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## Effects of diesel exhaust particles on primary cultured healthy human conjunctival epithelium

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### ABSTRACT

**Background:** Air pollution from road traffic is a serious public health problem. Epidemiologic studies have demonstrated adverse health effects associated with environmental pollution. Diesel exhaust is a major contributor to ambient particulate matter air pollution. We studied the effects of exposure to diesel exhaust particles on allergic conjunctivitis using cultured conjunctival epithelial cells obtained from healthy people.

**Objective:** To identify the factors involved in the human conjunctival epithelial response to diesel exhaust in vitro.

**Methods:** Healthy individuals underwent conjunctival biopsy, and the samples were incubated on conjunctival epithelial sheets. We investigated the effects of exposure to diesel exhaust using GeneChip arrays. The adhesion molecules and cytokines showing increased expression on GeneChip arrays were verified by real-time reverse transcription polymerase chain reaction and enzyme-linked immunosorbent assay.

**Results:** The GeneChip array showed increased expression of adhesion molecules, cytokines, chemokines, and growth factors after exposure to diesel exhaust. Real-time reverse transcription polymerase chain reaction and enzyme-linked immunosorbent assay confirmed that the expression of intercellular adhesion molecule 1 and interleukin 6, in particular, were significantly upregulated.

**Conclusion:** Our experimental data confirm that exposure to diesel exhaust particles increases inflammatory factor expression in human conjunctiva and thereby contributes to allergic conjunctival responses.

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### Introduction

Allergic conjunctivitis is the most common ocular surface allergic disease and affects more than 20% of the population.<sup>1–3</sup> As a result of changes in the living environment and other factors, the incidence of allergic diseases, including atopic dermatitis and pollinosis, is progressively increasing. The medical community has been challenged to develop effective therapies for these allergic diseases.<sup>4</sup> To better understand the pathogenesis of allergic disease, researchers are increasingly focusing on the effects of environmental factors, such as exposure to atmospheric pollutants, UV light, and viral infection. Epidemiologic studies have found that the increased incidence of allergic diseases is at least partly due to increased exposure to atmospheric pollutants.

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The effects of diesel exhaust on the pathophysiology of allergic airway disease have been analyzed in animals and humans using in vitro and in vivo models. The components of diesel exhaust as environmental factors have received much attention in these studies. In fact, it was reported that diesel exhaust particles and carbon black can induce dust mite allergy in rats.<sup>5</sup> Exposure to diesel exhaust also enhances ozone-induced airway inflammation in healthy humans<sup>6</sup> and in people with asthma.<sup>7</sup> In the bronchial epithelium, diesel exhaust increases interleukin (IL) 8, growth-regulated oncogene (GRO)  $\alpha$ , and IL-13 and activates several transcription factors (eg, nuclear factor- $\kappa$ B and activator protein 1) and mitogen-activated protein kinases (eg, p38 and c-Jun N-terminal kinase).<sup>8,9</sup> Holgate et al<sup>10,11</sup> exposed healthy and asthmatic individuals to diesel exhaust for 2 hours and determined messenger RNA expression in bronchial wash fluid. Stenfors et al<sup>12</sup> determined cytokine production in lavage fluid after exposing asthmatic and healthy individuals to diesel exhaust for 6 hours. These findings suggest that oxidant pollutants, including diesel exhaust particles, cause allergic inflammation by activating allergic inflammatory response elements.

In this study, we hypothesized that the ocular surface is exposed to diesel exhaust particles, which enhance the expression of

cytokines and growth factors, leading to allergic conjunctival inflammation. Using a GeneChip array, we first investigated the effects of exposure to diesel exhaust *in vitro*.

## Methods

### Culture of human conjunctival epithelial cells

This study was approved by the Ethics Committee of Tokyo Dental College (Chiba, Japan). All experiments were conducted in accordance with principles of the Declaration of Helsinki. Written informed consent was obtained from all volunteers before participation. Human conjunctival samples were collected from 7 healthy volunteers using scissors under 2% Xylocaine anesthesia. To establish primary conjunctival epithelial cells, samples were cultured in supplemented hormonal epithelial medium supplemented with 10% heat-incubated fetal bovine serum. Specimens were cultured in complete medium at 37°C in a humidified atmosphere supplemented with 5% carbon dioxide in air for a few weeks. Once the epithelial cells had grown, they were transferred to a flask and incubated. Subconfluent epithelial cells were subcultured in 96-well plates at  $2 \times 10^5$  cells/mL. One day later, the cells were incubated with or without diesel exhaust particles (100 µg/mL) or tumor necrosis factor (TNF; 30 ng/mL). At the indicated times, culture supernatants were collected and the cytokine levels were determined. The conjunctival cells were also collected and gene transcription was measured. All experiments were performed in triplicate for each cell culture.

### Genechip expression analysis

Human gene expression was examined using the U95A probe array (GeneChip; Affymetrix, Santa Clara, California), which contains an oligonucleotide probe set for approximately 10,000 full-length genes. Gene expression was determined according to the manufacturer's instructions and previous reports.<sup>13</sup> Briefly, total RNA (3–10 µg) was extracted from approximately  $10^7$  cells after incubation for 6 hours. Double-stranded complementary DNA was synthesized and subjected to *in vitro* transcription in the presence of biotinylated nucleoside triphosphates. The biotinylated complementary RNA was hybridized with a probe array at 45°C for 16 hours, stained with streptavidin-phycoerythrin (Molecular Probes, Eugene, Oregon), and scanned using a Hewlett-Packard Gene Array Scanner. The fluorescence intensity of each probe was quantified using GeneChip Analysis Suite 5.0 software (Affymetrix). The expression level of individual messenger RNAs (mRNAs) was determined as the mean fluorescence intensity among the intensities for 6 pairs of probes (perfect-matched and single nucleotide-mismatched probes) consisting of 25-mer oligonucleotides. If the intensities of mismatched probes were very high, gene expression was judged to be absent even if a high average fluorescence was obtained with the GeneChip Analysis Suite 5.0 software. The level of gene expression was determined as the average difference using the GeneChip software. The percentages of the specific average difference level vs the mean average difference level of 16 probe sets for housekeeping genes ( $\beta$ -actin and glyceraldehyde-3-phosphate dehydrogenase) were then calculated.

### Real-time RT-PCR

Total RNA was obtained from isolated epithelial cells (>95% purity) using a total RNA isolation kit (RNeasy Mini Kit; Qiagen, Valencia, California) according to the manufacturer's protocol and was treated with DNase (Gibco-BRL) before undergoing reverse transcription. One microgram of each RNA sample was reverse transcribed using random hexamers and SuperScript III (Invitrogen, Carlsbad, California). The cDNAs were amplified and quantified on a LightCycler (Roche, Mannheim, Germany) using a QuantiTect

**Table 1**  
Primer Sequences

|               | Forward                | Reverse                  |
|---------------|------------------------|--------------------------|
| RANTES        | ATCCTCATTGCTACTGCCCTCT | CAATGTAGGCAAAGCAGCAGG    |
| ICAM-1        | CTGTGTCCCCCTCAAAGTCA   | ATACACCTCCGGTGTCTCC      |
| IL-8          | GTCTGCTAGCCAGGATCCACAA | GAGAAACCAAGGCACAGTGGAA   |
| IL-6          | CAATAACCACCCCTGACCCA   | GCGCAGAATGAGATGAGTTGTC   |
| GRO- $\alpha$ | ATTCACCCCAAGAATCATCA   | CACCACTGAGCTTCTCTCTC     |
| GRO- $\beta$  | GCAGGGAATTCACCTCAAGC   | AGCTTCTCTCTCTCTCTGG      |
| LARC          | TGTCAGTGCTGCTACTCCACCT | CTGTGTATCCAAGACAGCAGTCAA |

Abbreviations: GRO, growth-regulated oncogene; ICAM-1, intercellular adhesion molecule 1; IL, interleukin; LARC, liver and activation-regulated chemokine; RANTES, regulated on activation, normal T-expressed, and presumably secreted.

SYBR Green PCR kit. The primers used for real-time PCR are listed in Table 1. To ensure equal loading and amplification, all products were normalized for glyceraldehyde 3-phosphate dehydrogenase transcription as an internal control.

### ELISA

Human intercellular adhesion molecule (ICAM) 1, IL-6, IL-8, liver and activation-regulated chemokine (LARC), RANTES (regulated on activation, normal T-expressed, and presumably secreted), GRO- $\alpha$ , GRO- $\beta$ , and GRO- $\gamma$  concentrations were measured using ELISA kits (R&D Systems, Minneapolis, Minneapolis). The detection limits of these assay kits were approximately 3 pg/mL.

### Statistical analysis

Paired *t* tests were conducted on log-transformed data to determine differences in supernatant cytokine content and cytokine expression between cells with or without exposure to diesel exhaust or TNF- $\alpha$ . Values are presented as mean  $\pm$  SD, and *P* < .05 was considered statistically significant.

## Results

### Elevated transcripts in deactivated conjunctival cells

The optimal incubation time to measure the apparent increase of the mRNAs in the model was revealed as 6 hours after exposure among 1, 3, and 6 hours. In addition, 24 hours after exposure among 8, 24, and 48 hours was the optimal time to determine protein production.

We used a GeneChip array to examine the expression levels of approximately 12,000 genes in conjunctival cells exposed to diesel exhaust particles. The fold-increase in expression was determined by calculating the mean difference in expression in activated cells relative to resting cells. The mean fold-increase was then determined for each sample. After eliminating the redundant transcripts, we selected the transcripts showing the greatest upregulation in the cells. An adhesion molecule (ICAM-1), chemokines (LARC, RANTES, GRO- $\alpha$ , GRO- $\beta$ , and GRO- $\gamma$ ), cytokines (IL-6, IL-8, IL-1 $\beta$ ), and growth factors (endothelial cell growth factor and epidermal growth factor) were upregulated by more than 3-fold compared with control cells and were selected for further analyses (Table 2).

### Confirmation of mRNA expression in conjunctival epithelial cells by real-time RT-PCR

As shown in Figure 1, the gene expression of ICAM-1 (from  $16,911.7 \pm 4,914.3$  to  $28,930.9 \pm 14,204.3$  copies/ng of RNA; *P* = .04), IL-6 (from  $1.73 \pm 0.94$  to  $13.40 \pm 11.02$  copies/ng of RNA; *P* = .02), IL-8 (from  $11,411.0 \pm 7,907.8$  to  $73,509.9 \pm 5,172.9$  copies/ng of RNA; *P* = .003), and LARC (from  $9,780.5 \pm 5,084.9$  to  $22,804.4 \pm 11,346.3$  copies/ng of RNA; *P* = .03) increased significantly after 6 hours of incubation with diesel exhaust particles compared with

**Table 2**Transcripts showing increased expression in activated conjunctival cells<sup>a</sup>

| Target         | Fold-increase |
|----------------|---------------|
| ICAM-1         | 1,096         |
| IL-8           | 39            |
| LARC           | 15            |
| RANTES         | 3             |
| GRO- $\alpha$  | 3             |
| GRO- $\beta$   | 3             |
| GRO- $\gamma$  | 2             |
| CCR-5          | 1             |
| NF- $\kappa$ B | 3             |
| IL-6           | 3             |
| IL-1 $\beta$   | 2             |
| ECGF           | 2             |
| EGF            | 2             |

Abbreviations: CCR, chemokine receptor; ECGF, endothelial cell growth factor; EGF, endothelial growth factor; GRO, growth-regulated oncogene; ICAM-1, intercellular adhesion molecule 1; IL, interleukin; LARC, liver and activation-regulated chemokine; NF- $\kappa$ B, nuclear factor- $\kappa$ B; RANTES, regulated on activation, normal T-expressed, and presumably secreted.

<sup>a</sup>The fold-increase was corrected for the expression of glyceraldehyde 3-phosphate dehydrogenase (the expression after 6 hours of incubation divided by expression in the control).

control. By contrast, chemokines, such as RANTES, GRO- $\alpha$ , GRO- $\beta$ , and GRO- $\gamma$ , were not significantly upregulated (data not shown). IL-1 $\beta$  and growth factors were not detected by real-time RT-PCR.

#### Verification of protein production from conjunctival epithelial cells by ELISA

As shown in Figure 2, the protein expression levels of ICAM-1 (from  $4,897.0 \pm 3,727.9$  to  $11,798.0 \pm 4,719.6$  pg/mL;  $P = .02$ ), IL-6 (from  $46.0 \pm 19.4$  to  $505.7 \pm 175.1$  pg/mL;  $P < .001$ ), and IL-8 (from  $11,428.5 \pm 4,007.0$  to  $18,078.4 \pm 5,756.8$  pg/mL;  $P = .009$ ) increased significantly after 24 hours of incubation with diesel exhaust particles compared with control. On the other hand, LARC,

RANTES, GRO- $\alpha$ , GRO- $\beta$ , and GRO- $\gamma$  were not significantly produced (data not shown). The protein expression levels of IL-1 $\beta$  and growth factors were not measured in this experiment.

#### Discussion

There is growing evidence that particulate air pollution increases the incidence of allergy and asthma, in addition to augmenting the severity of asthma and allergic diseases.<sup>14–17</sup> For example, diesel exhaust was reported to increase histamine-induced IL-8 and granulocyte-macrophage colony-stimulating factor production in nasal epithelial cells and endothelial cells.<sup>18</sup> Diesel exhaust was also reported to induce cytokine expression in human bronchial epithelial cells.<sup>19</sup> Therefore, we hypothesized that exposure to diesel exhaust would enhance the expression and production of cytokines, chemokines, and adhesion molecules, which may play key roles in establishing allergic inflammation on the ocular surface. In this study, we investigated the direct effect of diesel exhaust particles on immune responses in allergic conjunctivitis.

Exposing primary cultured human conjunctiva to diesel exhaust stimulated the conjunctiva, similar to that induced by TNF. The chosen diesel exhaust level of 100  $\mu$ g/mL is almost equivalent to the maximum concentration in air in the Japanese environment since 1995.<sup>5,20</sup> We observed that exposure to diesel exhaust increased the expression of cytokines, chemokines, growth factors, and adhesion molecules from the conjunctiva, as detected with a GeneChip array. The increased gene and protein expression of several representative cytokines was verified by real-time RT-PCR and ELISA. GeneChip is a high-density oligonucleotide expression probe array that is designed to measure the absolute levels of more than 10,000 transcripts by using the same set of internal standards. Because competition with another cell type is not required for the GeneChip array,<sup>13</sup> it is possible to compare the expression levels of each transcript among different types of cells. In the current study, the genes coding 1 adhesion molecule, 7 chemokines, 2 cytokines,

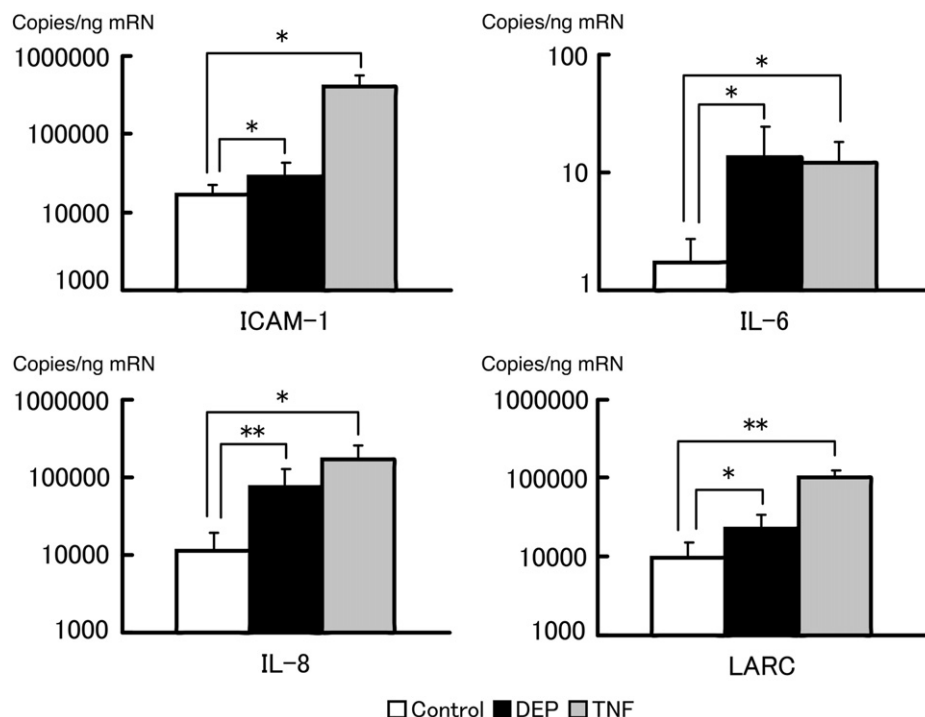


**Figure 1.** Gene expression in conjunctival epithelial cells. Cells were incubated with diesel exhaust particles or tumor necrosis factor for 6 hours. Values are mean  $\pm$  SD of 3 experiments. \* $P < .05$  and \*\* $P < .01$ .

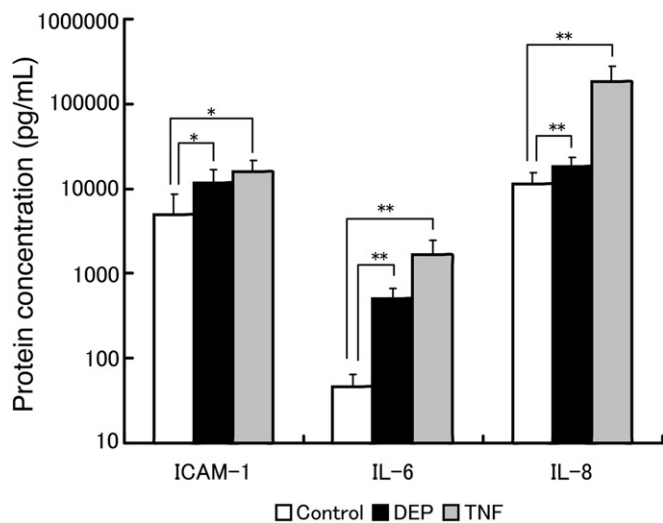


Figure 2. ■ ■ ■

and 2 growth factors were among the 30 genes showing greatest increases in expression in the conjunctival epithelial cells after exposure to diesel exhaust.

These findings indicate the importance of these genes in responses to environmental factors, including diesel exhaust. On the basis of these data, we have focused on specific adhesion molecules, chemokines, cytokines, and growth factors.

Similar increases in expression were observed in the lung. For example, in a previous study, exposure to diesel exhaust increased lung IL-6 and IL-8 expression.<sup>21</sup> Diesel exhaust also promoted angiogenesis/vasculogenesis activity by increasing vascular endothelial growth factor expression.<sup>22</sup> Increases in IFN- $\gamma$ , TNF, and IL-6 in delayed-type hypersensitivity in mice<sup>23</sup> and T<sub>H</sub>2 cell recruitment in nonatopic donors<sup>24</sup> have also been reported. The effects of diesel exhaust on pulmonary hyperresponsiveness, respiratory symptoms, allergic symptoms, and the epithelial tight junction have also been extensively studied.<sup>7,25–30</sup> Furthermore, diesel exhaust was shown to have adjuvant effects in terms of sensitization to common allergens and enhanced or had synergic effects on allergic symptoms.<sup>30–33</sup> The effects of diesel exhaust on cultured human conjunctival epithelia observed here strongly support the role of diesel exhaust as one of the most important air pollutants.

We first studied the effects of exposure to diesel exhaust using a GeneChip array in the model. GeneChip is a powerful tool for genomic analysis. It was used in the first screening to find the differentially regulated genes in conjunctival cells. Because GeneChip assay is a semiquantitative analysis (although it can measure expressions of numerous genes), we confirmed our findings using RT-PCR.

We are the first to confirm the expression of LARC, although expressions of IL-8, IL-6,<sup>34</sup> and GRO<sup>35</sup> have been reported in studies using lung cells, respiratory tract epithelial cells,<sup>36</sup> or cutaneous keratinocytes.<sup>37</sup> Expression of LARC is induced by TNF, as reported in this study, and also by stimulations via NF- $\kappa$ B, which has been reported previously. Therefore, enhancement of LARC expression in the human conjunctiva epithelial cells is reasonable. We also first reported the gene expression in human conjunctiva epithelial cells after exposure to diesel exhaust particles.

Although this model may not be a complete model of human conjunctiva tissue, we believe it is a useful model for in vitro studies. Diebold et al<sup>38</sup> established a cell line of cultured human conjunctiva epithelial cells and reported its characteristics. The crude human conjunctiva tissue includes goblet cells and

fibroblasts, but these cells were not found in the cell line because the cell line did not include MUC5AC, and therefore goblet cells and fibroblasts were not cultured. In our model, it was confirmed that fibroblasts were not detected before starting exposure. As a conclusion, our model includes the cultured conjunctiva epithelial cells without fibroblasts and goblet cells.

Interestingly, because 24 hours after incubation was the optimal time to determine protein production, the RANTES, GRO- $\alpha$ , GRO- $\beta$ , and GRO- $\gamma$  proteins were not detected at 8 or 48 hours, but increases in their protein expressions were apparent at other times. We also determined the mRNA expression levels of Toll-like receptors (TLRs), including TLR-2 and TLR-4, after 6 hours of incubation but found no increases in their expression levels. Therefore, further studies are needed to determine the appropriate incubation times and diesel exhaust concentrations to be able to detect changes in some mRNAs and proteins.

Adhesion molecules, such as ICAM-1, are important components of the inflammatory process. Exposure to diesel exhaust increased ICAM-1 expression in epithelial cells and may cause inflammation. IL-6 is involved in the growth of mast cells by promoting local mast cell growth and may cause allergic inflammation. Chemokines are mostly inducible and are selectively expressed in the conjunctiva. CC chemokines are mostly constitutive and are expressed in the epithelial cells. Chemokines seem to have selective functions with regard to ocular allergic inflammation and general defensive functions against insults, such as infections. LARC was the only CC chemokine detected in the epithelial cells in this study. LARC recruits T cells and dendritic cells via chemokine receptor (CCR) 6, CCR36, and CCR37, whereas RANTES attracts inflammatory cells (eg, eosinophils, basophils, T cells, monocytes, and dendritic cells, and neutrophils) via CCR1, CCR3, CCR4, CCR5, CCR30, and CCR38. Therefore, LARC and RANTES may have general functions unlike other CC chemokines. IL-8 is one of the chemokines produced by lymphoid cells and may recruit inflammatory cells to the ocular surface to establish inflammation, in addition to the activity of LARC.

Sunil et al<sup>21</sup> reported that IL-6 and IL-8 expression was increased in lung tissue exposed to diesel exhaust, whereas Sakai et al<sup>23</sup> reported that IL-6 production was increased in methyl-bovine serum albumin in mice. We speculate that the increased expression of IL-6 and IL-8 in the conjunctiva, as well as upregulation of ICAM-1, may contribute to the initial increase in inflammatory cells on the ocular surface. Increased expression of these proteins has been reported in neutrophils and mast cells and also supports mast cell growth. It is possible that activated neutrophils and mast cells cause a secondary increase in inflammatory cells and that inflammatory mediators induce conjunctival effects by binding to specific receptors on their target cells. These actions initiate downstream signaling pathways to enhance functional activation and ultimately contribute to allergic inflammation of the ocular surface.

Olopatadine is a clinically effective ophthalmic antihistamine drug that significantly decreased anti-immunoglobulin E mast cell supernatant-mediated upregulation of ICAM-1 expression on human conjunctival epithelial cells in vitro.<sup>39</sup> Olopatadine also attenuated histamine-stimulated phosphatidylinositol turnover and the secretion of IL-6 and IL-8 from human conjunctival epithelial cells.<sup>40</sup> Considering that exposure to diesel exhaust stimulates ICAM-1, IL-6, and IL-8 production in cultured conjunctiva epithelial cells and that olopatadine suppresses the production of these proteins in allergic conjunctivitis,<sup>39</sup> we think that antihistamine or other antiallergic agents could be used clinically to prevent or treat allergic inflammation caused by exposure to diesel exhaust.

The findings of the present study support the connection or coexistence of allergic conjunctivitis and dry eye, as recently reported by Hom et al.<sup>41</sup> Many of the elevated inflammatory factors

found in our study were also found in past dry eye studies, particularly IL-6 and IL-8.<sup>42</sup>

The present studies revealed that exposure to diesel exhaust increases cytokine and adhesion molecule expression in the human conjunctiva. Together with the similar results from a recent study on sequential exposure to diesel exhaust, the present results reveal a need to consider the interactions between and cumulative effects of air pollutants. We conclude that diesel exhaust has significant potential for inducing allergic responses, suggesting that avoiding/reducing contact with diesel exhaust and other air pollutants by washing the eyes or wearing goggles is necessary for patients with allergic conjunctivitis. It would be interesting to characterize the relative selectivities or toxic effects of components of diesel exhaust fumes on selective upregulation of cytokines. Further studies are needed to provide more insight into the effects of environmental factors, including viruses, UV light, and other pollutants, on allergic conjunctivitis and their potential synergistic effects.

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