

Major grass pollen allergen Lol p 1 binds to diesel exhaust particles: implications for asthma and air pollution

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Summary

Background Grass pollen allergens are known to be present in the atmosphere in a range of particle sizes from whole pollen grains (approx. 20 to 55 μm in diameter) to smaller size fractions $< 2.5 \mu\text{m}$ (fine particles, $\text{PM}_{2.5}$). These latter particles are within the respirable range and include allergen-containing starch granules released from within the grains into the atmosphere when grass pollen ruptures in rainfall and are associated with epidemics of thunderstorm asthma during the grass pollen season. The question arises whether grass pollen allergens can interact with other sources of fine particles, particularly those present during episodes of air pollution.

Objective We propose the hypothesis that free grass pollen allergen molecules, derived from dead or burst grains and dispersed in microdroplets of water in aerosols, can bind to fine particles in polluted air.

Methods We used diesel exhaust carbon particles (DECP) derived from the exhaust of a stationary diesel engine, natural highly purified Lol p 1, immunogold labelling with specific monoclonal antibodies and a high voltage transmission electron-microscopic imaging technique.

Results DECP are visualized as small carbon spheres, each 30–60 nm in diameter, forming fractal aggregates about 1–2 μm in diameter. Here we test our hypothesis and show by *in vitro* experiments that the major grass pollen allergen, Lol p 1, binds to one defined class of fine particles, DECP.

Conclusion DECP are in the respirable size range, can bind to the major grass pollen allergen Lol p 1 under *in vitro* conditions and represent a possible mechanism by which allergens can become concentrated in polluted air and thus trigger attacks of asthma.

Keywords: grass pollen, allergen, Lol p 1, monoclonal antibody, immunogold labelling, diesel exhaust, fine particles, $\text{PM}_{2.5}$

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Introduction

Proteins and glycoproteins derived from pollen grains are the major source of allergens in the external environment in temperate climates and between 10 and 25% of the population show symptoms of hayfever and allergic asthma [1]. Grass pollen allergens occur in the atmosphere in pollen grains (20 to 55 μm in diameter) as well as being associated

with fine particles less than 10 μm in size [2]. These fine particles are also referred to as suspended particulate material [3], PM_{10} or $\text{PM}_{2.5}$ [4,5,6] and pauci-micronic particles [7,8] and are airborne fractions derived mainly from industry, vehicle exhaust emissions, rubber tyres and products of combustion of coal and wood. We have recently identified a natural source of grass pollen allergen-containing fine particles, the starch granules (0.5 to 2.5 μm in diameter) released from within pollen grains when they rupture during rainfall [9,10], and these fine particles are associated with epidemics of thunderstorm asthma [11,12].

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European studies have shown that fine particles associated with air pollution can interact with allergenic pollen, possibly modifying the release of allergens [13]. There is a difficulty in identifying the precise origin of such airborne fine particles, but there is support for the notion that allergens derived from pollen can be associated with fine particles in the atmosphere [7,8,14,15]. However, there is little or no real experimental evidence. We propose a reductionist approach, attempting to simplify complex environmental interactions to analyse experimentally whether highly purified grass pollen allergens can bind to a defined source of fine particles similar to those present in the air of polluted cities. Diesel exhaust carbon particles (DECP) from diesel exhaust emissions can readily be obtained from a stationary diesel engine [16]. These have been shown to have an enhancing effect on IgE antibody production in mice to a known antigen [3] and have been associated with serious health risks in humans in air-polluted cities, including increased morbidity [17] and asthma [18]. These effects are distinct from the well-characterized effects of pollutant gases, such as nitrogen and sulphur oxides, on exacerbating existing asthma [19–22].

If we assume that free grass pollen allergen molecules, derived from whole pollen, ruptured grains and starch granules [9,10], become dispersed in microdroplets of water in the atmospheric aerosol during periods of rainfall or high relative humidity, then the following hypothesis is one explanation of the fate of grass pollen allergen molecules in the environment. We propose that the grass pollen allergen molecules dispersed in the aerosol bind to atmospheric fine particles, DECP, a mechanism that could concentrate the molecules on respirable-sized particles. Here, we report results of *in vitro* experiments to test our hypothesis, in which two purified natural allergens, Lol p 1 and Lol p 5, have been loaded separately onto DECP collected under defined conditions from diesel exhaust emissions. Binding of the allergens has been detected by specific monoclonal antibodies and immunogold labelling followed by high resolution electron microscopic imaging.

Materials and methods

High resolution TEM imaging of DECP

DECP were collected on 1 μm teflon filters at 50°C, for well-characterized engine operating conditions [16]. Specimens were prepared for high voltage transmission electron microscopy by scratching DECP deposits from the teflon filter,

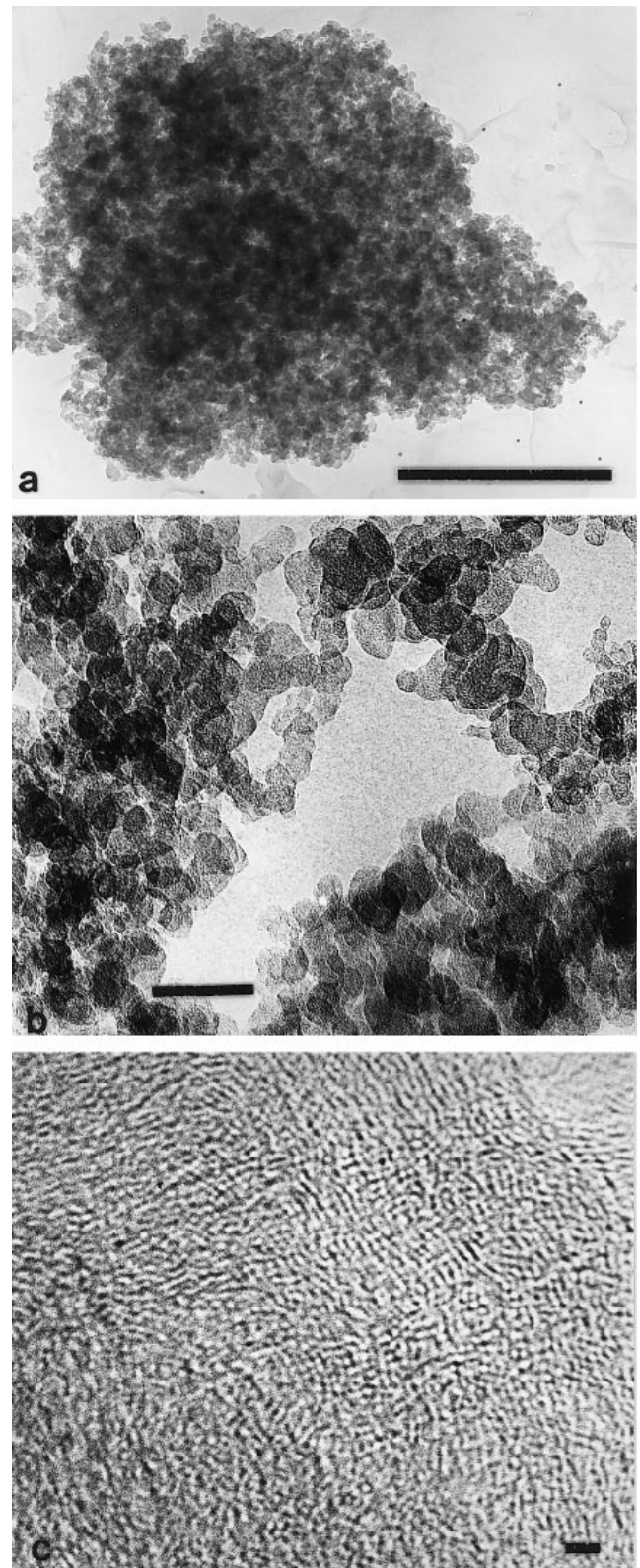
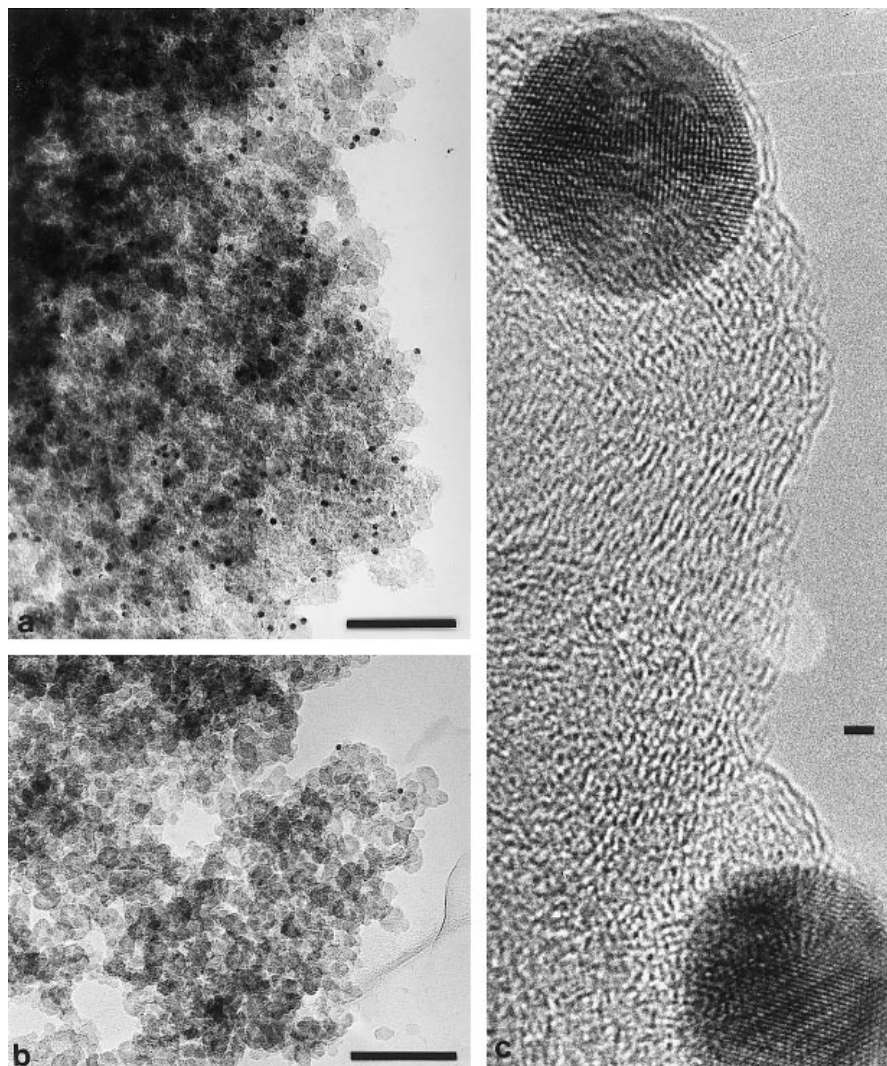


Fig. 1. Images of DECP. (a) HRTEM image of typical DECP fractal aggregate. Scale bar = 1 μm . (b) Individual particles have diameter of approx. 33–60 nm. Scale bar = 100 nm. (c) Detail of one DECP; disordered graphitic sheets are indicated by fringe spacings of approx. 0.345 nm. Scale bar = 2 nm.

Fig. 2. Interactions of DECP with natural rye-grass pollen allergens. (a) Aggregates of DECP after exposure to Lol p 1 and immunogold labelling of the allergen molecules. Note gold particles, imaged as 10 nm electron opaque discs decorating the DECP, which locate binding of allergen to carbon particles. Scale bar = 2 nm. (b) Detail of allergen binding sites on surface of DECP showing the fine structure of the 10 nm gold particles which label allergen/carbon binding sites for Lol p 1. Fringes indicate lattice planes of gold particles. Scale bar = 2 nm. (c) Aggregates of DECP after exposure to Lol p 5 and immunogold labelling. Note that in this case few binding sites are present under these experimental conditions. Scale bar = 200 nm.



mounting the dry powder onto 400-mesh copper grids. Images were recorded as described by Bursill *et al.* [23] using a JEOL-4000EX HRTEM operated at 400 keV.

Allergen purification, loading and immunogold labelling of DECP

Two of the most clinically significant allergens of rye-grass pollen were chosen for this study, namely Lol p 1 and Lol p 5. Lol p 1 is an abundant acidic glycoprotein of relative molecular mass $M_r = 35$ kDa, $pI = 5.5$ whereas Lol p 5 has $M_r = 28$ – 32 kDa and $pI = 9.0$. Lol p 1 and Lol p 5 were purified as previously reported [24, 25] and used at a concentration of approx. $20 \mu\text{g/ml}$ in PBS, pH 6.8. Briefly, total proteins were first separated according to protein charge by cation exchange chromatography (Mono S HR 5/5, Pharmacia) and the proteins eluted with a salt (1M NaCl) and pH gradients. The collected fractions were

analysed with dot-immunoblots using the allergen-specific monoclonal antibodies and human IgE as probes. The positive fractions were resolved by SDS-PAGE to check purity and the proteins subjected to *N*-terminal sequencing to confirm correct allergen groups. Following ion-exchange, fractions of interest were pooled, filter-concentrated and separated according to protein molecular mass on a gel filtration chromatography column (Superdex 75 HR 10/30, Pharmacia). Fractions were once again analysed to check purity and re-concentrated if needed.

DECP were carefully scraped from a teflon filter, dispersed in one drop of distilled water which was allowed to dry in air onto 400-mesh copper grids. Drops of allergen solution were placed in contact with the DECP-loaded grids and incubated for 30 min at room temperature. Excess (unbound) allergen was then washed away using PBS (5x for 5s each). Control grids were incubated in PBS, i.e. no allergen contact was made. All grids (including controls)

were then placed in specific MAbs FMC A1 (specific for Lol p 1) and FMC A7 (specific for Lol p 5) antibody supernatant solutions (PBS/BSA 1:1) for 1 h. Grids were washed and incubated for 1 h in goat anti-mouse IgG labelled with 10 nm colloidal gold, diluted 1:50 in PBS/BSA. Grids were then washed in PBS and double-distilled water before air drying prior to examination by HRTEM.

Results

DECP

Fig. 1a shows typical DECP, which comprise a more or less continuous fractal aggregate of individual carbon spheres, ranging in diameter from 30 to 60 nm (Fig. 1b). HRTEM images (Fig. 1c) reveal a disordered graphitic texture, resembling pyrolytic graphite in which the (0002) planes of graphite are indicated by a complex pattern of fringes having spacings of approx. 0.345 nm that is typical of carbon. The fine structures of DECP from different types of combustion appeared similar. Thick black deposits were produced under conditions of combustion in which the fuel was less completely burnt, while those from more completely burnt samples were less dense but showed no difference in structure or size of DECP.

Thus, we show that with high resolution imaging, DECP are solid carbon spheres, each approximately 60 nm in size, forming fractal aggregates of respirable size, typically between 1 and 2 μm in diameter.

Allergen-loaded DECP

Fig. 2a shows the results of the interaction between DECP and natural Lol p 1, as detected after washing by immuno-gold labelling. The 10 nm diameter gold particles (black circular contrast) decorate the DECP. HRTEM images (Fig. 2b) show details of the gold particles sitting on the graphitic DECP. The DECP have survived the labelling procedure intact without significant changes in structure (c.f. Fig. 1b).

There was virtually no labelling of Lol p 5, perhaps because of charge or other physicochemical differences and these images proved a positive control of antibody specificity of labelling. Control grids in which incubation in allergen was omitted, followed by MAb and gold treatments, all showed no labelling of DECP or on the carbon support films.

Thus, we show *in vitro* that the major rye-grass pollen allergen, the acidic glycoprotein Lol p 1 (Group 1 allergen) binds to DECP and is not removed by washing.

Discussion

Use of high resolution imaging techniques has revealed the

structure of DECP, while immuno-gold labelling techniques have shown that the major rye-grass pollen allergen Lol p 1 becomes loaded on DECP *in vitro*.

High resolution imaging of DECP shows that they comprise solid carbon spheres, 30 to 60 nm in diameter, forming fractal aggregates up to 1 or 2 μm in diameter, within the respirable size range [26]. These carbon spheres have previously been reported to be larger, 0.01 to 0.08 μm in diameter, forming solid particle agglomerates, 0.05 to 1.0 μm in diameter containing adsorbed hydrocarbons, liquid condensed hydrocarbon particles, and sulfates with hydration [27].

We show here that DECP can bind to the major grass pollen allergen Lol p 1 under *in vitro* conditions. While these results are qualitative, they indicate that allergens, such as Lol p 1, could be loaded on DECP in aerosols during or following periods of rainfall or high relative humidity associated with episodes of air pollution, when DECP will be concentrated in the air. A major epidemic of thunderstorm asthma occurred in London in July 1994 [12], with more than 1000 asthmatics reported to hospital emergency departments, many coming from more polluted areas [28]. Under these conditions, allergen-loaded starch granules provide a major source of $\text{PM}_{2.5}$. In polluted areas, $\text{PM}_{2.5}$ will be more concentrated, as well as higher levels of pollutant gases, such as oxides of nitrogen and sulfur, which are known to exacerbate asthma [20–22]. If allergen-loading of DECP occurs in the natural environment, as the experiments reported here suggest, then our results provide the mechanism for a causal link between grass pollen allergens, thunderstorms, air pollution and asthma. Further investigations of aerosols during the grass pollen season in polluted and less-polluted urban areas are now needed to confirm the identity of airborne DECP loaded with allergens.

Other types of fine particles than DECP are present in high concentrations in the urban atmosphere of cities, for example carbon particles from other kinds of combustion and rubber tyre dust. The latter particles naturally contain latex allergens [29], but carbon black (also a form of fine carbon particles, [30]) is added during manufacturing, and thus we predict may also become loaded with grass allergens in the same way as DECP.

The immunogold imaging methods reported here provide a useful means to identify allergen binding to fine particles collected on filters from air samplers in city atmospheres. The problem with the natural environment is to determine the precise identity of the diverse kinds of fine particles but high resolution imaging techniques offer one means of particle identification. Here, we have overcome this difficulty by using a defined source. Similar immunogold labelling techniques have been used to determine the sites of Fel d 1, the major cat allergen, in discrete components of house dust

samples [31]. Fel d 1 was associated with carbon particles assumed to originate from combustion in the home.

These experiments have been made possible by the availability of two purified major natural rye-grass pollen allergens, Lol p 1 and Lol p 5 as well as a source of DECP. Lol p 5 showed essentially no binding, providing a useful positive control of labelling specificity and its absence of binding is probably related to the loading mechanism. As an acidic glycoprotein, Lol p 1 may bind to the carbon particles because of charge. Lol p 1 would be negatively charged at the pH of 6.8 employed in our *in vitro* tests, while the DECP would be positively charged. One explanation why Group 5 allergen showed little or no binding is that as a basic protein it was positively charged at this pH. Alternatively, Lol p 1 may have bound to DECP because it is glycosylated, and the absence of glycan chains in Lol p 5 may explain why there was no binding. These possibilities can be tested in future experiments, by changing the pH of binding or by using deglycosylated allergen which in the case of Lol p 1 has been reported to retain its allergenic activity [32]. The possibility that hydrophobic interactions are involved needs also to be explored.

In the atmosphere, the allergen molecules will be dispersed in the aerosol, while binding to DECPs will have the effect of concentrating many allergen molecules on one particle. In our studies, the labelling was confined to the surface of the clusters of carbon spheres, probably an artifact of the technique used to detect allergen binding, as the allergen molecules are assumed to be able to interact within the carbon spheres. This possibility could be tested by sectioning of the spheres using resin as a matrix for immunogold labelling experiments.

Further investigations are needed to quantitatively detect Lol p 1 interactions with DECP *in vitro*. One or only a few DECPs may be sufficient to cause an allergic response at the cellular level in the airways. This is an important topic for future investigations. Skin prick tests and challenge tests are now needed to determine if Lol p 1-loaded DECP confer a bronchoconstrictive property in grass pollen-sensitive asthmatics.

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References

- 1 Wüthrich B, Schindler C, Leuenberger P, Ackermann-Lieblich U, and the SAPALDIA-Team Prevalence of atopy and pollinosis in the adult population of Switzerland (SAPALDIA study). *Int Arch Allergy Immunol* 1995; 106:149–56.
- 2 Spieksma FThM, Kramps JA, Van Der Linden AC et al. Evidence of grass-pollen allergenic activity in the smaller micronic atmospheric aerosol fraction. *Clin Exp Allergy* 1990; 20:273–80.
- 3 Takafuji S, Suzuki S, Koizumi K et al. Enhancing effect of suspended particulate matter on the IgE antibody production in mice. *Int Arch Allergy Appl Immunol* 1989; 90:1–7.
- 4 Pope CA III, Dockery DW, Spengler JD, Raizenne ME Respiratory health and PM10 pollution. A daily time series analysis. *Am Rev Respir Dis* 1991; 144:666–74.
- 5 Pope CA III, Dockery DW Acute health effects of PM10 pollution on symptomatic and asymptomatic children. *Am Rev Respir Dis* 1992; 145:1123–8.
- 6 Dockery DW, Pope CA III Acute respiratory effects of particulate air pollution. *Ann Rev Public Health* 1994; 15:107–32.
- 7 Spieksma F Th M, Nikkels BH, Dijkman Seasonal appearance of grass pollen allergen in natural, pauci-micronic aerosol of various size fractions. Relationship with airborne grass pollen concentration. *Clin Exp Allergy* 1995; 25:234–9.
- 8 Emberlin J. Plant allergens on pauci-micronic airborne particles. *Clin Exp Allergy* 1995; 25:202–5.
- 9 Suphioglu C, Singh MB, Taylor P et al. Mechanism of grass-pollen-induced asthma. *Lancet* 1992; 339:569–72.
- 10 Knox RB Grass pollen, thunderstorms and asthma. *Clin Exp Allergy* 1993; 23:354–9.
- 11 Bellomo R, Gigliotti P, Treloar A et al. Two consecutive thunderstorm associated epidemics of asthma in the city of Melbourne: the possible role of rye-grass pollen. *Med J Aust* 1992; 156:834–7.
- 12 Wallis DN, Webb J, Brooke D et al. A major outbreak of asthma associated with a thunderstorm: experience of accident and emergency departments and patients' characteristics. *BMJ* 1996; 312:601–4.
- 13 Behrendt H, Becker WM, Friedrichs KH, Darsow U, Tomingas R. Interaction between aeroallergens and airborne particulate matter. *Int Arch Allergy Immunol* 1992; 99:425–8.
- 14 Ong EK, Singh MB, Knox RB. Aeroallergens of plant origin: molecular basis and aerobiological significance. *Aerobiologia* 1995; 11:219–29.
- 15 Knox B, Suphioglu C. Environmental and molecular biology of pollen allergens. *Trends in Plant Sciences* 1996; 1:156–63.
- 16 Desai R. Oxygen enrichment of the diesel engine. PhD thesis, The University of Melbourne, Melbourne, 1995.
- 17 Dockery DW, Pope CA, Xu X-P et al. An association between air pollution and mortality in six U.S. cities. *N Engl J Med* 1993; 329:1753–9.
- 18 Dockery DW, Pope CA, Xu X-P, Pope III CA, Dockery DW, Spengler JD, Raizenne ME. Respiratory health and PM10 pollution. A daily time series analysis. *Am Rev Respir Dis* 1991; 144:666–74.
- 19 Department of Health. Non-biological particles and health. London: Department of Health, HMSO, 1995.
- 20 Ostro BD, Lipsett MJ, Wiener MB, Selner JC Asthmatic responses to airborne acid aerosols. *Am J Public Health* 1991; 81:694–702.

- 21 Devalia JL, Rusznak C, Herdman MJ et al. Effect of nitrogen dioxide and sulphur dioxide on airway response of mild asthmatic patients to allergen inhalation. *Lancet* 1994; 344:1668–71.
- 22 Rusznak C, Devalia J, Davies R The impact of pollution on allergic disease. *Allergy* 1994; 49:21–7.
- 23 Bursill LA, Peng J-L, Fan X-D Cross-sectional high resolution transmission electron microscopy study of the structures of carbon nanotubes. *Philosophical Magazine* 1995; A 71:1161–76.
- 24 Suphioglu C, Singh MB, Knox RB. Peptide mapping analysis of Group 1 allergens of grass pollens. *Int Arch Allergy Immunol* 1993; 102:144–51.
- 25 Suphioglu C, Singh MB, Simpson RJ, Ward LD, Knox RB Identification of canary grass (*Phalaris aquatica*) pollen allergens by immunoblotting: IgE and IgG binding studies. *Allergy* 1993; 48:273–81.
- 26 Schlesinger RB. Biological disposition of airborne particles: basic principles and application to vehicular emissions. In: *Air pollution, the automobile and public health*. Washington, DC: Health Effects Institute, National Academy Press, 239–98.
- 27 Sawyer RF, Johnson JH. Diesel emissions and control technology. In: *Diesel exhaust: a critical analysis of emissions, exposure, and health effects*. Cambridge, MA: Health Effects Institute, 1995: 67–81.
- 28 Gruner P. Hunt for 1,000 hit by pollution. *London: Evening Standard* Thursday 21 July 1994, p.5.
- 29 Brock Williams P, Buhr MP, Weber RW et al. Latex allergen in respirable particulate air pollution. *J Allergy Clin Immunol* 1995; 95:88–95.
- 30 Wolff RK, Bond JA, Henderson RF, Harkema JR, Mauderly JL. Pulmonary inflammation and DNA adducts in rats inhaling diesel exhaust or carbon black. *Inhalation Toxicol* 1990; 2:241–54.
- 31 Ormstad H, Namork E, Gaarder PI, Johansen BV. Scanning electron microscopy of immunogold labeled cat allergens (Fel d 1) on the surface of airborne house dust particles. *J Immunol Methods* 1995; 187:245–51.
- 32 Johnson P, Marsh DG. Allergens from common rye grass pollen. II. The allergenic determinants and carbohydrate moiety. *Immunochemistry* 1966; 3:101–10.