Molecular mechanisms in allergy and clinical immunology

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Biology of diesel exhaust effects on respiratory function

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In recent decades, clinicians and scientists have witnessed a significant increase in the prevalence of allergic rhinitis and asthma. The factors underlying this phenomenon are clearly complex; however, this rapid increase in the burden of atopic disease has undeniably occurred in parallel with rapid industrialization and urbanization in many parts of the world. Consequently, more people are exposed to air pollutants than at any point in human history. Worldwide, increases in allergic respiratory disease have mainly been observed in urban communities. Epidemiologic and clinical investigations have suggested a strong link between particulate air pollution and detrimental health effects, including cardiopulmonary morbidity and mortality. The purpose of this review is to provide an evidence-based summary of the health effects of air pollutants on asthma, focusing on diesel exhaust particles (DEPs) as a model particulate air pollutant. An overview of observational and experimental studies linking DEPs and asthma will be provided, followed by consideration of the mechanisms underlying DEP-induced inflammation and a brief discussion of future research and clinical directions. (J Allergy Clin Immunol 2005;115:221-8.)

Key words: *Air pollution, diesel exhaust particles, allergy, asthma, oxidative stress, respiratory effects*

Abbreviations used

AHR: Airway hyperreactivity
BEC: Bronchial epithelial cell
DEP: Diesel exhaust particle
GST: Glutathione-S-transferase
ICAM-1: Intercellular adhesion molecule 1
KLH: Keyhole limpet hemocyanin
PM: Particulate matter
PM_{10}: Particulate matter with diameter ≤ 10 μm
ROS: Reactive oxygen species

In 1873, when Charles Harrison Blackley reported that ''hay-fever'' or ''hay-asthma'' was actually caused by grass pollen, he also observed that it was more common in urban than in rural settings, an observation since confirmed in numerous studies.[1-4] Although the hygiene hypothesis might explain this in part, it is clear that it does not hold in all instances, and other factors are at work. In the last decade, evidence has accumulated from various disciplines that have implicated particulate air pollution in general and diesel exhaust in particular in the initiation and exacerbation of allergic airway disease. As we will review, epidemiologic studies have shown clear associations; human exposure and challenge studies have provided biologic plausibility, whereas animal and cellular studies have delineated putative molecular mechanisms.

Motor vehicle emissions are a major source of airborne pollutants. The combustion of fossil fuels produces a number of unhealthy substances, including carbon monoxide, nitrogen oxides, benzene, sulfur dioxides, and particulate matter (PM). The largest single source of airborne PM from vehicles is derived from diesel exhaust. Advances in technology have resulted in diesel engines that produce less nitrogen oxide and particulate mass (but higher particle numbers). However, diesel engines continue to emit higher emissions of gaseous substances and up to 100 times more particles compared with gasoline engines equipped with modern exhaust treatment systems. In addition, with the high cost of fuel in many countries, use of diesel engines has increased because of their superior energy efficiency and endurance. For example, in 1997-1998, diesel engines powered 75% of trucks, 98% of buses, and 12% of passenger cars in Japan, with 18% of all motor vehicles being diesel powered.[5] These diesel

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vehicles produced 75% of the nitrogen oxide emissions in that nation and nearly 100% of vehicular PM emissions.

Diesel fuel combustion results in the production of diesel exhaust particles (DEPs), as well as gaseous compounds, including nitrogen oxides and precursors of ozone. DEPs consist of an elemental carbon core with a large surface area to which hundreds of chemicals and transition metals are attached. Most mechanistic studies have attributed the proinflammatory and adjuvant effects of DEPs to these chemical constituents.[6] The majority of DEPs are classified as fine (2.5-0.1 μm) or ultrafine (<0.1 μm) particles, but these primary DEPs can coalesce to form aggregates of varying sizes. It has been postulated that because smaller particles have a greater relative surface area, they should carry proportionally more chemicals and have greater biologic effects.[7,8] If proved, this would necessitate a revision of current regulatory standards that currently do not include ultrafine particles.

OBSERVATIONAL STUDIES ON HEALTH EFFECTS OF DIESEL EXHAUST

Numerous studies have linked air pollution to human morbidity and mortality.[9-13] Increased ambient PM has been linked to observed increases in adverse cardiovascular and respiratory health events. In addition to the adverse respiratory effects, which are the focus of this review, the cardiovascular effects of air pollution are particularly alarming because of the associated high mortality. Increased rates of myocardial infarction and hospitalization for cardiac events have been associated with higher air pollution levels.[14-16] Numerous studies have shown associations between increased symptoms of cough, bronchitis, asthma, and chronic obstructive pulmonary disease and increased air pollutant levels.[17-19] Studies on diesel effects per se have been few. One group studied the respiratory mucus membranes of Swiss custom officers solely occupied with clearing of diesel trucks for 40 hours a week over 5 years and showed that they experienced goblet cell hyperplasia compared with officers employed in offices.[20] Another study showed increased IgE levels among nonatopic dock workers regularly exposed to diesel emission from forklifts or trucks in the ship holds.[21] The paucity of these studies is because accurate measurements of human *in vivo* ambient diesel exposures have been hampered by the lack of suitable biomarkers. Therefore levels of PM or exposure to road traffic have often been used as proxies for exposure.[22] Several studies demonstrate a consistent effect of long-term exposure to car traffic on nonspecific respiratory symptoms and lung function.[23,24] Particulate air pollution specifically has been shown to triple chronic cough and nocturnal cough in Swiss children living in communities with the highest PM_{10} levels compared with those children living in the least exposed areas.[25] Similarly, a large study of 6 US studies demonstrated a 3-fold increase in chronic cough with increased exposure to particulate pollution.[26] Many studies have shown that proximity to traffic is a risk factor for wheezing, asthma severity, outcomes, and prevalence.[27-33] For example, physician-diagnosed asthma has been reported to be more frequent in children living within 100 m of a freeway,[34] and preschool children are more likely to be hospitalized for asthma if they live in areas with high vehicular traffic.[35] Comparable results between atopy and road traffic have been observed.[36-39] For example, higher rates of allergic sensitization are found in children playing more than 1 hour a day near major traffic thoroughfares.[30]

EXPERIMENTAL STUDIES ON ALLERGY AND ASTHMA HEALTH EFFECTS OF DIESEL PARTICLES

The specific effects of diesel exhaust and its particles on allergic respiratory disease have been explored in a number of animal, *in vitro*, and human clinical studies. The most consistent finding in these investigations is the profound adjuvant effects of DEPs on the development and intensity of allergic inflammation. Animal studies have demonstrated an increase in total and antigen-specific IgE levels, as well as increases in IL-4, IL-5, and GM-CSF levels in response to DEP exposure.[40-43] In addition, DEPs reproducibly induce increased airway eosinophilic inflammation, goblet cell hyperplasia, and airway hyperreactivity (AHR) in murine models of asthma.[44-46] Rats kept in a polluted environment demonstrate increased airway hyperresponsiveness to methacholine, which improves when the rats are moved to an area with nonpolluted ambient air.[47] Intranasal or intratracheal sensitization with grass pollen and DEPs in rats induces inflammatory reactions in the lung, with infiltration of macrophages and eosinophils.[48] Guinea pigs exposed to diesel exhaust at levels encountered in polluted areas experience increased sneezing with repeated nasal allergen administration, increased nasal secretions, and increased numbers of nasal epithelial and subepithelial eosinophils.[49] Additional studies with guinea pigs show that a 4-week exposure to diesel exhaust results in enhanced nasal hyperresponsiveness and augmented sneeze responses to histamine.[50]

In vitro studies with DEPs show that they can target multiple cell types (Table I) and can potentially act at several important steps of the allergic cascade. Human B cells cultured with IL-4 and CD40 mAbs in the presence of DEP-derived chemicals demonstrate up to a 360% increase in IgE production.[51] DEPs also enhance histamine-induced IL-8 and GM-CSF levels from human airway epithelial cells *in vitro*.[52] Extracts from DEPs effectively enhance human eosinophil adhesion to nasal epithelial cells and induce eosinophil degranulation.[53] In addition, PBMCs from allergic subjects cocultured with DEPs and allergen show synergistic increases in IL-8, RANTES, and TNF-α production.[54] These cytokines are overexpressed in asthmatic bronchoalveolar lavage fluids and are believed to enhance airway inflammatory responses.[55,56] *In vitro* studies also suggest that bronchial epithelial cells (BEC) are profoundly affected by DEP exposure,

TABLE I. Direct effects of DEPs and their extracts on multiple cell types

A. Bronchial and nasal epithelial and endothelial cells:
Increase expression of chemokines and cytokines (IL-8, eotaxin, RANTES, GM-CSF, and IL-6)
Increase expression of histamine 1 receptor
Upregulate expression of adhesion molecules (ICAM-1)
Increase phase 2 enzyme expression
B. Eosinophils
Enhance adhesion to nasal epithelial cells
Induce eosinophil degranulation
C. Mast cells
Enhance IgE-mediated histamine release
Enhance cytokine production (IL-4, IL-6)
D. Basophils
Induce histamine release in the absence of IgE
Enhance cytokine production (IL-4)
E. PBMCs
Induce chemokine production (IL-8, RANTES)
Synergize with allergen to increases in IL-8, RANTES, and TNF-α production
F. B cells
Enhance IgE production after IL-4 and anti-CD40 stimulation
G. Monocytes-macrophages
Modulate cytokine production (eg, inhibits IL-12p40 production)
Inhibit prostaglandin E_2 release
Increase phase 2 enzyme expression

TABLE II. Clinical effects of diesel exhaust in human controlled exposure studies

A. Diesel exhaust effects on healthy subjects
Increased number of inflammatory cells (neutrophils, B cells, T cells, mast cells) in the airways
Increased circulating neutrophils and platelets
Increased histamine levels
Increased cytokines (IL-6) and CXC chemokines (IL-8 and GrO-α)
Increased expression of adhesion molecules ICAM-1 and VCAM-1
Decreased macrophage function
Increased airway resistance
B. Diesel exhaust effects on subjects with mild asthma
Increased hyperresponsiveness to methacholine
Increased airway resistance
Increased sputum IL-6 levels
No apparent airway inflammation
Increased epithelial staining for IL-10

VCAM-1, Vascular cell adhesion molecule 1.

particularly in asthmatic individuals. Cultured BECs from asthmatic patients constitutively release greater amounts of IL-8, GM-CSF, RANTES, and soluble intercellular adhesion molecule 1 (ICAM-1) compared with levels in nonasthmatic individuals. Exposure to low DEP concentrations (10 μg/mL) significantly increases release of these cytokines from BECs of asthmatic subjects, whereas BECs of nonasthmatic subjects require higher concentrations of DEPs (50-100 μg/ml) to cause significant increases in IL-8 and GM-CSF production.[57] *In vivo* studies support a proinflammatory role for DEPs. Increases in lymphocytes, monocytes-macrophages, and neutrophils are observed after nasal challenge of subjects with DEPs, and this is accompanied by an increase in levels of the CC chemokines RANTES, macrophage inflammatory protein 1α (MIP-1α), and monocyte chemoattractant protein 3 (MCP-3).[58]

Given these effects on proinflammatory mediators and chemokines, it is unsurprising that short-term exposure of healthy human subjects to diesel exhaust at high concentrations induces airway inflammatory responses. In these controlled chamber exposure experiments, diesel exhaust increases circulating neutrophils and platelets, sputum neutrophil counts, and bronchial tissue mast cell, neutrophil, and lymphocyte counts. In addition, IL-6 and IL-8 expression is increased, as is expression of the adhesion molecules ICAM-1 and vascular cell adhesion molecule 1.[59-61] Such studies suggest that DEPs might contribute significantly to the pulmonary inflammation associated with allergic asthma. Similar approaches have demonstrated that diesel exhaust exposure can increase airway hyperresponsiveness to methacholine and airway resistance in patients with mild asthma.[62-64] However, at least one recent study has suggested that healthy individuals experienced airway inflammation 6 hours after diesel exhaust exposure, whereas asthmatic subjects did not show similar inflammation with the same exposure.[61] This occurred despite the fact that both healthy and asthmatic individuals showed similar increases in airway resistance after diesel exhaust exposure.

A sizeable body of *in vivo* human experimental data supports the detrimental effects of DEP exposure on clinical allergy. In subjects challenged nasally with 0.3 mg of DEPs (equivalent to 40 hours of ambient exposure in Los Angeles) increased IgE isotype switching *in vivo* results in increased total IgE levels.[65-67] In addition, DEPs can interact with allergen to augment allergen-induced responses, so that allergen-specific IgE levels are up to 50-fold greater in allergic subjects challenged with DEPs plus allergen than in those receiving allergen alone. In addition, diesel particles have been shown to broadly stimulate cytokines when administered alone, but when administered with allergen, they induce a T_H2 cytokine profile and a concomitant decrease in IFN-γ levels in the nasal environment. DEPs also appear to affect immediate responses through direct effects on mast cell and basophils.[68,69] Increased histamine levels are seen in the bronchoalveolar lavage fluid of healthy individuals exposed to diesel exhaust.[59] Moreover, nasal histamine levels after challenge of dust mite–sensitive subjects with dust mite are increased 3-fold when DEPs are coadministered with the allergen.[70] This boost in histamine release manifests itself also in significant increases in symptom scores and a reduction of the allergen threshold necessary to produce symptoms. Thus if administered with DEPs, only 20% of the amount of intranasal dust mite allergen normally required will result in a symptomatic response. In the real world this effect is probably compounded because DEPs can also increase histamine 1 receptor mRNA

TABLE III. Clinical effects of DEPs in nasal provocation studies

A. Immediate-phase response (minutes)
Increases allergen-induced histamine release and symptoms
B. Short-term response (hours)
Increases release-production of C-C chemokines
Increases cellular inflammation
Induces a potent T_H2 cytokine milieu in the presence of allergen (eg, increased IL-4 and decreased IFN-γ levels)
C. Intermediate-term response (days)
Enhances total and allergen-specific IgE responses to allergen
Increases number of IgE-secreting cells in nasal mucosa
D. Long-term response (days)
Enhances primary allergic sensitization

expression in human airway epithelial and endothelial cells.[52] Subsequent studies have focused on the role of DEPs in increasing the rate of primary allergic sensitization.[71] Repeated nasal immunization with the classic immunogenic neoantigen keyhole limpet hemocyanin (KLH) led to local production of anti-KLH mucosal IgG and IgA but not IgE. In contrast, if subjects were pre-exposed to DEPs before each KLH exposure, 60% produced mucosal anti-KLH IgE. These subjects also had increased IL-4 levels in nasal lavage fluid. Rechallenge of subjects with KLH months later resulted in rhinitic symptoms and production of KLH IgE. Theoretically, the capacity of DEPs to increase rates of allergic sensitization and to worsen symptoms, thereby turning what normally would be a mild response into one that is clinically relevant, might account for the higher allergic prevalence associated with road traffic.

A summary of the clinical effects of diesel in human subjects is shown in Tables II and III.

An important caveat to all experimental studies is that the composition of diesel exhaust varies greatly depending on the engine load conditions and other factors. Differences in generation of particles or exhaust might well explain some discrepancies between studies. For example, cultured normal BECs treated with extracts from diesel particles generated under different conditions exhibit differences in IL-8 production and prostaglandin E_2 release.[72] A closer collaboration between our own discipline and that of toxicologists, chemists, and engineers should be encouraged so that we can model as accurately as possible the conditions used in experimental setups to what we actually breathe in the real world.

MECHANISMS OF DEP EFFECTS

The underlying mechanisms by which DEPs exert biologic effects is an area of ongoing investigation. Most of the focus has been in the role of reactive oxygen species (ROS) generated on exposure to diesel exhaust or particles and the subsequent generation of oxidative stress within exposed cells. ROS, such as superoxide, hydrogen peroxide, and hydroxyl radical, are reactive with proteins, lipids, and DNA, leading to cellular damage. It is important to realize that inflammation per se is an oxidative event. ROS are produced by macrophages, neutrophils, and eosinophils in the airway. However, ROS might also play an important role in originating pulmonary inflammation.[73] Superoxide generation has been shown at sites of allergen challenge in the human lung,[74] whereas in animal models oxygen radicals can contribute to antigen-induced AHR.[75,76] Oxidative stress is defined as a depletion of reduced glutathione in exchange for an increase in oxidized glutathione.[77] Noninvasive markers of oxidative stress, such as hydrogen peroxide, nitric oxide, carbon monoxide, and 8-isoprostane released in expired air correlate with the degree of airway inflammation in asthmatic subjects.[78-81] Oxidative damage to airway epithelium also produces AHR in human subjects.[82] The importance of oxidative stress in asthma is further supported by the fact that asthmatic subjects have altered levels of lung antioxidant enzymes.[83,84] Thus the hypothesis has arisen that pollutants, such as DEPs, can have a ''double whammy'' effect by increasing oxidative stress directly through the induction of ROS and indirectly through the resultant enhanced inflammation, which thus generates additional ROS and further inflammation.

In vitro, animal, and human studies demonstrate the importance of oxidative stress in mediating the inflammatory and adjuvant effects of DEPs. Tissue culture macrophages and BECs generate ROS with exposure to DEPs or DEP extracts.[85,86] Likewise, incubation of lung microsomes with organic DEP extracts generate Superoxide.[87] N-acetylcysteine, a thiol antioxidant, suppresses the oxidative stress effects of DEP exposure in macrophages but not in epithelial cells.[86] In murine studies intratracheal DEP administration causes increased nitric oxide production, along with cellular infiltration, increased mucus production, and increased AHR.[88,89] Aerosolized DEPs in mice have adjuvant effects on ovalbumin-induced allergic responses that are suppressed by thiol antioxidants.[90] Hydrogen peroxide has been visualized *in vivo* by means of chemiluminescence in the lungs and mediastinal fields of rats exposed to concentrated ambient particles.[91] To date, *in vivo* human studies have shown indirect evidence of ROS production with DEP exposure. Controlled DEP exposure with exposure chambers leads to airway inflammation, with increased sputum neutrophils and myeloperoxidase and increased carbon monoxide in exhaled air.[92] Lipid peroxidation markers have also been identified in the urine of human subjects exposed to DEPs.[93] As previously mentioned, current evidence suggests that the chemicals contained on DEPs, rather than the carbon core, induce ROS production. Intact DEPs induce ROS production, but extracts made from these particles also induce ROS production in macrophages, epithelial cells, and lung microsomes. Comparison studies of urban PM, DEPs, and carbon black particles on a human BEC line demonstrate that DEPs and, to a lesser extent, PM induce the release of GM-CSF and increase intracellular peroxide production. Carbon black particles (which contain essentially no chemicals) have no such effects.[94]

The cycle of ROS generation and inflammation can normally be curtailed by the cell's cytoprotective defenses. However, when these defenses are overwhelmed, an imbalance resulting in oxidative stress arises. On the basis of this paradigm, it is likely that individuals who are exposed to high oxidant insults or who are unable to generate suitable antioxidant defenses might be at increased risk of DEP-induced airway inflammation and a resulting increased risk of asthma. Current evidence suggests a 3-tiered oxidative stress model describing cellular responses to DEP exposure.[95] Phase 2 drug metabolizing and antioxidant enzymes, such as NAD(P)H:quinone oxidoreductase (NQO1), glutathione-S-transferase M1 (GSTM1), and heme oxygenase-1 (HO-1), are the first and most sensitive responses to oxidative stress. If these enzymes fail to neutralize the effects of ROS, mitogen-activated protein kinase and nuclear factor κB activation occurs, leading to expression of factors involved in airway inflammation, such as proinflammatory cytokines, chemokines, and adhesion molecules. Very high levels of oxidative stress result in cell apoptosis or necrosis. In support of this model, there is strong evidence that PM, DEPs, and their resident chemicals can induce mitogen-activated protein kinase and nuclear factor κB activation[96-99] and that this can result in gene transcription for IL-4, IL-5, IL-13, TNF-α, ICAM-1, and vascular cell adhesion molecule 1.[100] Furthermore, this activation is inhibited by pretreatment with an antioxidant.[101,102]

Given the heterogeneous nature of diesel exhaust and the hundreds of chemicals present on its particle, it is unlikely that other mechanisms are not also present. For example, in a murine model DEPs will strongly inhibit the LPS-induced IFN-γ response by interfering with cytokine signaling pathways that stimulate natural killer and natural killer T cells to produce IFN-γ. Unlike DEP-induced IgE production, this effect is not altered by pretreatment with antioxidants.[103] In addition to these molecular effects, DEPs might physically increase the allergen dose that reaches the lungs. DEPs have been shown to adsorb antigens onto their surface and thus might act as carriers of allergens, increasing their deposition in the respiratory tract.[104] The ultrafine particle fraction of diesel exhaust might also exert biologic effects independent of chemical composition through penetration of cellular components, such as mitochondria.[105]

FUTURE STUDIES AND DIRECTIONS

Continued research is necessary to further detail the mechanisms by which DEPs and other air pollutants cause adverse health effects. However, presently, there is widespread recognition that particulate air pollution has significant detrimental health effects. Such consensus affords the opportunity to intervene and potentially prevent pollutant-induced health complications. These opportunities currently include 3 major areas of focus. First, regulations governing the production of harmful air pollutants should reflect the known health risks of pollutant exposure. This is a matter of public policy and governmental legislation of which clinicians and scientists should be an integral part. Going forward, several areas need to be studied so that better policy can be implemented. It will be of great importance to define the adverse effects of the abundant ultrafine particles and to identify suitable biomarkers for diesel exposure and clinical markers for oxidative stress in human subjects. Such markers will be extremely useful in detecting susceptible individuals and in testing the effectiveness of therapeutic interventions.

A second area of considerable importance is identifying populations at increased risk from DEP exposure. Certainly, given the weight of human clinical studies implicating asthma as a risk factor, these individuals should be advised to monitor air quality reports, particularly in urban areas, and minimize outdoor activities during periods of especially poor air quality. However, recent investigations have started to recognize that susceptibility to the adverse risks from pollutants is an intrinsic trait most probably related to genotype. Glutathione-s-transferases (GSTs) are a family of phase 2 enzymes. In clinical studies individuals with genotypes resulting in an impaired ability to make 2 antioxidant members of this family (GSTM1 and GSTP1) were shown to have enhanced nasal allergic responses to DEPs.[106] When challenged with allergen plus DEPs, these individuals made significantly larger increases in IgE and histamine compared with patients with functional forms of the genes. It should be mentioned that these GST polymorphisms have previously been shown to be associated with asthma.[107-110] It is conceivable that the asthma risk identified by such polymorphisms primarily represents susceptibility to the harmful respiratory effects of particulate air pollution. Given that it is improbable that phase 2 enzyme genes are the only ones involved in determining susceptibility to pollutants, the search for other candidate genes will be an exciting new avenue of research.

The final area of focus is the development of therapeutic interventions. Our knowledge of putative mechanisms and susceptible populations would lead one to speculate that the best therapeutic strategy would be to enhance the antioxidant defenses of the human airway. To date, antioxidant therapy for human asthma has been disappointing, as evidenced by the lack of clinical effect seen with various antioxidant regimens.[111-113] Thiol antioxidants, such as N-acetylcysteine, which showed promise in animal studies, have not shown significant *in vivo* protective effects against DEP-induced airway oxidative stress in our human studies (personal observation). However, the discovery of individual genotypic susceptibility to pollutant-induced oxidative stress might allow for more focused investigation of such therapies in a selected population. A recent report has described the beneficial effects of dietary antioxidant supplementation among genetically susceptible children in a highly polluted environment, suggesting such strategies might be effective.[114] In addition, therapy directed at upregulating phase 2 enzyme gene expression might be useful in

offsetting the inflammatory effects of ROS and oxidative stress. We are currently performing early-phase human trials to investigate the effectiveness of select phase 2 enzyme-inducing compounds that successfully abrogate the adjuvant effects of DEPs in animal and *in vitro* studies. Whether such chemopreventative strategies will prove clinically useful remains to be seen. However, effectively reducing the inflammatory effects of DEPs might mark a significant step in reversing the rapidly increasing prevalence of asthma and allergy witnessed in past decades. The expanding knowledge of the health effects of DEPs might ultimately have wider implications for understanding the effect of other air pollutants and for unraveling the complex factors leading to asthma pathogenesis and exacerbation.

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