

Scanning electron microscopy of immunogold labeled cat allergens (Fel d 1) on the surface of airborne house dust particles

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Abstract

This study investigated the ability of an immunogold labeling technique to demonstrate the presence of Fel d 1 (domestic cat) allergens on the surface of particles in samples of airborne house dust. Suspended particulate matter was sampled from ten Norwegian households, five with and five without a domestic cat. The specimens were immunogold labeled and examined in the backscatter electron imaging mode of the scanning electron microscope and in the transmission electron microscope. X-ray microanalysis was also applied to execute element analysis of the suspended particulate matter. The gold probe was mainly detected on carbon particles in the suspended particulate matter, both on small ($< 1 \mu\text{m}$) and larger carbon aggregates ($1\text{--}10 \mu\text{m}$). The present method may be useful in studying the localisation of different allergens on airborne house dust particles of various sizes and composition.

Keywords: Airborne house dust; Immunolabeling; Cat allergen; Backscatter electron imaging; Transmission electron microscopy; X ray microanalysis

1. Introduction

There is considerable interest in the relation of ambient air pollution to asthma and other respiratory diseases. Particulate air pollution has been associated with increased respiratory symptoms (Ostro et al., 1991; Pope et al., 1991; Pope and Dockery, 1992).

The question of how particulate matter in the air contributes to asthma and respiratory symptoms is, however, still largely unanswered. Dust particles may possibly act as carriers of allergens (Behrent et al., 1992; Luczynska et al., 1990; Spiekma et al., 1990), or may by some mechanism modify the immune response, either by enhancing IgE production as found in mice (Takafuji et al., 1989a), or by causing nonspecific irritation in the airways, thereby contribute to bronchial hyperresponsiveness.

Two terms are frequently used when referring to particulate matter less than $10 \mu\text{m}$ in aerodynamic diameter: suspended particulate matter (SPM) and particulate matter $< 10 \mu\text{m}$ (PM_{10}). This fraction is further divided into a coarse fraction ($> 2.5 \mu\text{m}$), mostly containing mechanically generated particles,

Abbreviations: BEI, backscatter electron image/imaging; BSA, bovine serum albumin; DEP, diesel exhaust particles; ELISA, enzyme-linked immunosorbent assay; IgE, immunoglobulin E; RAST, radio-allergosorbent test; SEI, secondary electron image/imaging; SEM, scanning electron microscope/microscopy; SPM, suspended particulate matter; TEM, transmission electron microscope/microscopy; XRMA, X-ray microanalysis.

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and a fine fraction ($< 2.5 \mu\text{m}$), mostly containing exhaust particles and secondary particles generated by chemical reactions in the atmosphere after being emitted as gaseous compounds (Takafuji et al., 1989a). During inhalation, the bulk of the coarse particles will be retained in the upper airways, whereas the fine particles may penetrate more deeply into the respiratory tree, and thus more readily provoke asthmatic reactions. A correlation of daily changes in SPM with respiratory symptoms and asthma has been reported (Pope et al., 1991; Pope and Dockery, 1992).

The primary aim of this study was to investigate the ability of an immunogold labeling technique to demonstrate the presence of cat (Fel d 1) allergens on the surface of SPM particles. The method described combines the principles of the 'immunonegative stain technique' for transmission electron microscopy (TEM) (Beesley, 1986) with visualization in the scanning electron microscope (SEM) using backscattered electrons. In the backscatter electron imaging (BEI) mode the contrast is generated by atomic number differences in the specimen, which makes it ideal for the detection of gold probes labeling cell surfaces (De Harven et al., 1984; Nava et al., 1984). Comparison of the topographic image (secondary electron image (SEI)) and the atomic number contrast image (BEI) of the SPM particles was performed to elucidate which type of SPM particles bound Fel d 1 allergens. TEM was carried out on the same specimens, to confirm the morphology of the SPM particles at a higher resolution. X-ray microanalysis (XRMA) was used in order to obtain a chemical element analysis of the SPM particles.

2. Materials and methods

2.1. Air sampling of SPM

Samples were obtained from ten households, five with and five without a cat. A Dymax 30 vacuum pump (Austen pumps, UK) was used to collect airborne particles. The sampling proceeded for 24 h with a flow of 4 l/min. The low flow was used to make sure that the dust collected was truly airborne and not sedimented. The airborne particles were collected on a 25 mm diameter polycarbonate filter,

pore size $0.4 \mu\text{m}$ (Millipore), in a filter cassette of polypropylene (Millipore). The polycarbonate filter was backed by a $5 \mu\text{m}$ pore size cellulose filter and a cellulose pad (both Millipore) to ensure an even flow distribution over the main filter area. During sampling, the filter cassette was located about 1 m above the floor, with an open face 45° downwards. In our experience, this sampling condition and geometry result in collection of dust particles mainly $< 10 \mu\text{m}$ in diameter (unpublished results). The particles collected during this sampling will be referred to as SPM particles.

2.2. Immunogold labeling

Discs of 3 mm in diameter were punched out from the SPM exposed filters and floated face down for 10 min on 15 μl drops of 0.1 M ammonium acetate (AA) containing 1% bovine serum albumin (BSA) and 0.02% NaN_3 , pH 7.4 (AA/BSA) to block nonspecific binding of proteins. The specimens were then transferred to 15 μl drops of primary antibody diluted in AA/BSA for incubation overnight in a humid chamber at 4°C . The primary antibody used was a monoclonal mouse IgG1 anti-Fel d 1 (clone 6F9 A4-H1, obtained from M. Chapman, University of Virginia, USA). As negative controls, specimens were incubated either without antibody or with four different monoclonal mouse IgG1 antibodies to human leucocyte antigens. The concentration of the antibodies used was 10 $\mu\text{g/ml}$. After incubation the specimens were washed on four consecutive drops of AA/BSA for 15 min each and drained by touching to the tip of a filter paper. The specimens were then incubated for 90 min at room temperature on 15 μl drops of goat anti-mouse IgG conjugated with 30 nm gold particles (Bio Cell Research Laboratories, Cardiff, UK) diluted 1/40 in AA/BSA. After incubation the specimens were again washed with four drops of AA/BSA, drained on filter paper, and air dried. Optimal incubation times and concentrations of primary antibody and gold conjugate (probe) were found in preliminary experiments. The specimens were finally mounted on brass stubs using carbon paint, and coated with a thin layer of evaporated carbon before examination in the SEM (SEI and BEI modes). Immunolabelings were performed in duplicates, i.e. on two discs from each SPM filter.

2.3. Preparation for TEM

After examination in the SEM, the specimens were carefully removed from the stub, using a scalpel,

and transferred to 3 mm 200 mesh copper grids. The grids were placed in a Jaffe washer containing chloroform to remove the polycarbonate part of the specimens (Jaffe, 1948). The specimens were kept

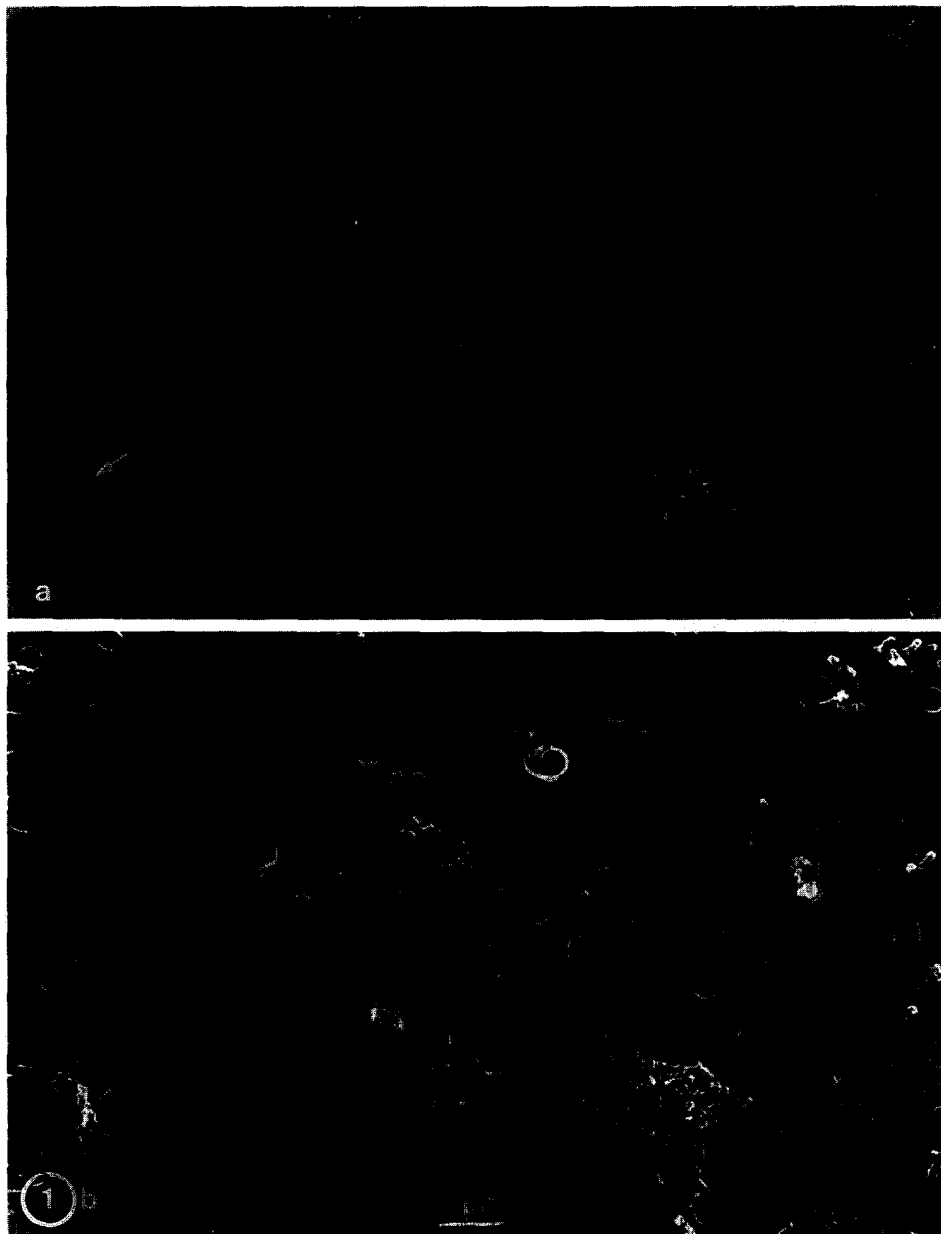


Fig. 1. Backscatter (a) and secondary (b) electron micrographs of carbon aggregates in an airborne dust sample from a household with a cat, labeled with mouse IgG1 anti-Fel d 1. The probe is goat anti-mouse IgG conjugated with 30 nm gold particles. Note the gold labeling also on the smaller carbon aggregates ($< 1 \mu\text{m}$) (arrows). $\times 13\,000$.

just below the chloroform surface level for 24 h. This treatment results in an extraction replica of SPM particles on the grid.

2.4. Electron microscopy

2.4.1. SEI / BEI

The specimens were examined in a JSM-840 scanning electron microscope equipped with a conventional tungsten hairpin electron emitter and an annular solid state backscatter electron detector. The microscope was operated at 15 keV, with a probe current of 1×10^{-10} A and a working distance of 7–8 mm (Namork, 1991). The microscope was operated in the BEI mode to detect the immunogold labeling, and in the SEI mode to visualize the topography of the labeled SPM particles. Micrographs in both modes were recorded at primary magnifications of 7000–8000 $\times$.

2.4.2. TEM

The specimens were examined in a JEM-100 CX transmission electron microscope, operated at 80 keV. The SPM particles were imaged at magnifications between 10 000 and 30 000 $\times$.

2.4.3. XRMA

Element analysis of the SPM particles was carried out in TEM using an energy dispersive X-ray spectrometer (Link ISIS system). The specimens were contained in a graphite specimen holder and tilted to 35° during analysis.

3. Results

The gold probes on the surface of the SPM particles were easily detected as white spots in the BEI mode of the SEM (Fig. 1a). The topographic morphology of the labeled SPM particles was visualized in the SEI mode (Fig. 1b). Based on TEM morphology, it is apparent that the gold probe was labeling the carbon aggregates (soot) in the SPM sample. Both small ($< 1 \mu\text{m}$) and larger aggregates (1–10 μm) were labeled by the gold probe. The gold probe appearing in the background of Fig. 1a, was always seen to label small carbon aggregates when imaged in the SEI mode (Fig. 1b, arrow). No significant gold

binding was found to other types of either organic or inorganic particles in the SPM mixture (e.g. Fig. 2). Due to differences in the imaging process of the two instruments, the gold probe labeling the carbon aggregates was not as easily detected in TEM as compared to the BEI mode of the SEM (Fig. 2). On the other hand, the higher resolution of the TEM instrument discriminates structural details better than the SEM. This can be seen in Fig. 2 which shows the gold probe labeling the carbon aggregate (C) and not the sulphur containing particle (S) often found in SPM samples.

When analysing gold labeled SPM particles in the XRMA, no energy peaks were present apart from a small gold peak ($M\alpha 1$), representing the gold label. The fact that these spectra do not contain any elements with excitation energies above fluorine (F), supports the hypothesis that the gold labeled SPM particles are carbon (soot) aggregates.

Gold labeling of carbon aggregates was found in all household samples studied, both with (Figs. 3a and 3b) and without a cat (Fig. 3c). Based on subjective estimates, however, specimens from households with a cat showed more densely labeled aggregates. Similar results were found in all parallel experiments from the SPM filters with regard to both the density of labeling and the kind of particles

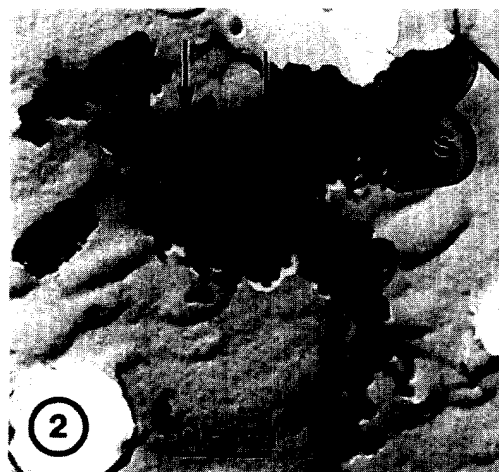


Fig. 2. Transmission electron micrograph of the same SEM specimen as in Fig. 1, showing the characteristic morphology of a carbon aggregate (C). The gold probe (arrows) is shown to label the carbon aggregate, but not the sulfur particle (S). $\times 30\,000$.

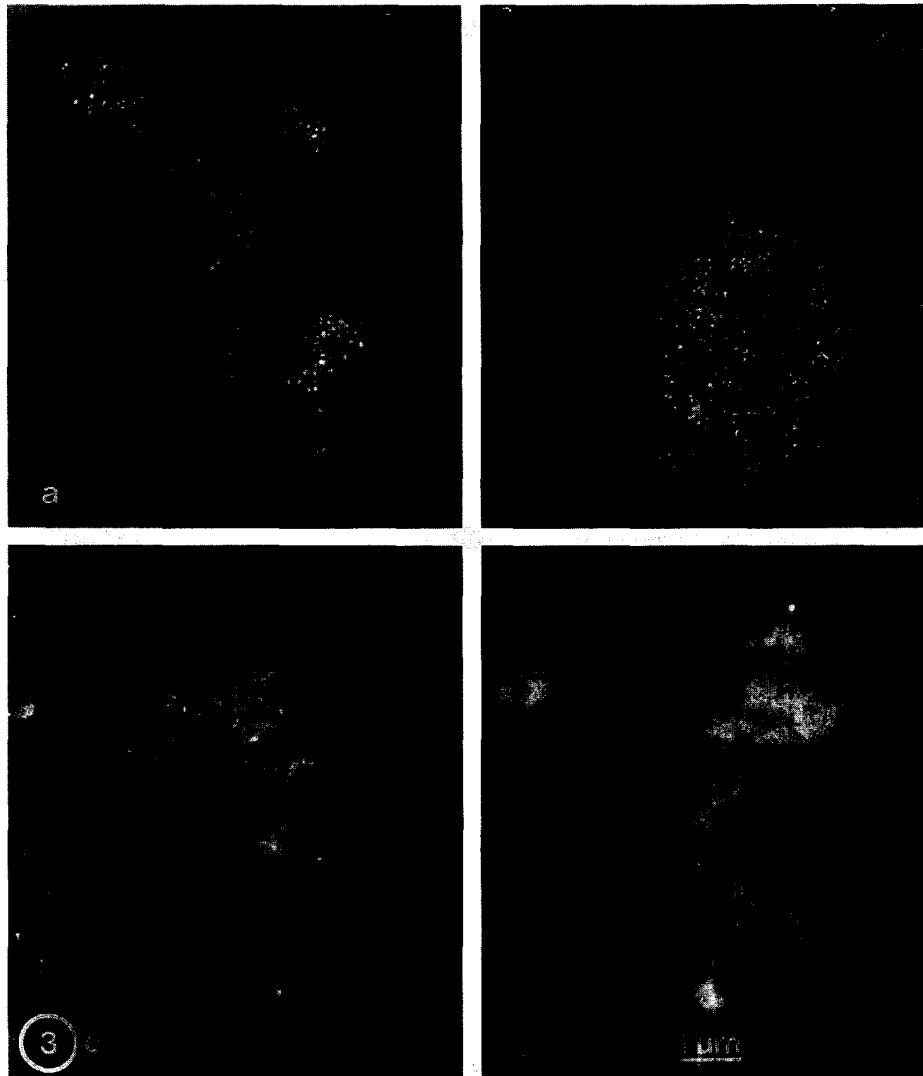


Fig. 3. Backscatter electron micrographs of carbon aggregates in airborne dust samples from different households. *a* and *b*: preparations from households with a cat; and *c*: preparation from a household without a cat – all three labeled with mouse IgG1 anti-Fel d 1; and *d*: preparation from a household with a cat and labeled with a mouse IgG1 anti-human leucocyte as a negative control. The probe is goat anti-mouse IgG conjugated with 30 nm gold particles. $\times 10\,500$.

which were labeled by the gold probe. The labeling was considered to be specific, since the negative controls showed no labeling (e.g. Fig. 3d).

4. Discussion

In TEM it was difficult to visualize the gold probe on the electron dense SPM particles, since both the

probe and the particles scatter electrons to angles outside the objective lens aperture. Because of the higher resolution, however, TEM proved useful to verify the characteristic morphology of the carbon aggregates (Vostal, 1980). In the SEM, the backscatter electrons can be used more efficiently by electronically adjusting the image contrast and brightness. The BEI mode of the SEM thereby proved useful for visualizing gold probe labeling in SPM

samples. The gold labeling and the topography of the SPM particles had to be recorded separately in BEI and SEI modes, respectively, since signal mixing of the two modes gave a secondary electron signal which was too weak to satisfactorily visualize the morphology of the carbon aggregates.

Methods such as ELISA or RIA are commonly used to demonstrate and quantify allergens in house dust. However, the present method, permits both the visualization of the allergen on SPM particles and the elucidation of the nature of allergen binding particles. The present method also has an advantage in requiring a much smaller quantity of house dust than ELISA. This makes it easier to study airborne house dust sampled with a low flow, which is more in accordance with human respiration. After extraction of three different 3 mm discs (two from households with a cat and one from a household without a cat) each in 300 μ l buffer (Tovey et al., 1981), the Fel d 1 concentrations measured by the Fel d 1 specific ELISA (de Blay et al., 1991) were consistently below the detection limit of 1.2 ng/ml (Ormstad et al., unpublished results). However, a limitation of the present method is that it is not quantitative. The apparent density of gold labeling in the specimen will, amongst other factors, depend on the amount and the bulkiness of carbon aggregates in the SPM sample.

Fel d 1 allergens were also detected in households without a cat, although the labeling density in these specimens was considered to be lower than in households with a cat (Figs. 3a–3c). The finding of Fel d 1 allergens in houses without a cat has also been reported by Wood et al. (1988) and Sakaguchi et al. (1993), examining settled house dust, using ELISA. In the latter investigation, Sakaguchi et al. suggested that the finding of a considerable amount of Fel d 1 allergens in households without a cat was due to cat dander being brought into the home on human clothes.

The presence of allergenic activity has been demonstrated in various size fractions of airborne particles, using ELISA (Rantio-Lehtimäki et al., 1994) and RAST inhibition assay (Spieksma et al., 1990). Spieksma et al. found grass pollen allergenic activity in the aerosol fraction containing particles $< 0.6 \mu$ m. This shows that airborne pollen allergen activity can be associated with particles much smaller

than intact pollen grains (10–80 μ m in diameter). Luczynska et al. (1990) found cat allergens, but not mite allergens, associated with small airborne particles, using a radioimmunoassay.

In the present study Fel d 1 allergens were found to be adsorbed to carbon aggregates in SPM samples, which is supported by earlier observations showing that SPM and diesel exhaust particles (DEP) are able to adsorb proteins. Takafuji et al. (1989a) found SPM to have the ability to adsorb proteins, the smallest particles being most efficient. They also found that SPM has an enhancing effect on the ovalbumin specific IgE antibody production in mice. When analysing SPM particles in TEM, a considerable content of soot particles is observed, mostly $< 1 \mu$ m with the characteristic structure of clusters of smaller particles with round shapes. Larger aggregates (up to 10 μ m), are also observed (Ormstad et al. to be published). These soot particles are thought to consist of elemental carbon, showing no energy peak in XRMA. The observed morphology, size and amount of these carbon aggregates, are in accordance with reports concerning DEP (Muranaka et al., 1986; Vostal, 1980). Diesel exhaust is reported to have a greater content of carbon particles than exhaust of engines run on petrol (Muranaka et al., 1986; Vostal, 1980). The authors further describe DEP to have the characteristic structure of clusters of small subunits with round shapes, providing a large surface area. The majority of DEP (95%) have a diameter $< 1 \mu$ m, the mean mass diameter being 0.2–0.3 μ m (Vostal, 1980). Like SPM, DEP have also been found to both enhance IgE production in mice and adsorb proteins (Muranaka et al., 1986). Furthermore, Takafuji et al. (1989b) discussed the possibility that pollen allergens may be carried on DEP in the atmosphere. It has been estimated that up to 30% of inhaled DEP can be deposited in the alveolar region of the respiratory tract (Vostal, 1980) and a large proportion of this will be phagocytosed by alveolar macrophages. Thus carbon particles (soot), which constitute a substantial amount of the fine fraction of SPM, may be of considerable importance in the provocation of allergic airway responses, contributing both by carrying allergens into the airways and by causing increased production of specific IgE.

It has previously been found that the sources of Fel d 1 allergens are the sebaceous glands (Bartho-

lome et al., 1985; Charpin et al., 1991) and saliva (Anderson and Baer, 1981; Brown et al., 1984). It is possible that the allergens become airborne as droplets of saliva, and are adsorbed to the surface of the carbon particles in the air.

In summary, this study shows that the immunogold labeling technique described is applicable for the detection of Fel d 1 allergens on the surface of SPM particles. The results indicate that the Fel d 1 allergens are mainly adsorbed to the surface of the carbon aggregates in SPM samples. The method may be a useful tool in studying the presence of different allergens of relevance to asthma on SPM particles of various sizes and composition. Further studies along these lines are in progress.

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