Diesel Exhaust Particles Block Induction of Oral Tolerance in Mice

SHIN YOSHINO, MOTOYASU OHSAWA and MASARU SAGAI

Department of Microbiology, Saga Medical School, Saga 849-8501 (S.Y.); Department of Environmental Toxicology, Faculty of Pharmaceutical Sciences, Teikyo University, Sagamiko, Kanagawa 199-0195 (M.O.) and Research Team for Health Effects of Air Pollutants, National Institute for Environmental Studies, Tsukuba, Ibaraki 305 (M.S.), Japan

Accepted for publication July 2, 1998

ABSTRACT

We investigated the effect of diesel exhaust particles (DEP) on oral tolerance. Oral tolerance was induced by feeding mice with 10 mg of hen egg lysozyme (HEL) daily over a period of 5 days before immunization with the antigen. Varying doses of DEP were orally administered immediately before each feeding of HEL. The results showed that oral administration of HEL significantly suppressed production of anti-HEL IgG, IgG1 and IgG2a antibodies, delayed-type hypersensitivity and proliferative responses of lymph node cells to the antigen. The suppression of these immune responses to HEL by the oral antigen was associated with a marked decrease in secretion of interferon-γ and interleukin-4 from the lymphoid cells. Administration of DEP dose-dependently blocked suppression by oral HEL of antigen-specific IgG, IgG1 and IgG2a antibody production, delayed-type hypersensitivity and lymphoid cell proliferation. The suppression by the fed antigen of secretion of interferon-γ and interleukin-4 was also markedly diminished by the particles. Thus, DEP appear to be effective in blocking induction of oral tolerance.

It is long recognized that oral administration of antigen induces immunological unresponsiveness to the antigen termed oral tolerance (Mowat, 1987; Weiner, 1994). It is thought to contribute to the prevention of food hypersensitivity (Mowat, 1994). Previous studies also indicated that feeding pathogenic antigens was effective in suppressing a variety of autoimmune disorders including experimental autoimmune encephalomyelitis (Higgins and Weiner, 1988), collagen-induced arthritis (Nagler-Anderson, 1986) and experimental autoimmune uveoretinitis (Nussenblatt, 1990). It is long recognized that oral administration of antigen induces immunological unresponsiveness to the antigen termed oral tolerance (Mowat, 1987; Weiner, 1994). It is thought to contribute to the prevention of food hypersensitivity (Mowat, 1994). Previous studies also indicated that feeding pathogenic antigens was effective in suppressing a variety of autoimmune disorders including experimental autoimmune encephalomyelitis (Higgins and Weiner, 1988), collagen-induced arthritis (Nagler-Anderson, 1986) and experimental autoimmune uveoretinitis (Nussenblatt, 1990). Although the exact mechanism underlying induction of oral tolerance still remains obscure, possibilities include deletion (Chen et al., 1995) and anergy (Whitaacre et al., 1991) of antigen-specific lymphocytes and suppression by inhibitory cytokines including transforming growth factor-β and IL-4 secreted from regulatory T cells (Chen et al., 1994; Khoury et al., 1992).

DEP generated by diesel engine-powered cars have been implicated in the incidence of allergic respiratory diseases including asthma (Diaz-Sanchez, 1997; Sagai, 1996). DEP enhance antigen-specific IgE antibody production in serum (Diaz-Sanchez, 1997; Takafuji et al., 1987; Tsien et al., 1997) and increase local and systemic secretion of proinflammatory mediators including oxygen free radicals (Sagai et al., 1993; Kumagai et al., 1997) and of various cytokines such as IL-1, IL-4, IL-5, IL-6, IL-8, IL-10 and IL-13 (Fujimaki et al., 1994; Diaz-Sanchez et al., 1997; Takano et al., 1997). Takafuji et al. (1987) demonstrated that 125I-ovalbumin given to mice through their noses reached not only the lung via the airway but also the gut via the esophagus. We also observed marked deposits of DEP in intestinal tissues after exposure to the airborne particulates (Sagai, unpublished data). These data suggest that DEP might modulate oral tolerance.

We show that oral administration of DEP blocked suppression by feeding HEL of anti-HEL IgG, IgG1 and IgG2a antibody production, DTH and proliferative responses to the antigen in mice. The suppression of secretion of IFN-γ and IL-4 by the oral antigen was also significantly diminished by DEP.

Methods

Animals. Male DBA/1J mice, 8 to 9 wk of age, were used in all experiments. The mice were bred in the animal breeding unit of Saga Medical School, Saga, Japan. They were maintained in a temperature- and light-controlled environment with free access to standard rodent food and water.

Immunization. Mice were immunized s.c. at the base of the tail with 100 μg of HEL (Sigma Chemical Co., St. Louis, MO) dissolved in PBS, phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay.

ADDITIONAL INFORMATION: DEP, diesel exhaust particles; HEL, hen egg lysozyme; DTH, delayed-type hypersensitivity; IFN-γ, interferon-γ; IL-4, interleukin-4; PBS, phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay.

ABBREVIATIONS: DEP, diesel exhaust particles; HEL, hen egg lysozyme; DTH, delayed-type hypersensitivity; IFN-γ, interferon-γ; IL-4, interleukin-4; PBS, phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay.
50 μl of 0.9% NaCl and emulsified with an equal volume of complete Freund’s adjuvant (Difco laboratories, Detroit, MI) (day 0).

Induction of oral tolerance. Oral tolerance was induced by the methods described previously (Yoshino and Ohsawa, 1997). Briefly, mice were fed 10 μg of HEL dissolved in 0.25 ml of 0.9% NaCl through a syringe fitted with an 18-G ballpoint needle on days −5, −4, −3, −2 and −1 before immunization with HEL. As controls, 0.25 ml of 0.9% NaCl and 0.25 ml of 0.9% NaCl containing 10 μg of limulus polyphemus hemocyanin (Sigma) were given orally daily on the above days.

Administration of DEP. DEP were generated by a four-cylinder diesel engine and collected on a glass filter in a constant-volume sampler system as described previously in detail (Sagai et al., 1993). The mean of the diameter of DEP was 0.4 μm, 0.01, 0.1 and 1 mg of DEP suspended in 0.25 ml of PBS containing 0.01% Tween 20 (PBS) were orally administered immediately before each feeding of HEL. A total of 0.25 ml of PBS was given as a control.

Measurement of DTH. On day 12 after immunization, 10 μg of HEL dissolved in 20 μl of PBS were injected s.c. into the right footpad. As a vehicle control, 20 μl of PBS were injected into the left footpad. The thickness of the right and left footpad were measured using a dial gauge caliper calibrated with 0.01-mm graduations (Ozaki MFG, Tokyo, Japan) immediately before and 48 hr after the challenge injection. The increase in left footpad thickness was subtracted from the increase in right footpad thickness to give the value due to the specific response to the antigen. In unsensitized mice, responses to HEL and PBS were essentially equivalent.

Measurement of antibodies to HEL. Blood was collected on day 21 after immunization and sera were heat inactivated at 56°C for 30 min. IgG, IgG1 and IgG2a antibodies to HEL were measured using ELISA (Yoshino, 1998). In brief, 96-well flat-bottomed microtiter plates were incubated with 100 μl/well of HEL (100 μg/ml) at 37°C for 1 hr and washed three times with PBS containing 0.05% Tween 20. The wells were then blocked by incubation with 100 μl of PBS containing 1% ovalbumin (Sigma) at 37°C for 1 hr. After washing, the plates were incubated with 100 μl of a 1:1,000 dilution of each serum sample at 37°C for 30 min. The plates were washed, and 100 μl/well of a 1:10,000 dilution of rat anti-mouse IgG, IgG1 or IgG2a labeled with alkaline phosphatase (PharMingen, San Diego, CA) were added and incubated at 37°C for 1 hr. After washing, 100 μl of 3 mM of p-nitrophenylphosphate (Bio-Rad Laboratories, Hercules, CA) were added per well and the plates were incubated in the dark at room temperature for 15 min. The absorbance was then measured at 405 nm in a Titertec Multiscan spectrophotometer (EFLAB, Helsinki, Finland). The results were expressed as absorbance units at OD 405 ± S.E.M.

Proliferation assay. Mice were killed 14 days after immunization and single cell suspensions were prepared from their inguinal lymph nodes. A total of 5 × 10^6 cells in 100 μl of RPMI 1640 (Flow Laboratories, Inc., McLean, VA) containing 1 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 5 × 10^−5 M 2-mercaptoethanol and 1% heat-inactivated autologous mouse serum were added to each microwell followed by the addition of 100 μl of 25, 50 and 100 μg/ml of HEL. The cells were cultured for 72 hr. Each well was pulsed with 0.5 μCi of tritiated thymidine, and the cells were cultured for another 16 hr. The cultures were harvested onto fiberglass filters using a multiharvester and counted using standard liquid scintillation techniques. Results, expressed in cpm, are the average of quadruplicate cultures of cells pooled from four mice.

Cytokine measurement. Single cell suspensions from inguinal lymph nodes were prepared as described above and 5 × 10^6 cells/ml were cultured in 1-ml aliquots in 24-well tissue culture plates either in medium alone or with 50 μg/ml of HEL. Forty-eight hours later, supernatants were harvested and stored at −70°C until assayed. Secreted TNF-γ and IL-4 was quantified using sandwich ELISA techniques. The ELISA kits for these cytokines were commercially available from Funakoshi Co., Tokyo, Japan.

Results

Effect of DEP on humoral immune responses to HEL in orally tolerized mice

To investigate the effect of DEP on humoral immune responses in orally tolerized animals, mice were fed HEL before immunization with the antigen. As shown in figure 1, feeding HEL was followed by significant suppression of production of anti-HEL IgG antibodies. LPH given orally as a feeding control failed to modulate the antibody production. Oral administration of 0.01, 0.1 and 1 mg of DEP resulted in diminution of the suppression of anti-HEL IgG antibody production by oral HEL in a dose-related fashion. Administration of 1 mg of DEP plus 0.9% NaCl showed a serum level of anti-HEL IgG antibodies similar to that in animals given PBS plus 0.9% NaCl.

Anti-HEL IgG2a and IgG1 antibodies were measured to examine the effects of DEP and HEL given orally on Th1 (Burnstein and Abbas, 1993) and Th2 (Isakson et al., 1982) helper T cell-mediated immune responses, respectively. The results are shown in figure 2. Significantly decreased production of anti-IgG1 antibodies was observed in mice fed HEL with PBS. Oral administration of HEL with DEP dose-dependently blocked the suppression of anti-HEL IgG1 antibody production by the oral antigen. Production of anti-HEL IgG2a antibodies was also decreased by feeding the antigen, although the suppression of IgG2a antibody production by HEL appeared to be much greater than that of anti-HEL IgG1 antibody production. Administration of DEP was followed by marked abrogation of suppression of anti-HEL IgG2a antibody production by the oral antigen.

Effect of DEP on cellular immune responses to HEL in orally tolerized mice. To learn whether DEP modulated cellular immune responses in orally tolerized animals, DTH to HEL was induced in the footpad of mice fed the antigen. As shown in figure 3, footpad DTH to HEL was markedly suppressed by feeding the antigen. Administration of DEP was followed by blockade of suppression of the T cell-mediated responses in a dose-related manner.

Effect of DEP on proliferative responses of lymphoid cells to HEL in orally tolerized mice. The effect of DEP on proliferative responses to HEL in mice fed the antigen was examined in vitro. Feeding HEL resulted in marked suppression of proliferation of lymph node cells to HEL (table 1). Its maximum suppression was 91% when the lymphoid cells...
respectively. Administration of DEP also diminished the decrease in IL-4 production by the oral antigen down to 42, 21 and 13% by 0.01, 0.1 and 1 mg of the particles, respectively.

**Adjuvant effect of DEP on humoral immune responses.** DEP are known to enhance antigen-specific IgE antibody production, indicating that the airborne particles have adjuvant activity (Sagai et al., 1996; Takafuli et al., 1997; Tsien et al., 1997). However, as shown in our experiments, immune responses to HEL in mice administered with DEP plus 0.9% NaCl before immunization with the antigen were similar to those in animals given PBS plus 0.9% NaCl. Therefore, DEP appeared to lack adjuvancity in our experimental system used. Subsequently, further experiments were carried out to seek conditions in which DEP acted as an adjuvant. Mice were given DEP without HEL orally daily on days −5 to −1 before immunization with HEL, days 0 to 5, respectively.

**Effect of DEP on secretion of INF-γ and IL-4 in orally tolerized mice.** INF-γ and IL-4 that are known as Th1 and Th2 cytokines (Diamantstein et al., 1988; Mu and Sewell, 1994), respectively, were also measured to examine whether the blockade by DEP of suppression of immune responses to HEL in orally tolerized mice was associated with Th1 and Th2 type of CD4⁺ T cell responses. Feeding HEL markedly suppressed IFN-γ (93% suppression) as shown in figure 4. Moderate suppression of IL-4 production was observed in the HEL-fed animals (46% suppression). Treatment with DEP diminished the suppression of IFN-γ secretion by HEL fed down to 90, 84 and 62% by 0.01, 0.1 and 1 mg of DEP, respectively. Administration of DEP also diminished the decrease in IL-4 production by the oral antigen down to 42, 21 and 13% by 0.01, 0.1 and 1 mg of the particles, respectively.
days 0 to 10 and days 0 to 20 after immunization with the antigen. The results are shown in Table 2. Again, DEP given on days −5 to −1 failed to affect anti-HEL IgG antibody production. In contrast, administration of DEP on days 0 to 10 as well as on days 0 to 20 significantly enhanced the antibody production.

**Discussion**

Our study shows that DEP blocked suppression by feeding HEL of immune responses to the antigen including IgG, IgG1 and IgG2a antibody production, DTH and proliferative responses, suggesting that the airborne particulates produced by diesel engine-powered cars may be effective in abrogating oral tolerance. DEP have been implicated in the incidence of allergic airway disease (Diaz-Sanchez, 1997; Sagai et al., 1996). However, few studies demonstrated previously that airborne pollutants including DEP affected oral tolerance that was thought to play a role in the prevention of food allergy (Mowat, 1994).

DEP consist of carbon cores containing organic compounds such as polyaromatic hydrocarbons (Schuetzle, 1983; Draper, 1986). DEP carbon cores also contain a trace of heavy metals including iron (Vouk and Piver, 1983). Some of these organic compounds and metals have been shown to be cytotoxic (McClellan, 1987; Handa et al., 1993). Therefore, the blockade of induction of oral tolerance by DEP may be due to cytotoxic effects of the airborne particulates on immune cells such as lymphocytes and macrophages responsible for induction of tolerance. However, this does not appear to be the main reason for the blocking effect of DEP on oral tolerance because mice given DEP plus PBS had antigen-specific antibody production, DTH responses and proliferative responses similar to those given PBS plus 0.9% NaCl. DEP were orally administered at 0.01, 0.1 and 1 mg for a period of 5 days before immunization with HEL. However, the doses of DEP and the administration period do not appear to affect the immune system considerably.

DEP are also demonstrated to have adjuvant activity because the airborne particulates can enhance antigen-specific IgE antibody production (Diaz-Sanchez, 1997; Takafuli, 1987; Tsien et al., 1997). Therefore, the prevention of oral tolerance by DEP might have associated with their capacity as an adjuvant. However, this is also unlikely because DEP given before immunization with HEL themselves failed to affect immune responses to HEL. DEP acted significantly as an adjuvant when they were administered daily at least for a period of 10 days after immunization with HEL.

**TABLE 2**

Enhancement of anti-HEL IgG antibody production by DEP administered after immunization with HEL.

<table>
<thead>
<tr>
<th>Duration</th>
<th>PBS</th>
<th>DEP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Days 0–5</td>
<td>0.53 ± 0.06</td>
<td>0.53 ± 0.06</td>
</tr>
<tr>
<td>Days 0–10</td>
<td>0.62 ± 0.07</td>
<td>0.62 ± 0.07</td>
</tr>
<tr>
<td>Days 0–20</td>
<td>0.88 ± 0.07</td>
<td>1.22 ± 0.09</td>
</tr>
</tbody>
</table>

Daily mice were given PBS or 1 mg of DEP orally on the indicated days before or after immunization with HEL on day 0. Serum samples were collected on day 21 and assayed for anti-HEL IgG antibodies by ELISA as described in “Materials and Methods.” Values are shown as mean ± S.E.M. of five mice.

DEP are known to facilitate secretion of Th2 cytokines including IL-4 (Fujimaki et al., 1994; Diaz-Sanchez et al., 1997) that is dependent on Th2 helper T cells (Isaekson et al., 1982). We recently found that the airborne pollutants also increased IFN-γ production (Yoshino, unpublished data) that is Th1 cell-dependent (Diamantstein et al., 1988). In our study DEP blocked suppression of secretion of both IL-4 and IFN-γ by feeding HEL. Because IFN-γ has been shown to play a role in both IgG2a antibody production (Boom et al., 1988) and DTH (Fong and Mosmann, 1989), the blockade by DEP of suppression of IFN-γ secretion seen in HEL-fed mice might have led to the enhancement of production of anti-HEL IgG2a antibodies as well as DTH to HEL in the fed animals. IL-4 secretion is shown to play a role in IgG1 antibody production (Estes et al., 1995). Therefore, the blocking effect of DEP on suppression of IL-4 secretion by oral HEL might have resulted in an increase in the level of anti-HEL IgG1 antibodies in mice fed the antigen. Taken together, the ability of DEP to enhance secretion of these cytokines might have at least in part contributed to the blockade of induction of oral tolerance by the airborne particulates.

Although the exact mechanism by which oral tolerance is induced is not defined well, possibilities include deletion (Chen et al., 1995) and anergy (Whitacre et al., 1991) of antigen-specific lymphocytes, and suppression by inhibitory cytokines including IL-4 secreted from regulatory T cells (Khouri et al., 1992; Chen et al., 1994), depending on the dosage and the nature of antigen fed, and the frequency of antigen administration (Gregerson et al., 1993; Friedman and Weiner, 1994; Garside et al., 1995). For instance, Friedman and Weiner (1994) demonstrated that low doses (less than 1 mg) of oral antigen upregulate secretion of inhibitory cytokines including IL-4 involving in active suppression, although high doses (more than 5 mg) appeared to induce anergy. However, Garside et al. (1995) showed that feeding 25 mg of ovalbumin reduced production of Th2 cytokines including IL-4 as well as Th1 cytokines such as IFN-γ. Similar results were seen in our previous (Yoshino and Ohswara, 1997) and also in our studies in which 10 mg of HEL were orally administered. Furthermore, Melamed et al. (1996) reported that continuous feeding of ovalbumin decreased secretion of inhibitory cytokines including IL-4. This, thus, feeding large amounts of antigen appears to suppress IL-4 secretion although this cytokine appears to be augmented by low doses of antigen given orally.

Epidemiological studies provided indirect evidence for an increased incidence of asthma and atopy linked to airborne pollution (Rusznak et al., 1994). There is also evidence that DEP cause asthma like symptoms in mice (Sagai et al., 1996) It is of note that 6% of patients with asthma and 5 to 6% of atopic dermatitis patients are associated with food allergy (Sabbah et al., 1997). A significant amount of DEP was observed not only in the lung but also in the gut after exposure to the airborne particulates (Takafuli, 1987). These findings and our data showing the blockade of induction of oral tolerance by DEP suggest that the diesel engine-derived particulates may act as one of external substances that play a role in induction of food allergy in humans.

**References**
