



Diesel exhaust particles and allergenicity of pollen grains of *Lilium martagon*

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Abstract

Diesel exhaust particles are considered as the most important parts of air pollutants. Diesel exhaust particles have been shown to express both adjuvant activity for sensitization against common allergens and enhancing effects on allergic symptoms in sensitized individuals. In this research, pollen grains of *Lilium martagon* that are known as a non-allergic substance were collected and exposed to DEP 5 and 10 days. The allergy potency of different pollen extracts were compared by means of skin test, as well as analyses blood eosinophil numbers and IgE levels in the treated animals. Normal and DEP-exposed pollen grains were examined by scanning electron microscopy. Pollen extracts were also studied by SDS–PAGE for DEP-induced changes in protein profiles. Allergic bands were also studied and checked by using immunoblotting method. The results of the investigated allergy tests showed that DEP-exposed pollen grains are effective in inducing allergic symptoms. According to our microscopic observations, organic substances that exist in the DEP, mediate agglomeration of particles on the pollen surface. In appropriate conditions, water-soluble components of DEP may induce changes that affect the release of pollen proteins. SDS–PAGE showed protein profiles of pollen grains were changed and some new bands appeared in DEP-exposed pollen grains. Immunoblotting studies showed a new band in DEP-exposed pollen grains that react strongly with anti-IgE, but there is no allergenic band in normal pollen grains. On the other hand, diesel exhaust particles can carry pollen allergen molecules, induce new proteins (allergens), and also act as adjuvant for allergens.

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1. Introduction

There has been a dramatic increase in the incidence of allergy and asthma over the last century (Ishizaki et al., 1978; Hwang et al., 2005). The factors underlying this phenomenon are clearly complex; however, this rapid increase in atopic disease has undeniably occurred in parallel with rapid industrialization and urbanization in many parts of the world (Riedl and Diaz-Sanchez, 2005). Among many hypotheses, the idea that environmental pollutants may play important role has gained substantial public and scientific attention (Brun-Fahrlander et al., 1992; Rusznak et al., 1994; Ring and Behrendt, 1993; Behrendt et al., 1997). Persistent exposure to traffic-related air pollution and especially particulate matter from motor

vehicles has often been discussed as one of the factors responsible for this increase (Heinrich and Wichmann, 2004). Ising et al. (2003) conclude that the pathogenesis of allergies can be stimulated by adjuvant effects of diesel exhaust particles (DEP). DEP appear to play a role in respiratory and allergic disease (Pandya et al., 2002).

Especially DEP has been shown to express both adjuvant activity for sensitization with common allergens and enhancing effects on allergic symptoms in sensitized individuals (Riedel and Kramer, 1988; Holt et al., 1986; Matsuamara, 1970; Helander et al., 1997; Ito et al., 1996; Kainka-Stanicke et al., 1989). By attaching to the surface of pollen grains and of plant-derived particles of paucimicronic size, several types of pollutants have been shown to modify not only the morphology of these antigen-carrying agents but also their allergenic potential (Chehregani et al., 2004a,b; Amato et al., 2005). In addition, by inducing airway inflammation, which increases airway permeability,

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pollutants overcome the mucosal barrier and could be able to prime allergen-induced responses (Amato et al., 2005).

Diesel exhausted particles may affect pollen grains indirectly via stress on the growth on the plant or directly through contamination of the anthers on the plant or during the flight of pollen grains through the air (Emberlin, 1998). Some studies showed that air pollutants could induce the breakage of proteins or the formation of new proteins in pollen (Ruffin et al., 1983; Chakraborty et al., 1996; Chehregani et al., 2004a, b). A decrease of total pollen protein in polluted areas was reported (Behrendt et al., 1997; Chehregani et al., 2004a, b).

In conclusion, the evidence for an increased risk for allergy and asthma is still weak (Heinrich and Wichmann, 2004). In this report, the allergenicity of *Lilium* pollen grains were examined following exposure to DEP to detect if changes in the power of pollen or associated proteins occurred. In addition, the allergy potency of those pollen grains was investigated to determine what the DEP might affect the induction of allergic responses via exogenous modification of the allergen(s) itself.

2. Materials and methods

2.1. Sampling

Lilium martagon (belonging to *Liliaceae* family) is a member of the decorative plants, which are found in different parts of Iran. Planted samples in Iran's central regions were collected and pollen grains were removed from growing plants directly and harvested by passing the plant material through mesh with 30–40 µm pores. Some of the harvested pollen grains were used as fresh, and others were exposed to approximately same weight of DEP for 5 and 10 days. DEP were prepared from an old Mercedes bus (Model 302) that is a popular bus in Iran. Incubation of pollen grains with DEP was taken at room temperature (25 ± 2) with humidity of about 40%. Incubation condition was the same for normal and experimental groups.

2.2. Animals

Male Balb/C Mice (8–10 weeks of age) were obtained from the Razi institute and housed in an air-conditioned room at a temperature of 25 ± 2 °C and provided a standard laboratory diet and given water (with added Vitamins). The animals were sensitized by intraperitoneal injection of 50 µL pollen extract (containing about 20 µg protein in phosphate saline buffer (PBS)) prepared from fresh and DEP-exposed pollen grains (Sheldon et al., 1967). In each case, $\text{Al}(\text{OH})_3$ (at same level) was employed as an adjuvant. Treatments were performed six times, i.e., twice a week and two period of 3 weeks.

2.3. Skin tests

One week after latest sensitization, the mice were utilized in skin tests. Each animal was tested with 30 µL of the total extract (containing 20 µg protein with adjuvant) diluted with 0.05 M phosphate buffered saline (pH 7.4). Some animals were treated by water-soluble part of DEP. For this aim, 20 µg DEP was dissolved in saline buffer and used for treatment after passing through Millipore 0.2 µm. The negative control was buffered saline and the positive control was histamine acid phosphate. The skin reactions at the on abdomen site were read after 30, 60, and

120 min from the start of the test and quantified on the basis of wheal diameter.

2.4. Clinical and serological tests

Blood samples were drawn from the sensitized mice (harvested at eye win) 1 week after the skin test (Aberg, 1989). Serum from each sample is isolated by centrifugation and then stored at -76 °C. The sera of 15 *Lilium*-allergic sensitized animals were tested for IgE reaction against untreated *Lilium* pollen grains. The sera of 14 sensitized animals were tested for pollen grains, which were exposed to DEP for 5 days. Fifteen animals were tested for pollen grains, which were exposed to DEP for 10 days and 15 animals were tested for DEP without pollen extract. Sera from 12 animals, (not sensitized to *Lilium* pollen grains and not treated by DEP), served as the control sera in parallel. In each analysis, total serum IgE was evaluated by using ELISA for the blood of mice. The standard Pharmingen mouse IgE protocol for sandwich ELISA was used to quantify the total amount of IgE. The serum IgE levels were expressed in nanogram per milliliter after comparing with mouse IgE standards (Pharmingen, Calif, USA). Peripheral blood smears from both control and experimental animals were also prepared for examination of blood cell differentials (Mondal et al., 1997).

2.5. Electrophoresis studies

Pollen protein extractions of normal and polluted pollen grains were carried out at 4 °C in Tris–HCl buffer (pH 7.6). SDS–polyacrylamide gel electrophoresis over a 12% gel was performed on the soluble proteins according to the method of Laemmli (1970). The extraction of soluble proteins was made in sample buffer (0.125 M Tris–HCl, pH 6.8, 4% SDS, 20% glycerol, 10% β -mercaptoethanol, 0.1% bromophenol blue dye) with heating for 3–4 min at 100 °C before loading. The amount of protein was 10 µg per lane; protein marker standards (Sigma, St. Louis, MO) were run in parallel.

2.6. IgE-specific immunoblotting

Normal and polluted pollen proteins which are subjected to SDS–PAGE were then transferred electrophoretically to 0.2 µm PVDF (polyvinylidene difluoride) membranes (Bio Rad) for 1 h at 100 V in an electro-transfer unit (Bio Rad) (Robtorn Janson et al., 2002). The membranes were then blocked over night at 4 °C in PBS/5% non-fat dry milk/0.05% $\text{Na}_2\text{S}_2\text{O}_3$. Diluted sensitized or treated mouse sera (1:5 v:v in PBS/1% non-fat dry milk/0.01% $\text{Na}_2\text{S}_2\text{O}_3$) were placed with membranes and incubated overnight at 4 °C. The membranes were then washed for 3 times (20 min each) with PBS/0.1% Tween 20 and then incubated overnight at 4 °C with polyclonal Goat anti-mouse IgE (Abcam, Cambridge, UK); diluted 1:400 in non-fat milk buffer. The membranes were then washed for 3 times (20 min) and incubated 1 h at room temperature with peroxidase-conjugated rabbit anti-goat IgG(H+L) (Jackson Immunosci Research, USA) diluted 1:2000 in non-fat milk buffer. The membranes were then washed 5 times (20 min) and incubated 1 min in enhanced chemiluminescence plus (Amersham Bioscience, USA). The membranes were then exposed to Kodak medical X-ray film for 3 min and developed in a Fuji X-ray film processor model FPM 700. The pre-stained protein ladder for western blot was purchased from New England Biolabs Company.

2.7. Scanning electron microscopy (SEM)

Polluted and normal pollen grains were studied by scanning electron microscopy. After coating with gold, samples were analyzed by using a SEM model SEM-EDS, EX 300 (Link, UK). The purified pollen grains were fixed by glutaraldehyde, post-fixed with osmium, dehydrated, and dried prior to gold coating.

3. Results

3.1. Skin test

The results of skin tests for the different samples are shown in Table 1. These results indicate that pollen extracts, which were prepared from fresh pollen grains, had no significant allergenic effects but pollen grains that were exposed to DEP could induce skin reactions. Pollen that had been exposed to DEP for 10 days showed the maximum allergenic activity; the mice that received this preparation displayed average wheal diameter of 5 cm. Allergy reactions were also relatively large in pollens that were exposed to DEP for 5 days (average wheal diameters = 4.2 cm). Injection of DEP extracts also induced skin reactions by themselves; in these cases, average wheal diameters were 1.2 cm. In contrast, injection of pollen extracts prepared from non-polluted pollen grains did not elicit clear skin reactions (average wheal diameter was 0.3 cm). Statistical analysis showed that the effect of the polluted pollen extracts was about 3–4 times more than that of the water-soluble extract of the DEP and about 7–8 times more than that of the normal pollen extract. Injection of the pollutant-treated pollen extracts caused the formation of a deep wound at the injection site after 24 h, whereas injection of DEP extract or the normal pollen extract did not.

3.2. Clinical and serological tests

The results of the analyzed blood smears prepared from each of the different treatment groups are also mentioned in Table 1. The analysis showed that the amounts of eosinophils and neutrophils were increased in animals that were treated with the polluted pollen extracts. The quantity of eosinophils in animals that were treated by polluted pollen was about 2–3 times more than those treated with DEP extract only, and about 5–6 times more than the group that were treated by normal pollen grains.

Examination of total IgE levels in the blood of mice in the different groups (Table 1) represented that all polluted pollen extracts and the DEP extract itself caused an

increase in the levels above background. The results indicate that the amount of IgE was increased significantly in mice that were treated with the DEP-exposed pollen extract.

3.3. Protein analyses

The protein profiles of the different pollen samples (normal and DEP-exposed) are shown in Fig. 1. The pollen grains exposed to DEP for 10 days have some different bands that were not present in the extracts of untreated pollens. Especially two additional bands, with molecular weight of about 35 and 65 kDa, were visible. Interestingly, in all polluted pollen grains, total protein content was decreased in response to DEP (data not shown). This decrease is more obvious in groups that were exposed to DEP for 5 days.

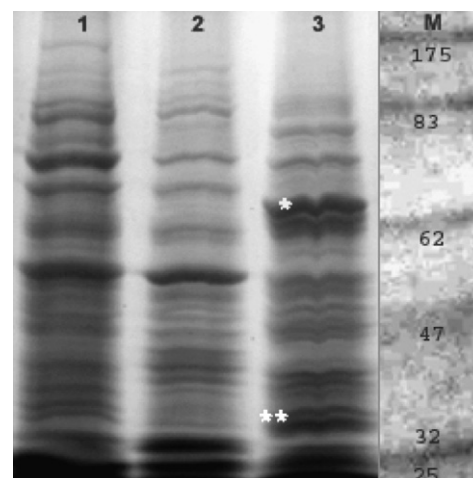


Fig. 1. Protein profiles of different pollen grains. Pollen grains that were exposed to diesel exhaust particles for 10 days have two bands (*, 35 and **, 65 kDa) that clearly differed from those extracted from normal pollen grains. The bands with molecular mass of 35 and 65 kDa are extra bands that are not visible in normal plants. Line 1, bands of normal pollen grains collected from non-polluted area; Line 2, bands of pollen grains exposed to DEP for 5 days; Line 3, bands of pollen grains exposed to DEP for 10 days; M, molecular weight standard markers.

Table 1

Comparison of allergic reaction by means of skin test (wheal diameter), blood eosinophil, neutrophil, and IgE levels in control and treated mice

Samples	C	N	5P	10P	DEP
Wheal diameter (cm)	0.4±0.2	0.6±0.3	4.2±0.7*	5.2±1.3*	1.2±0.4
Eosinophil number (× 10 ⁴ cells/mL blood)	5.5±1.3	9.4±4.2	47.6±4.9*	54.2±5.6*	10.6±3.8
Neutrophil number (× 10 ⁴ cells/mL blood)	35.0±1.9	38.5±3.4	84.2±2.8**	93.4±3.7**	42.3±3.4
IgE (ng/mL)	0.56±0.20	2.75±1.18	12.82±1.46*	14.6±1.80*	3.48±2.35

Data showed in all groups that treated with polluted pollen extracts skin reaction, amount of eosinophils, and IgE level increased considerably. C, control group that treated with saline; N, the group that treated with normal pollen extract; 5P, the group that treated with pollen extract that exposed to DEP for 5 days; 10P, the group that treated with pollen extract that exposed to DEP for 10 days; DEP, the group that treated with diesel exhaust particles. Differentiations between the 5–10 days exposed groups with control and normal groups are significant, but between DEP and normal group with control ones are not significant ($P < 0.05$). Each group represents the mean ± S.E. 12–15 animals.

* $P < 0.01$.

** $P < 0.05$.

3.4. IgE-specific immunoblotting

For detection of allergen proteins by allergen-sensitive sera, immunoblotting was carried out with the pollen extracts. As Fig. 1 clearly shows, there are some different bands of pollen-soluble proteins, with about 14 distinct protein bands which were detected by Comassi blue staining. To identify bands corresponding to the allergen(s), IgE-binding bands were visualized by immunoblotting using sera from animals sensitized with the *Lilium* normal and DEP-treated pollen extracts. After immunoblotting, a band with molecular mass of about 35 kDa was observed in DEP-exposed pollen grains (Fig. 2). Intense reactivity was observed with the sera of 13 out of a total of 15 sensitized animals. There were differences between animals that were treated by normal and DEP-exposed pollen grains.

3.5. SEM analysis

Ultra-structural analysis of pollen grains of *L. martagon* L. showed that they are ovular in shape, 30–35 μm in diameter, 60–70 μm in length, and had a thick exin with one visible colpe. SEM analysis showed that the tectum on pollen grains is reticulate (Fig. 3). On the surface of non-polluted pollens neither atmospheric fine dust nor agglomeration is present. After contamination with DEP, the color of the pollen grains changed dramatically and they darkened considerably. The pollen grains became folded and vermicular particles accumulated on the surface of

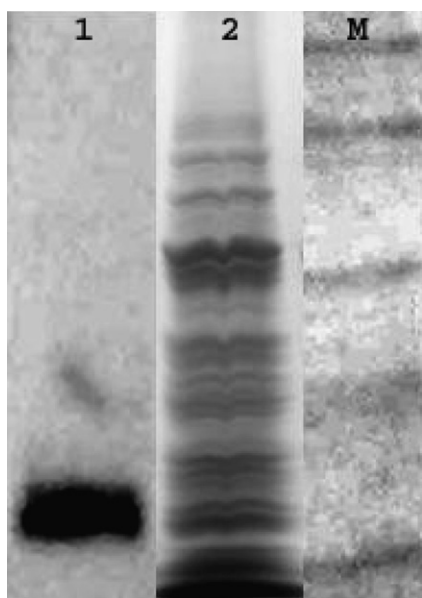


Fig. 2. Results of immunoblotting showed that DEP-treated pollen grain of *Lilium* has an allergenic protein with a molecular mass of 35 kDa. We could not detect any allergenic band in normal pollen grains. M, pre-stained molecular mass markers: Line 1, protein band probed with pooled positive sera. The dark spot indicate reactivity of IgE antibodies with corresponding protein bands acting as allergen. Line 2, protein bands of pollen extract stained with Comassi Brilliant blue.

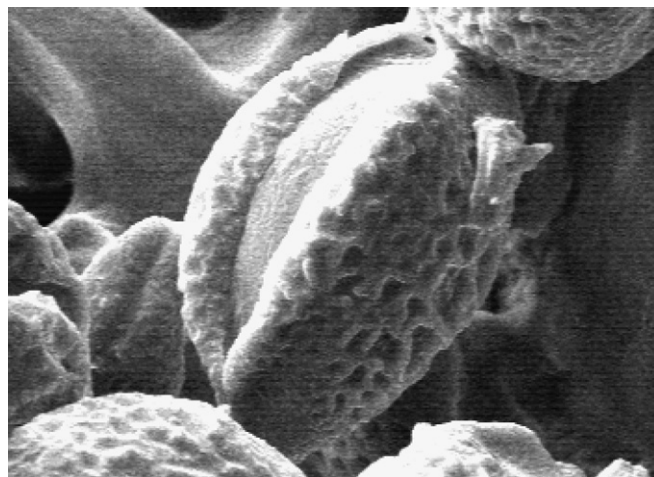


Fig. 3. Scanning micrograph of *Lilium martagon* pollen grains collected from non-polluted regions. Pollen grains are ovular having reticulate sculpturing as tectum (1500 $\times$).

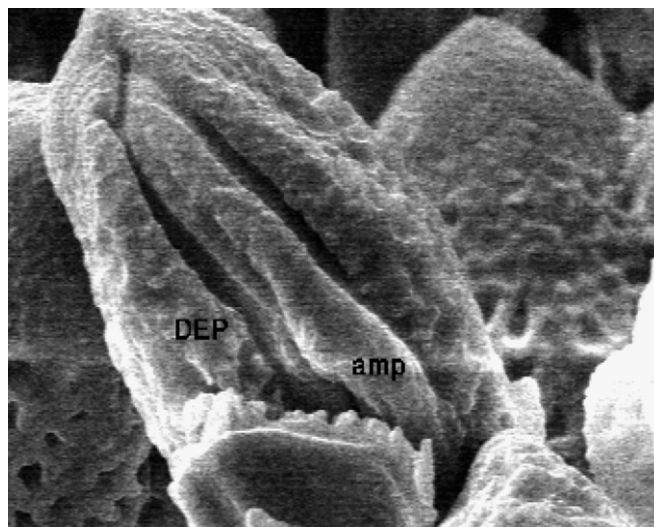


Fig. 4. Scanning micrograph of *Lilium martagon* pollen grains exposed to DEP for 5–10 days. Pollen grains are folded, tectum disrupted, and air born particles are accumulated on the surface of pollen. DEP, diesel exhausts particles; amp, agglomerated pollen's material (1500 $\times$).

pollen grains (Fig. 4). The pollen grains were covered with high amounts of DEP and the tectum was disrupted. The interaction between pollen grains and particulate pollutants caused pollen constituents to be released and an agglomeration of organic particles on the pollen surface forms (Fig. 4).

4. Discussion

DEP have different detrimental effects on organisms. It has been demonstrated that diesel emission increase allergic reactions as rhinitis and asthma (Mehiri Ben Rhoma et al., 2005). DEP have been shown to express both adjuvant activity for sensitization against common allergens and enhance allergic symptoms in sensitized individuals (Riedel

et al., 1998; Holt et al., 1986; Matsuromura, 1970; Helander et al., 1997; Ito et al., 1996; Kainka-Stanicek et al., 1989; Streereberg et al., 2003). In this state, the allergenic potential of polluted and non-polluted pollens was assessed by meaning inducibility wheals, changes in leukocyte number, and the amounts of the total IgE in the blood of mice treated with pollens. Our clinical and serological results showed that abundance of eosinophils, average wheal diameter, and amount of total IgE in animals that were treated with extracts of fresh pollen were near control group levels (Table 1). Based on this, we were able to conclude that *L. martagon* is not an allergenic plant.

However, these results clearly showed that the allergic potential of these pollen grains was increased when they were exposed to DEP. This finding is in accordance with some prior research by other investigators (Rusznak et al., 1994; Ito et al., 1996; Chakraborty et al., 1996; Behrendt et al., 1997; Knox et al., 1997; Majd and Kiabi, 1997; Emberlin, 1998; Jae-Kyoung et al., 2001; Chehregani et al. 2004a, b). Our studies indicate that DEP itself was not the cause of increasing the allergic reactions; the effect of a DEP extract were several times less than that from the DEP-treated pollen grains with regard to induction of allergy symptoms in treated animals (Table 1).

SDS-PAGE studies showed that DEP treatment caused the increased expression of novel proteins in pollen grains (Fig. 1). This effect is in accordance with the findings of Ruffin et al. (1983) but not with those of Behrendt et al. (1997) and Chehregani et al. (2004a, b) about effects of air pollution. Immunoblotting studies indicated that one of the new bands apparently had allergenic properties in situ. Formation of these novel proteins (or breakage of large proteins to small ones) seems to be effect of the DEP exposure. The finding that the band with molecular mass of 35 kDa could react with anti IgE strongly (Fig. 2) is novel with regard to what is known about the effects of DEP on pollen and its proteins. Since all DEP-exposed pollen grains could induce allergic reaction but newly formed protein were illustrated in the groups that were exposed to DEP for 10 days, it seems that formation of allergic protein is in common in both 5 and 10 days DEP-exposed group, but the amount of this in 5 days DEP-exposed group is less than the 10 days exposed ones. We can conclude that our protein analysis methods were not sufficient (low sensitive) to detection of low concentration of 35 kDa protein in the 5 days exposed pollen grains but allergy results indicate that this protein should be also formed in 5 days exposed group.

Ultra-structural studies (Figs. 3 and 4) showed that DEP have affected pollen grains. On the surface of non-polluted pollen grains, neither atmospheric fine dust nor agglomeration was visible (Fig. 3). When pollen grains were exposed to DEP, they became folded, and vermicular particles accumulated on the surface of pollen. Pollen grains that exposed to DEP for 5 and 10 days were covered with high amounts of DEP (Fig. 4). Agglomeration of air pollutants was seen on the surface of pollen grains; this is in agreement with observations which were reported by some

researchers (Behrendt et al., 1992, 1997; Knox and Suphioglu, 1996; Chehregani et al. 2004a, b).

According to our observations, DEP absorbed into air pollutants mediate the agglomeration of particles on the pollen surface followed by local pre-activation of the coated pollen grains. Under appropriate conditions (i.e., humidity) DEP can act as detergent and water-soluble compounds of DEP may induce local allergen release and agglomeration of pollen material including pollen proteins on the surface of pollens (Fig. 4). A consequence of the release of allergen molecules as aerosols is that the molecules are free to interact with other types of air borne particles, such as DEP. Findings of several researchers support this idea (Knox and Suphioglu, 1996 and Behrendt et al., 1997). Environmental factors such as humidity, thunderstorms, and air pollutants have been shown to influence allergen liberation in complex manner. Among pollutants, SO₂ has a clear-cut inhibitory effect on allergen release whereas DEP has been shown to activate pollen and induce protein and allergen releases (Behrendt and Becker, 2001). In this condition, sensitized individuals are in more contact with pollen proteins (allergens) than in the non-polluted condition.

5. Conclusion

Allergy frequency was increased dramatically in the last few years. It seems that air pollution has responsibility in this phenomenon. DEP is the most important part of pollutants in polluted cities. DEP have some well-known effects such as increasing allergy potency of pollen grains. Based on this research, in conclusion, DEP not only have toxic effect on organisms regarding induction of allergy, but also they are capable of causing allergen release, and induction of new pollen protein (allergen) formation.

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Hereby I confirm that all our methods of study were conducted in accordance with national and institutional guidelines for the protection of human subjects and animal welfare.

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