrendt *et al.,* 1997). This is in contrast to pollutants such as sulfur dioxide ($SO_2$) and larger dust particles, which appear to affect mostly the upper airways, causing bronchitis-like disease.

This distinction was determined from the fact that in the severely polluted ($SO_2$, larger dust particles) former East Germany the prevalence of respiratory allergy is lower than in former West Germany (high levels of ultrafine particles and $NO_2$) (von Mutius *et al.,* 1992). The fact that after the unification the prevalence is increasing in the former East indicates that some factors in Western lifestyle play a role (von Mutius *et al.,* 1998). Also within the same country and even the same region differences in prevalence can be found,

for example Swedish children with an antroposophic lifestyle (Alm et al., 1999) or children who spend their first years of life in a rural area show a lower prevalence of atopy than in comparable urban populations (Nilsson *et al.,* 1999).

Probably a combination of several of the above mentioned factors will determine the development of allergy, causing an increased risk of developing an IgE-mediated allergy for a person in a Westernized society who is exposed to a "Western" diet, "Western" air pollution, and a "Western" infection pattern.

**Particulate air pollutants**

Particulate air pollution is known to play an important role in the exacerbation of existing airway disease (Jacobs *et al.,* 1997, Pope and Dockery 1992, Lipsett *et al.,* 1997) and the induction of immunological alterations in humans (Hadnagy *et al.,* 1996). Road transport is a major source of the smallest class of particles and gaseous pollutants. The presence of heavy car traffic, producing large amounts of diesel exhaust particles (DEP), coincides with (new cases of) allergic diseases of the airways (Ishizaki *et al.,* 1987, Aarts *et al.,* 1999). Epidemiological evidence suggests that an increase in liquid petroleum derived pollutants, a/o. fine particles, $O_3$, and $NO_x$ is associated with exacerbation of allergic airway disease (Devalia *et al.,* 1996, Maynard and Waller 1996, Lipsett *et al.,* 1997).

*Particulate matter*

Particulate matter has gained a lot of attention over the past 10 years, and is recognized as an important causative agent of acute and probably also long-term pulmonary effects (Dockery and Pope 1994). Yet, the exact role of PM is not clear (Wardlaw 1992), and because PM is a heterogeneous agent, it is not sure which component of PM is responsible for the effects. PM is usually characterized according to its size, PM10 representing particles up to 10 μm. The coarse fraction with diameters between 2.5 and 10 μm mainly consists of inorganic minerals such as wind blown dust. Silicates are mainly present in this coarse fraction (Ormstad *et al.,* 1998, Churg and Brauer, 1997). Crystalline silica particles (SIP) are known for their adjuvant activity (Mancino *et al.,* 1983) as well as their fibrogenic potential, while amorphous SIP are nonfibrogenic and the inflammatory response they induce after one dose is transient (Yuen *et al.,* 1996). Little is known about the immunomodulatory potential of SIP in relation to respiratory allergy.

The fine (0.1-2.5 μm) and ultrafine (<0.1 μm) fractions predominantly consist of carbonaceous particles like DEP that are composed of carbonaceous particle cores to which polycyclic aromatic hydrocarbons are attached. Carbon black particles (CBP) lack these

substances, but they are possibly not as inert (Løvik *et al.,* 1997, Nilsen *et al.,* 1997) as they were once thought to be (Crosbie 1986). DEP, however, are known from both epidemiological as well as experimental studies in humans and mice (Saxon and Diaz-Sanchez 2000) to have effects on lung function and respiratory allergy.

*Diesel exhaust particles*

DEP make up an important part of PM and are most likely responsible for the increase in respiratory symptoms observed in persons exposed to high levels of PM in the vicinity of freeways (van Vliet *et al.,* 1997). From a Japanese study it was concluded that the presence of DEP plays a crucial role in the increasing prevalence of respiratory allergy to a common allergen (Ishizaki *et al.,* 1987). In animal studies the immunomodulating activity of DEP has been shown in several models. In mice the adjuvant activity has been shown after immunization with DEP and ovalbumin (OVA) via various routes. After intraperitoneal injection of DEP and OVA, the primary IgE antibody responses are higher than after injection of OVA alone (Muranaka *et al.,* 1986). Also exposure to a combination of antigen and DEP via the inhalatory route was shown to result in higher local and systemic levels of cytokines (IL-4, IL-5, IL-10, GM-CSF), higher IgE and IgG titers in blood and aggravated airway inflammation (eosinophils, neutrophils, lymphocytes) in mice (Takano *et al.,* 1997, Fujimaki *et al.,* 1994 and 1997). Similar results were obtained after intranasal exposure (Takafuji *et al.,* 1987, Nilsen *et al.,* 1997) and in a rat model (Steerenberg *et al.,* 1999a).

In humans Diaz-Sanchez *et al.* (1997a) showed that subjects who are allergic to ragweed display enhanced specific IgE production and skewed Th2-cytokine patterns after combined intranasal exposure to allergen and DEP. This confirms the finding in epidemiological studies that allergic persons show worsening of respiratory symptoms during spells of severe air pollution. In healthy subjects acute short-term DEP exposure caused a pulmonary and systemic inflammatory response. The fact that by means of standard lung function measurements (peak expiratory flow rate, forced expiratory volume, etc) this response cannot be observed may explain why this inflammatory response has not been noted in earlier studies (Salvi *et al.,* 1999 and 2000). In 1999 Diaz-Sanchez *et al.* also showed that the induction of allergy to a new antigen (keyhole limpet hemocyanin [KLH]) was facilitated by coexposure to DEP. This finding supports the few epidemiological studies that report enhanced risk on sensitization for subjects who are exposed to (particulate) pollution.

## Interactions between particulate air pollution and the immune system

### *Mechanisms of interaction*

Mechanisms by which particles enhance the immune response to common allergens could act at different levels. Particles and allergens could already interact in the outside air. Particles in the aqueous phase can interact with pollen *in vitro*, causing allergens to be concentrated on respirable size particles (Knox *et al.,* 1997), which function as allergen carriers (Ormstad *et al.,* 1998). The organic substances adsorbed on particles could play a role in preactivation of pollen, which under appropriate conditions could release its, possibly altered, allergens (Behrendt *et al.,* 1992). Also the presence of endotoxin in the air or on particles could stimulate IgE-mediated immune responses (Wan et al., 2000).

After inhalation, the particle-allergen complex may deposit in a different site as compared to a protein alone. Allergen may be slowly released locally in the lung, resembling "classical" adjuvant function (Janeway and Travers 1994). Particles could also change the microenvironment within the lung and act as adjuvant by causing local airway irritation and inflammation, as has been shown for DEP (Siegel *et al.,* 1997, Gilmour 1995) and residual oil fly ash (ROFA) (Lambert *et al.,* 1999). Locally the particles are taken up by macrophages (Kobzik 1995, Palecanda *et al.,* 1999) in which the production of cytokines like TNFα and IL-1 and mediators such as nitrogen oxide (NO) can be induced (Becker *et al.,* 1996). Epithelial cells also take up particles, and produce proinflammatory mediators like IL-6 and IL-8 (Steerenberg *et al.,* 1998, Bayram *et al.,* 1998). Cytokine release by epithelial cells of asthmatic patients is increased as compared to non-asthmatic subjects, indicating that individuals with airway disease are more vulnerable to the adverse effects of air pollution (Devalia *et al.,* 1999). Together possible damage of the epithelial barrier and a decrease in ciliary activity may cause impaired clearance of antigen (Devalia *et al.,* 1997), increasing the amount of antigen available for cells of the immune system.

Another possibility may be that the presence of particles downregulates the normal suppressor activity of macrophages on DC antigen presentation (Thepen *et al.,* 1992, Holt *et al.,* 1993). Macrophages are able to transport particles to the draining lymph nodes of the lung (Harmsen *et al.,* 1985, Lehnert *et al.,* 1986), but it is unknown if immunomodulation can also be performed locally in the draining lymph nodes. Although cytokine production is observed in the lymph nodes, it is less likely that antigen presentation by DC will be influenced in the PBLN since during migration DC become unresponsive to stimuli from the environment.

*Crucial particle characteristics*

Which characteristic of a particle is crucial in adjuvant activity remains unclear. The adsorbed substances as present on DEP appear to play an important role, at least in *in vitro* systems (Takenaka *et al.,* 1995, Bayram *et al.,* 1998, Yang *et al.,* 1997, Ohtoshi *et al.,* 1998, Tsien *et al.,* 1997), but the (hard core of) the particle itself also contributes to the adjuvant activity. This was shown in the primary popliteal lymph node assay (PLNA) by Løvik *et al.* (1997) who demonstrated that CBP, representing the non-extractable particle core of DEP, injected subcutaneously (sc) with OVA caused an increase in PLN weight, cell numbers, cell proliferation and IgE-levels. Yet, CBP were slightly less potent than DEP. In addition to that, also clean, synthetic particles like polystyrene particles are able to adjuvate the immune response via different routes of administration (Granum *et al.,* 2000b).

Size is probably an important characteristic because it plays a crucial role in determining whether and where particles are deposited after inhalation. While particles larger than 10 μm are deposited in the upper airways and are subsequently removed by the mucus-escalator and swallowed, particles of 2.5 μm reach conducting airways and the alveolar region and stay present in the alveoli for a long time (Churg and Brauer 1997, Ferin *et al.,* 1992). In addition to that, number (Granum *et al.,* 2000b), surface reactivity (Fubini 1997) and charge (Oortgiesen *et al.,* 2000) appear to be important determinants. An order of importance for the afore mentioned characteristics seems impossible to give.

**Mouse models on respiratory allergy and pollution**

Animal models are necessary for research on the immune system and allergy, because they give crucial information in addition to *in vitro*, human and epidemiological studies. Whereas *in vitro* assays are necessary to investigate a mechanistical aspect of the immune response, the interactions that take place in the complete and complex immune system cannot be reproduced *in vitro*. Also, as a consequence of ethical considerations, studies on human volunteers can only give a certain amount of information because sampling from different organs and body fluids is limited and also because a risk of sensitization always exists. Also epidemiological studies cannot replace *in vivo* studies completely. Firstly because the effect of one particular pollutant is hard to deduce from the mixture of pollutants that humans are exposed to, secondly because they do not give information on mechanism.

Therefore animal models have been developed. Most models are mouse models (Wills-Karp 2000), which is a logical choice of animal since it is a small animal, of which the immune system is similar to the human system and a lot of monoclonal antibodies are available (Karol 1994). Also many inbred and knockout strains are available (Wills-Karp 2000). However, administration of an antigen via the relevant inhalatory or intranasal route easily induces tolerance (suppression of IgE responses) in mice. To achieve sensitization, antigen can be administered by means of injection and/or use of adjuvants (Kung *et al.,* 1994). Injection models such as the PLNA do not require special equipment, and both the assay itself as well as the read out of the immune response is relatively easy to perform. But although the PLNA is a suitable screening assay, the injection in the paw makes research beyond some basic immunomodulating mechanisms irrelevant for real life situations. Other models combine injections with inhalatory exposures (Hessel *et al.,* 1995), but even though those models effectively mimic respiratory allergy and/or asthma, they do not fully take the natural route of exposure into account. Because sensitization is achieved in an artificial way, only the effects of pollutants on sensitized animals can be assessed, while the effects of pollutants on the sensitization process remain undetermined.

Models which only use inhalatory or intranasal exposure (Renz *et al.,* 1992) are rare and often use long protocols, and in addition the pollutant is given during the whole period, irrespective of the phase of the immune response (Fujimaki *et al.,* 1997, Maejima *et al.,* 1997, Nilsen *et al.,* 1997, Takafuji *et al.,* 1987). Only recently a simple model was developed in the rat in which exposure to the pollutant ROFA took place exclusively 1 day before sensitization, and the adjuvant effect was described extensively (Lambert *et al.,* 1999).

**Scope of this thesis**

The aim of this thesis was to develop a model to assess the immunomodulating capacity of particulate air pollution and to unravel underlying mechanisms. Our hypothesis was that exposure of naïve subjects to particulate air pollution during sensitization to an antigen leads to an increased risk of allergic sensitization.

Our first question was: do different components of particulate air pollution possess any immunomodulating capacity? Therefore we determined the adjuvant activity of different types of particles in a subcutaneous injection model in mice. In this model the influence of the particles on the primary and secondary immune response was investigated (Chapter 2). Our second question was: do these particles modulate functions of the alveolar macrophage, one of the main cell types they come in contact with after inhalation?

Therefore we assessed the effects of the particles on a mouse alveolar macrophage cell line *in vitro* (Chapter 3). To find out whether the particles adjuvated the immune response, and especially allergic sensitization, after exposure via the relevant route *in vivo*, an intranasal exposure model was developed in mice (Chapter 4). To determine whether this adjuvant effect acted exclusively during the sensitization phase, we assessed the influence of the presence of particles during the different phases of the immune response in the intranasal exposure model (Chapter 5). In Chapter 6 we determined whether coadministration of particles and antigen was necessary for adjuvant activity by separating antigen and particle doses in the intranasal model. To evaluate different methods of antigen exposure, an inhalatory exposure model was developed in addition to the intranasal model (Chapter 7). Results as obtained in these studies are summarized and discussed in Chapter 8.

# CHAPTER 2

# DIESEL EXHAUST, CARBON BLACK, AND SILICA PARTICLES DISPLAY DISTINCT TH1/TH2 MODULATING ACTIVITY

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## ABSTRACT

Certain particulate air pollutants may play an important role in the increasing prevalence of respiratory allergy by stimulating T helper 2 cell (Th2)-mediated immune responses to common antigens. The study described here examined different particles, diesel exhaust particles (DEP), carbon black particles (CBP) and silica particles (SIP), for their immunomodulating capacity in both primary and secondary immune responses in female BALB/C mice. The primary response was studied after subcutaneous injection of one milligram (mg) of particle together with 10 microgram (μg) of reporter antigen TNP-OVA (2,4,6-trinitrophenyl coupled to ovalbumin) into the hindpaw. Interferon γ (IFNγ) and interleukin 4 (IL-4) production was assessed in the popliteal lymph node (PLN) at Day 2 and Day 5 after injection by flowcytometry and ELISA. The number of IL-4 containing CD4+ T cells increased between Day 2 and Day 5 in DEP- and CBP-exposed mice, in contrast to SIP-treated animals. IL-4 production by cultured PLN cells was also significantly increased for DEP- and CBP-treated animals. The secondary response was studied in different organs after an intranasal challenge with TNP-OVA (50 μg) which was given 4 weeks after the initial subcutaneous injection. Five days after challenge the number of antibody forming cells (AFC) was assessed in peribronchial lymph nodes (PBLN), spleen, bone marrow and PLN, and antibody levels were determined in blood samples obtained weekly. It appeared that all particles acted as adjuvant, but the different particles stimulated distinct types of immune responses to TNP-OVA. DEP-treated animals show high IgG1 and IgE levels in serum and high IgG1 and IgE forming AFC numbers in PBLN, bone marrow and spleen. CBP-treated animals show even higher IgG1 and IgE levels and AFC numbers, and in addition display IgG2a production. SIP-injected animals display predominantly IgG2a responses. It is concluded that DEP are able to skew the immune response toward the T helper 2 (Th2) side, whereas SIP stimulate a Th1 response and CBP have a mixed activity, stimulating both Th1 and Th2 responses in this model.

## INTRODUCTION

The prevalence of allergic airway diseases has increased during the past few decades in Western and Westernized societies (Schäfer and Ring, 1997, Lundbäck 1998, ISAAC 1998). Since the genetic makeup of a diverse population cannot change so rapidly, the cause(s) will most likely be found in our changing lifestyle and environment. Different factors (Hopkin 1997) associated with Western lifestyle, such as smaller family size (Matricardi *et al.,* 1998), childhood infections (Cookson 1997), and Western diet (Black and Sharpe 1997) and lifestyle-associated environmental changes such as air pollution (Frew and Salvi, 1997) have been proposed to explain the increasing prevalence.

The role of air pollution, however, is not completely clear. First, air pollution has not been proven to increase the risk of sensitization of naïve subjects, although it clearly plays a role in the exacerbation of existing disease (Jacobs *et al.,* 1997, Pope and Dockery 1992, Lipsett *et al.,* 1997, Duhme *et al.,* 1996). Second, a distinction must be made between different types of air pollution. Pollutants such as (ultra) fine particles and oxidant gases such as $NO_2$ and $O_3$ appear to influence the functioning of the immune system, thereby possibly playing a role in airway allergy (Devalia *et al.,* 1996, Behrendt *et al.,* 1997). This is in contrast to pollutants such as $SO_2$ and larger dust particles, which appear to affect mostly the upper airways, causing bronchitis-like disease. This distinction has become clear from the fact that in the more-polluted former East Germany the prevalence of respiratory allergy is lower than in former West Germany (Mutius *et al.,* 1992). Combined with the increasing prevalence in the former East after the unification, this indicates that some factors in Western lifestyle play a role (Mutius *et al.,* 1998). One of those factors may be “Western pollution”.

Fine particles, also called particulate matter (PM) in relation to airway diseases, have gained much attention over the past 10 years and are recognized as an important causative agent of acute and probably also long-term pulmonary effects (Dockery and Pope 1994). Yet, the exact role of PM is not clear (Wardlaw 1992), and because PM is a heterogenic agent, it is not evident which component of PM is responsible for the effects. DEP make up an important part of PM (Bérubé et al., 1997), and are most likely responsible for the increase in respiratory symptoms observed in persons exposed to high levels of PM in the vicinity of freeways (van Vliet *et al.,* 1997). A Japanese study concluded that the presence of DEP plays a crucial role in the increasing prevalence of respiratory allergy to a common allergen (Ishizaki *et al.,* 1987).

In accordance with this, DEP have been found to adjuvate in particular Th2-like immune responses in humans and mice. Intranasal exposure to allergen in combination

with DEP stimulates a Th2 type cytokine pattern and enhances specific IgE production in humans allergic to ragweed (Diaz-Sanchez *et al.,* 1997a). In mice the adjuvant activity of DEP has been shown after immunization via various routes with DEP and ovalbumin. After intraperitoneal injection of DEP and OVA the primary IgE antibody responses were higher than after injection of OVA alone (Muranaka *et al.,* 1986). Also exposure to a combination of antigen and DEP via the inhalatory route was shown to result in higher local and systemic levels of cytokines (IL-4, IL-5, IL-10, GM-CSF), higher IgE and IgG titers in blood, and aggravated airway inflammation in mice (Takano *et al.,* 1997, Fujimaki *et al.,* 1994, Fujimaki *et al.,* 1996). Similar results were obtained after intranasal exposure of mice (Takafuji *et al.,* 1987, Nilsen *et al.,* 1997) and in a rat model (Steerenberg *et al,* 1999a).

PM is usually characterized according to its size, PM10 representing particles up to 10 μm. Size is crucial in determining whether and where particles are deposited in the lung; (ultra) fine particles (<2.5 μm) seem to be able to reach the alveoli and stay present for a long time (Churg *et al.,* 1997, Ferin *et al.,* 1992). However, size may not be the (only) critical characteristic, others like surface chemistry may be equally important (Murphy *et al* 1998). At least in *in vitro* systems substances that are present on DEP can modulate the response (Takenaka *et al*. 1995, Bayram *et al.,* 1998, Yang *et al*.1997, Ohtoshi *et al.,* 1998, Tsien *et al.,* 1997), in addition to the (hard core of the) particle that also contributes to the adjuvant activity. The latter was shown in the primary popliteal lymph node assay (PLNA) by Løvik *et al*. (1997), who demonstrated that carbon black particles (CBP), representing the nonextractable particle core of DEP, injected subcutaneously (sc) with OVA caused an increase in PLN weight, cell numbers, cell proliferation (inflammatory response) and IgE-levels. Yet, they were slightly less potent than DEP. Carbonaceous particles like DEP and CBP are present in the ultrafine (<0.1 μm) and fine (0.1-2.5 μm) fractions of particulate matter. Silicates are also present in PM, but mainly in the coarse fraction, with diameters between 2.5 and 10 μm (Ormstad *et al.,* 1998, Churg and Brauer 1997). Crystalline silica particles (SIP) are known for their adjuvant activity (Mancino *et al.,* 1983) as well as their fibrogenic potential, while amorphous SIP are nonfibrogenic and the inflammatory response they induce after one dose is transient (Yuen *et al.,* 1996). Little is known about the immunomodulatory potential of SIP in relation to respiratory allergy.

In this study the carbonaceous particles DEP and CBP were compared to amorphous SIP. From the comparison between DEP and CBP an idea about the role of adsorbed substances can be formed. The capacity of these three particles, differing in size and origin, to modulate the obligatory costimulation and type of immune response was studied by measuring different types of costimulatory molecules, cytokines and antibodies. Cytokines

are among others produced by T helper cells (CD4+) which play a role in determining the direction of the immune response. Th1 and Th2 subsets can be distinguished based on their cytokine patterns. Th1 cells produce IFNγ, which results in a cellular response and IgG2a production. Th2 cells produce IL-4, which stimulates a humoral response, characterized by IgG1 and IgE production. Cytokines are also produced by cytotoxic T cells (CD8+), effector cells of the cellular response. In the primary response, we determined IL-4 and IFNγ containing T cells and production, and the expression of CD80, CD86, and CD40 at 2 and 5 days after subcutaneous injection of particle together with the antigen TNP-OVA. The secondary response was studied after an intranasal challenge with antigen alone, which was given 28 days after the subcutaneous (sc) injection. The capacity of the different particles to stimulate antibody production in different organs during the secondary response was examined 5 days after challenge. In both models we compared the different particles with regard to their capacity to adjuvate the immune response to TNP-OVA, while the emphasis was on determining Th1/Th2 characteristics of the response.

## MATERIALS AND METHODS

### Animals

Female, specific pathogen-free BALB/c mice (6-8 weeks of age) were obtained from the Utrecht University breeding facility. Mice were housed under hygienic barrier conditions in filter-topped macrolon cages with bedding of wood chips, a temperature of 23±2$^{o}$C, 50-60% relative humidity and a 12-h light/dark cycle. They received standard lab chow and acidified tap water *ad libitum*. The experiments were approved by an ethical committee and conducted in accordance with the Guiding Principles in the Use of Animals in Toxicology.

### Chemicals and reagents

SIP ($SiO_2$) were obtained from Sigma Chemical Company (St. Louis, MO) and CBP from Brunschwich Chemie (Amsterdam, The Netherlands). DEP were a friendly gift from dr. P. Scheepers (Nijmegen University, The Netherlands). The antigen TNP-OVA was prepared as described by Albers *et al*. (1997). Immobilon-P membranes were obtained from Millipore (Etten-Leur, The Netherlands), and alkaline-phosphatase-conjugated goat αmouse IgG1, IgG2a and IgE antibodies from Southern Biotechnology Associates (Birmingham, AL). Antibodies for IgE and cytokine sandwich ELISA; αmouse IgE (02111D), αmouse IFNγ (18181D) and αmouse IL-4 (18191D) plus their biotinylated counterparts (188112D and 18042 D), and αmouse IFNγ (19301T) and IL-4 (19231V), were obtained from Pharmingen (Hamburg, Germany). ELISA plates (highbond 3590) were obtained from Costar (Cambridge, MA). All reagents for the TNP-specific ELISA

were all obtained from Sigma. Streptavidine coupled to horseradish peroxidase was obtained from CLB (Amsterdam, the Netherlands). Surface markers for flowcytometry (Pharmingen) were αCD3-FITC (01084D), αCD4-FITC (01064D), αCD8-FITC (01044D), αCD80-FITC (09604D), αCD86-FITC (09274D), αCD40-FITC (09664D), αCD4-PE (01075A) and αCD19-PE (09655B). For intracellular labeling αIFNγ-PE (18115A) and αIL-4-PE (18195A) were used.

**Treatment of the mice**

Particle suspensions (20 mg/ml) were prepared in saline containing 0.2mg/ml TNP-OVA and 10% normal mouse serum. The latter was added to facilitate suspension of the hydrophobic particles, while they stirred for 18 h (Løvik *et al*. 1997). A total volume of 50 µl was sc injected into the right hind footpad of naive BALB/c mice. To determine the primary response, mice were terminated 2 or 5 days after injection and the right PLN was taken out. The secondary response was determined 5 days after an intranasal challenge, which took place 4 weeks after injection. This challenge was performed by pipetting 50 µl of saline containing TNP-OVA (1 mg/ml) on the nostrils under light ether anesthesia. Blood was collected weekly by orbita punction under ether anesthesia; sera were prepared and frozen individually. Five days after challenge mice were terminated and right PLN, bone marrow, spleen and PBLN were isolated. PLN, PBLN and spleen were minced to prepare single cell suspensions. Erythrocytes in spleen suspension were lysed using a 0.83% (w/v) ammonia buffer and cells were washed 2 times in PBS/1% BSA.

**ELISPOT assay**

These assays were essentially performed as described previously (Schielen *et al.,* 1995). Briefly, from each organ $0.5*10^6$ cells in 500 µl PBS/1% BSA were incubated (4 h, 37$^o$C) in wells containing TNP-BSA coated (overnight [o.n.] 4$^o$C) Immobilon-P membranes as bottom. Thereafter, membranes were washed and incubated with optimal dilutions of isotype-specific alkaline phosphatase-conjugated αmouse Ig antibodies in PBS/T (o.n. 4$^o$C). After washing, spots were developed by incubation with 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium (BCIP/NBT). Specific AFCs per $10^6$ cells were calculated from spot numbers counted with the aid of a stereo microscope.

**TNP-specific IgG1 and IgG2a ELISA**

Plates were coated (o.n. 4$^o$C) with TNP-BSA (20 µg/ml) in 0.05 M carbonate buffer pH 9.6, and blocked with PBS/1% BSA (1 h, room temperature [RT]). Serial dilutions of sera were incubated (1 h, RT) and plates were washed and incubated with an optimal dilution of alkaline phosphatase-conjugated anti-mouse IgG1 or IgG2a (1 h, RT), followed by p-nitrophenyl phosphate (1 mg/ml in diethanolamine buffer, pH 9.8) for 30 min. Absorbance

was measured at 405 nm. Titers were calculated by means of the sample dilution at which extinctions were higher than background+2*SD.

**TNP-specific IgE ELISA**

Plates were coated (6 h, first hour at 37$^{o}$C, last hours at RT) with αIgE, 2 µg/ml in 0.05 M carbonate buffer, pH 9.6. Serial dilutions of sera were incubated (o.n. 4$^{o}$C) and plates were washed and incubated with an optimal dilution of alkaline phosphatase-conjugated TNP (1 h, RT). See preceding section for last steps of the procedure.

**Flowcytometry**

For analysis of surface markers, single cell suspensions (5*10$^{6}$/ml) in RPMI/10% FCS were incubated with combinations of FITC and PE-conjugated αCD3, αCD4, αCD8, αCD19, αCD80, αCD86, and αCD40 at predetermined dilutions (30 min, RT). Cells were washed in PBS and stored for up to 24 h in PBS/0.1% paraformaldehyde prior to analysis. *In vitro* restimulation and intracellular staining were essentially performed as described by Openshaw *et al. (*1995). In short, freshly isolated single cell suspensions (5*10$^{6}$cells/ml) were stimulated with 50 ng/ml PMA and 500 ng/ml ionomycin in the presence of 10 µg/ml Brefeldin A for 4 h before an equal volume of PBS/4% paraformaldehyde was added. After fixing (20 min, RT), cells were washed in PBS and stored o.n. in PBS. Cells were washed, resuspended in PBS/1% BSA/0.5% saponin, and incubated for 10 min (RT). Next, PE-conjugated αIFNγ, αIL-4, or isotype control was added at 5 µg/ml in PBS/1% BSA/0.5% saponin (30 min, RT). Cells were washed twice with PBS/1% BSA/0.5% saponin, once with PBS/1% BSA, and incubated for 10 min in this buffer to reseal cell membranes. Finally, surface staining was performed using FITC-conjugated αCD3, αCD4 and αCD8. Samples were analyzed on a FACScan flowcytometer (Becton Dickinson, San Jose, CA) with thresholds set on the isotype-control stainings that were included for every suspension.

**Cell culture**

A 100 µl sample from the cell suspensions (1*10$^{6}$/ml) was incubated with 50 µl stimulus (concanavaline A, 50 µl of 15 µg/ml) o.n. at 37$^{o}$C, 5% $CO_2$. After centrifugation (10 min at 1000 rpm) supernatant was collected and stored at -20$^{o}$C for analysis. After analysis, the results were corrected for the differences in T-cell percentages as assessed by FACS analysis. For example, although controls contain an average of 60% T cells, CBP-treated animals show an average of only 34%, resulting in a multiplication of CBP results with a correction factor of 60/34=1,8.

**Sandwich ELISA for IL-4 and IFNγ**

Plates were coated (o.n., 4$^{o}$C) in 0.05 M carbonate buffer pH 9.6 with 1 µg/ml αmouse IFNγ or IL-4, and after washing were blocked with PBS/T/1% casein (4 h, RT).

Supernatants were then incubated o.n. at 4°C . Dilutions of mouse IFNγ or IL-4 were included on every plate for standard curve production. Plates were washed and incubated with 0.25 µg/ml biotine-conjugated αmouse IFNγ or IL-4 in PBS/T/1% casein (1 h, RT). After washing, streptavidine coupled to horseradish peroxidase (dilution 1:10,000) in PBS/T/1%casein was added (3/4 hr, RT). After washing, TMB (0.1 mg/ml) substrate was added, and the color reaction was stopped after 10 min with $H_2SO_4$. Absorbance was measured at 450 nm.

**Statistics**

Preceding statistical analysis, ELISPOT data (number of AFCs) were transformed to log10 values to homogenize variance. Differences between group means were analyzed using one-way ANOVA with Scheffe post-hoc test for contrasts ($p<0.05$ or $p<0.01$). In the case of nonsufficient homogeneity of variances, a Kruskal-Wallis ranking test was performed ($p<0.05$).

## RESULTS

### Cell number, costimulation molecules, and cytokine and antibody production in PLN during the primary response

At Day 2 and Day 5 after subcutaneous injection of particles together with TNP-OVA in the hind footpad of mice, various parameters were assessed to evaluate early events during the primary immune response. All particles induced clear changes in PLN cell number and cell types from Day 2 onward (Fig. 1).

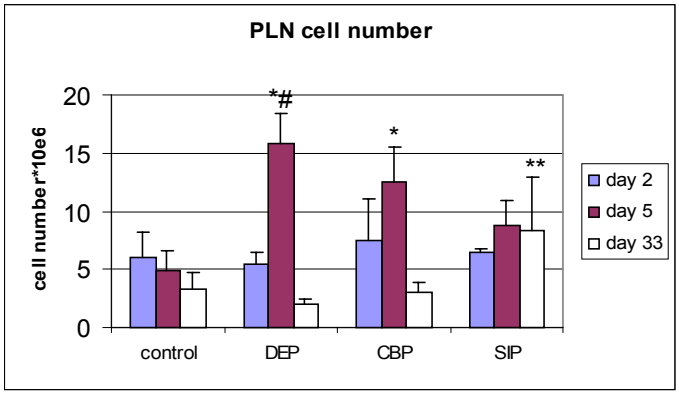


**Figure 1:** Cell numbers of PLN at Day 2 and 5 after injection and at Day 33 after injection and intranasal challenge. Depicted are average ±SD *$p<0.01$ vs. control group. **$p<0.01$ vs. control, DEP, CBP. #$p<0.05$ vs. SIP group. All determined by ANOVA followed by Scheffe's post-hoc test.

At Day 5, cell numbers of the CBP and DEP-treated groups were increased significantly, while at Day 33 only the cell number of the SIP-treated group was elevated. The percentage of B cells was increased at Day 5 (Fig. 2A) and the percentage of T cells was decreased. Using the cell numbers given in Fig. 1 for calculations, it appeared that at Day 5 a significant increase in absolute B cell numbers was observed in all particle-treated groups compared to controls (Fig. 2B). The DEP-injected group also showed a significantly higher increase compared to the SIP-injected group. Absolute T cell numbers did not change significantly in the particle-treated groups. The number of B cells, expressing costimulatory molecule CD86 (B7.2), was significantly increased in the DEP and CBP-treated groups compared to the SIP-injected and the control group. A slightly, but not significantly, increased expression was observed for CD80 (B7.1) in all particle-injected groups. The DEP-injected group showed significantly increased CD40 expression, whereas this was less pronounced in the CBP and SIP-treated groups (Fig. 2B).

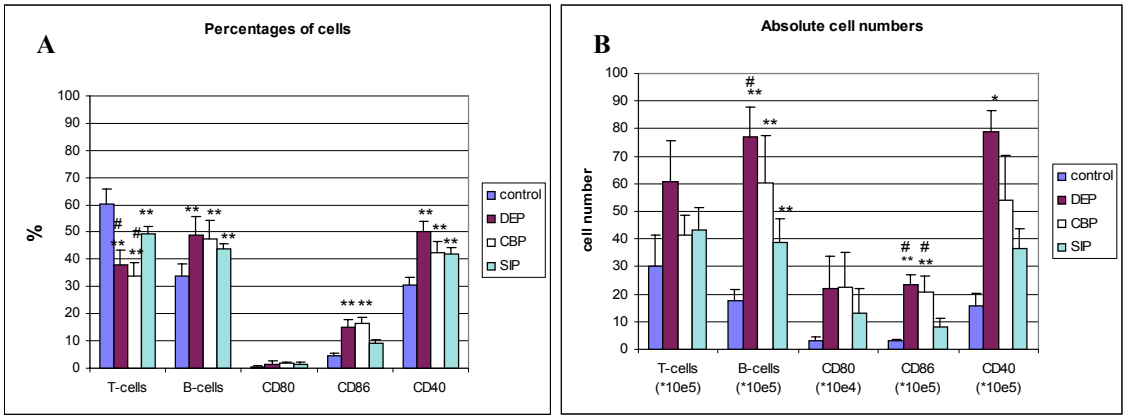


**Figure 2:** Numbers of T cells, B cells and B cells expressing costimulatory molecules at Day 5 after injection of particles and TNP-OVA during the primary response as determined by flowcytometry. Cells were double stained for combinations of CD3, CD4, CD8, CD19, CD80, CD86, and CD40. A: numbers on the y-axis are percentages. B: numbers on the y-axis represent absolute numbers per animal, which have to be multiplied by either $10^4$ or $10^5$ as is noted on the x-axis.. Depicted are average ±SD *$p<0.05$ vs. control group, determined by Kruskal-Wallis ranking test. **$p<0.01$ vs. control group, #$p<0.01$ vs. SIP group, determined by ANOVA followed by Scheffe's post-hoc test.

No significant changes in T helper cell cytokine pattern as determined by FACS analysis were observed at Day 2 (Fig. 3A), but all particle-treated groups contained a somewhat higher number of IFNγ-containing T helper cells than IL-4-containing cells. At Day 5,

however, the ratio of IL-4 to IFNγ completely turned around for the DEP- and CBP-treated groups, from <1 at Day 2 to >1 at Day 5. This was caused by a significant increase in the absolute number of IL-4 containing CD4+ T cells in these groups. IFNγ-containing cells also increased in the DEP-treated group. The absolute number of IFNγ containing CD8+ T cells was significantly elevated in all particle-treated groups at Day 5 (Fig. 3B).

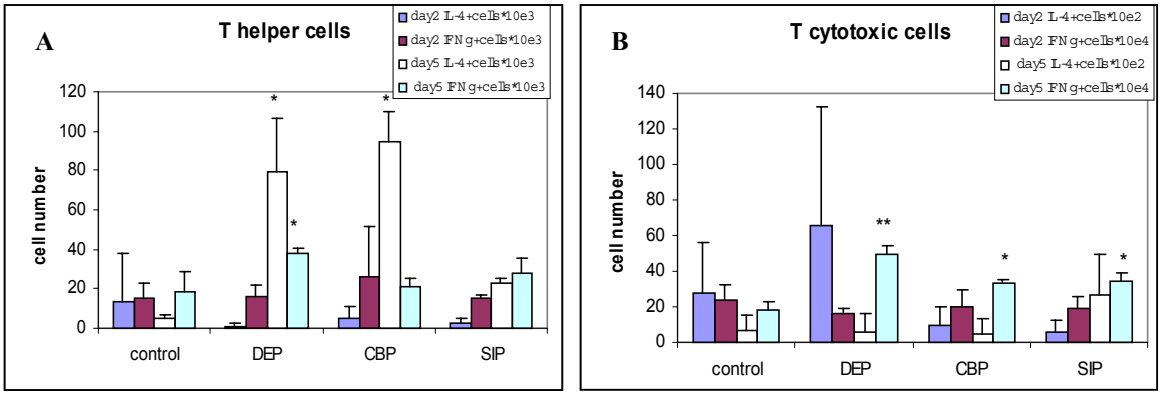


**Figure 3:** Cytokine containing T cells in the PLN during the primary response at Day 2 and 5, as determined by intracellular staining for IL-4 and IFNγ, in combination with surface staining for CD3 and CD4, and analysed by flowcytometry. Depicted are average ±SD. Figure 3A shows T helper cell data, *p<0.05 vs. control group, determined by ANOVA followed by Scheffe's post-hoc test for IL4, or determined by Kruskal-Wallis ranking test. Figure 3B shows cytotoxic T cell data, *p<0.05 vs. control group, **p<0.01 vs control and p<0.05 vs. CBP and SIP, all determined by ANOVA followed by Scheffe's post-hoc test.

In addition to the number of cytokine-containing cells, actual cytokine production was assessed (Fig. 4). ELISA was performed on supernatants of overnight cultures of PLN cells, which were isolated 5 days after injection. A correction was made for the decrease in T cell percentage as observed in particle-treated groups, as indicated under the Materials and Methods. PLN cell cultures from CBP and DEP-treated groups produced significantly higher levels of IL-4 than controls, whereas IFNγ production was significantly enhanced only in the CBP-treated group. Cytokine production in SIP-treated animals did not show significant changes (Fig. 4).

At Day 7 after injection, ELISPOT on cultures of PLN cells was performed. Hardly any antibody-forming cells were observed in PLN cells, and small numbers of IgG1-AFCs were found only in the CBP-injected group (not shown).

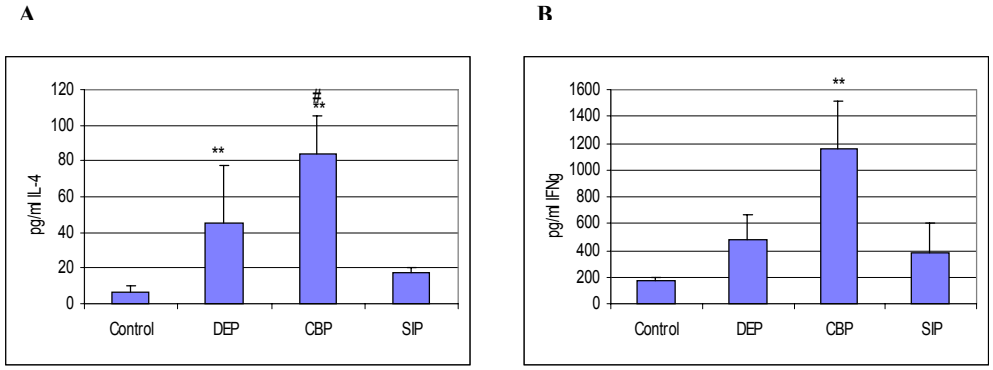


**Figure 4:** Overnight cytokine production by PLN cells (removed at Day 5 after injection) as determined by ELISA, and corrected for T cell percentage. Depicted are average ±SD. Figure A: **p<0.01 vs. control, #p<0.05 vs. SIP, determined by ANOVA followed by Scheffe's post-hoc test. Figure B: ** p<0.01 vs. all other groups, determined by ANOVA followed by Scheffe's post-hoc test.

**Levels of TNP-specific IgG1, IgG2a and IgE antibody titers in serum during the secondary response**

Additional information about the immunomodulating activity of the different particles was obtained during the secondary response. Four weeks after the subcutaneous injection, an intranasal challenge with TNP-OVA alone was given, and at Day 33, 5 days after challenge, AFC numbers were determined in different organs. Immunoglobulin levels were assessed in sera obtained weekly. After injection, immunoglobulin levels could already be detected at Week 1 and increased steadily thereafter. After intranasal challenge at Week 4, a memory response was initiated, indicated by the rapidly increasing immunoglobulin levels (Fig. 5).

Regarding the different isotypes, IgG1 levels in the DEP and the CBP-treated groups increased after injection at Day 0 and remained significantly elevated in comparison to control levels for 5 weeks. The IgG1 levels in the DEP and CBP-injected groups were significantly higher than those of the SIP-treated group, which remained at control levels. From Week 2 on, IgG2a levels of the SIP-treated group were significantly higher than the levels of control animals. IgG2a levels of both SIP and CBP-treated groups were significantly higher than those in the DEP-injected group, which were comparable to, or even significantly lower (in Week 3 and Week 4), than control levels. Specific IgE levels initially remained unchanged after injection, but were significantly enhanced in the CBP and the DEP-treated group after challenge (Week 5).

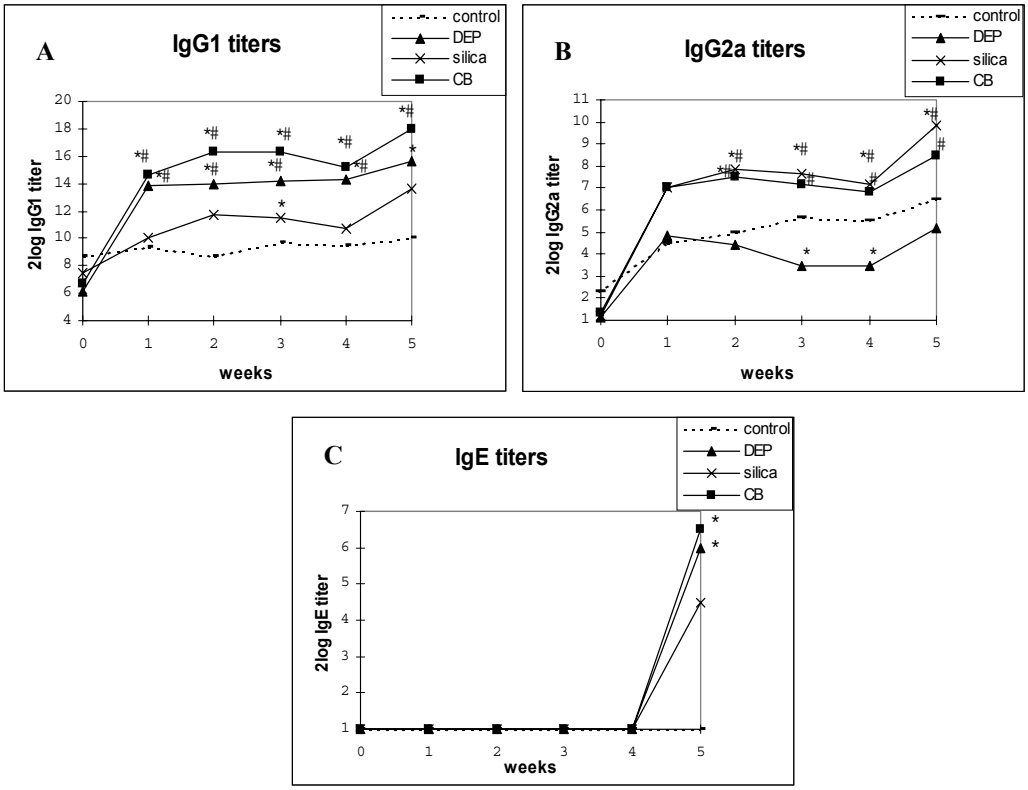


**Figure 5:** TNP-specific antibody titers in serum during the secondary response, challenge at week 4. Depicted are average ±SD. A: IgG1, B: IgG2a, C: IgE. *p<0.05 vs. control, for IgG1: #p<0.05 vs SIP, for IgG2a: #p<0.05 vs DEP, determined by ANOVA followed by Scheffe's post-hoc test, or by Kruskal-Wallis ranking test for IgE.

**Number of TNP-specific antibody forming cells in the secondary response.**

At Day 33 the numbers of AFC was assessed in different organs. In all particle-injected groups, the number of IgG1-AFC was significantly enhanced in PBLN and spleen as compared to controls (Fig. 6). But IgG1-AFC numbers in the SIP-treated group were significantly less enhanced than those in the CBP-injected group. In bone marrow and PLN only the DEP and the CBP-injected groups, but not the SIP-injected group, had significantly higher IgG1-AFCs than the controls. The number of IgE-AFCs in PBLN and spleen were significantly increased in the CBP and DEP-injected groups compared to those

in the control and the SIP-treated group. In the PBLN, the numbers of IgG2a-AFCs were enhanced in the CBP and the SIP-treated group compared to the control group.

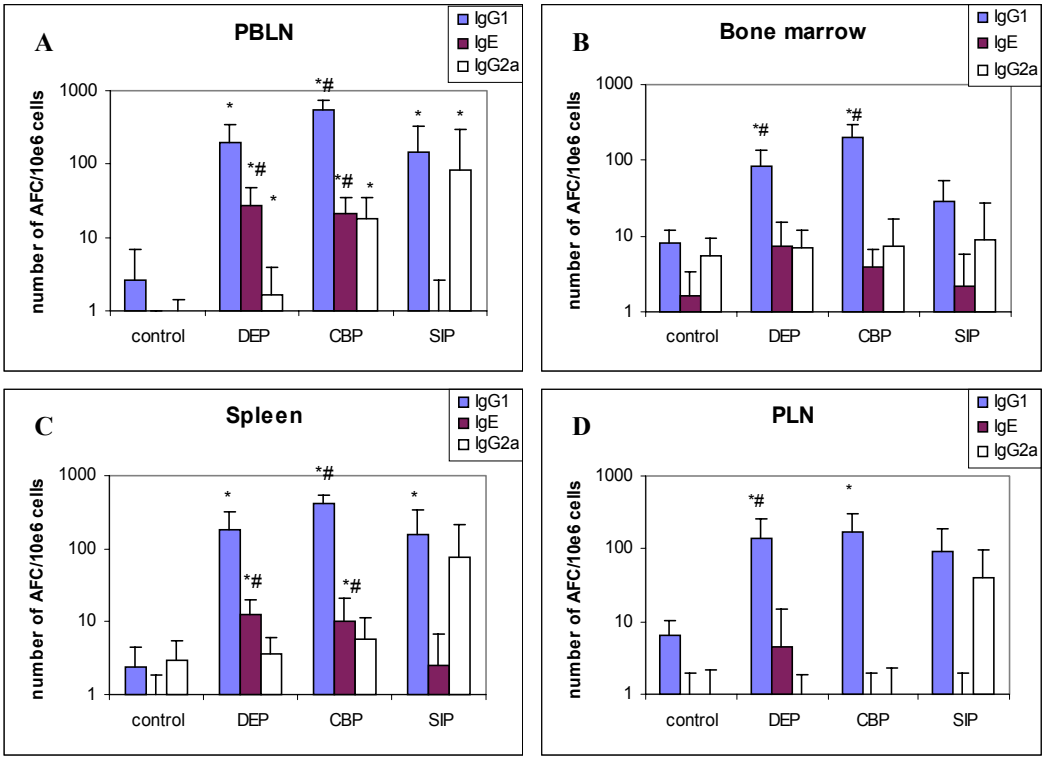


**Figure 6:** Number of TNP-specific antibody forming cells per $10^6$ cells in PBLN, spleen, bone marrow and PLN, as determined by ELISPOT at Day 33 in the secondary response. Depicted are average ±SD. *p<0.05 vs. control, #p<0.05 vs. SIP, determined by ANOVA followed by Scheffe's post-hoc test, or by Kruskal-Wallis ranking test for PBLN IgE and IgG2a.

## DISCUSSION

Particulate air pollution has been shown to play a role in the exacerbation of respiratory allergy. However, its influence on the induction of the immune response is unknown (Diaz-Sanchez *et al.,* 1997b). The present study intended to give more insight into the capacity of different particles to modulate the induction of an immune response. An overview of the results obtained in this study is given in Table 1. It readily appears that characteristics of a Th2 response, such as IL-4-containing and -producing T helper cells and IgG1 and IgE

production, are all increased in DEP- and CBP-treated animals and not in SIP-treated animals. In addition, CBP-treated animals also show increased IgG2a production. Although it is known that Th2-mediated responses are involved in respiratory allergy to common allergens, carbonaceous particles like DEP and also CBP could play a stimulatory role in this process.

**Table 1:** Summary of data. Particle-treated groups were compared to the control group.

| | **Costimulatory molecules** | | **Cytokines** | | | | | | **Antibodies** | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Expression | | Production | | | | Excretion | | Excretion | | | Present in serum | | |
| | Bcell-CD40 | Bcell-CD86 | CD4 -IL-4 | CD4-IFNγ | CD8 IL-4 | CD8 IFNγ | IL-4 | IFNγ | AFC IgG1 | AFC IgE | AFC IgG2a | IgG1 | IgE | IgG2a |
| DEP | + | ++ | + | + | - | ++ | + | - | + | + | - | + | + | - |
| CBP | +/- | ++ | + | - | - | + | ++ | + | + | + | + | + | + | + |
| SIP | +/- | - | - | - | - | + | - | - | - | - | + | - | - | + |

= no increase
+/- = non-significant increase
+ = significant increase $p<0.05$
++ = significant increase $p<0.01$
All determined by ANOVA followed by Scheffe's post-hoc test, or by Kruskal-Wallis ranking test (see figures for details).

The different characteristics of the immune responses stimulated by the particles are already illustrated in the primary response, during which differences are seen in expression of costimulatory molecules and cytokine production between the groups treated with different particles. At Day 5 after injection, a higher expression of the costimulatory markers CD40 and CD86 was observed in the groups treated with carbonaceous particles as compared to the SIP-treated group. CD80 expression was not enhanced, indicating a difference in expression kinetics or a more prominent role of CD86 in these Th2-mediated immune responses, as was also observed in an experimental allergic encephalomyelitis model (Kuchroo *et al.,* 1995).

During the primary response the IL-4/IFNγ ratio in T helper cells of both DEP and CBP-treated animals changed from <1 at Day 2 to >1 at Day 5. The effector cytotoxic T cells revealed an increased IFNγ production in all particle-treated groups. The higher number of IL-4-containing T helper cells is reflected by a higher level of IL-4 in culture

supernatant of DEP and CBP-treated animals. The increased number of IFNγ-containing cytotoxic T cells is reflected by a significantly increased level in supernatant of CBP animals, although the increase of the levels of DEP and SIP-treated animals was not significant. In our model the IFNγ content of a cell possibly does not correlate completely with actual excretion. Together, these data on the primary response indicate a difference in kinetics of the adjuvant activity between SIP, CBP and DEP.

Differences in immunomodulation were even more obvious in the secondary response after the intranasal challenge with TNP-OVA. In the SIP-treated group high IgG2a levels and AFC numbers were found. A Th1-mediated immune response was also observed after inhalation of silica (Davis *et al.,* 1999). In contrast, in CBP and DEP-treated animals high IgE and IgG1 levels and AFC numbers were found, indicative of a Th2-stimulating capacity of the carbonaceous particles. The adjuvant activity and Th2-skewing capacity of DEP have been shown in different animal models and in man. DEP and CBP clearly showed adjuvant activity in our model, but the character of the adjuvant activity differed slightly. CBP-treated animals showed IgG2a production, whereas IgG2a levels in DEP-treated animals seemed even somewhat suppressed compared to control levels. The immune response induced by the presence of DEP was, although somewhat less powerful, in the end more clearly of the Th2 type than the response after CBP injection. The attached substances that are present on DEP, consisting mostly of polyaromatic hydrocarbons (PAH-DEP), are often suggested as being responsible. In our model the attached substances of DEP could possibly be involved in this skewing toward the Th2 side, as has also been shown by others (Takenaka *et al.,* 1995, Tsien *et al.,* 1997).

Nevertheless, attached substances are apparently not responsible for the entire adjuvant activity, since two particles lacking attached substances, CBP and SIP, also acted as adjuvant. Evidently the presence of any particle core is crucial. However, SIP, size 1-5 μm, consisting of oxidized silica, induced a type of immune response different from CBP (0.3 μm), which have a carbonaceous core. Particle core characteristics like size and material probably play a crucial role in determining the type of immune response that is initialized.

In this study, different types of particles were able to adjuvate the primary immune response, with effects that were also clearly measurable during the secondary response. Modulation of sensitization may also take place in humans who are coexposed to allergens and pollutants. Although so far only epidemiological evidence exists for the involvement of particulate pollutants in the exacerbation of respiratory allergy, our present results may imply the facilitated initiation of allergy by the presence of particles as well. In contrast to our model, in real life the lung is exposed to antigen and particles. Whether particles also

act as adjuvant in the initiation phase of an immune response after administration via the relevant route, and which factors are crucial then are the subjects of investigation.

## ACKNOWLEDGMENTS

The authors thank Prof. Dr. J. G. Vos and Prof. Dr. W. Seinen for critically reading the manuscript.

# CHAPTER 3

# MODULATORY EFFECTS OF PARTICULATE AIR POLLUTANTS ON ALVEOLAR MACROPHAGE FUNCTION *IN VITRO*

Maaike van Zijverden, Wendy van Dalen, Raymond Pieters, Flemming Cassee, Jan Dormans, Henk van Loveren, Peter Steerenberg

## ABSTRACT

Particulate air pollution is known to affect human health. Its role in the aggravation of existing respiratory allergy is evident, and particulate matter may also play a role in the increasing prevalence of the disease. When particulate air pollution is inhaled by man, especially the (ultra) fine (<2.5 μm) particles are deposited deeply into the lung, where they are phagocytized by alveolar macrophages. In addition to clearing foreign material, these cells are important immune regulators. In the present study we therefore determined effects on cell damage and functional effects of exposure to different types of particles in a murine alveolar macrophage cell line (CRL 2019). Silica (SIP), diesel exhaust particles (DEP) from two different sources (DEP1 and DEP3), and carbon black particles (CBP), all smaller than 5 μm, were tested at a concentration of 0.11 and 0.33 mg/ml.
Our results demonstrate that this selection of particles had diverse effects on cell damage, phagocytosis and cytokine production. Cell damage ranged from extensive cell damage (indicated by LDH release) as induced by CBP, moderate damage by DEP1 and SIP, to no damage by DEP3. All particles caused a decrease in phagocytosis, from moderate decreases induced by SIP and DEP1 to large decreases induced by CBP and DEP3. TNFα production was concentration-dependent for all particles and DEP1 and CBP stimulated the highest absolute levels. Effects on IL-1β and IL-6 were measurable only after LPS-priming of the macrophages, production was not dependent on particle concentration. No correlation appeared to exist between inducing cell damage, blocking phagocytosis and stimulating production of cytokines. In addition, we tested the effect of ozone pre-exposure of DEP. We observed that after exposure of DEP3 to ozone, ozone-pretreated DEP3 displayed significantly higher TNFα production and complete phagocytosis inhibition at lower particle concentrations compared to non-exposed DEP3, whereas LDH release was not affected. The increased production of TNFα and the impairment of phagocytosis *in vitro* are in line with a role for alveolar macrophages in the mechanism of sensitization to common allergens in subjects exposed to PM.

## INTRODUCTION

The prevalence of asthma and respiratory allergy has been increasing at a high rate in Western industrialized countries (Lundbäck 1998, Schäfer and Ring 1997). This trend has been shown to coincide with the increase in fossil fuel combustion and emission of (ultrafine) particulate matter (PM) (Peterson and Saxon 1996). Diesel exhaust particles (DEP) make up an important part of ambient PM. In animal studies the immunomodulating activity of DEP has been shown to result in cytokine production, enhanced IgE and IgG levels, and increased airway inflammation (Takano *et al.,* 1997, Fujimaki *et al.,* 1994 and 1997, Takafuji *et al.,* 1987, Nilsen *et al.,* 1997, Steerenberg *et al.,* 1999a). In humans, exposure to DEP has been shown to cause aggravation of symptoms in subjects already suffering from respiratory allergy (van Vliet *et al.,* 1997). In healthy subjects, DEP cause an inflammatory response (Salvi *et al.,* 1999 and 2000) and facilitate mucosal sensitization to a new antigen (Diaz-Sanchez *et al.* 1999). Taken together, it is hypothesized that PM, and especially its major constituent DEP, facilitates the development of respiratory allergy.

The ultrafine fraction (PM<0.1μm) mainly consists of carbonaceous particles originating from combustion processes (e.g. DEP) in developed countries, whereas in the coarse (2.5-10μm) fraction inorganic minerals such as silica, and larger carbon aggregates are found (Ormstad *et al.,* 1997). Larger particles are deposited in the conducting airways, from which they can be cleared by mucociliary clearance. In contrast, particles of 2.5 μm and smaller are effectively deposited in the alveolar region of the lung (Churg and Brauer 1997, Ferin *et al.,* 1992). Alveolar macrophages can take up particles by a receptor-mediated mechanism (Kobzik 1995, Palecanda *et al.,* 1999), and this phagocytosis capacity has been shown to decrease during exposure to particles (Becker and Soukup, 1998).

In addition to effects of particles on innate macrophage functions like phagocytosis, the production and secretion of regulatory molecules such as cytokines and chemokines involved in acquired immunity may be affected by the presence of PM (van Rooijen and Sanders 1997). Resident alveolar macrophages are crucial in downregulation of antigen presenting cell functions of pulmonary dendritic cells (Poulter and Burke 1996). Depletion of macrophages leads to an increase of (IgE-mediated) immune responses to inhaled allergen (Thepen *et al.,* 1992, de Haan *et al.,* 1996, Leenaars *et al.,* 1997), suggesting that disturbance of macrophage function may play a role in asthma and respiratory allergy. Macrophages spontaneously secrete low levels of IL-1β and somewhat higher levels of TNFα, IL-6 and IL-8. This production is increased after exposure to particles like urban air particles (Dong *et al.,* 1996, Becker *et al.,* 1996, Ning *et al.,* 2000) and DEP *in vitro* (Yang

*et al.,* 1997). The oxidant stress caused by the generation of ROS and NO in response to particle exposure is (partly) responsible for the cytokine production (Hiura *et al.,* 1999, Barrett *et al.,* 1999).

Taking into account the diverse and important functions of the alveolar macrophage, changed macrophage functions as can be induced by particles may play a role in enhanced allergic sensitization. Increased levels of (pro)inflammatory cytokines may lead to inflammation. TNFα produced by alveolar macrophages acts as the main trigger for the upregulation of several cytokines and adhesion molecules by means of nuclear factor (NF)κB activation in the lung, finally leading to inflammation (Lentsch *et al.,* 1999). Moreover, in a rat model TNFα was shown to play a crucial role in adjuvant activity of residual oil fly ash (ROFA) particles on the immune response to a protein antigen (Lambert *et al.,* 2000a). A decline in phagocytosis capacity may cause coadministered antigen to escape uptake and degradation by alveolar macrophages. This may cause larger quantities of antigen to contact immune cells such as DC, a process that is known to enhance sensitization and inflammation (MacLean *et al.,* 1996). The cytokine-rich environment together with enhanced antigen presentation could hypothetically lead to enhanced risk of allergic sensitization.

In the present study we tested the effect of particles on both phagocytosis and cytokine production of an alveolar macrophage cell line of BALB/c mouse origin. Cells were incubated with different types of PM, either or not after priming with lipopolysaccharide (LPS), which has been shown to cause an enhanced cytokine response to particles in alveolar macrophages (Imrich *et al.,* 1999, Ning *et al.,* 2000). In the present study we used amorphous silica (SIP) representing the coarse fraction of PM, and DEP, representing the (ultra-)fine fraction. In addition, (ultra-)fine carbon black particles (CBP) were used that lack the attached chemicals as found on DEP. DEP from different sources were used (DEP1 and DEP3). Moreover, we pre-exposed DEP3 to ozone for 1, 24 or 48h, and tested the effect of ozone treatment of DEP in relation to macrophage function. After overnight incubation of cells and particles, culture supernatant was removed on which IL-1β, IL-6, TNFα and LDH assays were performed, whereas total LDH content and phagocytosis capacity was quantified (Burleson *et al.,* 1987) in the remaining macrophages.

## MATERIALS AND METHODS

**CRL2019**

The murine alveolar macrophage cell line CRL 2019 was obtained from ATCC (Manassas, VA). Cells were cultured and maintained in 75 $cm^2$ culture flasks (Nuclon, Nunc, Roskilde, Denmark) in complete RPMI 1640 (Gibco BRL, Breda, the Netherlands) supplemented with 10% fetal calf serum (FCS), 2% penicilline-streptomycine (SVM, Bilthoven, the Netherlands), 2% sodium bicarbonate BRL, 1% sodium pyruvate and 1.25% Glucose (Gibco BRL). The macrophages were removed from the culture flasks by trypsinization (3 min). Trypsine solution was diluted 10 times with phosphate buffered saline (PBS), and sodium hydrogen carbonate colution (0.75 g/l), 1% penicilline-streptomycine and 0.05% EDTA (all derived from SVM). CRL 2019 macrophages were cultured until the $20^{th}$ passage.

**Particle collection and preparation of the suspensions**

Silica particles (SIP) were approximately 1–5 µm (S-5631, Sigma Chemical Company, St. Louis, MO). Carbon Black Particles (CBP, 65 – 110 nm) were obtained from Brunschwich Chemie (06-0025, Amsterdam, the Netherlands). Diesel Exhaust Particles (DEP) type 1 (65-160 nm) were generated by 1980 Model 5.7 L Oldsmobile V-8 engines containing D-2 diesel control fuel. DEP1 were collected via the exhaust pipe and were diluted 1:10 with filtered air (Mauderly *et al.,* 1987). DEP3 (35-55 nm) were a friendly gift from F. Cassee (RIVM, The Netherlands). They were collected from a diesel motor Cummins type 6 BT 5-9 with a capacity of 113 kW. All carbonaceous particle suspensions (CBP, DEP1 and DEP3) contained a mixture of single particles as well as larger aggregates (1-10µm).

Ozone was generated by irradiation of oxygen with ultraviolet light ($3\ O_2 \rightarrow 2\ O_3$). The generated ozone was led through a column filled with 25 mg DEP3. The concentration of the ozone treatment was +/- 10 ppm in 2.7 l/min compressed air. In our experiments we used DEP3 exposed to 0, 1, 24 and 48 hours of ozone.

DEP1, DEP3 and CBP were suspended in plane RPMI 1640 medium (without serum) and sonicated for 6×30 seconds. SIP were suspended by shaking in complete RPMI 1640 medium. All particles were tested for endotoxin content by means of a Limulus amebocyte lysate (LAL) test (Limusate, Sigma), none reached detection level (0.025 ng/ml).

**Scanning electron microscopy of particle suspensions**

The ultrasonicated suspensions were dehydrated by means of a graded ethanol series, followed by two 5 min treatments in 100% hexamethyldisiazane (HMDS, no. 804324, Merck, Darmstadt, Germany). After the final HMDS treatment samples were air dried on a glass slide. Subsequently samples were coated with a thin layer of gold using a sputter

coating unit (Polaron, Watford, UK) and size was determined in a scanning electron microscope (PSEM 525, Philips, Eindhoven, the Netherlands).

**Exposure of the cells**

Cells ($0.5*10^6$ cells per well) were transferred to 6-wells flat bottom plates (Costar, New York, NY). Next day, 2 ml of fresh complete medium was added with or without (100 ng/ml) lipopolysaccharide (LPS, Brunschwig). After 3 hours cells were washed 3 times with plane RPMI 1640 medium to remove excess LPS. The different particle suspensions (CBP, DEP1, SIP and DEP3 +/- 0, 1, 24 and 48 hours ozone pre-exposure) were administered at a concentration of 0 mg/ml (negative control), 0.11 mg/ml or 0.33 mg/ml for overnight (o.n.) incubation. After collection of the supernatants, cells were washed 2 times with plane RPMI 1640 medium to remove excess particles. For measurement of intracellular amounts of LDH, IL-1β, IL-6 and TNFα, 0.5% Triton X-100 (Merck) was administered for 1 hour to destroy cell membranes.

**Phagocytosis**

Fluorescent polymer microspheres (diameter 2 μm, Duke Scientific, Palo Alto, CA) were added for 3 hours to assess phagocytosis at a temperature of 37°C or 0°C (negative control). After washing 2 times with plane RPMI 1640 medium, adhered cells were removed by brief trypsinization, and after centrigation (800 RPM, 5 min), 100 μl of each sample was used to prepare a cytospin preparation. After fixation (5 min methanol) and staining (5 min Giemsa and 20 min May-Grünwald (Merck)), phagocytosis was measured by counting the number of microspheres inside 100 cells using a fluorescent microscope. The macrophages were divided into 2 groups: cells that phagocytized less than 20 microspheres, and cells that phagocytized over 20 microspheres (positive cells).

**Cytokine assay**

Intracellular and extracellular cytokines (IL-1β, IL-6 and TNFα) were measured by ELISA. In the general procedure, plates were coated with rabbit-anti-mouse antibody (o.n. 4°C). After washing 6 times with PBS/0.1% Tween 20 and blocking (2 h) at room temperature (R.T.) with PBS/1% bovine serum albumine (BSA), plates were washed and incubated with the samples (1.5 h, RT). After washing, biotinylated antibody was administered (1 h, RT). After washing, streptavidin-HRP-conjugate was administered (1 h, RT), followed by tetramethylbenzidine substrate (10 min RT, in the dark). The coloring reaction was stopped by the addition of sulfuric acid. The plates were read at 450 nm. The cytosets, CMC0814 (IL-1β), CMC0064 (IL-6) and CMC3014 (TNFα), were obtained from Biosource (Ettenleur, the Netherlands). The minimal detectable concentration was 2 pg/ml for all cytokines. All cytokines were expressed as pg per unit LDH, resulting in arbitrary units, to compensate for the slightly variable amount of cells present in each well.

**Lactodehydrogenase (LDH) measurement**

LDH levels in supernatants and in suspension after lysis of the cells were measured with a spectrophotometer (Hitachi 912, Rosche, Almere, the Netherlands). To determine damage of the cells, the amount of the extracellular LDH was divided by the total values of the intracellular and extracellular LDH, and was expressed as percentage.

**Statistical analysis**

Data were expressed as the mean +/- SD. Statistical analysis was performed using a two-tail Student's *t*-test.

## RESULTS

**LDH levels**

Total LDH represents the number of cells present in a well. After exposure to 0.11 and 0.33 mg/ml of respectively SIP, CBP, DEP1 and DEP3, cells preactivated with LPS showed a dose-dependent decrease in total amount of LDH of 7% and 11%, 22% and 32%, 16% and 66%, and 7% and 6% respectively (not shown). Cells that were not preactivated by LPS treatment showed lower total amounts of LDH (not shown), probably caused by a lower level of activation and/or proliferation.

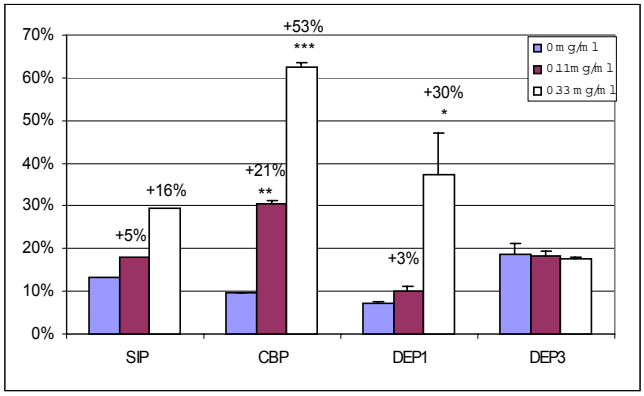


**Figure 1:** LDH release after exposure to SIP, CBP, DEP1 and DEP3 at 0, 0.11 and 0.33 mg/ml. Depicted are average ± standard deviation of duplicate or triplicate measurements. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, all versus 0 mg/ml, as determined by two-tail Student's *t*-test.

Release of LDH from cells represents cell damage, indicating toxicity of the particles. We noticed an increase in LDH release after administration of SIP, CBP, DEP1 and DEP3 to preactivated cells. As shown in Fig. 1, without exposure to particles cells

have a spontaneous release of approximately 12±5%. Administration of SIP (0.11 and 0.33 mg/ml) led to an increase of LDH release to 18% and 29% respectively. After exposure to CBP or DEP1 (0.11 and 0.33 mg/ml), the LDH release increased to 30% and 62% and 10% and 37% respectively (Fig.1, and an overview of results of preactivated cells is given in Table 1). Administration of DEP3 did not influence LDH release. In non-preactivated cells increase of LDH release was roughly the same after CBP exposure, whereas SIP and DEP1 stimulated approximately half of the release as compared to preactivated cells. DEP3 did not influence the LDH release (an overview of results of non-preactivated cells is given in Table 2).

**Table 1:** LDH release, phagocytosis and increase of cytokine production in LPS-preactivated alveolar macrophages after exposure to SIP, CBP, DEP1 and DEP3.
**Table 2:** LDH release, phagocytosis and increase of cytokine production in non-preactivated alveolar macrophages after exposure to SIP, CBP, DEP1 and DEP3.

**Table 1:**

| | | Increase of LDH release | Inhibition of phagocytosis | TNFα | IL-6 | IL-1β |
|---|---|---|---|---|---|---|
| SIP | 0.11mg/ml<br>0.33mg/ml | 5%<br>16% | 21%<br>40% | 28<br>15 | -7<br>0 | 3<br>68 |
| CBP | 0.11mg/ml<br>0.33mg/ml | 21%<br>53% | 74%<br>100% | 53<br>77 | 7<br>15 | 63<br>96 |
| DEP1 | 0.11mg/ml<br>0.33mg/ml | 3%<br>30% | 35%<br>62% | 29<br>149 | 26<br>85 | 76<br>46 |
| DEP3 | 0.11mg/ml<br>0.33mg/ml | 0%<br>0% | 62%<br>92% | 5<br>28 | 26<br>20 | -<br>- |

**Table 2:**

| | | Increase of LDH release | Inhibition of phagocytosis | TNFα | IL-6 | IL-1β |
|---|---|---|---|---|---|---|
| SIP | 0.11mg/ml<br>0.33mg/ml | 2%<br>9% | 44%<br>61% | 14<br>47 | -<br>- | -<br>- |
| CBP | 0.11mg/ml<br>0.33mg/ml | 6%<br>54% | 59%<br>94% | 2<br>7 | -<br>- | -<br>- |
| DEP1 | 0.11mg/ml<br>0.33mg/ml | 2%<br>16% | 37%<br>68% | 1<br>7 | -<br>- | -<br>- |
| DEP3 | 0.11mg/ml<br>0.33mg/ml | 0%<br>1% | 20%<br>100% | 3<br>6 | -<br>- | -<br>- |

LDH release (%): particle-treated sample – control sample.
Phagocytosis (%): decrease of cells containing > 20 fluorescent microspheres.
TNFα, IL-6, IL-1β: Absolute increase per unit LDH. - = No production

**Phagocytosis**

As shown in Fig. 2, the percentage of preactivated cells phagocytizing over 20 fluorescent microspheres was 27±3%. After exposure to SIP (0.11 and 0.33 mg/ml), the percentage of preactivated cells phagocytizing over 20 fluorescent microspheres was reduced to 20% and 15% respectively (Fig. 2). After exposure to CBP (0.11 and 0.33 mg/ml) the percentage of cells phagocytizing over 20 microspheres decreased from 31% to 8% and 0% respectively. Administration of DEP1 reduced phagocytosis from 26% to 17% and 10% at concentrations of 0.11 mg/ml and 0.33 mg/ml, whereas preactivated cells exposed to DEP3 (0.11 mg/ml and 0.33 mg/ml) showed a decline of cells phagocytizing over 20 microspheres from 26% to 10% and 2%, respectively (Fig. 2).

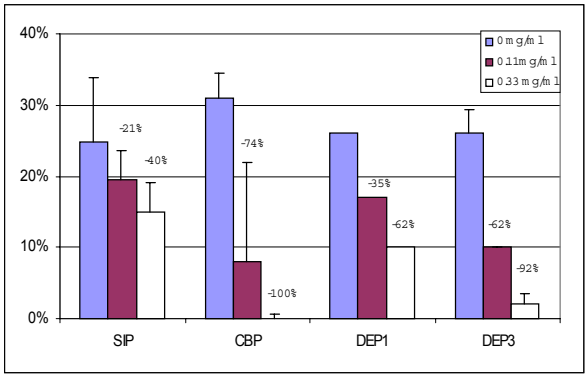


**Figure 2:** Phagocytosis of fluorescent probes after particle exposure. The percentage of macrophages containing >20 probes after exposure to SIP, CBP, DEP1 and DEP3 at 0, 0.11 and 0.33 mg/ml. Depicted are average ± standard deviation of 6 measurements.

Phagocytosis was almost completely inhibited when cells were incubated with microspheres at 0°C (not shown). Non-preactivated cells showed a slightly lower percentage of cells phagocytzing over 20 microspheres 18±5% (not shown) compared to LPS primed cells when no particles were administered. Also in non-preacivated cells phagocytosis was reduced after particle exposure (Table 2).

**TNFα production**

After exposure of preactivated cells to 0.11 and 0.33 mg/ml CBP, DEP1 and DEP3, TNFα production (expressed per unit LDH, measured in supernatant) showed a concentration-dependent increase (Fig. 3). After CBP exposure, TNFα production increased approximately 6- and 9-fold for 0.11 and 0.33 mg/ml, whereas after DEP1 exposure it increased 2- and 8-fold (Fig. 3).

Preactivated cells exposed to 0.11 and 0.33 mg/ml SIP failed to show an increase of TNFα production expressed in unit LDH (Fig. 3, Table 1). A concentration-dependent increase was also observed in non-preactivated cells, although absolute levels (not shown) and absolute increases (Table 2) were lower for all particles except SIP.

We also measured the TNFα production expressed per unit LDH intracellularly. The intracellular TNFα production increased during exposure to the different particles comparable to the increase of the extracellularly detected TNFα (not shown).

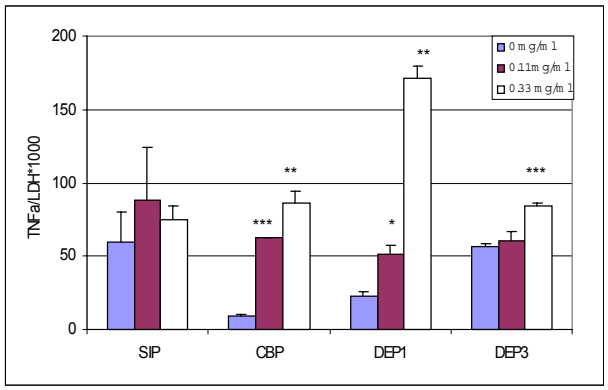


**Figure 4:** TNFα release per unit LDH after SIP, CBP, DEP1 and DEP3 at 0, 0.11 and 0.33 mg/ml. Depicted are average ± standard deviation of duplicate or triplicate measurements. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, all versus 0 mg/ml, as determined by two-tail Student's *t*-test.

**IL-6 production**

Only preactivated cells show IL-6 production. As shown in Table 1, exposure to CBP, DEP1 and DEP3 led to an increase compared to the control sample, whereas IL-6 production was not influenced by exposure to SIP.

**IL-1β production**

Cells that were not preactivated by administration of LPS showed no production of IL-1β. Preactivated cells, however, produced IL-1β when exposed to 0.11 and 0.33 mg/ml SIP, CBP or DEP1. No IL-1β production was measured when cells were exposed to DEP3 (Table 1).

**DEP1 versus DEP3**

Although DEP1 and DEP3 are both diesel exhaust particles and have approximately the same size as examined by scanning electron microscopy (not shown), CRL 2019 macrophages showed some important differences in response (Table 1 and 2). After DEP1 exposure both LDH release and TNFα production increased strongly compared to DEP3

exposure. Exposure to DEP3, however, led to a stronger reduction of the phagocytosis compared to DEP1. It is known that DEP in ambient air can be altered by other air pollutant components, such as ozone. Therefore we pretreated DEP3 with ozone during 1, 24 and 48 hours as described in Materials & Methods. This treatment did not lead to morphological changes of the particles as examined by scanning electron microscopy (not shown).

**LDH levels (ozone-exposed DEP3)**

In both preactivated and non-preactivated cells, total amount of LDH (not shown) after exposure 1, 24 and 48 hours ozone-exposed DEP3 (DEP3 $_{ozone\ 1}$ , DEP3 $_{ozone\ 24}$ and DEP3 $_{ozone\ 48}$) was hardly influenced compared to the control sample. Also the release of LDH in preactivated cells (an overview of results of preactivated cells is given in Table 3) and non-preactivated cells (Table 4) was unaltered by ozone exposed DEP3.

**Phagocytosis (ozone-exposed DEP3)**

Whereas preactivated cells showed a decrease from 26% to 10% and 2% when exposed to 0.11 and 0.33 mg/ml untreated DEP3 respectively, exposure to DEP3 $_{ozone\ 1}$, DEP3 $_{ozone\ 24}$ and DEP3 $_{ozone\ 48}$ inhibited phagocytosis to 5% (0.11 mg/ml) and to 0% (0.33 mg/ml) as shown in Fig. 4. Numbers of non-preactivated cells phagocytizing over 20 fluorescent microspheres decreased when exposed to untreated DEP3. Inhibition increased from 20% to 100% for 0.11 and 0.33 mg/ml respectively (Table 4). For DEP3 $_{ozone\ 1}$ this was respectively 55% and 100%, whereas phagocytosis was completely blocked when the cells were exposed to DEP3 $_{ozone\ 24}$ and DEP3 $_{ozone\ 48}$ (Table 4).

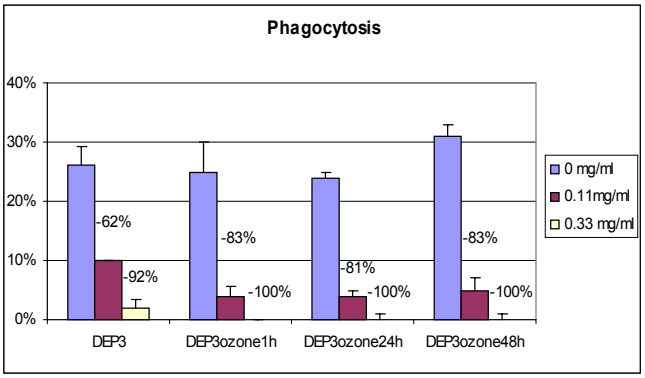


**Figure 4:** The percentage of macrophages containing >20 probes after exposure to DEP3, $DEP3_{ozone1}$, $DEP3_{ozone24}$ and $DEP3_{ozone48}$ at 0, 0.11 and 0.33 mg/ml. Depicted are average ± standard deviation of 6 measurements.

**Table 3:** LDH release, phagocytosis and increase of cytokine production in LPS-preactivated alveolar macrophages after exposure to DEP3, $DEP3_{ozone1}$, $DEP3_{ozone24}$, and $DEP3_{ozone48}$.
**Table 4:** LDH release, phagocytosis and increase of cytokine production in non-preactivated alveolar macrophages after exposure to DEP3, $DEP3_{ozone1}$, $DEP3_{ozone24}$, and $DEP3_{ozone48}$.

**Table 3:**

| | | Increase of LDH release | Inhibition of phagocytosis | TNFα | IL-6 |
|---|---|---|---|---|---|
| DEP3 | 0.11mg/ml<br>0.33mg/ml | 0%<br>0% | 62%<br>92% | 5<br>28 | 26<br>20 |
| DEP3 ozone 1 | 0.11mg/ml<br>0.33mg/ml | 0%<br>8% | 83%<br>100% | 22<br>38 | 16<br>3 |
| DEP3 ozone24 | 0.11mg/ml<br>0.33mg/ml | 0%<br>8% | 81%<br>100% | 56<br>72 | 17<br>59 |
| DEP3 ozone48 | 0.11mg/ml<br>0.33mg/ml | 0%<br>6% | 83%<br>100% | 40<br>70 | 61<br>42 |

**Table 4:**

| | | Increase of LDH release | Inhibition of phagocytosis | TNFα | IL-6 |
|---|---|---|---|---|---|
| DEP3 | 0.11mg/ml<br>0.33mg/ml | 0%<br>1% | 20%<br>100% | 3<br>6 | -<br>- |
| DEP3 ozone 1 | 0.11mg/ml<br>0.33mg/ml | 0%<br>0% | 55%<br>100% | 2<br>3 | -<br>- |
| DEP3 ozone24 | 0.11mg/ml<br>0.33mg/ml | 0%<br>4% | 100%<br>100% | 7<br>23 | -<br>- |
| DEP3 ozone48 | 0.11mg/ml<br>0.33mg/ml | 0%<br>4% | 100%<br>100% | 20<br>49 | -<br>- |

LDH release (%): particle-treated sample – control sample.
Phagocytosis (%): decrease of cells containing > 20 fluorescent microspheres.
TNFα, IL-6, IL-1β: Absolute increase per unit LDH.
-: No production

**TNFα, IL-6 and IL-1β production (ozone-exposed DEP3)**

Exposure to 0.33 mg/ml DEP3 of preactivated cells led to a significant increase in TNFα production compared to its control (Fig. 5).

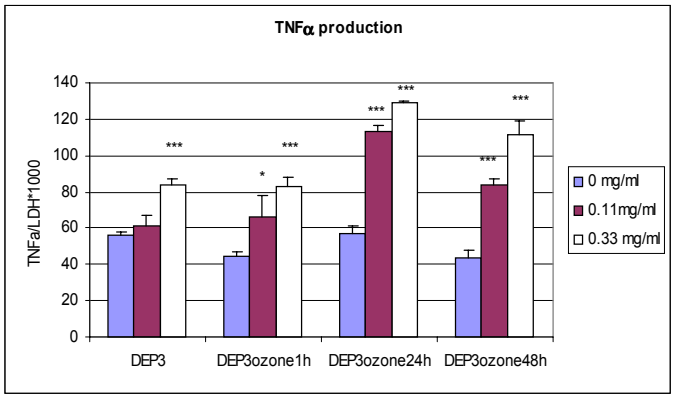


**Figure 5:** TNFα release per unit LDH after exposure to DEP3, $DEP3_{ozone\ 1}$, $DEP3_{ozone24}$ and $DEP3_{ozone\ 48}$ at 0, 0.11 and 0.33 mg/ml. Depicted are average ± standard deviation of duplicate or triplicate measurements. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, all versus 0 mg/ml, as determined by two-tail Student's *t*-test.

For $DEP_{ozone\ 1}$ TNFα production increased significantly at both 0.11 and 0.33 mg/ml. Exposure to 0.11 and 0.33 mg/ml $DEP3_{ozone\ 24}$ and $DEP3_{ozone\ 48}$ significantly stimulated TNFα production (Fig. 7). In non-preactivated cells TNFα also increased concentration-dependently and more pronouncedly after exposure to ozone-exposed DEP as compared to untreated DEP (Table 4).

Preactivated cells showed a spontaneous production of IL-6. No increased production of IL-6 was observed after exposure to ozone- exposed DEP3 (Table 3). Non-preactivated cells show no IL-6 production at all (Table 4). Preactivated and non-preactivated cells exposed to the different ozone-exposed DEP3 particles did not produce IL-1β at any time (not shown).

## DISCUSSION

Outdoor air consists of a complex mixture of particulate and gaseous pollutants. In the present study we examined the effects of several particulate air pollutants on cell damage, phagocytosis and production of proinflammatory mediators in a murine alveolar macrophage cell line. In addition, the effect of a "secondary" particle, ozone-exposed DEP,

was assessed. The present study shows that different types of particles have distinct effects on cell damage, phagocytosis and cytokine production by alveolar macrophages. Moreover, ozone-exposed DEP show more pronounced effects on phagocytosis and cytokine production compared to untreated DEP.

SIP, DEP1 and especially CBP caused cytotoxicity in the macrophages, indicated by the LDH release that dose-dependently increased upon particle exposure, as was also found by others (Kim *et al.,* 1999). In contrast to DEP1, DEP3 did not cause any increase in LDH release, but DEP3 did have the most profound inhibitory effect on phagocytosis. Therefore it can be concluded that the increase in cell damage is not related to the decrease in phagocytosis. CBP also inhibited phagocytosis almost completely, whereas DEP1 and SIP had the least effect. Ultrafine particles have been shown to inhibit phagocytosis when they occupied 2.6% of macrophage volume (Oberdörster *et al.,* 1994). Particles may downregulate the expression of macrophage receptors involved in phagocytosis (Becker and Soukup 1998). The fact that alveolar macrophages employ distinct receptor-mediated mechanisms for different particles, as was described for SIP versus DEP (Kobzik 1995, Palecanda *et al.,* 1999), may play a role in the observed differences.

IL-6 and IL-1β production were not clearly particle concentration-dependently stimulated, and production was only observed after LPS preactivation of the cells, which has also been shown by others to amplify the cytokine response of macrophages (Imrich *et al.,* 1999). On the contrary, TNFα was spontaneously released and displayed a clear particle-concentration-response relationship. CBP stimulated the highest cytokine levels, followed by DEP1, DEP3 and SIP respectively. This order correlates with adjuvant potential in an *in vivo* model (van Zijverden *et al.,* Chapter 4). Overall it appears that increased cytokine production does not correlate with increased LDH release and inhibition of phagocytosis. The fact that cytotoxicity is not related to phagocytosis, has also been shown by others (Hadnagy and Seemayer, 1994).

The present study shows that DEP of different sources have distinct effects. An explanation may be found in differences in amount or composition of attached substances. Interaction of DEP with ozone is known to cause substantial changes in the composition of the attached polyaromatic hydrocarbons, increasing the number of chemically reactive groups (Madden *et al.,* 2000). This observation is highly relevant since ozone is a ubiquitous and reactive pollutant in ambient air, showing harmful effects on human health both alone (Hiltermann *et al.,* 1997) and in combination with PM (Korrick *et al.,* 1998). Recently, ozone-exposed DEP were shown to increase LDH content and polymorphonuclear cell infiltrate in lung lavage fluid when compared to untreated DEP in rat after *in vivo* administration (Madden *et al.,* 2000). The present study shows the effects

of ozone-exposed DEP3 on macrophage function, displaying a more powerful inhibition of phagocytosis already at lower particle concentration and stimulation of TNFα production compared to untreated DEP3.

Our results suggest that TNFα production by alveolar macrophages may represent an important determinant of adjuvant activity of PM *in vivo*. The concurrent severe decline in phagocytosis is in line with the hypothesis that a cytokine-rich environment in combination with augmented antigen presentation leads to increased risk of allergic sensitization. In humans exposed to a mixture of pollutants in outdoor air, the interaction of airborne particles with other pollutants such as ozone may pose an additional risk of sensitization.

## ACKNOWLEDGMENTS

The authors would like to thank P. Beekhof, R. van Loenen and P. Fokkens (RIVM) for excellent technical assistance and prof. Dr. J. G. Vos (RIVM) and prof. Dr. W. Seinen (RITOX) for critically reading the manuscript.

# CHAPTER 4

# PARTICULATE AIRBORNE POLLUTANTS ADJUVATE ALLERGIC SENSITIZATION

Maaike van Zijverden, Marianne Bol, Rob Bleumink, Anita van der Pijl, Joost Vos, Henk van Loveren, André Penninks, Raymond Pieters

## ABSTRACT

Particulate air pollution negatively affects human health. Whereas it is known to increase symptoms of existing respiratory allergy, its effect on sensitization to common allergens is unknown. For hazard identification a good animal model is needed to assess the possible immunomodulating effects of particles on the sensitization process and the further course of the immune response. Whereas existing models frequently make use of artificial methods like injection and/or adjuvants to facilitate sensitization, we have developed a model in which sensitization takes place via the relevant route of exposure. Antigen (2,4,6-trinitrophenyl coupled to ovalbumin [TNP-OVA], total dose 30 μg) and particles (total dose 200 μg) were administered intranasally to female BALB/c mice during Day 1,2,3, and at Day 10 a challenge with antigen alone (10 μg) was performed. The immune response was read out 5 days after this challenge. Immunoglobulin (Ig) levels were measured in serum, antibody-forming cells (AFC) were determined in the lymph nodes draining the lung (peribronchial lymph nodes [PBLN]), and cell types present in bronchial alveolar lavage (BAL) were differentiated. The effects of two types of diesel exhaust particles (DEP) from different sources (DEP1 and DEP2), carbon black (CBP) and amorphous silica particles (SIP) were tested. All particles except SIP were found to be able to adjuvate the immune response to TNP-OVA. DEP1 and CBP induced the highest Ig-levels in blood and AFC-numbers in PBLN, followed by DEP2, whereas the increases induced by SIP were not significant. CBP induced an increased number of eosinophils in BAL, whereas SIP stimulated neutrophils to infiltrate. The effect of an extra challenge at Day 17 was tested in animals exposed to CBP. At Day 22 these animals showed significantly higher IgG1-AFC and eosinophil numbers as well as IgG2a levels compared to Day 15. The fact that particles display persistent adjuvant activity when they are administered during sensitization points out that the risk of sensitization of naive subjects may be increased during coexposure to particles and allergen.

## INTRODUCTION

The prevalence of asthma and respiratory allergy has been increasing at a high rate in Western industrialized countries during the past few decades (Lundbäck 1998, Schäfer and Ring, 1997). Since these higher rates are exclusively observed in Westernized societies (ISAAC 1998, Jogi *et al.,* 1998, Beasley *et al.,* 2000) it is generally thought that they are associated with various aspects of Western lifestyle (Hopkin 1997, Popp *et al.,* 1989). Studies in (former) West and East Germany in the early 1990s clearly display the higher prevalence in the West (von Mutius *et al.,* 1992). However, recent studies after unification show an increasing rate also in the former East (von Mutius *et al.,* 1998). These epidemiological facts of asthma have resulted in different theories of asthma onset. One theory associates outdoor air pollution with asthma (Rusznak *et al.,* 1994). Epidemiological support is for example found in studies showing differences in the prevalence of childhood asthma between rural and urban centers in several countries (Goren *et al.,* 1988, Goren and Hellmann 1988, Weinberg 2000, Beasly *et al.,* 2000), and the fact that in recent years asthma is increasing in cities of developing countries (Weinberg 2000). Both observations may partly be associated with air pollution produced by road transport (Duhme *et al.,* 1996, van Vliet *et al.,* 1997). The fact that humans are exposed to a complex mixture of pollutants and innumerable other environmental factors makes it hard to extract the responsible factor from epidemiological studies. Diesel exhaust particles (DEP) are one of the main suspects, because they make up a large part of air pollution in Western cities. Moreover, in already sensitized subjects DEP are known to be able to both increase symptoms (Pope and Dockery 1992 ) and IgE production (Diaz-Sanchez *et al.,* 1997a), and cause inflammation (Salvi *et al.,* 1999 and 2000) and even adjuvate mucosal sensitization to a neoantigen in naïve subjects (Diaz-Sanchez *et al.,* 1999).

DEP and other carbonaceous particles smaller than 2.5 μm are part of the (ultra)fine fraction of so called particulate matter (PM). The coarse fraction (2.5-10 μm) of PM mainly consists of inorganic wind blown dusts such as silica and large carbon aggregates (Churg and Brauer 1997, Ormstad *et al.,* 1997). Size is crucial in determining whether and where particles are deposited in the lung. Ultrafine and fine particles are able to penetrate deep into the lung and stay present for a long time (Churg and Brauer, 1997, Ferin *et al.,* 1992). Other particle characteristics such as attached chemicals and surface area probably also play a role in the adjuvant activity.

The mechanism by which particles modulate the immune response is incompletely known (Salvi and Holgate 1999), but inflammation probably plays a central role (Saxon and Diaz-Sanchez 2000). Information on these mechanisms is indispensable for estimating

human risks of particle exposure (McClellan 1997). Therefore a good model is needed in which the effect of particles on sensitization and on the further course of the immune response can be assessed. Present models displaying important effector phase characteristics like specific IgE and eosinophils almost all use injections and/or adjuvantia to sensitize (Kung *et al.,* 1994, Yamada *et al.,* 1994, Hessel *et al.,* 1995). Hence they do not reflect real life exposure and its unique characteristics (Constant *et al.,* 2000). In addition, models in which particle and antigen exposure are combined are often long and complicated (Takafuji *et al.,* 1987), and particle dosing is not related to the phase of the immune response but rather takes place continuously (Fujimaki *et al.,* 1997, Takano *et al.,* 1997, Maejima *et al.,* 1997). A recent promising rat model by Lambert *et al.* (1999) is relatively short (2 weeks) and particle exposure is limited to one dose.

In the model presented in this study we intranasally sensitized mice with TNP-OVA together with different particles during 3 consecutive days. The capacity of the particles to adjuvate an immune response to the antigen was compared after an antigen challenge at Day 10. We compared the immunomodulating capacity of two types of DEP from different sources (DEP1 and DEP2), CBP and amorphous silica particles (SIP). DEP and CBP represent particles from the (ultra)fine fraction, but whereas DEP are known for their immunomodulating capacity, CBP are possibly not as inert (Løvik *et al.,* 1997, Nilsen *et al.,* 1997) as they were once thought to be (Crosbie 1986). This was also shown in a previous study using subcutaneous injection (van Zijverden *et al.,* 2000), in which the carbonaceous particles DEP and CBP were compared with amorphous SIP, of which the immunomodulatory capacity in relation to respiratory allergy is unknown. All types of particles were able to act as adjuvant in this injection model, but the alteration in immune response with respect to Th1/Th2 differentiation differed with the different particles.

In the present intranasal model, the immune response was assessed at Day 15, five days after challenge. In addition, the effect of an extra challenge at Day 17 was tested in animals exposed to carbon black particles (CBP), and the response was determined at Day 22. In all animals antibody levels in blood and antibody forming cells (AFC) in peribronchial lymph nodes (PBLN) were assessed. Also cell infiltrates in the lung were characterized in bronchial alveolar lavage (BAL).

## MATERIALS AND METHODS

### Animals

Female, specific pathogen-free BALB/c mice (6-8 weeks of age) were obtained from the Utrecht University breeding facility. Mice were housed under hygienic barrier conditions

in filter-topped macrolon cages with bedding of wood chips, a temperature of 23±2$^{o}$C, 50-60% relative humidity and a 12-h light/dark cycle. They received standard lab chow and acidified tap water *ad libitum*. The experiments were approved by an ethical committee and conducted in accordance with the Guiding Principles in the Use of Animals in Toxicology.

**Chemicals and reagents**

Silica (amorphous, microcrystalline $SiO_2$ [SIP]) was obtained from Sigma Chemical Company (St. Louis, MO) and CBP from Brunschwich Chemie (Amsterdam, The Netherlands). DEP1 and DEP2 were generous gifts from respectively Dr. P. Steerenberg (RIVM, the Netherlands) and Dr. P. Scheepers (Nijmegen University, the Netherlands). The antigen TNP-OVA (2,4,6-trinitrophenyl coupled to ovalbumin) was prepared as described by Albers *et al*. (1997). Immobilon-P membranes were obtained from Millipore (Etten-Leur, The Netherlands), and alkaline-phosphatase (AP)-conjugated goat- anti-mouse IgG1 and IgG2a antibodies from Southern Biotechnology Associates (Birmingham, AL). Anti-mouse IgE (02111D) for IgE ELISA was obtained from Pharmingen (Hamburg, Germany). All reagents for ELISA were obtained from Sigma Chemical Company (St. Louis, MO), whereas the plates (highbond 3590) were obtained from Costar (Cambridge, MA).

**Treatment of the mice**

Particle suspensions (2.67 mg/ml) were prepared in saline containing 0.4 mg/ml TNP-OVA and 5% heat inactivated normal BALB/C mouse serum. The latter was added to facilitate suspension of the hydrophobic particles, while they stirred for 18 h (Løvik *et al*. 1997). Naïve female BALB/c mice (n=8) were intranasally instilled by pipetting a total volume of 25 µl on the nostrils under light ether anesthesia. Sensitization took place at Days 1, 2 and 3, adding up to a total TNP-OVA dose of 30 µg and a total particle dose of 200 µg. A challenge dose of 10 µg TNP-OVA was given at Day 10 (Table 1). Blood was collected by orbita punction under ether anesthesia 3 days before the experiment and 5 days after challenge (Day 15), just prior to dissection, at which also BAL was obtained and PBLN, spleen and bone marrow were taken out. In a separate experiment the effect of an extra challenge at Day 17 was tested in animals exposed to CBP (n=4). Blood was drawn 3 days before the start of the experiment, and at Day 15 and Day 22, at which the dissection took place (Table 1).

**Table 1:** Treatment groups in the intranasal model (n=4-6). Blood samples were taken at Day –2 and at the end of the experiment at either Day 15 or Day 22.

| group | Sensitization (Day 1,2,3) | Challenge (Day 10) | End of experiment (Day 15) | Challenge (Day 17) | End of experiment (Day 22) |
|---|---|---|---|---|---|
| Control | TNP-OVA | TNP-OVA | yes | - | - |
| Particle treatment | TNP-OVA+ particles (total dose 200 μg) | TNP-OVA | yes | - | - |
| Control | TNP-OVA | TNP-OVA | no | TNP-OVA | yes |
| Particle treatment | TNP-OVA+ particles (total dose 200 μg) | TNP-OVA | no | TNP-OVA | yes |

**TNP-specific IgG1 and IgG2a ELISA**

Plates were coated (overnight [o.n.] 4$^{o}$C) with TNP coupled to bovine serum albumin ([BSA] TNP-BSA, 20 μg/ml) in 0.05 M carbonate buffer pH 9.6, and blocked with phosphate buffered saline containing 0.05% Tween and 1% BSA ([PBS/T/BSA], 1 h, RT). Serial dilutions of sera were incubated (1 h, room temperature [RT]) and plates were washed and incubated with an optimal dilution of AP-conjugated anti-mouse IgG1 or IgG2a (1 h, RT), followed by p-nitrophenyl phosphate (1 mg/ml in diethanolamine buffer, pH 9.8) for 30 min. Absorbance was measured at 405 nm. Titers were calculated by means of the sample dilution at which extinctions were higher than background+2*SD. Background levels at Day 0 were subtracted from Day 15 and Day 22 data to obtain increases in titer.

**TNP-specific IgE ELISA.**

Plates were coated (6 h, first hour at 37$^{o}$C, last hours at RT) with αIgE, 2 μg/ml in 0.05 M carbonate buffer pH 9.6. After washing, serial dilutions of sera were incubated (o.n. 4$^{o}$C) and plates were washed and incubated with an optimal dilution of a biotin-TNP-BSA complex (1h, RT). After incubation (45 min, RT) with poly-HRP-streptavidine (CLB, Amsterdam, The Netherlands), tetramethylbenzidine (1 mg/ml) was added (15 min, RT). The coloring reaction was stopped with $H_2SO_4$ and absorbance was measured at 450 nm. Titers were calculated by means of the sample dilution at which extinctions were higher than background+2*SD. Background levels at Day 0 were subtracted from Day 15 and Day 22 data to obtain increases in titer.

**ELISPOT assay**

These assays were essentially performed as described previously (Schielen *et al*., 1995). Briefly, organs were minced to prepare single cell suspensions, and 0.5*10$^{6}$ cells in 500 μl PBS/1% BSA were incubated (4 h, 37$^{o}$C) in wells containing TNP-BSA (20 μg/ml) coated

(o.n. 4°C) Immobilon-P membranes as bottom. Thereafter, membranes were washed and incubated with optimal dilutions of isotype-specific AP-conjugated anti-mouse Ig antibodies in PBS/T (o.n. 4°C). After washing, spots were developed by incubation with 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium. Specific AFCs per $10^6$ cells were calculated from spot numbers counted with the aid of a stereo microscope.

**Bronchial alveolar lavage and cell differentiation**

The lungs of the mice were lavaged 3 times with 1-ml aliquots of PBS (37°C). The bronchial alveolar lavage was kept on ice until it was centrifuged (1000 g, 5 min). The pellet was resuspended in 250 µl of cold PBS. For differential cell counts cytospin preparations were made and stained 7 min with May-Grünwald (Merck, Darmstad, Germany), and subsequently 20 min with Giemsa staining (Merck). After coding, the cytospin preparations were evaluated using oil immersion microscopy. Cells were identified and differentiated into macrophages, eosinophils, neutrophils and lymphocytes by standard morphology. Macrophages containing one of the carbonaceous particles (DEP1,2 and CBP) could be detected because of the black color of the particles, and were indicated by M+. Per cytospin preparation at least 200 cells were counted, and the percentages of the various cell types were calculated.

**Statistics**

Preceding statistical analysis, ELISPOT data (the number of AFCs) were transformed to log10 values to homogenize variance. Differences between group means were analyzed using one-way ANOVA with Bonferroni's post-hoc test for contrasts ($p<0.05$ or $p<0.01$). In the case of nonsufficient homogeneity of variances, a Kruskal-Wallis ranking test was performed.

## RESULTS

The different particles were administered on 3 consecutive days together with antigen during the sensitization phase, up to a total dose of 200 µg/animal. At Day 10 a challenge with antigen was given, and at Day 15 the immunomodulatory capacity of different particles was assessed. In an additional experiment, CBP-exposed mice were given an additional antigen-challenge at Day 17, and the immune response was determined at Day 22.

**Levels of TNP-specific IgG1, IgG2a and IgE antibodies in serum**

Immunoglobulin levels were determined in sera obtained at Day 0 and at the end of the experiment at Day 15 or Day 22. Day 0-background levels were subtracted from levels found at the end of the experiment. At Day 15 all particle-treated groups except SIP

showed significant increases in IgG1 titers compared to TNP-OVA controls. Levels of DEP1-treated animals were also significantly enhanced compared to those in SIP-treated animals (Fig. 1A). IgG2a was significantly enhanced in DEP1-treated animals compared to those in TNP-OVA controls and SIP (Fig. 1B). IgE was enhanced significantly in CBP and DEP1-treated animals compared to controls (Fig. 1C).

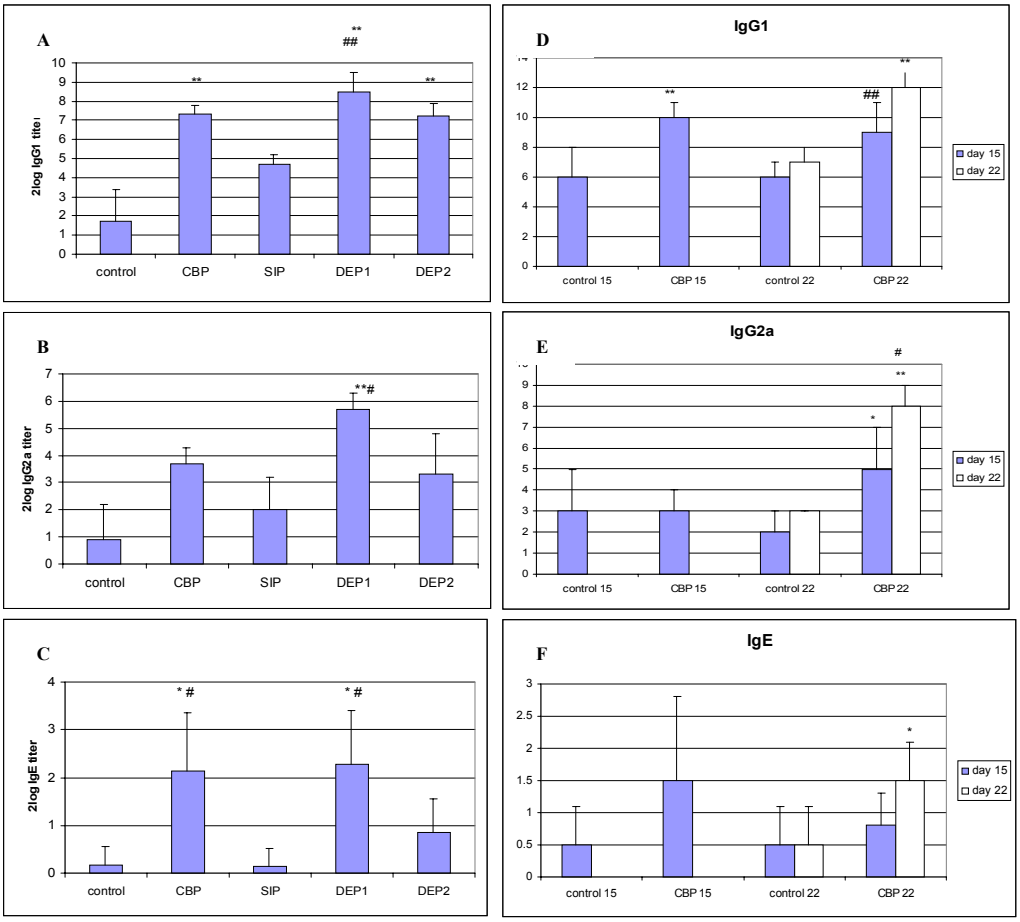


**Figure 1:** The increase in TNP-specific antibody titers in serum between Day 0 and Day 15 after exposure to CBP, SIP, DEP1 and DEP2 during sensitization with TNP-OVA (A,B,C) and between Day 0, 15 and 22 after exposure to CBP during sensitization with TNP-OVA (D,E,F). Depicted are averages ± standard deviations [SD]. Figure 1A: IgG1 titers. Figure 1B: IgG2a titers. Figure 1C: IgE titers. * = $p<0.05$ versus TNP-OVA control, ** = $p<0.01$ vs TNP-OVA control, # = $p<0.05$ vs SIP, ## $p<0.01$ vs SIP. Figure 1D: IgG1 titers. Figure 1E: IgG2a titers. Figure 1F: IgE titers. * = $p<0.05$, ** $p<0.01$ vs all TNP-OVA control groups, ## = $p<0.01$ versus all TNP-OVA Day 15 data, # $p<0.05$ vs CBP Day 15 data from the same group, as determined by ANOVA followed by Bonferroni's post hoc test.

At Day 22 IgG1 levels in CBP-treated animals were even more pronouncedly enhanced (Fig. 1D), whereas IgG2a was significantly higher at Day 22 compared to Day 15 (Fig. 1E). IgE was significantly enhanced at Day 22 compared to controls (Fig. 1F).

**Number of TNP-specific antibody forming cells in PBLN**

The number of Ig-producing cells was assessed in PBLN, spleen and bone marrow. In the latter two organs hardly any AFC were identified (not shown). In PBLN IgG1 but no IgG2a and IgE-AFC could be detected. This is in accordance with IgG1 being the isotype detected in highest levels in serum (not shown). At Day 15 IgG1-AFC were found in significantly increased numbers in CBP- and DEP1-treated animals as compared to SIP, whereas numbers in CBP-treated animals were also significantly higher than TNP-OVA control level (Fig. 2A). At Day 22 this increase was even more pronounced, and significantly higher than TNP-OVA controls and Day 15 numbers of CBP-treated animals (Fig. 2B). The AFC numbers at Day 15 of the CBP-treated group in Fig. 2B appeared higher than those in Fig. 2A. However, this difference was not statistically significant and is considered to be due to inter-experimental and intra-species variation.

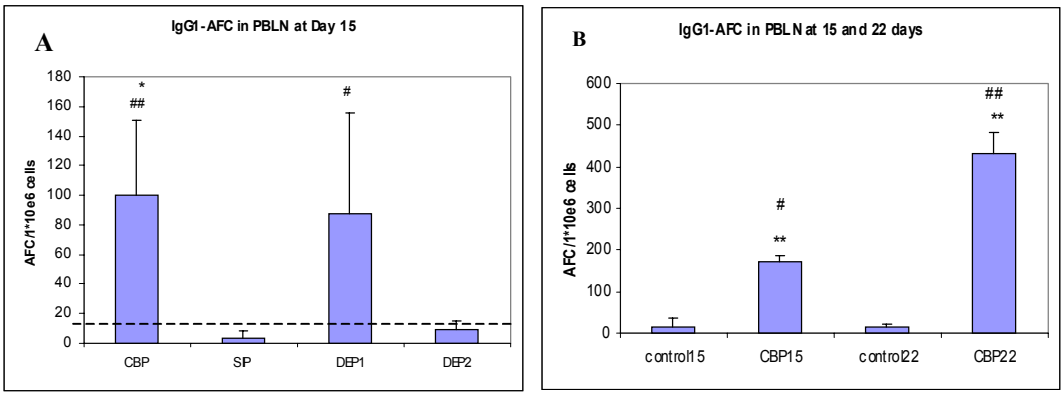


**Figure 2:** Number of TNP-specific IgG1-forming cells per $10^6$ cells, at Day 15 after exposure to CBP, SIP, DEP1 and DEP2 (Figure 2A) and at Day 15 and 22 after exposure to CBP (Figure 2B) (averages ± SD).
Figure 2A: The dashed line represents TNP-OVA control level. * = $p<0.05$ vs control, # = $p<0.05$ vs SIP, ## = $p<0.01$ vs SIP, as determined by ANOVA followed by Bonferroni's post hoc test.
Figure 2B: ** = $p<0.01$ vs TNP-OVA control Day 15, # = $p<0.05$ vs TNP-OVA control Day 22, ## = $p<0.01$ vs TNP-OVA control Day 22, as determined by ANOVA followed by Bonferroni's post hoc test.

**Cell differentiation of BAL cells**

BAL cells from a representative TNP-OVA control animal are shown in Figure 4A. More than 95% of the cells are macrophages. BAL cells from the CBP- or DEP1/2-exposed animals showed that approximately 50% of the macrophages present was colored black (M+) (Fig. 3A and 4B). Phagocytosis of the white SIP could not be scored accurately. In addition, the CBP-treated group showed a significant eosinophil-infiltrate. The infiltrate of SIP-treated animals mainly consisted of neutrophils. Non significant infiltrates were observed in DEP1 and DEP2-treated animals (Fig. 3A). At Day 22 the eosinophil infiltrate in CBP-treated animals was significantly enhanced as compared to TNP-OVA controls and Day 15 levels of CBP-treated mice (Fig. 3B).

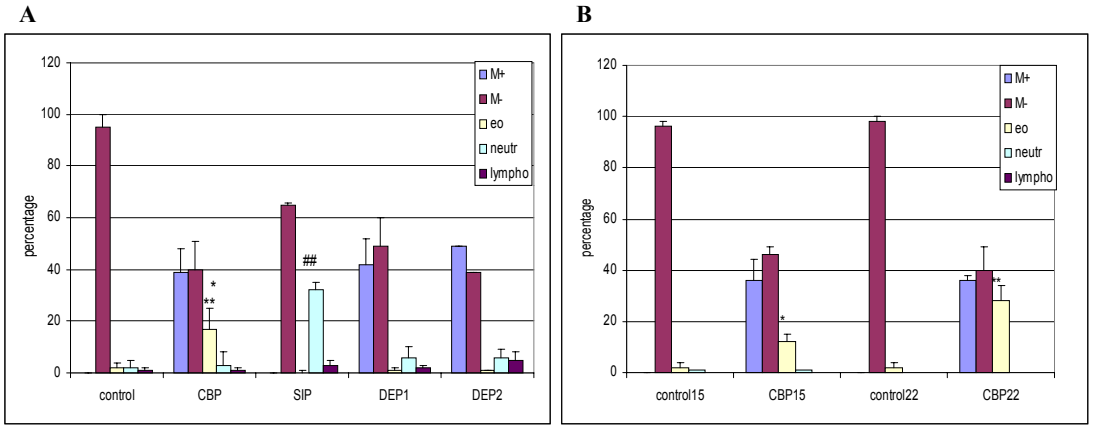


**Figure 3:** Percentages of various cell types in bronchial alveolar lavage at Day 15 after exposure to CBP, SIP, DEP1 and DEP2 (Figure 3A) and at Day 15 and 22 after exposure to CBP (Figure 3B) (averages ± SD). "M+" = macrophages containing carbonaceous particles (SIP was not visible), "M" = macrophages not containing particles, "eo" = eosinophil, "neutr" = neutrophil, "lympho"= lymphocyte.
Figure 3A: * = $p<0.05$ versus DEP2, ** = $p<0.01$ vs TNP-OVA control and SIP, as determined by Kruskal-Wallis ranking test, and ## = $p<0.01$ vs TNP-OVA control, SIP, DEP1 and DEP2, as determined by ANOVA followed by Bonferroni's post hoc test.
Figure 3B: * = $p<0.05$ vs TNP-OVA control Day 15 and control Day 22, ** = $p<0.01$ vs CBP Day 15, TNP-OVA control Day 15 and TNP-OVA control Day 22, as determined by ANOVA followed by Bonferroni's post hoc test.

**Figure 4A:** Brochial alveolar lavage cells from a representative control animal (cytospin preparation after May Grünwald Giemsa staining). Arrow indicates macrophage.
**Figure 4B:** Brochial alveolar lavage cells from a representative CBP-exposed animal, (cytospin preparation after May Grünwald Giemsa staining). Arrow1 indicates macrophage, arrow2 indicates macrophage with particles, arrow3 indicates eosinophil. (N.B: Pictures will be professionally edited for the final manuscript)

## DISCUSSION

Particulate air pollution causes exacerbation of symptoms in allergic and asthmatic subjects (Pope and Dockery, 1992, Lipsett *et al.,* 1997). However, the mechanism behind the general immunomodulatory capacity of PM and especially its influence on the sensitization process are unknown.

In the study described here we developed a model in which the influence of different components of PM on both the systemic as well as the local immune response could be determined. The coadministration of particles during sensitization clearly adjuvated the immune response to the antigen TNP-OVA in this model. This immunostimulatory capacity was irrespective of the type of antigen, because the model could also be performed with OVA (unpublished results). Administration of an extra antigen challenge at Day 17 to CBP-treated animals revealed the persistent character of the adjuvant activity. This was illustrated by the ongoing increase of IgG1 and the significantly enhanced IgG2a levels and

IgG1-AFC and eosinophil numbers measured at Day 22 compared to Day 15. This deterioration of the overall effects may be caused by the persistent adjuvant effect of the particles.

Strikingly, all the different carbonaceous particles displayed adjuvant activity, whereas SIP were less effective. CBP and DEP1 were the strongest adjuvants, as can be concluded from high antibody levels and AFC numbers. Moreover, SIP-exposure induced a neutrophil infiltrate, as was also reported by others (Bissonnette and Rola-Pleszczynski 1989, Yuen *et al.,* 1996), whereas CBP-treatment caused an infiltrate of eosinophil. The particle size difference between SIP and the other particles probably results in a different particle distribution in the lungs (Churg and Brauer, 1997, Ferin *et al.,* 1992), possibly causing the difference in adjuvant activity. The observed difference in adjuvant activity between the two types of equally sized DEP as observed in our model indicates that DEP derived from different sources may have distinct biological effects. This may be caused by a difference in amount or composition of attached substances (Takenaka *et al.,* 1995, Tsien *et al.,* 1997), but other particle surface characteristics such as charge may also play a role (Oortgiesen *et al.,* 2000). However, from the fact that also CBP, which lacks attached substances, adjuvates the response, it may be concluded that the mere presence of a particle core is sufficient to adjuvate an immune response, which is in agreement with other studies (Løvik *et al.,* 1997, van Zijverden *et al.,* 2000).

Intranasal coadministration of particles and antigen was effective in adjuvating the immune response in our model. This resembles the real life situation in which subjects breathe DEP complexed to allergen (Ormstad *et al.,* 1998, Behrendt *et al.,* 1992). The response of CBP-treated animals showed characteristics of an immediate allergic response, e.g. the presence of IgE and eosinophils (Coyle *et al.,* 1996). This indicates that particles may play a role not only in the aggravation but also in the induction of respiratory allergy. The effect of exposure during distinct phases of the immune response is presently subject of investigation.

## ACKNOWLEDGMENTS

The authors thank Prof. Dr. W. Seinen and Prof. Dr. J. G. Vos for critically reading the manuscript.

# CHAPTER 5

# PARTICULATE AIRBORNE POLLUTANTS ADJUVATE BOTH PRIMARY AND SECONDARY ANTIGEN-SPECIFIC IMMUNE RESPONSES AFTER INTRANASAL ADMINISTRATION

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## ABSTRACT

Particulate airborne pollution triggers symptoms in persons already suffering from respiratory allergy, but its effect on the sensitization process and consequently its possible role in the increasing incidence of respiratory allergy is unknown. The study described here examined different particles, diesel exhaust particles (DEP) from 2 different sources, carbon black particles (CBP), and amorphous silica particles (SIP), for their immunomodulating capacity after intranasal administration during different phases of the immune response to a protein antigen. The intranasal exposure regimen in female BALB/c mice consisted of a sensitization phase of 3 consecutive doses of 10 µg of reporter antigen TNP-OVA (2,4,6-trinitrophenyl coupled to ovalbumin) at Day 1, 2, and 3, followed by a challenge with TNP-OVA (10 µg) at Day 10. To study the effect of particles on the sensitization process, they were coadministered with TNP-OVA at Day 1, Day 2 and Day 3 (total particle dose: 200 µg). The influence of particle exposure during the challenge was assessed in mice that were coadministered particles (67 µg) only with the challenge of TNP-OVA, while a third group of mice was exposed during both phases. TNP-specific antibody forming cells (AFCs) in peribronchial lymph nodes (PBLN) and immunoglobulin (Ig) levels in blood were measured at Day 15 by respectively ELISPOT and ELISA. Groups receiving particles during both sensitization and challenge showed the highest increases in serum IgG1, IgE and to a lesser extent IgG2a levels. Also an increase in IgG1-AFC number was observed in lymph nodes draining the lung. Moreover, particles were able to stimulate the immune response after administration exclusively during sensitization. This may imply that exposure to particulate air pollution is involved in the facilitated initiation of allergy and supports the notion that particles contribute to the increasing prevalence of respiratory allergy.

## INTRODUCTION

Exposure to pollutants has been shown to trigger clinical asthmatic symptoms in an individual who is already sensitized and hyperreactive (Pierson and Koenig, 1992, Devalia *et al.,* 1996). For instance hospital admission of asthmatics increases during spells of severe air pollution (Lipsett *et al.,* 1997, Korrick *et al.,* 1998, Jacobs *et al.,* 1997). Although air pollution clearly plays a role in the exacerbation of respiratory symptoms, the relationship between pollution and initial sensitization is still open to debate (Wardlaw 1992). There are some studies showing a higher incidence of asthma and bronchial hyperresponsiveness in subjects living near busy roads (Ishizaki *et al.,* 1987, van Vliet *et al.,* 1997, Wjst *et al.,* 1993). In animal studies pollutants like diesel exhaust particles (DEP) (Diaz-Sanchez 1997b, Nilsen *et al.,* 1997, Steerenberg *et al.,* 1999a, Takano *et al.,* 1997, Takafuji *et al.,* 1987, Fujimaki *et al.,* 1994, Maejima *et al.,* 1997, Muranaka *et al.,* 1986), $O_3$ (Biagini *et al.,* 1986), $SO_2$, $NO_2$ (Siegel *et al.,* 1997, Gilmour 1995) and residual oil fly ash (ROFA) (Lambert *et al.,* 1999) were shown to adjuvate the immune response (Gilmour 1995). Only few of these studies (Biagini, Siegel, Lambert) concern modulation exclusively of the primary immune response, exposing naïve animals to pollutants solely during the sensitization phase of the immune response.

Especially the role of airborne particulate matter (PM) has gained a lot of attention during the last decade, and it is recognized as an important causative agent of acute and possibly also long-term pulmonary effects (Dockery and Pope 1994). PM is a heterogenic agent, one of its main components in westernized societies are DEP. DEP have been shown in both humans (Diaz-Sanchez *et al.,* 1997a and 1999, Ishizaki *et al.,* 1987) and animals (Takano *et al.,* 1997, Takafuji *et al.,* 1987, Fujimaki *et al.,* 1994, Maejima *et al.,* 1997, Muranaka *et al.,* 1986) to stimulate immune responses to common allergens. In addition to carbonaceous components such as DEP and soot also silicates may be present in PM, of which the immunomodulatory potential in relation to respiratory allergy is unknown. PM is usually described according to its size; PM10 represent particles up to 10 µm. Size is a crucial characteristic in determining whether and where particles are deposited in the lung (Churg and Brauer 1997, Ferin *et al.,* 1992). For respiratory allergy, however, the exact role of PM and its crucial characteristic or its responsible component is unknown.

In a previous study (van Zijverden *et al*., Chapter 4) we developed an intranasal exposure model in mice in which we showed that different particles were able to act as adjuvant when coadministered during sensitization, leading to inflammatory cell infiltrates and IgE production. This was also shown in rat by Lambert *et al.* (1999), who showed that

exposure of rats to ROFA before sensitization with antigen adjuvates a Th2-like immune response.

The purpose of this study was to determine the adjuvant capacity of different types of particles after intranasal administration during different phases of the immune response. In our model, the antigen TNP-OVA was administered intranasally at Days 1, 2 and 3, and a challenge took place at Day 10. Particles were coadministered during the sensitization and/or challenge phase of the immune response. This enabled us to study the possible correlation of adjuvant activity with the phase of particle exposure. Two types of DEP were used, which differed in origin (DEP1 from a V8 motor, DEP2 from a 4 kW light duty engine). DEP1, DEP2 and carbon black (CBP) were compared to amorphous silica particles (SIP). The capacity of the four particles to modulate the type of immune response was studied by measuring Day 15-antibodies in serum and antibody forming cells (AFC) in lymph nodes draining the lung (PBLN).

## MATERIALS AND METHODS

### Animals

Female, specific pathogen-free BALB/c mice (6-8 weeks of age) were obtained from the Utrecht University breeding facility. Mice were housed under hygienic barrier conditions in filter-topped macrolon cages with bedding of wood chips, a temperature of 23±2$^{o}$C, 50-60% relative humidity and a 12-h light/dark cycle. They received standard lab chow and acidified tap water *ad libitum*. The experiments were approved by an ethical committee and conducted in accordance with the Guiding Principles in the Use of Animals in Toxicology.

### Chemicals and reagents

Silica (amorphous, microcrystalline $SiO_2$ [SIP]) was obtained from Sigma Chemical Company (St. Louis, MO) and CBP from Brunschwich Chemie (Amsterdam, The Netherlands). DEP1 was a friendly gift from Dr. P. Steerenberg (RIVM, the Netherlands) and was derived from a V8 motor (Mauderly *et al.,* 1987). DEP2 was obtained from Dr. P. Scheepers (Nijmegen University, The Netherlands), and was derived from a 4 kW light duty engine. The antigen TNP-OVA was prepared as described by Albers *et al*. (1997). Immobilon-P membranes were obtained from Millipore (Etten-Leur, The Netherlands), and alkaline-phosphatase-conjugated goat-anti-mouse IgG1, IgG2a and IgE antibodies from Southern Biotechnology Associates (Birmingham, AL). Anti-mouse IgE (02111D) for IgE ELISA was obtained from Pharmingen (Hamburg, Germany). ELISA plates (highbond 3590) were obtained from Costar, Cambridge, MA. All reagents for the TNP-specific ELISA were obtained from Sigma Chemical Company (St. Louis, MO).

**Treatment of the mice**

Particle suspensions (2.67 mg/ml) were prepared in saline containing 0.4 mg/ml TNP-OVA and 5% heat inactivated normal BALB/C mouse serum. The latter was added to facilitate suspension of the hydrophobic particles, while they stirred for 18 h. Naïve female BALB/c mice were intranasally instilled by pipetting a total volume of 25 µl on the nostrils under light ether anesthesia. Sensitization took place at Day 1, 2 and 3, adding up to a total TNP-OVA dose of 30 µg. A challenge dose consisting of 10 µg TNP-OVA was given at Day 10. Particles were administered during either sensitization, challenge, or both phases, and consisted of 67 µg of particle per dose (Table 1). Blood was collected by orbita punction under ether anesthesia at Day 0 and 5 days after challenge at Day 15. At dissection PBLN, spleen and bone marrow were taken out for ELISPOT.

**Table 1:** Treatment groups, n=4 (group 4, 7, 10, 13), n=10 (group 2, 3, 5, 6, 8, 9, 11, 12), n=18 (group 1). Blood samples were taken at Day 0 and at Day 15 when animals were sacrificed. TNP-OVA=10µg/dose, particles=67µg/dose.

| group | Sensitization (day 1, 2, 3) | Challenge (day 10) |
|---|---|---|
| 1 | TNP-OVA | TNP-OVA |
| 2 | TNP-OVA + CBP | TNP-OVA |
| 3 | TNP-OVA + CBP | TNP-OVA + CBP |
| 4 | TNP-OVA | TNP-OVA + CBP |
| 5 | TNP-OVA + DEP1 | TNP-OVA |
| 6 | TNP-OVA + DEP1 | TNP-OVA + DEP1 |
| 7 | TNP-OVA | TNP-OVA + DEP1 |
| 8 | TNP-OVA + DEP2 | TNP-OVA |
| 9 | TNP-OVA + DEP2 | TNP-OVA + DEP2 |
| 10 | TNP-OVA | TNP-OVA + DEP2 |
| 11 | TNP-OVA + SIP | TNP-OVA |
| 12 | TNP-OVA + SIP | TNP-OVA + SIP |
| 13 | TNP-OVA | TNP-OVA + SIP |

**TNP-specific IgG1 and IgG2a ELISA**

Plates were coated (overnight [o.n.] 4$^{o}$C) with TNP-BSA (20 µg/ml) in 0.05 M carbonate buffer pH 9.6, and blocked with PBS/T/1% BSA (1 h, room temperature [RT]). Serial dilutions of sera were incubated (1 h, RT) and plates were washed and incubated with an optimal dilution of alkaline phosphatase-conjugated anti-mouse IgG1 or IgG2a (1 h, RT), followed by p-nitrophenyl phosphate (1 mg/ml in diethanolamine buffer, pH 9.8) for 30 min. Absorbance was measured at 405 nm. Titers were calculated by means of the sample

dilution at which extinctions were higher than background+2SD. Background levels at Day 0 were subtracted from Day 15 levels to obtain the increase in IgG1/IgG2a.

**TNP-specific IgE ELISA**

Plates were coated (6 h, first hour at 37$^{o}$C, last hours at RT) with αIgE, 2 μg/ml in 0.05 M carbonate buffer pH 9.6. After washing, serial dilutions of sera were incubated (o.n. 4$^{o}$C) and plates were washed and incubated with an optimal dilution of a biotin-TNP-BSA complex (1h, RT). After incubation (45 min, RT) with poly-HRP-streptavidine (CLB, Amsterdam, The Netherlands), tetramethylbenzidine (1 mg/ml) was added (15 min, RT). The coloring reaction was stopped with $H_2SO_4$ and absorbance was measured at 450 nm. Titers were calculated by means of the sample dilution at which extinctions were higher than background+2*SD. Background levels at Day 0 were subtracted from Day 15 levels to obtain the increase in IgE.

**Calculation of Stimulation Indices**

The increase in titers of a particle-treated group divided by the increase in titers as observed in the control group gives the stimulation index (SI) of a certain immunoglobulin isotype. The average of the SIs as observed in all groups treated during one particular phase of the immune response (either during sensitization, sensitization and challenge, or challenge phase) represents the overall SI for particle administration during that phase.

**ELISPOT assay**

These assays were essentially performed as described previously (Schielen *et al*. 1995). Briefly, organs were minced to prepare single cell suspensions, and $0.5*10^6$ cells in 500 μl PBS/1% BSA were incubated (4 h, 37$^{o}$C) in wells containing TNP-BSA coated (o.n. 4$^{o}$C) Immobilon-P membranes as bottom. Thereafter, membranes were washed and incubated with optimal dilutions of isotype-specific alkaline phosphatase-conjugated anti-mouse Ig antibodies in PBS/T (o.n. 4$^{o}$C). After washing, spots were developed by incubation with 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium (BCIP/NBT). Specific AFCs per $10^6$ cells were calculated from spot numbers counted with the aid of a stereo microscope.

**Statistics**

Preceding statistical analysis, ELISPOT data (number of AFC) were transformed to log10 values to homogenize variance. Differences between group means were analyzed using one-way ANOVA with Scheffe post-hoc test for contrasts ($p<0.05$ or $p<0.01$). In the case of nonsufficient homogeneity of variances, a Kruskal-Wallis ranking test was performed ($p<0.05$).

## RESULTS

**Levels of TNP-specific IgG1, IgG2a and IgE antibodies in serum**

To assess the immunomodulating activity of the different particles, immunoglobulin levels were determined in sera obtained at Day 0 and at the end of the experiment (Day 15). For every animal the background at Day 0 was subtracted from that at Day 15, giving the levels shown in Figure 1. IgG1 displayed the highest increase in titers between Day 0 and Day 15 in most groups, IgE the lowest.

**Modulation of the immune response by particles administered during sensitization**

When particles were administered exclusively during the 3 days of the sensitization phase of the immune response, both CBP and DEP1-treated animals showed significant increases in IgG1 and IgG2a between Day 0 and Day 15. IgE titers significantly increased in DEP1-treated mice. IgG1 levels in DEP2 and SIP-treated animals also showed a tendency to increase, whereas in these groups IgE and IgG2a remained at control levels (Fig. 1). The stimulation indices (SI) of the treatment groups for the different immunoglobulin isotypes are given in Table 2. Significantly higher SIs for the different isotypes support the relatively strong adjuvating activity of DEP1 and CBP.

**Modulation of the immune response by particles administered during sensitization and challenge**

The immune response was most clearly adjuvated when particles were administered in both sensitization and challenge phase. IgG1 was significantly enhanced in all particle-treated groups, but again most clearly in CBP and DEP1-exposed animals, which also showed significantly enhanced IgG2a levels. IgE was significantly enhanced in CBP and DEP1 treated animals (Fig. 1). Again high SIs (Table 2), reflect the strong adjuvating capacity of CBP and DEP1. DEP2 and SIP-treated animals also showed increased SIs for IgG1.

**Modulation of the immune response by particles administered during challenge**

Exposure to particles solely in the challenge phase adjuvated the immune response in the DEP1-treated group, showing significantly enhanced IgG1 and IgG2a levels, whereas also IgE showed a tendency to increase. However SIs in the challenge phase were significantly lower compared to SIs of animals treated with DEP1 during sensitization or during both phases (Table 2). IgG1 titers of CBP-treated animals also showed a tendency to increase (Fig. 1), but this SI was significantly lower compared to animals to which CBP was administered during the other phases. Neither for DEP2 nor SIP-treated animals significant differences were found between SIs of groups treated during different phases (Table 2).

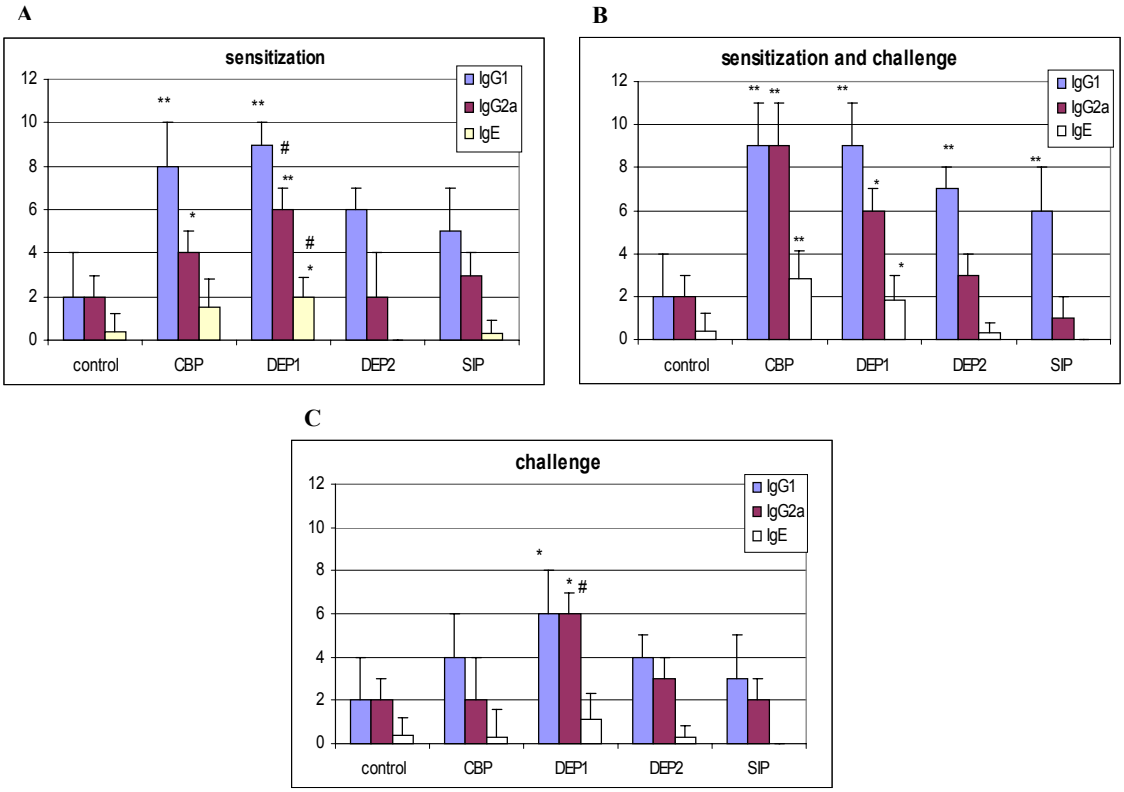



**Figure 1:** The increase in TNP-specific antibody $^{2}$log titers between Day 0 and Day 15 as determined by ELISA. A) Particle administration during sensitization. B) Particle administration during sensitization and challenge. C) Particle administration during challenge. Depicted is average +/- standard deviation.
IgG1: * = p<0.01 vs control, ** = p<0.01 vs control, as determined by ANOVA followed by Scheffe's post hoc test. Kruskal-Wallis ranking test was performed for "particles in sensitization-phase"
IgG2a: * = p<0.01 vs control, ** = p<0.01 vs control, # = p<0.05 vs SIP, "= p<0.01 vs CBP as determined by ANOVA followed by Scheffe's post hoc test. Kruskal-Wallis ranking test was performed for "particles in sensitization and challenge phase"
IgE: * = p<0.05 vs control, ** = p<0.01 vs control as determined by ANOVA followed by Scheffe's post hoc test.

**Table 2:** Treatment groups and their Stimulation Indices for the different immunoglobulin isotypes, based on increases in serum Ig-titers between Day 0 and Day 15 as determined by ELISA.

| Group | SI IgG1 | SI IgG2a | SI IgE |
|---|---|---|---|
| CBP sensitization | 3.9±0.9 [1, A] | 2.1±0.3 [3] | 3.8±3.2 |
| DEP1 sensitization | 4.5±0.4 [2, B] | 2.8±0.4 [4] | 5.0±2.2[6] |
| DEP2 sensitization | 2.7±0.7 | 0.9±0.9 | 0.0±0.0 |
| SIP sensitization | 2.6±0.8 | 1.3±0.8 | 0.8±1.4 |
| CBP sens + challenge | 4.6±1.1 [A] | 4.4±0.9 [5, A] | 7.0±3.3[7, A] |
| DEP1 sens + challenge | 4.6±1.2 [B] | 2.8±0.5 | 5.0±5.4 |
| DEP2 sens + challenge | 3.3±0.5 | 1.6±0.3 | 0.6±1.3 |
| SIP sens + challenge | 3.0±1.2 | 0.6±0.5 | 0.0±0.0 |
| CBP challenge | 1.8±0.4 | 0.8±0.8 | 0.8±1.4 |
| DEP1 challenge | 3.1±0.3 | 2.8±0.3 | 2.8±.6 |
| DEP2 challenge | 2.2±0.6 | 1.3±1.3 | 0.6±1.3 |
| SIP challenge | 1.5±1.1 | 0.9±0.9 | 0.0±0.0 |

As determined by ANOVA followed by Scheffe's post hoc test, or in case of non-sufficient homogeneity of variances, a Kruskal-Wallis ranking test was performed. Depicted are averages ± standard deviation:

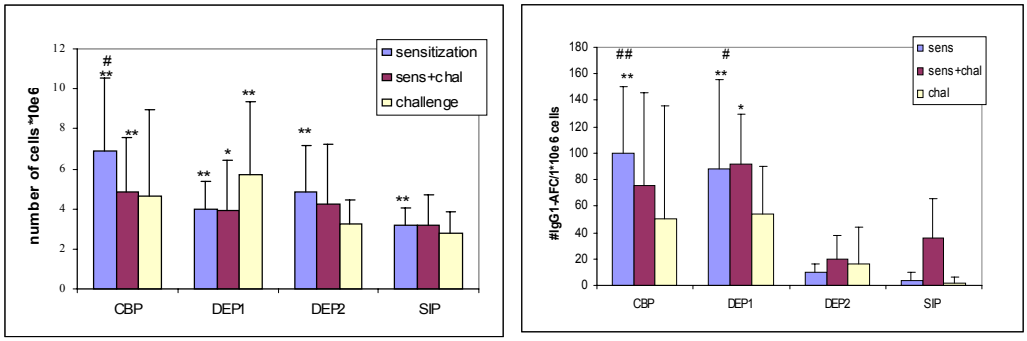
Differences **between particles** within each phase of exposure:
1= >SIP sensitization ($p<0.01$)
2= >DEP2 sensitization and SIP sensitization ($p<0.01$)
3= >DEP2 sensitization ($p<0.05$)
4= >DEP2 sensitization and SIP sensitization ($p<0.01$)
5= >DEP2 sens+challenge and SIP sens+challenge ($p<0.01$)
6= >DEP2 sensitization ($p<0.05$)
7= >DEP2 sens+challenge and SIP sens+challenge ($p<0.05$)

Differences **between phases** within each particle-treatment:
A= >CBP challenge ($p<0.05$)
B= >DEP1 challenge ($p<0.01$)

**Number of cells and TNP-specific antibody forming cells in PBLN at Day 15**

Significant increases in PBLN cell number were induced when particles were administered during sensitization (Fig. 2A). Cell numbers also increased significantly in CBP and in DEP1-treated animals when particles were administered during both phases, whereas in the latter group they also increased significantly when particles were only administered during challenge (Fig. 2A).

The number of specific AFC was assessed in PBLN, spleen and bone marrow. In the latter two organs hardly any AFC were identified (not shown). In PBLN only IgG1-AFC could be found, which is in line with IgG1 being the isotype detected in highest levels in serum. In groups receiving particles only during the sensitization phase, CBP and DEP1-treated animals showed significantly enhanced IgG1-AFC numbers, both were significantly higher than all other groups (Fig. 2B). Particle administration during both phases of the immune response caused significantly increased AFC-numbers in DEP1-treated animals, while CBP-treated animals also showed a tendency to increase. The single dose during the challenge phase caused no significant increase.




**A** **B**

**Figure 2A:** Cell number of PBLN at Day 15. The dotted line represents control level. Average +/- standard deviation are depicted. *p<0.05 vs. control, #p<0.05 vs. SIP, ##p<0.01 vs. SIP, as determined by ANOVA followed by Scheffe's post hoc test.
**Figure 2B:** Number of TNP-specific antibody forming cells per $0.5*10^{e}6$ cells as determined in PBLN by ELISPOT at Day 15. The dotted line represents control level
Average +/- standard deviation are depicted. *p<0.05 vs. control, , #p<0.05 vs. SIP, ##p<0.01 vs. SIP, as determined by ANOVA followed by Scheffe's post hoc test.

## DISCUSSION

The exposure of already sensitized subjects to particulate pollutants is known to aggravate respiratory symptoms (Lipsett *et al.,* 1997). In the present study, different types of particles are shown to adjuvate the immune response when administered during different phases of the immune response. To summarize and discuss the results, average SIs ((SI IgG1+SI IgG2a+SI IgE)/3) are calculated for every phase (Table 3). In combination with data as presented in Fig. 1 and Fig. 2, it becomes clear that adjuvant activity was best expressed when particles were administered during both sensitization and challenge phase (average SI

3.1). Particles also clearly adjuvated the immune response when they were administered only during sensitization (average SI 2.5), whereas the single challenge dose was the least effective (average SI 1.6, Table 3). Overall DEP1 and CBP adjuvated the immune response best, as can be concluded from the significantly highest antibody increases (Fig. 1), and IgG1-AFC numbers (Fig. 3), and is also indicated by the high average SIs (Table 3).

**Table 3:** Average Stimulation Indices

| Particle | SI sensitization | SI sensitization+ challenge | SI challenge |
|---|---|---|---|
| CBP | 3.3 | 5.3 | 1.1 |
| DEP1 | 4.1 | 4.1 | 2.9 |
| DEP2 | 1.2 | 1.8 | 1.4 |
| SIP | 1.6 | 1.2 | 0.8 |
| average | 2.5 | 3.1 | 1.6 |

Animals were exposed to different particles during distinct phases of the immune response. Shown are the average stimulation indices ((SI IgG1+SI IgG2a+SI IgE)/3) and the average stimulation indices for the different phases, based on increases in serum Ig-titers between Day 0 and Day 15 as determined by ELISA (Fig. 1) and the stimulation indices (Table 2).

In this model the adjuvant effect of particles administered exclusively in the challenge phase was not as striking as after exposure in the sensitization phase or in both phases. This may be caused by the fact that the particle doses administered during the 3 day-sensitization phase adds up to 200 μg, whereas the challenge phase comprises only one day and consequently one third of the sensitization dose. Still, this suffices to adjuvate the response in case of DEP1. In addition, the time between the challenge dose and the end of the experiment, although long enough to allow a secondary immune response to develop, may be too short for the adjuvant activity to be fully expressed. Moreover, this model is aimed at inducing a suboptimal immune response during sensitization in order to facilitate detecting small adjuvant effects, and therefore does not represent the situation in allergic subjects.

DEP1 appears to have a stronger adjuvant activity than DEP2. This difference in adjuvant activity may be explained by a difference in particle characteristics such as amount of attached substances. However, in our model CBP and DEP1 were both strongly immunostimulatory, indicating that the mere presence of a particle core is enough to adjuvate an immune response. The possible differences between DEP from distinct sources is important to bear in mind when comparing studies from literature in which DEP is used.

A small difference in size for example may result in a different particle distribution in the lungs (Churg and Brauer 1997, Ferin *et al.,* 1992).

Regarding the different isotypes produced in our model, particles administered into the lung mostly stimulate immune responses with Th2 characteristics. Overall, the increase in the Th2-mediated isotype IgG1 is more prominent than the increase in the Th1-mediated isotype IgG2a (Fig. 1), and in addition the Th2-mediated isotype IgE is also produced in response to CBP and DEP1. This appears to be partly in contrast with results from the subcutaneous model (van Zijverden *et al.,* 2000), in which the same particles stimulated both Th1 (SIP), Th2 (DEP) or mixed (CBP) reactions. This may reflect distinct default reactions in lung and skin; while in the skin a Th1 response arises to a particular antigen, in the lung the same antigen initiates a Th2 response (Constant *et al.,* 2000). Although respiratory allergy to common protein antigens is a Th2-mediated disease, a Th1-mediated reaction is not necessarily less harmfull. On the contrary, Th1 cells may not only fail to counterbalance the effects of Th2 cells (Hansen *et al.,* 1999), they actually may be indispensable and cooperate with Th2 cells in a murine model of eosinophilic airway inflammation (Randolph *et al.,* 1999). The inflammatory reaction initiated by Th1 cells plays a crucial role in this process. The fact that DEP1 and CBP stimulate IgG2a production in addition to IgG1and IgE in our model indicates that both Th1 and Th2-mediated reactions are taking place at the same time. This makes particles such as DEP1 and CBP likely candidates to play a stimulatory role in the process of respiratory allergy.

This study shows that intranasal administration of different types of particles in mice stimulates the immune response. The fact that particles also display adjuvant activity when they are administered exclusively during the sensitization phase may imply that exposure to particulate air pollution is able to cause facilitated initiation of allergy in humans. Although so far only epidemiological evidence exists for the involvement of particulate pollutants in the exacerbation of respiratory allergy, our present results may support a role for particles in the increasing incidence of allergy as well. The mechanism by which particles stimulate immunosensitization is presently subject of investigation.

## ACKNOWLEDGMENTS

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# CHAPTER 6

# COADMINISTRATION OF ANTIGEN AND PARTICLES OPTIMALLY ADJUVATES THE IMMUNE RESPONSE IN AN INTRANASAL ADMINISTRATION MODEL

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## ABSTRACT

Particulate matter is known to affect human health, yet the mechanism(s) by which it acts is largely unknown. One of the factors that may play a role in particle adjuvant activity is binding of allergen to particles. This may turn the particles into allergen carriers resulting in antigen deposition within the altered inflammatory microenvironment created by the particles. We compared the effectivity of simultaneous versus separate administration of antigen and particles during sensitization in an intranasal exposure model. Sensitization consisted of 3 doses (10 μg) of TNP-OVA at Days 1, 2 and 3. Two hundred μg of carbon black particles (CBP) were administered either 1 day before sensitization (Day 0), 1 day after sensitization (Day 4), or during sensitization. The latter was performed either at Day 1 (200 μg) or at Day 1, 2 and 3 (67 μg/day). At Day 10 a challenge with 10 μg of TNP-OVA was performed, and at Day 15 the immune response was assessed. The total number of cells as well as antibody forming cells (AFC) in lymph nodes draining the lung (peribronchial lymph nodes [PBLN]) were determined, and immunoglobulin levels in blood were assessed. Cell numbers of PBLN increased significantly in all particle-treated groups compared to controls. The number of TNP-specific IgG1-forming cells in the groups receiving particles during sensitization was significantly higher than control level. Only groups receiving particles during or before sensitization displayed significantly higher IgG1 levels than controls, in contrast to the group receiving particles after sensitization. TNP-specific IgE increased significantly compared to controls only in animals receiving 3*67μg doses during sensitization. IgG2a did not show significant differences, indicating that the response is predominantly Th2-mediated. These data indicate that coadministration of particles at all time points of antigen dosing constitutes as the most effective way to adjuvate an immune response in our model, as opposed to separate particle and antigen dosing. Also administration shortly before antigen administration was effective, suggesting that time-dependent processes are involved in adjuvant activity of particles, supporting the important role of the altered inflammatory microenvironment created by the particles.

## INTRODUCTION

Particulate matter (PM) is recognized as an important causative agent of acute and probably also long-term pulmonary health effects (Dockery and Pope 1994). One of these effects is the aggravation of symptoms of respiratory allergy, and possibly also facilitated sensitization to common allergens like pollen. Diesel exhaust particles (DEP), which make up an important part of PM, are likely to play an important role in these effects (van Vliet *et al.,* 1997, Ishizaki *et al.,* 1987). The immunomodulatory capacity of DEP has been shown in several animal models (Takano *et al.,* 1997, Fujimaki *et al.,* 1997, Takafuji *et al.,* 1987, Nilsen *et al.,* 1997) and also in experimental human studies (Diaz-Sanchez *et al.,* 1997a and 1999).

The exact mechanism by which particles enhance the immune response is unknown. Presumably, particles act at different levels (Salvi and Holgate 1999). Particles could interact with allergens in the outside air, turning respirable size particles into allergen carriers (Ormstad *et al.,* 1998, Knox *et al.,* 1987, Behrendt *et al.,* 1992) that deposit concentrated amounts of possibly altered (Behrendt *et al.,* 1997) allergen deeply into the lung. Once deposited, particles may alter macrophage (Yang *et al.,* 1997) and epithelial cell (Steerenberg *et al.,* 1998) function (Salvi *et al.,* 1999), thereby changing the microenvironment of the lung and possibly leading to modified antigen presentation and allergy.

So far, however, it is unknown whether both interaction and simultaneous deposition of the particle and allergen are necessary for adjuvant activity. A direct comparison within the same model between simultaneous and separate administration of antigen and particles has never been made. In the majority of the studies DEP and antigen are administered together, either via intratracheal or intranasal administration (Takano *et al.,* 1997, Fujimaki *et al.,* 1994, Takafuji *et al.,* 1987, van Zijverden *et al.,* Chapter 4). This appears to be an effective way to induce adjuvant activity in naïve mice, as measured by airway inflammation, cytokine production and IgG1 and IgE production. In sensitized human volunteers who were exposed to both ragweed and DEP simultaneously, increasing IgE and Th2 cytokines were observed (Diaz-Sanchez *et al.,* 1997a). However, separate dosing of DEP and antigen was effective in causing mucosal sensitization in (naïve) human volunteers (Diaz-Sanchez *et al.,* 1999) and elevated levels of IgE and IL-4 in mice (Fujimaki *et al.,* 1997). In rats, residual oil fly ash (ROFA), administered 1 day before the first antigen administration, was shown to adjuvate sensitization to antigen (Lambert *et al.,* 1999). This indicates that coadministration of and physical attachment between particle and antigen is not obligatory for the adjuvant activity.

Hence, so far no definite conclusions can be drawn on the effectivity of coadministration versus separate dosing of antigen and particles, especially because no direct comparison between the different models used hitherto can be made. Therefore we used an intranasal exposure model in which antigen and particles could be administered either together with or separate from antigen during sensitization. Sensitization comprised intranasal administrations of 10 µg TNP-OVA at Days 1, 2 and 3. Two hundred µg of carbon black particles (CBP) were administered either 1 day before sensitization (Day 0), 1 day after sensitization (Day 4), or during sensitization. The latter was performed either at Day 2 (200 µg) or at Day 1, 2 and 3 (67 µg/day). At Day 10 a challenge with 10 µg of TNP-OVA was performed, and at Day 15 the immune response was assessed. The number of cells and antibody forming cells (AFC) in lymph nodes draining the lung (peribronchial lymph nodes [PBLN]) was determined, and immunoglobulin levels in blood were assessed.

## MATERIALS AND METHODS

### Animals

Female, specific pathogen-free BALB/c mice (6-8 weeks of age) were obtained from the Utrecht University breeding facility. Mice were housed under hygienic barrier conditions in filter-topped macrolon cages with bedding of wood chips, a temperature of 23±2$^{o}$C, 50-60% relative humidity and a 12-h light/dark cycle. They received standard lab chow and acidified tap water ad libitum. The experiments were approved by the ethical committee for animal use of the Utrecht University, and conducted in accordance with the Guiding Principles in the Use of Animals in Toxicology.

### Chemicals and reagents

CBP was obtained from Brunschwich Chemie (Amsterdam, The Netherlands). The antigen TNP-OVA was prepared as described by Hudson and Hay (1989). TNP-BSA was coupled in an identical way. For TNP-specific IgE-ELISA, biotin was additionally coupled to TNP-BSA. Immobilon-P membranes were obtained from Millipore (Etten-Leur, The Netherlands), alkaline-phosphatase (AP)-conjugated goat-anti-mouse IgG1, IgG2a and IgE antibodies from Southern Biotechnology Associates (Birmingham, AL, USA). Anti-mouse IgE (02111D) for IgE ELISA was obtained from Pharmingen (Hamburg, Germany). ELISA plates (highbond 3590) were obtained from Costar, Cambridge, MA. All reagents for the TNP-specific ELISA were obtained from Sigma Chemical Company (St. Louis, MO, USA).

**Treatment of the mice**

Particle suspensions (2.67 mg/ml) were prepared in saline containing 5% heat inactivated normal BALB/C mouse serum and 0.4 mg/ml TNP-OVA in case of co-administration of antigen and particles. The mouse serum was added to facilitate suspending the hydrophobic particles, while stirring for 18 h. Naïve female BALB/c mice were intranasally instilled by pipetting a total volume of 25 µl on the nostrils under light ether anesthesia. Sensitization took place at Day 1, 2 and 3, adding up to a total TNP-OVA dose of 30 µg. A challenge dose consisting of 10 µg TNP-OVA was given at Day 10. Particles were administered either before, during, or after sensitization, and consisted of 3*67 µg of particles or 200 µg at once (Table 1). Control groups receiving only antigen, and only particles were added. Blood was collected by orbita punction under ether anesthesia at Day 0 and Day 15, 5 days after challenge and just prior to dissection, at which also bronchial alveolar lavage (BAL) was performed and PBLN were taken out.

**Table 1:** experimental setup.

| GROUP | Day 0 | Day 1 | Day 2 | Day 3 | Day 4 | Day 10 |
|---|---|---|---|---|---|---|
| | sensitization | sensitization | sensitization | sensitization | sensitization | challenge |
| 1: 3*67 group | - | 67µg CBP+ TNP-OVA | 67µg CBP+ TNP-OVA | 67µg CBP+ TNP-OVA | - | TNP-OVA |
| 2: 200dose group | - | 200 µg CBP+ TNP-OVA | TNP-OVA | TNP-OVA | - | TNP-OVA |
| 3: 200before group | 200 µg CBP | TNP-OVA | TNP-OVA | TNP-OVA | - | TNP-OVA |
| 4: 200after group | | TNP-OVA | TNP-OVA | TNP-OVA | 200 µg CBP | TNP-OVA |
| 5: control I | | TNP-OVA | TNP-OVA | TNP-OVA | - | TNP-OVA |
| 6: control II | saline | TNP-OVA | TNP-OVA | TNP-OVA | | TNP-OVA |
| 7: control III | - | TNP-OVA | TNP-OVA | TNP-OVA | saline | TNP-OVA |
| 8: particle-control | - | 67 µg CBP | 67 µg CBP | 67 µg CBP | - | saline |

Treatment groups (n=6-8). During sensitization groups 1and 2 receive particles (CBP) and antigen (TNP-OVA) together, in either 3 doses of 67µg (1) or 1 dose of 200µg (2). CBP is administered before TNP-OVA sensitization in group 3, whereas in group 4 CBP is dosed after sensitization. All control groups receive only antigen TNP-OVA. In addition, control group II and III receive a dose of saline to control for the extra dose before (group3) and after (group 4) the antigen administrations. A particle-control group is added, which only receives particles and no antigen.

**TNP-specific IgG1 and IgG2a ELISA**

Plates were coated (overnight [o.n.] 4°C) with TNP coupled to bovine serum albumin ([BSA] TNP-BSA, 20 μg/ml) in 0.05 M carbonate buffer pH 9.6, and blocked with phosphate buffered saline (PBS) containing 0.05% Tween20 (Brunschwich) and 1% BSA ([PBS/T/BSA]1 h, room temperature [RT]). Serial dilutions of sera were incubated (1 h, RT) and plates were washed and incubated with an optimal dilution of AP-conjugated anti-mouse IgG1 or IgG2a (1 h, RT), followed by p-nitrophenylphosphate (1 mg/ml in diethanolamine buffer, pH 9.8) for 30 min. Absorbance was measured at 405 nm. Titers were calculated by means of the sample dilution at which extinctions were higher than background+2*SD. Background levels at Day 0 were subtracted from Day 15 levels to obtain the increase in IgG1/IgG2a.

**TNP-specific IgE ELISA**

Plates were coated (6 h, first hour at 37°C, last hours at RT) with αIgE, 2 μg/ml in 0.05 M carbonate buffer pH 9.6. After washing, serial dilutions of sera were incubated (o.n. 4°C) and plates were washed and incubated with an optimal dilution of a biotin-TNP-BSA complex (1h, RT). After incubation (45 min, RT) with poly-HRP-streptavidine (CLB, Amsterdam, The Netherlands), tetramethylbenzidine (1 mg/ml) was added (15 min, RT). The coloring reaction was stopped with $H_2SO_4$ and absorbance was measured at 450 nm. A standard curve of TNP-specific mouse IgE was included for calculating IgE amounts.

**ELISPOT assay**

These assays were essentially performed as described previously (Schielen *et al.,* 1995). Shortly, PBLN were minced to prepare single cell suspensions, and $0.5*10^6$ cells in 500 μl PBS/1% BSA were incubated (4 h, 37°C) in wells containing TNP-BSA (20 μg/ml) coated (o.n. 4°C) Immobilon-P membranes as bottom. Thereafter, membranes were washed and incubated with optimal dilutions of isotype-specific alkaline phosphatase-conjugated anti-mouse Ig antibodies in PBS/T (o.n. 4°C). After washing, spots were developed by incubation with 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium (BCIP/NBT). Specific AFCs per $10^6$ cells were calculated from spot numbers counted with the aid of a stereo microscope.

**Statistics**

Preceding statistical analysis, ELISPOT data (the number of AFCs) were transformed to log10 values to homogenize variance. Differences between group means were analyzed using one-way ANOVA with Scheffe post-hoc test for contrasts ($p<0.05$ or $p<0.01$). In case of non-sufficient homogeneity of variances, a Kruskal-Wallis ranking test was performed ($p<0.05$).

## RESULTS

Different time points of particle administration were compared: either during the 3 day antigen sensitization (antigen at Day 1,2 and 3, 200 μg particles at Day 1), before sensitization (Day 0), or afterwards (Day 4). In addition, particles were also administered during sensitization in three small doses (67 μg at Day 1,2,3) to compare the effect of the number of doses. All groups were challenged with antigen alone at Day 10, and the immune response was determined at Day 15. For all treatments, separate control groups were included in the experiment. However it appeared that all antigen controls, i.e. animals receiving antigen and no particles (Table 1, groups 5,6,7) showed the same results. This indicates that an extra dose of saline and its concurrent anesthesia at Day 0 or Day 4 did not modulate the immune response, and we have therefore decided to consider all antigen control animals as one group. The control animals receiving only particles and no antigen did not show any immune response at all.

**Number of cells and TNP-specific IgG1 antibody forming cells in PBLN at Day 15**

Cell numbers increased significantly in all particle-treated groups compared to antigen controls (Fig. 1A). Cell numbers of animals receiving only particles and no antigen showed a comparable increase (not shown). In addition, the cell number of the group receiving 3 particle doses (67 μg) was significantly greater than the cell number of the group receiving one dose of particles (200 μg) during sensitization.

The number of TNP-specific antibody forming cells in the 3*67dose and the 200dose group was significantly higher than antigen control level. In addition, the 3*67dose group was higher than the groups receiving 200 μg doses before and after sensitization, but only significantly higher than the 200before group.

**TNP-specific antibody titers in serum**

For IgG1 and IgG2a, background levels were determined in sera obtained at Day 0 and subtracted from levels found at the end of the experiment at Day 15. This difference between Day 15 and Day 0 is depicted. For IgG1, groups receiving 3 small doses (3*67dose group) or one dose during (200dose group) or before (200before group) sensitization all displayed significantly higher increases than antigen controls (Fig 2A). IgG2a did not show significant differences (Fig 2B). TNP-specific IgE increased significantly only in the 3*67dose group compared to antigen controls (Fig 2C).

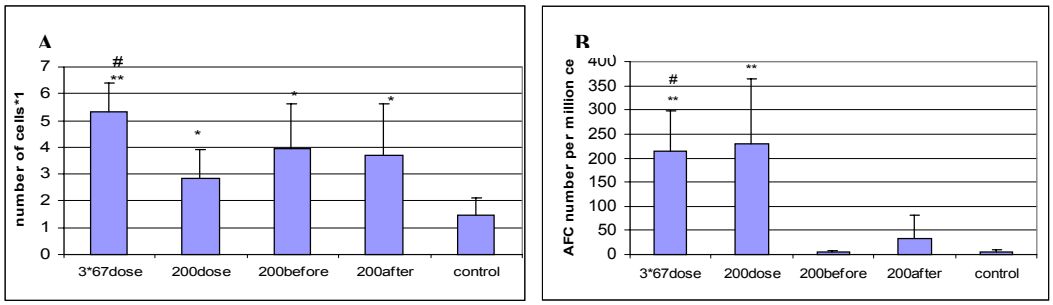


**Figure 1:** A: Number of cells in peribronchial lymph nodes after particle dosing during sensitization (3*67dose and 200 dose groups), before sensitization (200before group) and after sensitization (200after group). ** $p<0.01$ vs control, # $p<0.05$ vs 200dose group.
B: Number of TNP-specific IgG1-forming cells in PBLN after particle dosing during sensitization (3*67dose and 200 dose groups), before sensitization (200before group) and after sensitization (200after group). ** $p<0.01$ vs control, # $p<0.05$ vs 200before group.

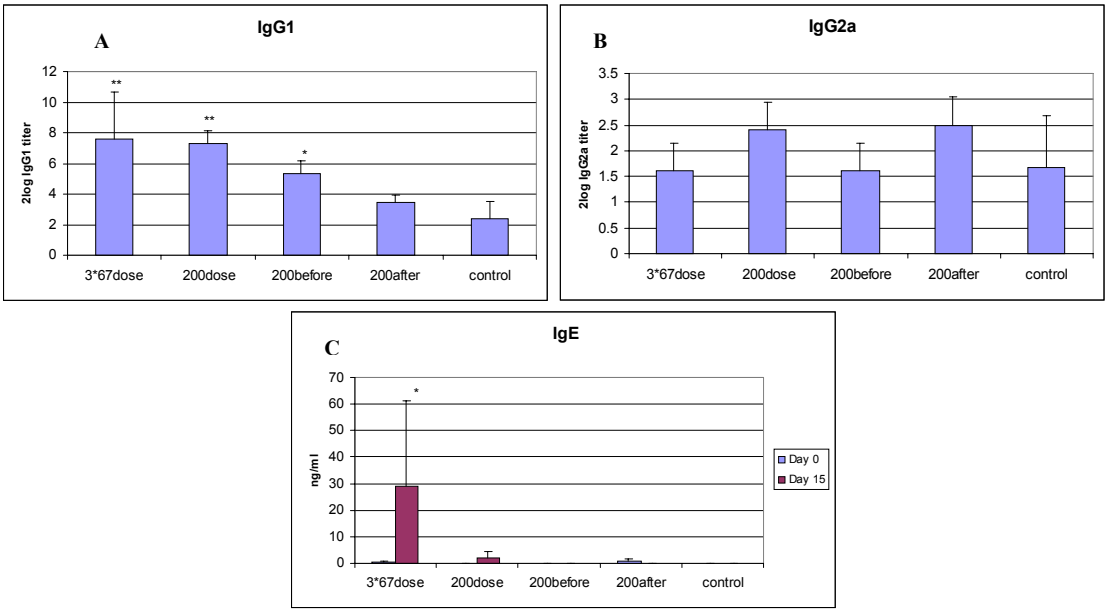


**Figure 2:** A: The increase in titers of TNP-specific IgG1titers between Day 0 and Day 15 after particle dosing during sensitization (3*67dose and 200 dose groups), before sensitization (200before group) and after sensitization (200after group). ** $p<0.01$, * $p<0.05$ vs control.
B: The increase in titers of TNP-specific IgG2a between Day 0 and Day 15 after particle dosing during sensitization (3*67dose and 200 dose groups), before sensitization (200before group) and after sensitization (200after group). C: TNP-specific IgE (ng/ml) at Day 0 and Day 15 after particle dosing during sensitization (3*67dose and 200 dose groups), before sensitization (200before group) and after sensitization (200after group). * $p<0.05$ vs control.

## DISCUSSION

We tested the hypothesis that particulate matter is involved in facilitation of sensitization to common allergens. Therefore we compared the effect of separate versus simultaneous administration of antigen and particle on sensitization in an intranasal exposure model. We found that co-administration of 3 small doses of particles during sensitization adjuvated the immune response best. One large dose during sensitization also adjuvated the IgG1 response, but did not stimulate IgE production. In addition, a particle dose one day before the start of the antigen administrations adjuvated the IgG1 response, but no significant AFC numbers could be detected. Finally, a particle dose 1 day after the antigen administrations was not effective as adjuvant.

It appears that the immune response can be modified by the relative times of particle and antigen exposure. Time dependency is demonstrated by the fact that alltogether, coadministration emerges as the most effective way to adjuvate an immune response in our model. Particles dosed in 3 doses simultaneously with antigen display the clearest adjuvant activity, even inducing IgE as opposed to one large co-administered dose. This indicates that a continuous particle exposure, possibly by stimulating an ongoing inflammatory response in close proximity of the co-administered antigen, favors adjuvant activity.

The importance of the relative times of particle and antigen exposure is demonstrated by the fact that also administration of particles 1 day before antigen caused an enhanced immune response (as was also shown by Lambert *et al.,* 1999), but IgG1 levels and AFC numbers were not as high as in co-administered groups. In contrast, particle dosing 1 day after the antigen administrations did not adjuvate the immune response. In order to adjuvate the immune response, particles apparently have to be present in the lung at the same time or shortly before antigen is administered. During this time the particles are phagocytized by macrophages, which are known to produce proinflammatory mediators in response to particles (Yang *et al.,* 1997). Moreover, particles induce a decrease in macrophage phagocytosis capacity (Becker and Soukup 1998, van Zijverden *et al.,* Chapter 3), possibly decreasing antigen breakdown by macrophages thereby increasing the amount of antigen available for presentation by dendritic cells. Time dependency was also shown in an intraperitoneal injection model by Granum *et al.* (2000a), who demonstrated that adjuvant activity is lower with increasing time (from 1 to 3 days) between particle and antigen injection. In an inhalatory model a gaseous pollutant, $NO_2$, was also shown to adjuvate the immune response to antigen that was administered 24 h later (Siegel *et al.,* 1997), as opposed to antigen that was administered 7 days later.

Overall, adjuvant activity of particles appears to be a time-dependent process, suggesting that an inflammatory microenvironment as created by the particles is crucial for adjuvating sensitization by particles.

## ACKNOWLEDGMENTS

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# CHAPTER 7

# INTRANASALLY ADMINISTERED PARTICULATE AIRBORNE POLLUTANTS ADJUVATE THE IMMUNE RESPONSE IN AN INHALATORY ANTIGEN EXPOSURE MODEL

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## ABSTRACT

Particulate air pollution negatively affects human health. It is known to increase symptoms of existent respiratory allergy, but its effect on sensitization to common allergens is not clear. Therefore a good animal model is needed to assess the possible immunomodulating effects of particles on the immune response. Whereas existing models frequently make use of artificial methods like injection and/or adjuvants to facilitate sensitization, we modified an existing model (Hessel *et al.,* 1995) to study effects of exposure to particles when antigen exposure took place via the relevant route. In BALB/c mice the original model, using 7 intraperitoneal (i.p.) injections on alternating days (1-13), was compared to the adapted model in which the i.p. injections were replaced by 7 ovalbumin (OVA) aerosols. Three weeks later all mice were exposed to 8 OVA aerosols on consecutive days. In addition, the effect of weekly intranasal administrations of 100 µg of particles was assessed in the completely aerosol-treated animals. The effects of diesel exhaust (DEP1), carbon black (CBP) and silica particles (SIP) were tested. The immune response was read out 1 day after the last aerosol (Day 42). Immunoglobulin (Ig) levels were measured in serum and antibody-forming cells (AFC) were determined in the lymph nodes draining the lung (peribronchial lymph nodes [PBLN]), spleen and bone marrow. Cells in bronchial alveolar lavage fluid (BAL) were differentiated. In comparison to the i.p. injected group, replacement of injections by aerosols led to a greater variability in response and lower average immunoglobulin levels in the aerosol exposed group. In all particle-treated groups, the number of animals that responded per group was increased, and they also showed higher IgG1 and IgG2a levels in serum. Specific IgE levels only increased in the CBP-treated group. No effect of treatment on BAL cell differentiation and AFC-numbers in PBLN was observed. Altogether, the immune response to OVA is differently adjuvated by the presence of different particles. Whereas IgG1 and IgG2a production are stimulated by all types of particles, only CBP stimulated the production of IgE. This warrants the importance of more research into the mechanism of particle-induced immunomodulation.

## INTRODUCTION

The prevalence of asthma and respiratory allergy has been increasing at a high rate in Western industrialized countries (Lundbäck 1998, Schäfer and Ring 1997). Epidemiological data have resulted in different theories of the increasing prevalence of asthma. One theory associates outdoor air pollution, and especially that produced by road transport, with asthma (Duhme *et al.,* 1996, van Vliet *et al.,* 1997, Rusznak *et al.,* 1994). The fact however that humans are exposed to a complex mixture of pollutants makes it hard to extract the responsible factor from epidemiological studies. Diesel exhaust particles (DEP) are one of the main suspects. They make up a large part of air pollution in Western cities and are known to increase symptoms (Pope and Dockery 1992) and IgE production (Diaz-Sanchez *et al.,* 1997a) in already sensitized subjects. Moreover they are able to cause inflammation (Salvi *et al.,* 1999, 2000) and adjuvate mucosal sensitization (Diaz-Sanchez et al., 2000) in naïve subjects. So far the mechanism by which particles modulate the immune response is incompletely known (Salvi and Holgate 1999).

To study the mechanisms underlying asthma and respiratory allergy, animal models are required. Models displaying important effector phase characteristics like specific IgE and eosinophils almost all use injections of allergenic materials and often adjuvantia for sensitization (Hessel *et al.,* 1995, Kung *et al.,* 1994, Yamada *et al.,* 1994, van Zijverden *et al.,* 2000). Despite the fact that these models effectively mimic respiratory allergy and/or asthma, they do not reflect real life exposure and its unique characteristics (Constant *et al.,* 2000), and are not suitable for studying the effect of air pollution on the sensitization process. This disadvantage has been overcome in other models in which the animals are exposed exclusively via the relevant route (Lambert *et al.,* 1999, van Zijverden et al., Chapter 4). However, the administration of antigen and particles via intranasal or intratracheal instillation of suspensions still differs greatly from real life inhalation exposure. Therefore we adapted an existing and well defined asthma model (Hessel *et al.,* 1995), in which all intraperitoneal (i.p.) injections during the sensitization phase were replaced by antigen aerosols. The adapted model consisted of 7 ovalbumin (OVA) aerosols on alternating days, which were 3 weeks later followed by 8 aerosols on consecutive days.

The immunomodulating effect of weekly intranasal doses of 100 µg of different particulate pollutants was tested in this model. DEP and carbon black (CBP), both organic carbonaceous particles (<0.1µm) and part of the (ultra)fine fraction of particulate matter (PM), were tested. Ultrafine particles are able to penetrate deep into the lung and stay present for a long time (Churg *et al.,* 1997, Ferin *et al.,* 1992). CBP represents the carbon core of DEP but lacks the attached chemicals as present on DEP. Whereas DEP are known

for their immunomodulating capacity, CBP are possibly not as inert (Løvik *et al.,* 1997, Nilsen *et al.,* 1997, van Zijverden *et al.,* 2000, Chapter 3,4) as they were once thought to be (Crosbie 1986). Also amorphous silica particles (SIP) were tested. The immunomodulatory capacity of SIP in relation to respiratory allergy is unknown. SIP (1-5 μm) predominantly belong to the coarse fraction of PM, which mainly consists of inorganic wind blown dusts and larger carbon aggregates (Ormstad *et al.,* 1997). We compared the capacity of the different particles to adjuvate the immune response to OVA by measuring antibody levels in blood, antibody forming cells (AFC) in PBLN, spleen and bone marrow, and differentiating cells in brochial alveolar lavage (BAL).

## MATERIALS AND METHODS

### Animals

Female, specific pathogen-free BALB/c mice (6-8 weeks of age) were obtained from the RIVM breeding facility. Mice were housed under hygienic barrier conditions in filter-topped macrolon cages with bedding of wood chips, a temperature of 23±2$^{o}$C, 50-60% relative humidity and a 12-h light/dark cycle. They received standard lab chow and acidified tap water ad libitum. The experiments were approved by an ethical committee and conducted in accordance with the Guiding Principles in the Use of Animals in Toxicology.

### Chemicals and reagents

Silica ($SiO_2$) was obtained from Sigma Chemical Company (St. Louis, MO) and CBP from Brunschwich Chemie (Amsterdam, The Netherlands). DEP1 was a friendly gift from Dr. P. Steerenberg (RIVM). The antigen ovalbumin (grade V) was obtained from Sigma. Immobilon-P membranes were obtained from Millipore (Etten-Leur, The Netherlands), alkaline-phosphatase (AP)-conjugated goat-anti-mouse IgG1 and IgG2a antibodies from Southern Biotechnology Associates (Birmingham, AL). All reagents for ELISA, except the OVA-specific IgE ELISA, were obtained from Sigma Chemical Company (St. Louis, MO), while the plates (highbond 3590) were obtained from Costar (Cambridge, MA). Plates for the OVA-specific IgE ELISA were obtained from Greiner Labortechnik (Frickenhausen, Germany), monoclonal rat anti-mouse was obtained from Zymed (clone LO-ME-2, Oxnard, CA). Blocking reagent and the reagent kit for labeling of OVA with digoxigenin (DIG antibody labelings kit) were obtained from Boehringer (Mannheim, Germany).

### Treatment of the mice

Particle suspensions (4 mg/ml) were prepared in saline and 5% heat inactivated normal BALB/C mouse serum. Naïve female BALB/c mice were weekly intranasally instilled by pipetting a total volume of 25 μl on the nostrils under halothane anesthesia (weekly dose:

100 µg, first dose at Day 1). Sensitization with antigen (Table 1) took place at Day 1,3,5,7,9,11 and 13 by exposing the mice (n=8-17) to an ovalbumin aerosol (2 mg/ml) for 5 min, in groups of maximal 6 mice. The aerosol was generated with an ultrasonic nebulizer (Medix 8001, particle size 3-5 µm), connected to a plexiglass exposure chamber (5 l). The challenge phase consisted of 8 aerosols on consecutive days (Day 34-41). Blood was collected by orbita punction under halothane anesthesia at Day 0, 21 and 42, just prior to dissection at which also BAL was performed and PBLN, spleen and bone marrow were taken out.

**Table 1:** Treatment groups in the inhalatory model (n=8-17). Blood samples were taken at Day 0, Day 21 and at the end of the experiment at Day 42.

| group | Weekly doses | Sensitization (day 1,3,5,7,9,11,13) | Boosters (day 34-41) |
|---|---|---|---|
| Positive control | - | i.p. OVA injections | OVA aerosol |
| Negative control | - | Saline aerosols | Saline aerosols |
| OVA-control | Saline | OVA aerosols | OVA aerosols |
| Particle treatment | 100 µg of particles in saline (start: day 1) | OVA aerosols | OVA aerosols |

**OVA -specific IgG1 and IgG2a ELISA**

Plates were coated (o.n. 4$^{o}$C) with OVA (20 µg/ml) in 0.05 M carbonate buffer pH 9.6, and blocked with phosphate buffered saline (PBS) containing 0.05% Tween20 (Brunschwig) and 1% bovine serum albumin ([PBS/T/1%BSA], grade V, Sigma) during 1 h at room temperature (RT). Serial dilutions of sera were incubated (1 h, RT) and plates were washed and incubated with an optimal dilution of AP-conjugated anti-mouse IgG1 or IgG2a (1 h, RT), followed by p-nitrophenylphosphate (1 mg/ml in diethanolamine buffer, pH 9.8) for 30 min. Absorbance was measured at 405 nm. Titers were calculated by means of the sample dilution at which extinctions were higher than background+2*SD. Background levels at Day 0 were subtracted from Day 21 or Day 42 levels to obtain the increase in IgG1/IgG2a.

IgG1-responders were defined as displaying an increase of >3 titers between Day 21 and Day 0, and >7 titers between Day 42 and Day 0. IgG2a-responders were defined as displaying an increase of >1 titer between Day 21 and Day 0, and >4 titers between Day 42

and Day 0. Averages of all animals together (responders and low-responders) were calculated.

**OVA-specific IgE ELISA**

Plates were coated (o.n. 4°C) with rat anti-mouse IgE (2 µg/ml) in 0.05 M carbonate buffer pH 9.6, and blocked with blocking reagent (Boehringer, 1h, 37°C). Serial dilutions of sera were incubated (1h, 37°C), and after washing an OVA-digoxigenin solution (0.01 µg/ml) in blocking reagent was added (1h, 37°C). Tetramethylbenzidine-substrate (0.1 mg/ml in sodiumacetate buffer pH 5.5) for 20 min, and after stopping the reaction with $H_2SO_4$ (2M), extinctions were measured at 450 nm. Titers were calculated by means of the sample dilution at which extinctions were higher than background+2*SD. The increase in titers between Day 42 and Day 0 was calculated. IgE-responders were defined as displaying an increase of >2 titers between Day 42 and Day 0. Averages of all animals together (responders and low-responders) were calculated.

**ELISPOT assay**

These assays were essentially performed as described previously (Schielen *et al.,* 1995). Shortly, PBLN, spleen and bone marrow were minced to prepare single cell suspensions, and $0.5*10^6$ cells in 500 µl PBS/1% BSA were incubated (4 h, 37°C) in wells containing OVA coated (20 µg/ml, o.n. 4°C) Immobilon-P membranes as bottom. Thereafter, membranes were washed and incubated with optimal dilutions of isotype-specific AP-conjugated anti-mouse Ig antibodies in PBS/T (o.n. 4°C). After washing, spots were developed by incubation with 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium (BCIP/NBT). Specific AFCs per $10*^6$ cells were calculated from spot numbers counted with the aid of a stereo microscope.

**Bronchial alveolar lavage and cell differentiation**

The lungs of the mice were lavaged 3 times with 1-ml aliquots of PBS (37°C). The bronchial alveolar lavage was kept on ice until it was centrifuged (1000 g, 5 min). The pellet was resuspended in 250 µl of cold PBS. For differential cell counts cytospin preparations were made and stained 7 min with May-Grünwald (Merck, Darmstad, Germany), and subsequently 7 min with Giemsa staining (Merck). After coding, the cytospin preparations were evaluated using oil immersion microscopy. Per cytospin preparation at least 200 cells were counted and differentiated into macrophages, eosinophils, neutrophils and lymphocytes by standard morphology.

**Statistics**

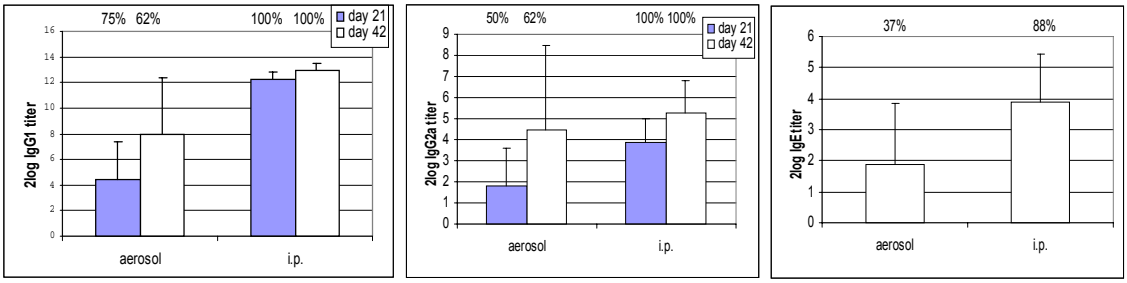
Preceding statistical analysis, ELISPOT data (the number of AFCs) were transformed to log10 values to homogenize variance. Differences between group means were analyzed using one-way ANOVA with Scheffe post-hoc test for contrasts ($p<0.05$ or $p<0.01$). In

case of not sufficient homogeneity of variances, a Kruskal-Wallis ranking test was performed ($p<0.05$).

## RESULTS

### OVA- specific antibody levels in original i.p. model versus accustomed inhalatory model.

Comparison of the i.p. sensitization model with our aerosol model shows that IgG1 levels in blood displayed a slower increase during aerosol exposure resulting in a lower level at Day 42 (Fig. 1A). Moreover, a greater variability in response was observed in the aerosol group for all isotypes, with the aerosol group containing both "responders" (62%) and "low-responders" (38%). IgG2a levels also increased slower in the aerosol-treated animals compared to the i.p. model (Fig. 1B). IgE increased greatly in 88% of the animals after i.p. exposure (Fig. 1C). In the inhalatory exposure group, this was only observed in 37% of the animals (Fig. 1C). This suboptimal stimulation of the humoral immune response appeared suitable for detecting particle adjuvant activity, therefore we proceeded by administering 100 µg of particles every week to aerosol-exposed animals (following section).




**A** **B** **C**

**Figure 1:** OVA-specific antibody levels in the original i.p. model compared to the accustomed inhalatory model (n=8). Indicated is the percentage of responders.
A: Increase in IgG1 titers at Day 21 and 42 in i.p. versus inhalatory exposed animals. Responders display an increase of >3 titers between Day 21 and Day 0, and >7 titers between Day 42 and Day 0.
B: Increase in IgG2a titers at Day 21 and 42 in i.p. versus inhalatory exposed animals. Responders display an increase of >1 titer between Day 21 and Day 0, and >4 titers between Day 42 and Day 0.
C: Increase in IgE titers at Day 42 in i.p. versus inhalatory exposed animals. Responders display an increase of >2 titers between Day 42 and Day 0.

**OVA- specific antibody levels, AFC numbers and BAL cells in the inhalatory model after weekly particle exposure.**

Hundred µg of particle was administered intranasally weekly during 42 days (total of 6 doses). Immunoglobulin levels were determined in sera obtained at Day 0, 21, and at the end of the experiment at Day 42. All particle-treated groups showed significant increases in IgG1 titers (Fig. 2A).

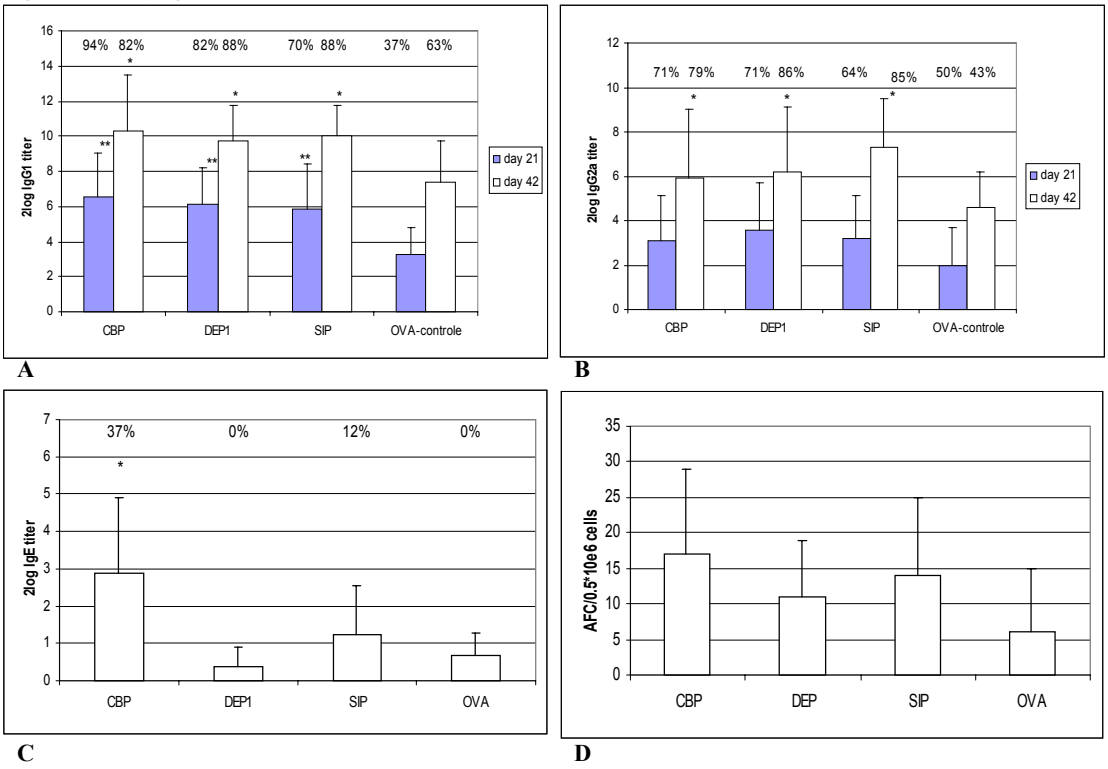



**Figure 2:** OVA-specific antibody levels in serum and OVA-specific IgG1-forming cells in PBLN in CBP, DEP1, SIP and OVA control-treated animals. n=(8-17). Indicated is the percentage of responders (A-C).
A: IgG1 titers at Day 21 and 42 in CBP, DEP1, SIP and control-treated animals. Responders display an increase of >3 titers between Day 21 and Day 0, and >7 titers between Day 42 and Day 0. * $p<0.05$, ** $p<0.01$ vs control.
B: IgG2a titers at Day 21 and 42 in CBP, DEP1, SIP and control-treated animals. Responders display an increase of >1 titer between Day 21 and Day 0, and >4 titers between Day 42 and Day 0. * $p<0.05$ vs control.
C: IgE titers at Day 42 in CBP, DEP1, SIP and control-treated animals. Responders display an increase of >2 titers between Day 42 and Day 0. * $p<0.05$ vs control.
D: IgG1-forming cells in PBLN at Day 42.

At Day 21 these differences were most pronounced compared to the OVA-control group, which contained only 37% responders compared to 70-94% in the particle-treated groups (Fig. 2A). Also at Day 42 these differences were still significant, but less pronounced compared to Day 21 because the number of responders in the OVA-control group increased to 63%. IgG2a titers were not significantly increased compared to controls at Day 21, but were significantly enhanced at Day 42 for all particle-treated groups (Fig. 2B). IgE increased in 37% of the CBP-treated animals as compared to OVA-treated mice, whereas only 12% of the SIP-treated and none of the DEP-treated animals showed an enhanced IgE response (Fig. 2C).
No antibody forming cells were detected in spleen and bone marrow (not shown). Statistically not significant increases in IgG1-AFC numbers were observed in PBLN (Fig. 2D). No effects on composition of BAL fluid were found.

## DISCUSSION

The present study intended to develop a model in which the immunomodulatory capacity of different components of PM could be tested after inhalatory antigen exposure. For this purpose, we modified an existing allergy model in the mouse (Hessel *et al.,* 1995) by replacing all i.p. OVA injections by aerosol exposures. As expected, in this new, completely inhalatory model average antibody levels did not increase to levels as high as after i.p. exposure. Remarkably, the inhalatory exposed animals showed a large variation in antibody titers; either animals responded with a large antibody increase (responders), or they hardly showed any antibody levels (low-responders). Since no intermediate responding animals were observed, animals could be divided into responding and low-responding animals. The inhalatory treatment appeared to be suitable for detecting adjuvant activity of particles. In the groups treated weekly with particles, it became clear that the different particles all displayed adjuvant activity on the humoral response. Not only did more animals per group respond, they also showed higher IgG1 and IgG2a levels compared to the OVA-controls. IgE increased in 37% of the CBP-treated animals.

In an intranasal exposure model (van Zijverden *et al.,* Chapter 4) we tested the same 3 types of particles and their effects on the immune response to a protein antigen (TNP-OVA). In this intranasal model, SIP displayed less pronounced adjuvant activity, IgG2a responses were in general less pronounced, and in addition to CBP also DEP stimulated IgE production. Moreover, inflammatory cells were found to infiltrate the lung, significantly elevated IgG1-AFC in PBLN were detected, and variations within each

treatment-group were not as pronounced as in the present inhalatory model. It is hard to directly compare the results from the intranasal and the inhalatory models since treatments differ greatly. However three factors can be appointed that possibly play a role in the observed differences. First, the role of the relative times of particle and antigen exposure has been shown in the intranasal model (van Zijverden *et al.,* Chapter 6) by comparing simultaneous and separate administration of antigen and particles. We showed that administration of particles one day before antigen was less effective in inducing IgE and IgG1-AFC compared to coadministration, indicating that particle adjuvant activity involves time-dependent processes. Although others (Lambert *et al.*, 1999) show that intranasal administration of residual oil fly ash particles 1 day before antigen is also effective in adjuvating the immune response, altogether adjuvant activity appears to be lower with increasing time between particle and antigen administration (Granum *et al.,* 2000a). This was also shown for a gaseous pollutant ($NO_2$) by Siegel *et al.,* 1997.

Second, in addition to separate times of administration, in the present inhalatory model antigen and particles were administered via different methods of administration (respectively inhalatory and intranasal). This may further reduce the chance of antigen deposition in close proximity of the particles, thereby decreasing the impact of the particle-induced inflammation on the immune response to the antigen. Third, the inhaled antigen dose is hard to control in whole body exposure and may not have been optimal in this model, indicated by the decreasing differences in IgG1 between controls and particle-treated animals after Day 21. In addition, the amount of antigen entering the whole body-exposed animal via the oral route, and the effect on possible tolerance induction, is unknown.

In real life, subjects may be exposed to both complexes of antigen and pollutant (Behrendt *et al.,* 1992, Ormstad *et al.,* 1998) as well as antigen and pollution at distinct time points. For the latter situation, this model appears to reflect real life fairly well, by showing that the number of animals responding with a humoral immune response increases when animals receive particle administrations in addition to antigen exposure. However, for studies on the mechanism of particle adjuvant activity and hazard identification, a simple and reliable intranasal model (van Zijverden, Chapter 4-6) may be more suitable than a model employing whole body, inhalatory antigen exposure route.

## ACKNOWLEDGMENTS

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# CHAPTER 8

# GENERAL DISCUSSION

## Introduction

The prevalence of respiratory allergy is increasing (Schäfer and Ring 1997, Lundbäck 1998, Beasley *et al.,* 2000). This increase appears to be correlated to Westernization and/or urbanization of countries. However it is unknown which factor in Western lifestyle is responsible. In general, factors concerning (patterns of) "infection" and "other factors in Western lifestyle" can be distinguished (Fig. 3 General Introduction). So far, vaccination against highly infectious airborne viruses has often been suggested to stimulate the development of allergy (Cookson 1997), but contradictory results have been found. Going through orofecal and foodborne infections appears to reduce the risk of allergy (Matricardi *et al.,* 2000). Hence "infection" appears to be a heterogeneous gathering of infections belonging to different subgroups, which need to be distinguished since they display distinct effects on the development of allergy. A similar distinction appears to be necessary in the air pollution theory. Air pollution is a heterogeneous mixture of gaseous ($NO_2$, $SO_2$, $O_3$) and particulate pollutants (particles from innumerable different sources). Although for a lot of compounds ($NO_2$, $O_3$, PM) a role in the aggravation of existing respiratory allergy is obvious, contradicting results are found in epidemiological studies on whether a high level of pollution also causes increased prevalence. The presence of mixtures complicates matters, making the effects of separate pollutants impossible to discern. Moreover, substances in the mixture may not all have different modes of action but rather show additive, antagonistic, or synergistic effects.

For all theories, from infection to pollution, the most important gap in the knowledge concerns the mode of action. For particulate air pollution inflammation appears to be crucial for adjuvant activity. A number of factors, ranging from particle shape (e.g. fibrogenous SIP) to surface charge (Oortgiesen et al., 2000), appear to be involved in the capacity of particles to cause inflammation. Since the mechanisms of particle adjuvant activity are largely unknown, our aim was to develop an (animal) model in which immunomodulation could be determined, and mechanisms could be studied. In the following paragraphs the different *in vivo* and *in vitro* models used in this thesis to evaluate adjuvant activity of particles are summarized.

## Summary of results

In **Chapter 2** a modification of the reporter antigen PLNA ([RA-PLNA] Albers *et al.,* 1997) was introduced. This model is relatively simple to perform, control and read out. After injection of 1mg of particle and 10 µg of antigen in the hind paw, different aspects of

the immune response can easily be determined in the draining popliteal lymph node. Our results indicate that different components of particulate matter, DEP, CBP and SIP, are all able to act as adjuvant in the immune response to the reporter antigen TNP-OVA. However, the different particles stimulate different types of immune responses to the same antigen. At Day 2 and 5 after injection the increase in the Th2-related cytokine IL-4 is largest for CBP and DEP compared to SIP and control. Intranasal challenge with the reporter antigen after subcutaneous sensitization in the hind paw allows for assessment of the immune response in lungs, bone marrow and spleen, indicating that systemic immunosensitization has occurred. The isotype patterns (based on serum levels and AFC in PBLN, bone marrow, spleen and PLN) observed after the intranasal challenge approach are indicative of a type 2 response after DEP exposure, whereas SIP induce more a type 1 response and CBP stimulate a mixed response to TNP-OVA.

In **Chapter 3** the *in vitro* effects of the various types of particles (DEP from various sources, SIP, CBP) on alveolar macrophages were assessed. All particles except DEP3 cause cell damage and they all induce a decrease in phagocytic activity of the macrophages. TNFα production is enhanced in a dose-dependent way in response to particles. IL-1β and IL-6 are only measurable after LPS prestimulation. In this *in vitro* model, DEP from different sources do not stimulate the same response (as was also found *in vivo* in Chapter 4-6). DEP1 stimulate more LDH release and TNFα production, whereas DEP3 block phagocytosis more effectively. Because they are both of similar size, we addressed the question whether a difference in surface chemical composition may cause such changes. Pre-exposure of DEP3 to O3 for more than 1 hour causes an increase in TNFα production, indicating enhanced immunomodulating capacity, and complete blockage of phagocytosis. Overall, it can be concluded that the different particles clearly modulate crucial macrophage functions. There appears to be no correlation between the different processes of inducing cell damage, blocking phagocytosis, and the production of proinflammatory cytokines in response to particle exposure. In addition, ozone-exposure of DEP appears to be able to enhance immunomodulating capacity of DEP.

A disadvantage of *in vitro* models is the fact that possibly important interactions between different cell types cannot be determined. The *in vivo* injection model, as presented in Chapter 1, also has a major drawback: it does not involve the real life route of exposure. To circumvent the disadvantages of *in vitro* and injection models, we developed a simple model using the relevant route of exposure. In **Chapter 4** a model was presented in which the antigen and particle were administered via the intranasal route. In this model both sensitization (Day 1-3) as well as challenge (Day 10) take place intranasally. Particles are co-administered with the antigen during the 3-day sensitization. At Day 15

immunoglobulins in blood and AFC in draining lymph nodes of the lung (PBLN) are determined, and leukocyte distribution in BAL is assessed. In this model CBP adjuvate the response most clearly, followed by DEP1, DEP2 and finally SIP which hardly adjuvate at all. The order of magnitude of these responses, which appear to be mostly Th2-mediated, for the different particles roughly resembles that seen in Chapter 2 and 3. The effect of an extra challenge with TNP-OVA (Day 17) in animals which received CBP during sensitization, results in even more pronounced immunoglobulin production and lung infiltrates at Day 22 compared to Day 15, stressing the persistent character of the particle adjuvant activity. Macrophages containing particles are still present in BAL at Day 22. The fact that the carbonaceous particles (CBP, DEP) are able to adjuvate sensitization to an antigen indicates the increased risk of sensitization to allergen for naïve subjects who are exposed to particulate matter.

In **Chapter 5** we used the intranasal model to further determine the effect of particle exposure during different phases of the immune response. From this chapter it can be concluded that administration of particles with antigen during both sensitization and challenge is most effective in adjuvating the immune response, whereas administration exclusively during sensitization is almost equally effective, and instillation exclusively during challenge hardly adjuvates the immune response. Exposure to particulate air pollution during both sensitization and challenge results in optimal adjuvation of an immune response to a co-administered antigen.

In **Chapter 6** we determined whether co-administration of antigen and particles is actually crucial for adjuvant activity. It appears that simultaneous administration of three small doses (67 μg) and antigen is most effective in adjuvating the immune response. One large co-administered dose (200μg) is also capable of adjuvating the immune response, but no IgE production is induced. Moreover, also particles that are separately administered one day before the administration of antigen are able to act as adjuvant. However, particles administered one day after antigen administration do not adjuvate the immune response. Altogether, coadministration emerges as the most effective way to adjuvate an immune response in our model. It appears that the immune response can be modified by the relative times of particle and antigen exposure.

In **Chapter 7** we determined whether the immune response after inhalation of an antigen aerosol could be adjuvated by intranasal particle administrations. Although adjuvant activity is detectable in this model, it is not accompanied by lung infiltrate as in the intranasal model. This may firstly be caused partly by the fact that antigen and particles are not simultaneously administered. Secondly, different methods of exposure are used for particles and antigen (intranasal and inhalatory respectively), thereby possibly inducing

distinct deposition patterns. Since inflammation causing adjuvant activity is a time-dependent process, this model may not provide optimal exposures.

**Different routes, different effects**

Different types of particles are obviously able to stimulate an immune response to antigen. However, they behave differently after administration via different routes. The responses in the intranasal model do not show the Th1/Th2 modulation as seen after injection (Chapter 2), but rather involve variations in strength of the Th2 response. The inhalation model, in turn, shows mixed responses for all types of particles.

At least two things may cause differences between the effects of injected particles and particles administered via the respiratory tract. Firstly, different responses may be a result of distinct deposition and retention patterns. Secondly, different cell types are present in skin versus lung, resulting in different biological responses. This is also illustrated by distinct default reactions in both organs, respectively Th1 in skin and Th2 in lung (Constant *et al.,* 2000). In this thesis, route differences are for example demonstrated by SIP that adjuvate mainly IgG2a production in the injection model (Chapter 2), whereas after intranasal administration (Chapter 4-5), SIP induce predominantly IgG1. Injection causes SIP to stay present subcutaneously for a long time (unpublished observation), possibly stimulating an ongoing cellular inflammatory response resulting in a high level of IgG2a. The fact that injection causes this kind of effects, emphasizes the limitation of this artificial route of administration. Irrespectively however, injection of particles using the RA-PLNA can be regarded as a simple prescreening tool for hazard identification of different particles. Apart from this possible application of the RA-PLNA, it is evident that more mechanistical research requires animal models employing the respiratory tract as route of exposure.

*Usefulness and relevance of the intranasal model*

With respect to the applicability of our relevant route of exposure models, it is important to consider the dose of particles used and compare it with human exposures. However, a direct comparison is impossible to make, since intranasal instillation of particles in a fluid matrix leads to unknown differences in deposition compared to real life particle inhalation. In the intranasal model, a particle dose of 67 µg per day is administered to the mice. In humans a 1-day stay in Los Angeles has been estimated to lead to a total exposure of 100 µg of particles (Saxon and Diaz-Sanchez 2000). This rough comparison shows that in our

model we are dealing with relatively high doses. Nevertheless, present studies (Chapter 4-6) demonstrate the usefulness of our intranasal model in mechanistical studies. Moreover, as it is also relatively simple to perform, apparently more robust than the inhalatory model, and allows grading of different particle adjuvant activities, the intranasal model seems to be advantageous over the inhalatory model at this moment (Chapter 7).

*Relevance of in vitro studies*

*In vitro* studies can give additional information on the exact response of a single cell type to a certain particle. The order of the potency of particles to induce production of proinflammatory cytokines, especially TNFα (CBP>DEP1>DEP3>SIP), in alveolar macrophages (Chapter 3) coincides with the strength of their adjuvating capacity in our *in vivo* intranasal model (Chapter 4-6). This, together with the fact that TNFα has been appointed as crucial in a comparable intranasal model of particle exposure (Lambert *et al.,* 2000a), suggests that TNFα production by alveolar macrophages represents an important determinant of adjuvant activity *in vivo*. In addition to the TNFα-driven mechanism, the severe decline in phagocytic capacity of the macrophages as observed *in vitro* may be relevant for the adjuvant capacity of particles *in vivo*. As a result of decreased phagocytic activity of alveolar macrophages, co- or subsequently administered antigen may escape uptake and/or degradation by these macrophages. As a result, a larger quantity of antigen may contact immune cells (such as interstitial DC). In addition, effects on the suppressive activity of the alveolar macrophage can not be excluded. In all, these *in vitro* studies suggest an important role for the alveolar macrophage, as it may contribute to a cytokine-rich environment and enhanced antigen presentation by DC.

**Particles**

In addition to variations in adjuvant activity caused by different routes of exposure, differences between particles are also observed within the same model. Overall, CBP display the strongest adjuvant activity in the intranasal model, and SIP the weakest (Chapter 4-5). *In vitro* measurements of TNFα production in macrophages also indicate this order of strength (Chapter 3). DEP from different sources show distinct adjuvant activities. Also completely different types of particles, from ROFA (Lambert *et al.,* 1999) to polystyrene particles (Granum *et al.,* 2000a), are able to adjuvate an immune response. Cellular and molecular effects of particles are still incompletely understood, but may be very complex. Moreover, which particle characteristics are crucial in determining the immunomodulatory potential is unknown (Fubini *et al.,* 1997).

*Crucial particle characteristics*

The differential roles of possible crucial particle characteristics (a random list is given in Table 1) such as size, surface area, charge and attached substances, are hard to determine. This is firstly due to the fact that these crucial particle characteristics are not independent of one another. An experimental set-up to determine the effect of smaller size causes a simultaneous increase in total surface area and number of administered particles. Correcting for these changes by maintaining a constant number causes a subsequent decrease in total weight of the administered dose, etc.

| Crucial particle characteristics | |
|---|---|
| Core | Attached substances |
| Size<br>Surface area<br>Charge<br>Shape<br>Number<br>Weight<br>Absorption capacity | Chemical composition<br>Oxidizing potential<br>Metals<br>Endotoxin |

**Table 1:** Different particle characteristics that play a role in adjuvant activity

Secondly, the importance of any particle characteristic for adjuvant activity is not constant over the entire process of respiratory sensitization. The different processes that play a role are depicted in Figure 1. From pre-exposure to exposure phase, and from retention on to effector phase, several mechanisms are at work, and many particle-cell interactions are involved. For these different phases, distinct particle characteristics can be appointed as crucial (Fig.1). The importance of every single particle characteristic can preferably be determined experimentally for the different phases, rather than for the complex immunosensitization process as a whole. For the pre-exposure phase, adsorption capacity is a crucial characteristic, determining the amount of substances that can become attached. In the exposure phase, weight as a conventional determinant of dose may preferentially be replaced by number, surface area or size (Granum *et al.,* 2000b, Fubini *et al.,* 1997). In determining site of deposition, size is a crucial characteristic. Particles smaller than 2.5µm are able to reach the conducting airways and the alveolar region (Anderson *et al.,* 1988).

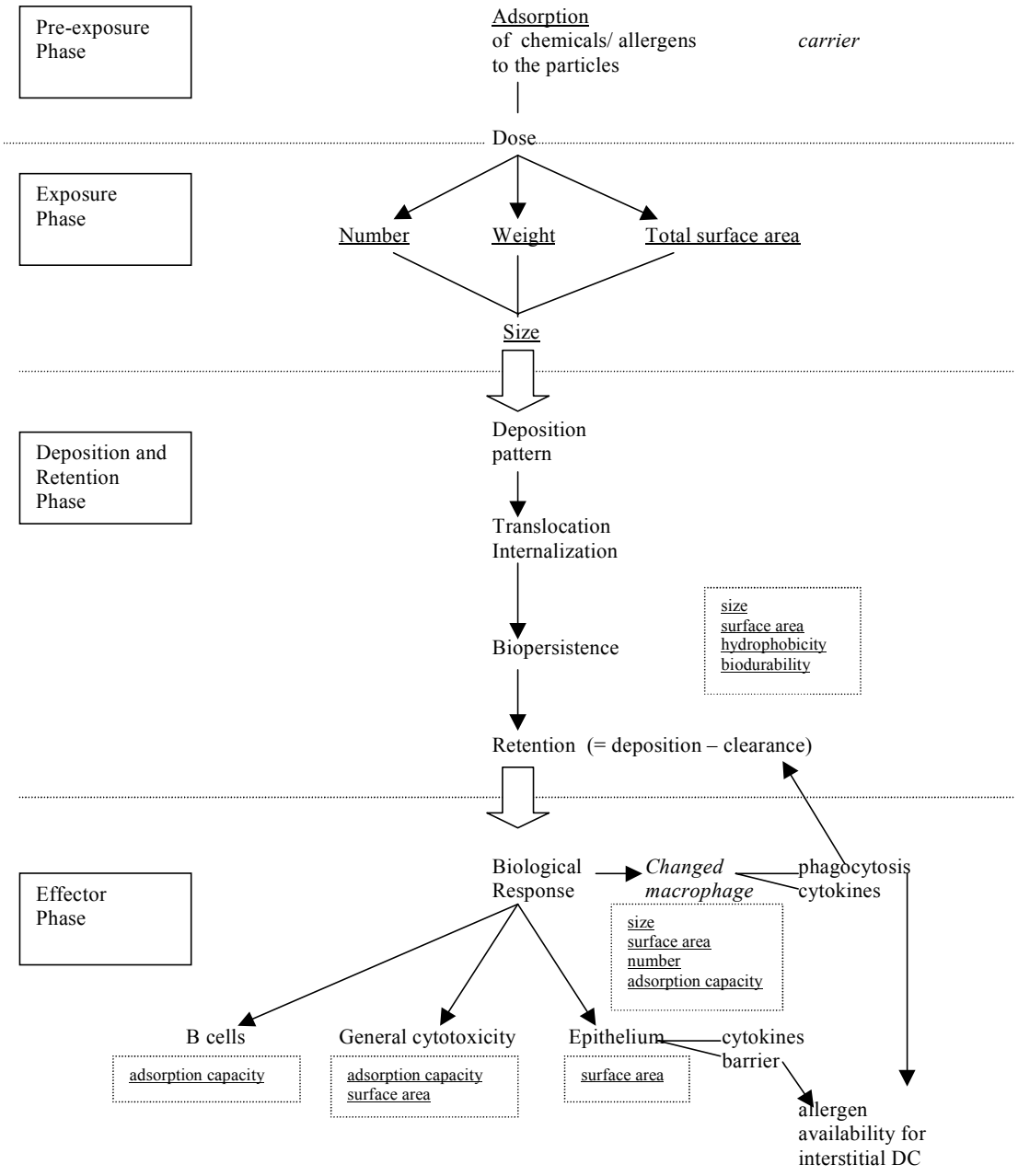
Pre-exposure Phase
Adsorption of chemicals/ allergens to the particles
carrier
Dose
Exposure Phase
Number
Weight
Total surface area
Size
Deposition and Retention Phase
Deposition pattern
Translocation Internalization
Biopersistence
size
surface area
hydrophobicity
biodurability
Retention (= deposition – clearance)
Effector Phase
Biological Response
Changed macrophage
phagocytosis
cytokines
size
surface area
number
adsorption capacity
B cells
adsorption capacity
General cytotoxicity
adsorption capacity
surface area
Epithelium
surface area
cytokines
barrier
allergen availability for interstitial DC
"Changed Microenvironment"

**Figure 1:** "Changed Microenvironment"
In time, distinct phases can be distinguished in the process leading to a changed microenvironment. In the **pre-exposure phase**, the adsorption capacity of a particle is crucial in determining whether or not the particle becomes a carrier (see text) of either chemicals or allergens. In the **exposure phase**, the dose is usually expressed as weight, but as weight is closely related to number, surface area and size, these are equally or even more important determinants of dose. In the **deposition and retention phase**, one of the main characteristics determining the pattern of deposition is size, whereas in addition surface area, hydrophobicity and biodurability are important in determining final retention. In the **effector phase**, many particle characteristics are involved in determining the reponse of different cell types. Of special interest are macrophages (see text), because they play a role in clearance of particles (and antigen) by means of phagocytosis, and in addition produce cytokines. Resuming, cytokines and cell damage together lead to inflammation. Inflammation on its turn leads, together with altered macrophage function and greater allergen availability, to a change in allergen responsiveness, ultimately possibly resulting in respiratory allergy.
*Italic* = mechanism as described in text
Underline = crucial particle characteristic

They stay present for a long time and are therefore in general considered more toxic than larger particles (Churg and Brauer 1997, Ferin *et al.,* 1992, Svartengren *et al.,* 1987). Final retention (deposition minus amount cleared), on its turn, is determined by numerous particle characteristics such as size, surface area, number, mass and hydrophobicity (Oberdörster *et al.,* 1994). Depending on the biological response process that is studied, one or a few particle characteristics can be regarded as most relevant. For (inhibition of) phagocytosis by macrophages for example, size and surface area (including attached substances) may be decisive (this thesis, Oberdörster *et al.,* 1994), whereas in addition number may be a crucial particle characteristic for subsequent activation (Fubini *et al.,* 1997). B cells are known to be affected by attached chemicals as present on DEP (Tsien *et al.,* 1997), indicating that for non-phagocytic cells especially surface characteristics causing general cytotoxicity may be important.

*Particles as carriers*

Adsorptive capacity is a particle characteristic that is crucial in the pre-exposure phase. Burning of fossil fuels produces both particles and chemicals, and depending on the type of particle the chemicals adsorb to the particles. Especially carbonaceous particles are known for their high adsorptive capacity, turning the particles into exquisite carriers. The capacity of particles to carry chemical substances is largely dependent on the surface characteristics of the particle, especially the material (e.g. silica versus carbon) and the micromorphology (smooth versus cavity-rich). The fact that particles lacking attached substances, such as

CBP, SIP (this thesis) and PSP (Granum *et al.,* 2000b) are able to adjuvate the immune response shows that characteristics of the particle core are important. Løvik *et al.* (1997) show in the PLNA that CBP were only slightly less potent than DEP in inducing an inflammatory response and systemic IgE to ovalbumin. However, both attached chemicals and metals (Lambert *et al.,* 2000b) are known to contribute significantly to the adjuvant capacity of particles. Diaz Sanchez *et al.* (1997b) shows that polyaromatic hydrocarbons (PAH) enhance ongoing IgE production, but not *de novo* synthesis. In addition to these quantitative effects, PAH also qualitatively alters the affinity of the IgE produced. Therefore attached chemicals seem to enhance the inflammatory potential of the particles, and modulate the nature of the adjuvant activity. Similar conclusions are drawn by Takenaka *et al.* (1995) and Tsien *et al.* (1997) from *in vitro* studies. Also the results presented in this thesis indicate that attached substances may play a role. The slight differences in character of the immune response induced by CBP and DEP (for example Chapter 2) and the differences between DEP from different sources (Chapter 3-5) may partly be caused by the modulating capacity of PAH. In Chapter 3 we show that DEP exposed to $O_3$ cause an increased TNFα release by alveolar macrophages. Increased toxicity of ozone-exposed DEP is also observed *in vivo*, and ozonation reaction products present on DEP are held responsible, because ozone-exposed CBP do not show altered toxicity (Madden *et al.,* 2000). Moreover, adsorbed chemicals influence surface characteristics such as charge, differences in which possibly modify cytokine responses of epithelium via the capsaicin receptor (Veronesi *et al.,* 1999). In a pilot *in vivo* experiment we observed slight differences between the adjuvant activity of negatively charged polymeric particles versus neutral ones (unpublished observation). However, in our *in vitro* model we observed that this charge difference did not influence phagocytosis or cytokine production by macrophages (unpublished observation).

A distinction can be made between carrying chemical substances and (protein) antigens. Whereas attached chemicals and metals add to the inflammatory capacity of the particle, carrying antigens may have quite different effects. Binding of allergen to (ultra) fine particles like DEP in outside air (Ormstad *et al.,* 1998) would concentrate the allergen on particles of respirable size (Knox *et al.,* 1997). This may facilitate subsequent deep deposition into the lungs, where the mucus escalator is not readily able to remove them. The fact that in our *in vivo* experiments (Chapter 4-5), the smallest (<1 μm), carbonaceous particles adjuvate the immune response better than SIP (1-5 μm) seems in line with this idea. However, co-administration of antigen and particles is not a prerequisite for adjuvant activity (this thesis, Lambert *et al.,* 1999, Granum *et al.,* 2000a). Finally, chemicals present

on particles may be involved in the modification of attached allergen (Behrendt *et al.,* 1992, 1997), possibly making the allergen more immunogenic.

**Particula(r)(te) mechanisms of immunomodulation**

In the effector phase (Fig.1), particles interact with different types of cells. In the next paragraph, the effect of particles on macrophage function is addressed. Thereafter, an overall mechanism of particle adjuvant activity is proposed.

*Changed macrophage function*

The alveolar macrophage belongs to the first line of defense in the lung, phagocytizing and degrading foreign bodies after deposition. Phagocytosis is severely impaired in alveolar macrophages after particle exposure (Chapter 3). *In vivo* this impairment of phagocytosis has already been demonstrated in macrophages in which ultrafine particles occupied only 2.6% of the cell volume (Oberdörster *et al.,* 1994). This decreased phagocytosis possibly causes antigen to escape degradation, thereby prolonging antigen presence and availability for cells of the immune system to mount a response.

In addition to their innate functions, macrophages also produce cytokines and other factors directly influencing adaptive immunity. For example T cells and DC are suppressed by macrophages (Holt 1993, Poulter 1994, Strickland 1996). In the PLN, macrophages have been shown to suppress the systemic immune response to a sc injected antigen, and deletion of these macrophages increases the number of AFC in the spleen (Delemarre *et al.,* 1990). Also in the lung depletion of macrophages has been shown to increase IgE synthesis (Thepen *et al.,* 1992). A decrease in suppressor activity has been suggested to be responsible for these effects. It is unknown whether exposure to particles affects suppressor activity, but the inflammatory microenvironment after entrance of particles may contribute to a breakdown of suppressive activity. The presence of GM-CSF for example has been shown to disrupt this suppression (Bilyk and Holt 1993, Holt 1996), and TNFα, a major proinflammatory cytokine produced by alveolar macrophages, has been shown to be crucial in the adjuvant activity of ROFA (Lambert *et al.,* 1999). TNFα is known to act as a trigger for the upregulation of several cytokines and adhesion molecules by means of NFκB activation in the lung, leading to inflammation (Lentsch *et al.,* 1999). These results suggest that the combination of a decrease in suppressor activity and an increase in production of proinflammatory cytokines by macrophages play an important role in particle adjuvant activity.

*Changed microenvironment: proposed mechanism*

Crucial processes in each of the described phases (Fig.1) contribute to what we call a "changed microenvironment in the lung". In short, in the pre-exposure phase particles interact with allergens and chemicals, and depending on their adsorption capacity they become carriers. During the exposure phase, weight, number, and total surface area are possible determinants of dose. These characteristics are strongly linked to size, the main characteristic determining the deposition pattern after dosing by inhalation. Small-sized particles are able to penetrate the epithelium and enter the interstitium, which is hypothesized to be crucial for inflammation (Oberdörster *et al.,* 1992). In addition, other characteristics such as charge and hydrophobicity may also influence deposition and translocation. Once deposited and retended, a biological response can take place. Particles carrying reactive chemicals as a consequence of their adsorption capacity, or particles having a reactive surface area by any other cause, can have cytotoxic effects on every cell present. Epithelium and macrophages make up the largest part of cells in the lungs, and are clearly affected. Both cell types produce cytokines in response to particle presence, and apart from macrophages, also epithelial cells take up particles (Steerenberg *et al.,* 1998). Cytotoxicity may negatively affect the barrier formed by the epithelium, causing nerve endings to be revealed and more allergen to cross the epithelial lining and contact for example DC. The decreased phagocytosis and suppressor capacity, together with increased production of proinflammatory mediators, may contribute to increased antigen presentation by DC. Central factors in the changed microenvironment are inflammation (cytokines, cell damage, nerve endings, infiltrating cells), allergen availability and changed macrophage (see previous paragraph) and APC function, which together could theoretically lead to a change in allergen responsiveness and development of respiratory allergy.

**Future directions**

A large amount of research remains to be performed on particle (immuno) toxicity. Several areas of interest can be indicated by means of Figure 1. Firstly, more knowledge is needed on the pre-exposure phase. Particles can interact with other substances, ranging from allergens to other pollutants, and too little attention is given to the particle acting as carriers. Carrying allergens does not only occur in obvious circumstances such as during pollen season with a simultaneous smog period. Also indoors, where fine particle concentrations are comparable to outdoor air, particles have been shown to carry allergens

(Ormstad *et al.,* 1998). The attached allergens have been shown to be of (sub) micronic size and display altered allergenicity (Emberlin 1995), which may be caused by interaction with other air pollutants. Also the interaction of the attached chemicals present on particles with other pollutants like $O_3$ is still a largely unexplored area, despite the fact that experimental and epidemiological studies showing the simultaneous effects of components in a mixture give rise to concern (Hoek *et al.,* 1997). In experimental studies the modes of action of different pollutants and emerging additive and synergistic effects on sensitization have to be determined. This would enable epidemiological exposure data of different pollutants to be added up in order to obtain a total personal dose.

Secondly, a more appropriate way to assess particle exposure dose may not be by measuring the usual "weight per $m^3$", but rather determine the number and size (Peters *et al.,* 1997), the two main determinants of total surface area. The exact influence of size, number and surface area on final retention is incompletely known, but may experimentally be determined by means of *in vivo* experiments with artificial particles such as polystyrene particles. They can be produced possessing all kinds of specific chemical and physical characteristics. This approach may result in a complete understanding of dosing, deposition and translocation leading to a computer model on particle kinetics, which can be applied to predict retention for every kind of environmental particle.

To determine whether a certain particle really poses a threat to human health, probably the most complex problem is determination of the biological response. This can be performed by a combination of *in vitro* and *in vivo* studies. For *in vitro* tests a critical endpoint parameter has to be defined which has been shown relevant for the *in vivo* situation. And therefore more knowledge is necessary on biological mechanisms of particle interaction with the different cell types in the lung. For example, do cytokines produced by epithelium actually contribute to the inflammatory response in the lung, or is break-down of its barrier function more crucial, preventing antigen to enter and causing sensory nerve ends to be exposed. Influence of particles on barrier function, but also the role of sensory nerves producing neuropeptides deserve more attention. In a comparable way different functions of the macrophage can be weighed. In addition to the production of regulatory substances, more attention should be given to the mechanism by which macrophages phagocytize particles. Implications of the fact that different mechanisms are used for different particle types (Kobzik 1995) are unknown. Tests with artificial particles may indicate which particle characteristic is crucial in influencing the most important cell functions. Simultaneously, *in vivo* tests are to be performed, both to determine the relevance of the *in vitro* findings, and to give overall mechanistical information. Cytokine

intervention studies, macrophage depletion studies, and different knockout mice can give highly necessary information.

Joined forces on elucidating pre-exposure interactions and dose measurement, supplemented with computer modeling for deposition, and knowledge on crucial particle characteristics for particle-cell-interactions, may ultimately lead to a situation in which determining the main characteristics of an unknown particle can be used for hazard identification.

**Concluding remarks**

The findings described in this thesis give further evidence for the hypothesis that the increased presence of DEP and similar airborne pollution may cause those with the appropriate genetic predisposition to become sensitized to allergens to which they may not otherwise have become sensitized. Coexposure to allergen, particulate air pollution and complexes of particles with (possibly altered) allergen contributes to this increased risk of sensitization. The intranasal model described in this thesis may substantially contribute to determination of sensitizing and adjuvating potential of unknown particulate pollutants, and the further elucidation of mechanisms of adjuvant activity. Moreover, in combination with chemical analysis of particles and epidemiological data, it may play an important role in hazard identification, dose-response determination, and ultimately risk assessment of PM.

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M. van Zijverden. The adjuvant activity of particulate pollutants: a modified popliteal lymph node assay and an intranasal exposure model. Oral presentation at satellite meeting on "Indoor environmental factors enhancing allergic immune responses", EUROTOX 99, Oslo, Norway, June 26, 1999.

M. van Zijverden. The adjuvant activity of particulate pollutants in an intranasal model. Oral presentation at Annual Meeting of the Society of Toxicology, Philadelphia, USA, March, 19-23, 2000.

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# Samenvatting

Allergische aandoeningen van de luchtwegen zoals hooikoorts komen steeds vaker voor. Bij allergie wordt een immuunreactie (= een reactie van de afweer) die normaliter tegen ziekteverwekkende organismen optreedt, ingezet tegen een "onschuldig" eiwit: het allergeen. Bekende allergenen zijn pollen van gras en bomen, huidschilfers van kat en andere huisdieren en de uitwerpselen van huisstofmijt. De toename van allergie beperkt zich tot westerse landen en landen met een vergelijkbare levensstijl. Dit geeft aanleiding tot veel epidemiologisch onderzoek naar aan levensstijl gerelateerde factoren die de afgelopen tientallen jaren veranderd zijn en mogelijk een cruciale rol spelen in het ontstaan van allergie, alsook. Dit is erg lastig onderzoek, aangezien de mens vanaf de conceptie al blootstaat aan ontelbare omgevingsfactoren. Zo levert onderzoek in voormalig Oost Duitsland zowel voor als na de hereniging veel informatie over allergie in een samenleving die "verwestert" . Ook onderzoek in gemeenschappen die qua levensstijl op een aantal punten duidelijk verschillen van de omringende westerse samenleving (bv. antroposofische) levert veel kandidaatfactoren. Deze kunnen grofweg worden ingedeeld in de categorieën infecties, dieet en luchtvervuiling. De eerste categorie is gebaseerd op het idee dat een verminderde blootstelling aan infecties het ontstaan van allergie zou bevorderen. Epidemiologisch onderzoek toont aan dat oudste kinderen, in het bijzonder van kleine gezinnen, vaker allergisch zijn. Dit geldt ook voor kinderen die niet naar de crèche gaan of door een andere reden weinig in contact komen met leeftijdgenoten. Jongere gezinsleden en crèchegangers daarentegen staan vanaf zeer jonge leeftijd vaker bloot aan onschuldige kinderziektes, wat hen zou beschermen tegen allergie. Ook wordt er met een beschuldigende vinger gewezen naar vaccineren tegen kinderziektes, maar op dit vlak bestaan veel tegenstrijdige onderzoeksresultaten. Een recent Italiaans onderzoek toont aan dat het doormaken van onschuldige infecties gerelateerd aan voedsel en hand-mond gedrag gecorreleerd is aan een kleinere kans op allergie. Dit geldt daarentegen niet voor ernstige infecties waartegen ingeënt wordt, zoals mazelen en de bof.

De tweede categorie factoren betreft ons eten. Onder andere de vetsamenstelling van ons "westerse" dieet is de laatste decennia erg veranderd, wat mogelijk onze afweer-reacties (waaronder allergie) beïnvloedt. Ook de samenstelling van onze darmflora verandert mee. Zo hebben kinderen uit Estland een andere darmflora dan Zweedse kinderen, die in vergelijking vaker allergisch zijn. Naast infectie-patroon en dieet vormt luchtvervuiling de derde categorie. De "klassieke" luchtvervuiling in het voormalige Oostblok maar ook bijvoorbeeld in veel ontwikkelingslanden bestaat uit roet en zwaveldioxide. Dit in tegenstelling tot de vervuiling in westerse landen waarin gassen als

stikstofdioxide en zeer fijne deeltjes (met een grootte van enkele micrometers tot veel kleiner) voorkomen. Fijne deeltjes in de lucht zijn afkomstig van verschillende bronnen, zowel natuurlijke (vulkanen, aardkorst, zeezout) als antropogene (industrie, verkeer). Zeer veel onderzoek wordt bijvoorbeeld gedaan naar de extreem fijne deeltjes die door dieselmotoren worden uitgestoten, en die vooral in westerse steden een belangrijk deel vormen van de luchtvervuiling. Dit blijft een actueel probleem aangezien de nieuwere dieselmotoren wel minder gewicht aan deeltjes uitstoten, maar dat deze reductie alleen de grotere deeltjes betreft, terwijl de kleinste deeltjes de lucht in blijven vliegen.

Deze fijne deeltjes en hun rol in het ontstaan van luchtwegallergie staan centraal in het onderzoek dat beschreven is in dit proefschrift. Terwijl van deeltjes al wel bekend is dat ze de symptomen van reeds bestaande allergie verergeren, staat nog niet vast dat deeltjes ook allergie veroorzaken in mensen die voorheen nog niet allergisch waren. Onze hypothese was dat in niet allergische mensen blootstelling aan deeltjes kan leiden tot het ontstaan van allergie tegen een willekeurig allergeen uit hun omgeving. Daarom was het doel van dit onderzoek een (dier- of cellijn-) model te ontwikkelen waarin de modulerende en/of stimulerende (=adjuvant) invloed van deeltjes op het ontstaan van allergie gemeten kan worden. Bovendien zou in dit model het achterliggende mechanisme onderzocht kunnen worden. In het hier beschreven onderzoek worden verschillende soorten dieseldeeltjes (DEP) vergeleken met koolstofdeeltjes (CBP) en silica deeltjes (SIP). Silica is een belangrijke component van de aardkorst en dus ook van opwaaiend stof en mijnstof. Dieseldeeltjes bestaan uit een koolstofkern waaraan allerhande chemicaliën geadsorbeerd zijn die tijdens de verbranding vrijkomen. Koolstofdeeltjes hebben eenzelfde soort kern maar bevatten geen aanhangende chemicaliën.

In het eerste experimentele hoofdstuk (**Chapter 2**) is getest of de verschillende modeldeeltjes DEP, CBP en SIP de immuunreactie tegen een willekeurig allergeen kunnen beïnvloeden (de allergische respons is altijd gericht tegen een allergeen en niet tegen de deeltjes). Hiertoe zijn de deeltjes samen met een allergeen ingespoten in de poot van muizen. Het inspuiten van het allergeen zonder de deeltjes gaf geen meetbare immuunrespons, evenmin als de deeltjes op zichzelf. Wanneer ze echter samen werden toegediend was er wel een duidelijke immuunrespons te meten: de deeltjes fungeerden als adjuvant. De eigenschappen van die immuunrespons bleken enigszins afhankelijk van welk type deeltje er werd meegespoten. Behalve stimulatie was er dus ook sprake van differentiële modulatie.

In **Chapter 3** hebben we bekeken of de deeltjes het functioneren van de macrofaag, de belangrijkste “opruim” cel in onze longen, *in vitro* (= in de reageerbuis, tegengesteld aan *in vivo* = in een levend organisme) beïnvloeden. Het bleek dat macrofagen werden

beschadigd door de deeltjes en hun capaciteit om deeltjes op te nemen (en zo op te ruimen) verminderde. Bovendien produceerden ze tijdens blootstelling aan de deeltjes stoffen die een ontsteking bevorderen. De modulatie van deze essentiële macrofaagfuncties speelt mogelijk een rol in de ontwikkeling van allergie.

De vorige twee hoofdstukken gaven aanleiding tot vermoedens over een mogelijke rol als adjuvant voor deeltjes in luchtwegallergie, maar uit de artificiële toediening (injectie) in Chapter 2 en de beperkte informatie uit *in vitro* metingen aan één enkel celtype (Chapter 3) konden geen harde conclusies getrokken worden. Daarvoor was een *in vivo* model nodig, waarin de toediening plaatsvindt via de luchtwegen, de manier waarop mensen ook in werkelijkheid blootgesteld worden. In **Chapter 4** worden de resultaten beschreven van intranasale blootstelling (via de neus) van muizen aan allergeen en deeltjes. De deeltjes werden alleen de eerste paar dagen van de allergeenblootstelling meegegeven, om na te gaan of het ontstaan van (nieuwe gevallen van) allergie wordt beïnvloed. Ook voor dit model gold dat allergeen en deeltjes afzonderlijk geen meetbare respons opwekten, maar dat er na gecombineerde toediening wel een meetbare immuunrespons optrad. Bovendien bleek de adjuvant werking na 3 weken nog steeds aanwezig en dus persistent van karakter te zijn. De deeltjes bleken dus ook na toediening via de relevante route (de luchtwegen) de immuunrespons te adjuveren (stimuleren). In **Chapter 5** dienden we de deeltjes behalve tijdens de eerste dagen ook later tijdens de reactie nog eens toe, en het bleek dat de adjuvant werking daar nog sterker door werd.

In **Chapter 6** wilden we testen of het essentieel was dat de deeltjes tegelijk met het allergeen werden gegeven, zoals we tot nu toe gedaan hadden, of dat de deeltjes ook wel wat eerder of later dan het allergeen toegediend konden worden om hun stimulerende werk te verrichten. Bovendien varieerden we het aantal deeltjesdoseringen, terwijl de uiteindelijk toegediende totale hoeveelheid deeltjes gelijk bleef. Het bleek dat het toedienen van kleine doses tegelijk met iedere allergeentoedoening het meest effectief was in het stimuleren van de immuunrespons. Vergeleken met deze kleine doseringen werkte één grote dosis iets minder goed. Als deze dosis reeds één dag voor het begin van de allergeentoedieningen werd gegeven, was de immuunstimulatie nog iets minder effectief. Een deeltjesdosering één dag na de allergeentoedieningen werkte helemaal niet.

In **Chapter 7** hebben we onderzocht of de deeltjes ook als adjuvant werken als het allergeen via een aerosol (= een wolk van allergeen) wordt toegediend. Aerosolinhalatie benadert de blootstelling in het dagelijks leven meer dan intranasale toediening. Het bleek dat de deeltjes ook in dit model als adjuvant werkten. Er kleefden echter een aantal bezwaren aan dit model, o.a. betreffende de lange duur van een experiment, dat het lastiger maakt om mee te werken dan het simpele en effectieve intranasale model.

Samenvattend kunnen we concluderen dat de verschillende typen deeltjes allemaal de immuunrespons in meerdere of mindere mate stimuleren. Er kan echter nog geen definitieve uitspraak gedaan worden over het mechanisme achter de adjuvant activiteit, omdat er zeer veel variabelen zijn. De deeltjes hebben zeer complexe chemische en fysische eigenschappen en bovendien spelen in de immuunrespons veel verschillende celtypen een rol.

In de algemene discussie (**Chapter 8**) wordt een hypothese geformuleerd over het ontstaan van een "veranderd micromilieu in de long" als oorzaak van allergie inductie. In het kort komt het erop neer dat deeltjes de long binnen komen na buiten het lichaam al dan niet al chemicaliën en allergeen gebonden te hebben. Hoeveel deeltjes er precies binnenkomen, en hoe diep ze de luchtwegen binnendringen, is onder andere afhankelijk van de grootte. Een fraktie van de deeltjes wordt, al dan niet met hulp van macrofagen, uit de longen verwijderd door middel van transport met het longslijm, dat met deeltjes en al wordt afgevoerd en doorgeslikt. De kleine deeltjes dringen door de eerste cellaag van de long heen, veroorzaken ontsteking en kunnen tientallen jaren in de long aanwezig blijven. Behalve de eerste cellaag van de long raken ook de macrofagen beschadigd door de aanwezigheid van de deeltjes en gaan ook de macrofagen stoffen produceren die ontsteking bevorderen. Door de inertheid van de deeltjes is de macrofaag niet erg effectief in het opnemen en opruimen van de deeltjes. In combinatie met de beschadiging van de eerste cellaag zorgt dit ervoor dat zowel deeltjes als ook allergeen makkelijker en in grotere hoeveelheden door de beschermende barrière van de long heendringen. Het allergeen komt op deze manier beter in contact met immuuncellen die makkelijk te aktiveren zijn in personen met de juiste genetische aanleg. Gesteund door de ontsteking en beschadiging van het micromilieu van de long wordt er eerder en heviger gereageerd op het allergeen. Het ontstaan van een nieuwe allergie is een feit.

Dit onderzoek ondersteunt de hypothese dat blootstelling aan DEP en vergelijkbare deeltjesvormige luchtvervuiling in personen met de juiste genetische achtergrond het ontstaan van allergie voor een willekeurig allergeen kan veroorzaken. Het intranasale model zoals beschreven in dit proefschrift zou kunnen bijdragen aan het ontrafelen van het achterliggende mechanisme. Bovendien zou het model in combinatie met chemische analyses van de deeltjes een belangrijke rol kunnen spelen bij de identificatie van de adjuverende potentie van onbekende deeltjesvormige luchtverontreiniging. Het vaststellen van een dosis-repons relatie en combinatie met gegevens uit de epidemiologie zou van betekenis kunnen zijn voor het inschatten van gezondheidsrisico's van deeltjesvormige vervuiling voor de mens.

# Curriculum Vitae

Maaike van Zijverden werd geboren op 16 januari 1973 te Reeuwijk. In 1991 behaalde zij het diploma Gymnasium β aan het Coornhert Gymnasium te Gouda, en in datzelfde jaar begon zij met de studie Biologie aan de Universiteit Utrecht. Tijdens de hoofdstage werd onderzoek verricht naar voedsel-allergie. De stage vond plaats bij TNO Voeding (immunotoxicologie) bij dr. E. C. de Jong onder supervisie van prof. Dr. W. Seinen (RITOX). Aansluitend op het behalen van het doctoraal diploma begon zij in september 1996 als assistent in opleiding aan het onderzoek beschreven in dit proefschrift. Het projekt werd gefinancierd door het UTOX, een samenwerkingsverband tussen het Research Instituut voor Toxicologie (RITOX), het Rijksinstituut voor Volksgezondheid en Milieu (RIVM), en TNO Voeding. Studies vonden plaats op het RITOX en het RIVM, begeleiding kwam van dr. R. Pieters en prof. dr. W. Seinen (RITOX), dr. H. van Loveren en prof. dr. J. G. Vos (RIVM), en dr. A. H. Penninks (TNO). In dezelfde tijd verdiepte zij zich door middel van cursussen en congressen in de toxicologie en immunologie, en schreef zij een rapport voor de Wetenschapswinkel Biologie en de Consumentenbond over allergie.

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