Enhancement of Collagen-Induced Arthritis in Mice by Diesel Exhaust Particles

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ABSTRACT
The present study was undertaken to investigate the effect of diesel exhaust particles (DEP) on collagen-induced arthritis (CIA), which is an experimental model of autoimmune disease, in mice. CIA was induced by s.c. injection of type II collagen (CII) emulsified with complete Freund’s adjuvant into the base of the tail (day 0) followed by a booster injection on day 21. Varying doses of DEP were intranasally administered every 2 days from days 0 to 20. The results showed that administration of DEP enhanced both the incidence and the severity of CIA. The enhancement of the disease was associated with pronounced production of anti-CII IgG and IgG2a antibodies. Treatment with DEP also augmented proliferative responses of spleen cells to CII. There was marked secretion of interferon-γ, interleukin (IL)-2, and IL-4 from the lymphoid cells in DEP-treated mice. Administration of DEP after onset of CIA was also effective in enhancing the severity of the disease as well as production of anti-CII IgG and IgG2a antibodies and secretion of interferon-γ, IL-2, and IL-4. These results suggest that exposure to DEP may influence autoimmune disease.

Materials and Methods

Animals. Male DBA/1J mice, 8 to 9 weeks 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 chow and water.

Induction of CIA. To induce CIA, 1 mg of CII extracted from native calf articular cartilage (Funakoshi Co., Tokyo, Japan) was dissolved in 1 ml of 0.1 N acetic acid and emulsified with an equal volume of complete Freund’s adjuvant (CFA; Difco Laboratories, Detroit, MI) and administered s.c. into the base of the tail of the mice. The antibody responses were determined by ELISA. The results showed that administration of DEP enhanced both the incidence and the severity of CIA.
Detroit, MI; Yoshino, 1998a). Fifty microliters of the emulsion containing 25 μg of CII was injected s.c. into the base of the tail (day 0). Twenty-one days later, the animals were given a booster injection of the same amount of the emulsion at the same site. To evaluate the severity of arthritis, the lesions of the four paws were each graded from 0 to 3 according to the increasing extent of erythema and edema of the periarticular tissue as described elsewhere (Yoshino and Cleland, 1992). The maximum possible score is 12.

**Administration of DEP.** DEP were generated by a diesel engine as described previously in detail (Sagai et al., 1993). The mean of the diameter of DEP was 0.4 μm. DEP (0.1, 0.3, and 1 mg/ml) suspended in 50 μl of PBS containing 0.01% Tween 20 were intranasally administered under anesthesia with sodium pentobarbital (Nacalai Tesque, Inc., Kyoto, Japan) every 2 days over a period of 20 days commencing on the day of immunization with CII. Fifty microliters of PBS alone were given as a control. In some experiments, the effect of DEP on existing CIA was examined. For this study, PBS and DEP were given daily intranasally to mice with CIA from days 31 to 45 after immunization with CII, and the severity of arthritis was determined on day 50. The mean joint scores of PBS and DEP treatment groups were between 2.12 and 2.17 on day 31 and there was no significant difference between the groups.

**Histology.** Mice were sacrificed on days 24 and 55 and hindpaws were amputated. The hindpaws were fixed in 4% formalin, sectioned at 4 μm, and stained with hematoxylin and eosin.

**Measurement of Antibodies to CII.** Blood was collected on day 25 after immunization with CII and sera were heat-inactivated at 56°C for 30 min. Anti-CII IgG and IgG2a antibodies were measured using an enzyme-linked immunosorbent assay (ELISA; Yoshino and Ohasawa, 1997). In brief, 96-well flat bottomed microtiter plates were incubated with 100 μl/well of CII (100 μg/ml) at 37°C for 1 h 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 h. After washing, the plates were incubated with 100 μl of a 1:600 dilution of each serum sample at 37°C for 30 min. The plates were washed, and 100 μl/well of a 1:1000 dilution of rat antimouse IgG or IgG2a labeled with alkaline phosphatase (Pharmingen, San Diego, CA) was added and incubated at 37°C for 1 h. After washing, 100 μl of 3 mM p-nitrophenylphosphate (Bio-Rad Laboratories, Hercules, CA) was 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 Titertek Multi-Scan spectrophotometer (EFLAB, Helsinki, Finland). The results were expressed as absorbance units at A405 ± S.E.M.

**Proliferation Assay.** Spleens were removed on day 25 after immunization with CII and cell suspensions prepared. Erythrocytes in the cells were lysed with Tris-NaCl. A total of 5 × 10^5 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 μM 2-mercaptoethanol, and 1% heat-inactivated autologous mouse serum were added to each microwell followed by the addition of 100 μl of 1:1000 dilution of each serum sample at 37°C for 24 h. The cells were then washed three times with PBS containing 0.05% Tween 20. The wells were incubated with 100 μl containing 25 μCi of tritiated thymidine. The cells were stimulated with CII (Yoshino, 1998b). Forty-eight hours later, supernatants were harvested and stored at −70°C until assayed. Cytokine production was quantified by ELISA. The ELISA kits for interferon (IFN)-γ, IL-2, and IL-4 were commercially available from Funakoshi Co. (Tokyo, Japan).

### Results

**Effect of DEP on CIA.** Two of 12 mice treated with PBS from days 0 to 20 showed signs of arthritis on day 27 (17% incidence; Table 1). The maximum incidence of joint inflammation in PBS-treated mice was 42% on day 40. When mice were treated with 0.1, 0.3, and 1 mg/ml of DEP, the incidence of arthritis increased in a dose-related fashion. All of the animals treated with 1 mg/ml DEP developed joint swelling by day 35. The effect of DEP on the severity of CIA was also examined. As shown in Fig. 1, treatment with 0.1 to 1 mg/ml of DEP was followed by augmentation of joint inflammation dose dependently. There was a significant increase in arthritis in mice treated with 0.3 and 1 mg/ml of DEP on days 27 to 55 and days 24 to 55, respectively. Normal non-CIA mice treated with 1 mg/ml of DEP developed no joint inflammation at least up to day 55 (data not shown).

**Effect of DEP on Histologic Changes in Joints.** Histologic evaluation of joints of mice treated with DEP was performed on days 24 and 55. On day 24, in mice treated with 1 mg of DEP there were marked edema of synovium and cell infiltrate in which neutrophils predominated (Fig. 2B), whereas no histologic changes were observed in PBS-treated mice (Fig. 2A). On day 55, DEP-treated animals had severely destroyed cartilage and subchondral bone accompanied by a large number of inflammatory cells (Fig. 2D). No such severe destruction of joints was seen in PBS-treated mice, although there was moderate erosion of cartilage in the control animals (Fig. 2C).

**Effect of DEP on Production of Anti-CII Antibodies.** To investigate the mechanism underlying the augmentation of CIA by DEP, anti-CII IgG antibodies and their isotype IgG2a that play a critical role in the disease (Stuart et al., 1982a; Hirofuji et al., 1985) were measured. The results are shown in Fig. 3. Treatment with 0.3 and 1 mg/ml of DEP was effective in increasing significantly anti-CII IgG antibody production. One milligram per milliliter DEP also significantly enhanced the level of anti-CII IgG2a antibodies.

**Effect of DEP on Proliferative Responses of Lymphoid Cells to CII.** The effect of DEP on proliferative responses of lymphoid cells to CII was investigated to learn whether peripheral immune cells were affected after exposure to the airborne pollutants. As shown in Table 2, treat-

<table>
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<th>Days after Immunization</th>
<th>0 (PBS)</th>
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<th>1 mg/ml</th>
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<td>2/12 (17)</td>
<td>3/12 (25)</td>
<td>8/12 (42)</td>
<td>8/12 (67)*</td>
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<td>6/12 (50)</td>
<td>8/12 (67)</td>
<td>10/12 (83)*</td>
</tr>
<tr>
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<td>5/12 (42)</td>
<td>7/12 (58)</td>
<td>8/12 (67)</td>
<td>12/12 (100)*</td>
</tr>
<tr>
<td>40</td>
<td>5/12 (42)</td>
<td>7/12 (58)</td>
<td>9/12 (75)*</td>
<td>12/12 (100)*</td>
</tr>
</tbody>
</table>

*Number of mice with arthritis/total number of mice used (% of incidence). Data are representative of three experiments.

*p < .05 versus PBS alone, χ-square test.

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**TABLE 1**

Effect of DEP on the incidence of collagen-induced arthritis in mice To induce CIA, mice were immunized with CII on day 0 and boosted on day 21 as described in Materials and Methods. Fifty microliters of PBS alone or 50 μl of PBS containing 0.1, 0.3, and 1 mg/ml of DEP were intranasally administered every 2 days from days 0 to 20. The incidence of arthritis was examined on the indicated days.
ment with DEP increased proliferation of spleen cells to the cartilage component. The increase rates of proliferative responses to 12.5, 25, and 50 μg/ml of CII in mice treated with 1 mg/ml of DEP were 85, 126, and 131%, respectively.

**Effect of DEP on Secretion of Cytokines.** To examine whether the enhancement of CIA by DEP was associated with secretion of cytokines, the Th1 cytokines IFN-γ and IL-2, and the Th2 cytokine IL-4 produced by spleen cells were measured. The results are shown in Fig. 4. Treatment with DEP was followed by a dose-dependent increase in all of the above Th1 and Th2 cytokines. The increase rates of IFN-γ, IL-2, and IL-4 in the animals given 1 mg/ml of DEP were 86, 68, and 65%, respectively.

**Comparison of DEP Effects on Cytokine Secretion between Mice Immunized with CII Alone and CII Plus CFA.** Previous studies demonstrated that DEP exposure increased the secretion of Th2 cytokines including IL-4, but not Th1 cytokines including IFN-γ (Fujimaki et al., 1995; Diaz-Sanchez et al., 1997). However, in those studies adjuvants including CFA were not used. Because CFA was used as an adjuvant in our experiments in which both Th1 and Th2 cytokines were markedly produced by DEP, studies on cytokine secretion in mice immunized with CII alone and CII plus CFA were performed. As shown in Table 3, when the animals were immunized with CII alone, the level of IL-4 was increased more than three times, whereas IFN-γ and IL-2 levels were unchanged. Exposure to DEP facilitated IL-4 secretion up approximately 2-fold. However, IFN-γ and IL-2 production was not affected by DEP treatment. Immunization with CII plus CFA markedly enhanced the secretion of IFN-γ and IL-2 as well as IL-2 and IL-4. Treatment with DEP was followed by greater enhancement of the production of both the Th1 and Th2 cytokines.

**Effect of DEP on Existing CIA.** The effect of DEP on existing CIA was also investigated. When mice with CIA were treated daily with DEP over a period of 15 days commencing on day 31, significantly enhanced arthritis was observed on day 50 (Table 4). The enhancement of joint inflam-
The present study demonstrates that DEP may play a role in autoimmune disease because the intranasal exposure to the airborne pollutants was followed by an increase in the incidence and severity of CIA in mice that was induced by immunization with the major cartilage component CII (Courtenay et al., 1980). DEP consist of carbon cores absorbing a wide variety of organic compounds including polyaromatic hydrocarbons, nitroaromatic hydrocarbons, heterocyclics, quinones, aldehydes, and aliphatic hydrocarbons (Schuetzle, 1983; Draper, 1986). The particles derived from diesel engine-powered cars also absorb a trace of heavy metals such as iron, copper, chromium, and nickel produced during the exhaust (Vouk and Piver, 1983). Some of these organic compounds and metals produced are cytotoxic and carcinogenic (Handa et al., 1983; McClellan, 1987). Accordingly, DEP were also demonstrated to be involved in allergic rhinitis (Diaz-Sanchez, 1994, 1997) and asthma-like symptoms (Sagai et al., 1996; Takano et al., 1997) because the airborne particulates enhanced antigen-specific IgE antibody production (Takafuji et al., 1987; Diaz-Sanchez, 1997; Tsien et al., 1997). However, it was not previously examined whether DEP affect autoimmune disease. To our knowledge, this is the first report of the modulation of autoimmune disease by DEP.

Increases in serum anti-CII IgG antibodies as well as the isotype IgG2a were observed in DEP-treated mice. These findings suggest that the enhancement of CIA by DEP may be due to the augmentation of humoral immune responses specific for the antigen by the airborne pollutants because a pivotal role for anti-CII antibodies in CIA was previously shown. For instance, CIA is passively transferred to naive recipients with sera from animals with the disease (Stuart et al., 1982a). In addition, injection of affinity-purified anti-CII IgG and IgG2a antibodies induces arthritis (Stuart et al., 1982a; Hirofujii et al., 1985).

Although enhanced production of serum anti-CII antibodies in mice exposed intranasally to DEP suggests that the airborne particles stimulate systemic immune systems, increases in the Th1 cytokines IFN-γ and IL-2 secreted from spleen cells and proliferative responses of the lymphoid cells to CII after DEP exposure also suggest their ability to stimulate immune responses systemically. Increased secretion of IFN-γ appears to have contributed to the increase in anti-CII IgG2a antibodies because this cytokine is an important mediator involved in IgG2a antibody production (Snapper and Paul, 1987). The enhanced secretion of IL-2, which plays a role in T cell activation, appears to support the result showing increased proliferation of spleen cells to CII seen in DEP-treated mice. Thus, increased secretion of IFN-γ and IL-2 might have contributed to the enhancement of CIA by the airborne particulates.

Unlike our results showing the enhancement of IFN-γ and IL-2 secretion by DEP, previous studies failed to demonstrate such effects of the airborne particles on Th1 cytokine secretion (Fujimaki et al., 1995; Diaz-Sanchez et al., 1997). This discrepancy appears to be due to the difference in the use of CFA as an adjuvant between the previous and present studies. Adjuvants including CFA were not used in the previous studies in which DEP enhanced allergic airway hypersensitivity, where CFA was used as an adjuvant to induce CIA in the present study. Unchanged levels of IFN-γ and IL-2 were also observed in DEP-treated mice in our experiments when the animals were immunized with CII alone as shown in Table 3, although immunization with CII without CFA itself failed to modulate production of the Th1 cytokines.

The level of the Th2 cytokine IL-4, which plays an impor-
IFN-γ mg/ml DEP were intranasally administered daily from days 31 to 45. The severity of CIA, production of serum anti-CII IgG and IgG2a antibodies, and in vitro secretion of Th1 and Th2 cytokines with 50 nmol of DEP act as adjuvants when given after immunization with anti-CII IgG and IgG2a antibodies. The augmentation of existing joint inflammation by DEP was associated with pronounced production of anti-CII IgG and IgG2a antibodies as well as IFN-γ, IL-2, and IL-4, suggesting that the enhancement of existing CIA in DEP-treated mice was also due to the ability of the airborne particulates to stimulate ongoing antigen-specific immune responses.

As shown in the present study, administration of DEP after the onset of CIA was followed by more severe joint inflammation. The augmentation of existing joint inflammation by DEP was associated with pronounced production of anti-CII IgG and IgG2a antibodies as well as IFN-γ, IL-2, and IL-4, suggesting that the enhancement of existing CIA in DEP-treated mice was also due to the ability of the airborne particulates to stimulate ongoing antigen-specific immune responses.

Alternatively, the increase in the severity of CIA in DEP-treated mice may be in part explained by activation of complement pathways that are important in the induction of CIA (Morgan et al., 1981) because Kanemitsu et al. (1998) demonstrated that DEP activated complement pathways. Furthermore, there is a possibility that proinflammatory mediators were involved in the enhanced joint inflammation in DEP-treated mice. For instance, DEP were shown to produce oxygen-free radicals (Sagai et al., 1993; Kumagai et al., 1997) and IL-1 (Yang et al., 1993; Kumagai et al., 1997) in vitro as well as in vivo.

Our recent studies showed that exposure to DEP before immunization with hen egg lysozyme blocked induction of oral tolerance to the antigen in mice (Yoshino et al., 1998). The blockade of oral tolerance by DEP appeared to be due to the abrogation by the airborne particulates of suppression of Th1 and Th2 cytokine secretion by oral administration of hen egg lysozyme, but not to the ability of DEP to enhance antigen-specific immune response, because there were no differences in immune responses to hen egg lysozyme between DEP- and PBS-treated mice when the air pollutants were given before immunization with the antigen. DEP appear to act as adjuvants when given after immunization with antigen as seen in the present study.

As shown in our experiments, mice were repeatedly treated with 50 μl of 0.3 and 1 mg/ml of DEP over a period of 20 days; this significantly increased the severity of CIA in mice. However, the amount of DEP exposure in the animals does not appear to be far from that in humans because Peterson and Saxson (1997) reported that 0.3 mg of DEP was equivalent to total exposure on 1 to 3 average days in Los Angeles.

Many features of CIA resemble those of human rheumatoid arthritis (RA) (Trentham, 1982; Stuart et al., 1982b). Although a key inducing agent in RA has not been identified, there are a number of studies demonstrating that autoimmunity is involved in the pathogenesis of the joint disease. For instance, elevated T cell reactivity and pronounced production of antibodies to autoantigens including CII are observed in RA (Stuart et al., 1983; Clague and Moore, 1984). Therefore, the enhancement of CIA by DEP suggests that RA might be also affected by exposure to the airborne pollutants.

### References

- Courtenay JS, Dallman MJ, Dayan AD, Martin A and Mosedale B (1980) Immuni-
- Handa T, Yamauchi T, Ohnishi M, Hisamatsu Y and Ishii T (1983) Detection and average content levels of carcinogenic and mutagenic compounds from the partic-

### Table 4

<table>
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<tr>
<th>DEP</th>
<th>Mean Joint Score</th>
<th>Anti-CII IgG (A405)</th>
<th>Anti-CII IgG2α (A405)</th>
<th>IFN-γ nmol</th>
</tr>
</thead>
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<td>0 (PBS)</td>
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<td>0.40 ± 0.03</td>
<td>227 ± 13.3</td>
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<td>0.3</td>
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<td>0.71 ± 0.06</td>
<td>0.44 ± 0.04</td>
<td>243 ± 22.3</td>
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<td>1</td>
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<td>1.08 ± 0.09*</td>
<td>0.67 ± 0.04*</td>
<td>365 ± 20.9*</td>
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<td>6.5 ± 0.6*</td>
<td>1.52 ± 0.12*</td>
<td>0.83 ± 0.06*</td>
<td>465 ± 36.5*</td>
<td></td>
</tr>
</tbody>
</table>

*p < .05 versus PBS alone, Mann-Whitney analysis.

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