

Adjuvant activity of diesel-exhaust particulates for the production of IgE antibody in mice

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The prevalence rate of allergic rhinitis caused by pollen has strikingly increased in Japan in the last three decades. The number of diesel cars in use has also rapidly increased in the country. This fact urged us to study the effects of particulates emitted from diesel cars on the production of IgE antibody. The primary IgE antibody responses in mice immunized with intraperitoneal injection of ovalbumin (OA) mixed with diesel-exhaust particulates (DEP) were higher than those in the animals immunized with OA alone. This effect of DEP on the production of IgE antibody in mice was also demonstrated when mice were immunized with repeated injections of dinitrophenylated-OA. In addition, persistent IgE-antibody response to major allergen of Japanese cedar pollen (JCPA), a most common pollen causing allergic rhinitis in Japan, was observed in mice immunized with JCPA mixed with DEP but not in the animals immunized with JCPA alone. The results do indicate that the adjuvant activity of DEP can not be excluded as a possible cause of the associated change in the number of diesel cars and allergic rhinitis caused by pollen in Japan. (J ALLERGY CLIN IMMUNOL 77:616-23, 1986.)

In recent years ARP has been the most common allergic disease in Japan as it has been in the United States. More than 20 different kinds of pollens have been demonstrated to cause the disease. AR caused by Japanese cedar pollen is the most prevalent.¹

Before 1950, however, ARP was extremely rare in Japan. It would be unreasonable to consider that grasses and trees and their airborne pollens increased dramatically in number recently to account for the striking increase in ARP patients in this country.

Since 1953, Kaneko et al.² have conducted a series of screening mass surveys of AR in school children aged 6 to 17 years in both rural and urban districts in Japan. They found that the prevalence rate of AR among school children in districts polluted by auto-

Abbreviations used

AR:	Allergic rhinitis
ARP:	Allergic rhinitis caused by pollens
DEP:	Diesel-exhaust particulates
OA:	Ovalbumin
KLH:	Keyhole limpet hemocyanin
JCPA:	Major allergen of Japanese cedar pollen
DNP-OA:	Dinitrophenylated-ovalbumin
HA antibody:	Hemagglutinating antibody
BBS:	Borate-buffered saline
PCA:	Passive cutaneous anaphylaxis

mobile exhaust was markedly higher compared to that in nonpolluted districts. The prevalence rate of AR among the children in some polluted districts reached 33.3% in 1980.

The number of diesel cars in use has rapidly increased in Japan from 14,897 in 1951 to 5,151,625 in 1983.^{3, 4} The area of Japan is approximately 370,000 square kilometers (143,706 square miles), only 25% of which is occupied by open fields. Automobile exhaust is one of the major causes of current air pollution in urban districts in Japan where its potential influence on the health of the inhabitants has presented many problems. A striking increase in the

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prevalence rate of ARP in air-polluted districts in Japan has coincided with the rapid increase in the number of diesel cars in use. This fact attracted our attention to automobile exhaust as one of the possible causes of ARP.

Automobile exhaust contains particulates as well as gaseous emissions. DEP from diesel cars weigh 20 to 100 times more than those particulates discharged from catalyst-equipped gasoline-powered cars of corresponding performance, although both types of vehicles emit similar amounts of nitrogen oxides and other gases.⁵⁻⁷

In experiments with mice, we have found that the DEP collected from exhaust of light-duty diesel cars have an enhancing effect on the IgE antibody production against not only OA but also allergenic substances isolated from Japanese cedar pollens.

MATERIAL AND METHODS

Mice

Female BDF₁ mice were obtained from Charles River Co., Atsugi, Japan. They were 8 to 10 weeks of age when they were first immunized.

Antigens

OA (six times crystalized) was purchased from Seikagaku Kogyo Co., Ltd., Tokyo, Japan. KLH was obtained from Calbiochem, La Jolla, Calif. DNP-OA or DNP-KLH was prepared by a method described by Ishizaka and Okudaira.⁸ The number of DNP groups coupled to one protein molecule were calculated to be 12 for OA and 18 for KLH.

JCPA was isolated from the pollens of the Japanese cedar (*Cryptomeria japonica*) by the method described by Yasueda et al.⁹ with slight modifications. Allergenic activity of JCPA was measured by quantitative intradermal skin test. Minimal concentrations of JCPA solution to elicit a positive reaction in five subjects sensitive to Japanese cedar pollen were 10^{-5} , 10^{-4} , 10^{-4} , 10^{-4} , and 10^{-5} μ g of protein per milliliter, respectively.

DEP

A 1980 Nissan Cedric with 4-cycle engine (displacement 2792 ml; type LD-28), a light-duty diesel-powered passenger car, was used to collect DEP. The engine was mounted on a chassis dynamometer to simulate loaded operation. Exhaust-particulate materials were collected into a cyclone equipped in the dilution tunnel constant-volume sampler system with loads ranging from 20 to 80 km/hr.¹⁰

The materials collected were examined by a scanning electron microscope (TEOL TSM-50A, Nihon-denshi, Tokyo, Japan) with an acceleration voltage at 25 kV. As illustrated in Fig. 1, the particulates have a rough surface. A part of the particulates appeared to be aggregated. The size distribution of the particulates was measured microscopically. Thirty-nine percent of the particulates was $<1 \mu$ m in diameter, 13% was 1 to 2 μ m, 13% was 3 to 5 μ m, and the other 35% was $>6 \mu$ m.



FIG. 1. Scanning electron micrograph of particulates emitted from diesel engine (original magnification $\times 30,000$). The samples were dusted onto double-sided sellotapes and coated with platinum films. The acceleration voltage was 25 kV.

Preparation of antigen-DEP mixture

A half volume of antigen solution in pH 8.0 of BBS was added dropwise to a 10 mg/ml suspension of DEP in BBS and stirred at room temperature for 15 minutes. The mixture of antigen and Al(OH)₃ gel (alum) was prepared in the same way.

Immunization

Twenty-two groups of mice were immunized following the schedule presented in Table I. Three groups of mice were injected intraperitoneally with 1 μ g of OA without adjuvant or mixed with alum or with DEP in 0.3 ml of BBS. The dose of alum or DEP was 2 mg per mouse. Three groups of mice were immunized with 10 μ g of OA without adjuvant or mixed with alum or DEP. In the same way, six groups of mice were immunized with 100 μ g or 1000 μ g of OA either alone or with alum or DEP. The animals were bled from the retroorbital plexus 7, 10, 14, 28, and 56 days after the first immunization. Sera obtained from mice in each group were pooled and stored at -75° C until the titration of antibodies.

The other four groups of mice were intraperitoneally injected with 1.0 μ g of DNP-OA without adjuvant or mixed with each one of three different doses of DEP, i.e., 20 μ g, 200 μ g, and 2 mg per mouse, respectively. The animals were boosted 4, 7, 10, and 13 weeks after the first immunization with the same doses of the antigen or the antigen-

TABLE I. Immunization schedule

Group of mice*	Antigen	Doses of antigen injected (μ g)	Adjuvant	Doses of adjuvant injected	Times of injection	Interval of injection (wk)
I	OA	1.0	—	—	1	—
II	OA	1.0	Al (OH) ₃	2 mg	1	—
III	OA	1.0	DEP†	2 mg	1	—
IV	OA	10	—	—	1	—
V	OA	10	Al (OH) ₃	2 mg	1	—
VI	OA	10	DEP	2 mg	1	—
VII	OA	100	—	—	1	—
VIII	OA	100	Al (OH) ₃	2 mg	1	—
IX	OA	100	DEP	2 mg	1	—
X	OA	1000	—	—	1	—
XI	OA	1000	Al (OH) ₃	2 mg	1	—
XII	OA	1000	DEP	2 mg	1	—
XIII	DNP-OA	1.0	—	—	5	3 or 4
XIV	DNP-OA	1.0	DEP	20 μ g	5	3 or 4
XV	DNP-OA	1.0	DEP	200 μ g	5	3 or 4
XVI	DNP-OA	1.0	DEP	2 mg	5	3 or 4
XVII	JCPA‡	1.0	—	—	5	4
XVIII	JCPA	1.0	Al (OH) ₃	2 mg	5	4
XIX	JCPA	1.0	DEP	2 mg	5	4
XX	JCPA	10	—	—	5	4
XXI	JCPA	10	Al (OH) ₃	2 mg	5	4
XXII	JCPA	10	DEP	2 mg	5	4

*Each group consisted of five mice.

†Diesel-exhaust particulates.

‡A major allergen of Japanese cedar pollen.

DEP mixture as the first immunization. Mice in each group were bled 2, 4, 5, 6, 8, 11, 13, 14, 15, 17, and 21 weeks after the first immunization.

Three other groups of mice were injected intraperitoneally with 1.0 μ g of JCPA without adjuvant or mixed with alum or with DEP. In the same way, three groups of mice were immunized with 10 μ g of JCPA either alone or with alum or DEP. They were boosted 4, 8, 12, and 16 weeks after the first immunization with the same doses of the antigen or the antigen with alum or with DEP. Mice in each group were bled 2, 4, 5, 6, 8, 9, 12, 13, 14, 16, 17, and 24 weeks after the first immunization. Sera obtained from mice in each group were pooled and stored at -75° C.

Measurement of antibodies

IgE antibodies were measured by the PCA assay in rats.¹¹ One tenth milliliter of serial twofold dilutions of the test sera was injected intracutaneously in the dorsum of 30-week-old Sprague-Dawley rats (Charles River Co.). Twenty-four hours after the passive sensitization, the rats were challenged intravenously with the antigens dissolved in 0.5 ml of saline and 0.5 ml of 2% Evan's blue dye. Doses of antigens used for the challenge were 4 mg for OA, 4 mg for DNP-KLH, and 40 μ g for JCPA. The test was carried out in duplicate, and the PCA titer was expressed as the

geometric means of the reciprocal of the maximal dilutions that elicited the reaction of 5 mm in diameter. HA antibody was detected in microtiter plates by the agglutination of antigen-coated erythrocytes prepared with glutaraldehyde.¹²

Examination of the capacity of DEP to adsorb soluble antigens

Five hundred micrograms of OA in 0.5 ml of BBS was added to 1.0 ml of DEP suspension (10 mg of DEP per 1.0 ml of BBS). After stirring for 1 minute, the preparation was centrifuged at $1500 \times g$ for 5 minutes. The amount of OA adsorbed to DEP was estimated from the protein concentration in the supernatant. The protein concentration was measured according to the method of Lowry.¹³ Similarly, 10 μ g of JCPA was mixed with 10 mg of DEP in 1.5 ml of BBS. After stirring for 1 minute, the preparation was centrifuged. The supernatant aspirated was filtered through a filter (Millex-GV, 0.22 μ m filter unit) to obtain supernatant I. The same procedure was carried out for the JCPA solution containing no DEP (10 μ g of JCPA per 1.5 ml of BBS) to obtain supernatant II.

The capacity of DEP to adsorb JCPA was estimated comparing the allergenic activities of the supernatant I and supernatant II. Allergenic activity of the supernatants was

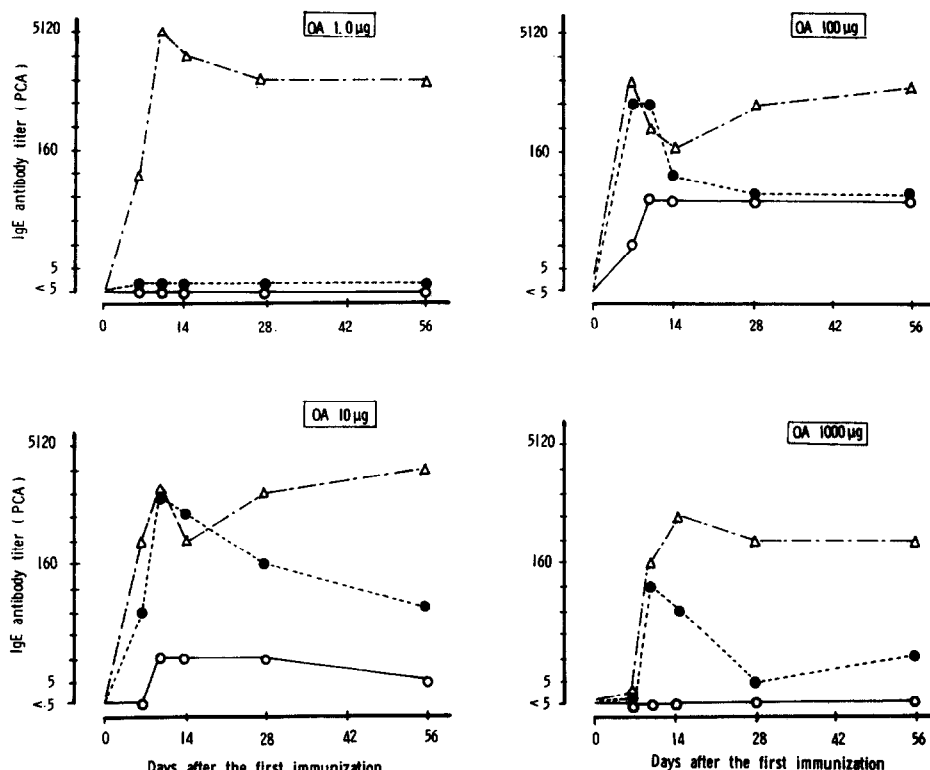


FIG. 2. Anti-OA IgE antibody response in mice immunized with a single injection of OA alone ($\circ$), OA plus 2 mg of $\text{Al}(\text{OH})_3$ (Δ), or OA plus 2 mg of DEP ($\bullet$). Doses of OA injected were 1.0 μg (upper left graph), 10 μg (lower left graph), 100 μg (upper right graph), and 1000 μg (lower right graph).

measured by quantitative intradermal skin test in the four subjects sensitive to Japanese cedar pollens.

Analysis

Statistical analyses of the obtained data were performed by the analysis of variance.

RESULTS

Primary IgE antibody responses to OA

The kinetics of the anti-OA IgE antibody responses in mice immunized with single intraperitoneal injection of OA without adjuvant or mixed with alum or DEP are presented in Fig. 2. IgE antibody response did not develop in mice immunized with 1.0 μg of OA plus DEP. The responses in animals immunized with either 10 μg or 100 μg of OA plus DEP peaked 10 days after immunization with PCA titer of 640 or 1280. These peak titers did not persist and declined to titers of 40 by day 56. Lower but similar IgE antibody response was observed in mice immunized with 1000 μg of OA plus DEP. The mice immunized with OA without adjuvant demonstrated no or minimal anti-OA IgE antibody responses. The difference in the magnitude of the responses between the mice im-

munized with OA plus DEP and those with OA alone was statistically significant ($p < 0.01$).

Anti-OA antibody response in mice immunized with 1 to 1000 μg of OA plus alum peaked 7 to 14 days after immunization with PCA titer of 640 to 5120. These peak titers persisted by 8 weeks. The magnitude of the response was greater than that in mice immunized with either OA alone or OA plus DEP ($p < 0.01$).

HA antibody titers in the sera obtained at various periods of immunization are listed in Table II. Mice immunized with OA plus DEP compared to mice immunized with OA plus alum demonstrated no or only low titer of HA antibody during the period of 8 weeks after immunization. DEP appeared to possess only weak adjuvant activity for the production of HA antibody.

Antibody responses to repeated injections of DNP-OA

Anti-DNP IgE (PCA) and HA antibody responses in mice that received five intraperitoneal injections of 1 μg of DNP-OA without adjuvant or mixed with DEP are presented in Fig. 3. The magnitude of anti-DNP IgE antibody response in mice immunized with DNP-

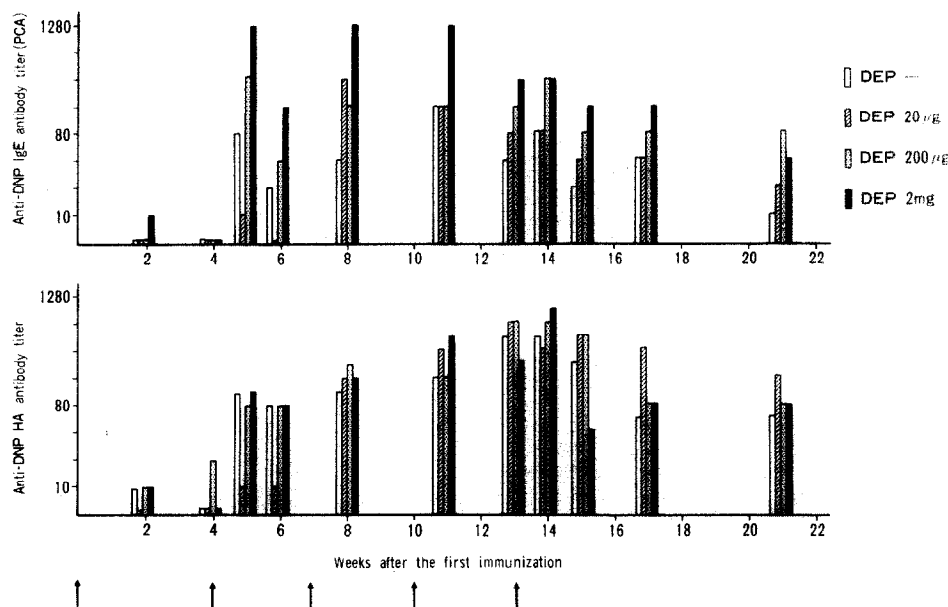


FIG. 3. Effect of various doses of DEP on the induction of anti-DEP IgE antibody response and anti-DEP HA antibody response in mice. Mice were immunized with repeated injections of 1 µg of DEP-OA alone, 1 µg of DEP-OA plus 20 µg of DEP, 1 µg of DEP-OA plus 200 µg of DEP, or 1 µg of DNP-OA plus 2 mg of DEP. Time of the immunizations (↑).

TABLE II. Results of anti-OA HA antibody titration of sera after immunization with OA alone, with OA plus AL (OH)₃, or with OA plus DEP

Group of mice	Immunization		Anti-OA HA antibody titer on day				
	Dose of OA (µg)	Adjuvant	7	10	14	28	56
I	1.0	—	<10	<10	<10	<10	<10
II	1.0	Al(OH) ₃	10	320	640	640	2560
III	1.0	DEP	<10	<10	<10	<10	<10
IV	10	—	<10	<10	<10	<10	<10
V	10	Al(OH) ₃	320	320	1280	2560	10240
VI	10	DEP	10	320	160	640	20
VII	100	—	40	160	320	160	320
VIII	100	Al(OH) ₃	160	160	640	10240	20480
IX	100	DEP	160	160	160	320	160
X	1000	—	<10	<10	<10	<10	<10
XI	1000	Al(OH) ₃	<10	<10	20	160	160
XII	1000	DEP	<10	<10	<10	<10	<10

OA plus 200 µg or 2 mg of DEP was greater than that in mice immunized with DNP-OA without adjuvant throughout the period of 21 weeks after the first immunization. The difference was statistically significant ($p < 0.05$). There were no remarkable differences between the magnitude of HA antibody responses in mice immunized with DNP-OA alone and in animals with DNP-OA plus DEP.

IgE antibody response to repeated injections of JCPA

Fig. 4 demonstrates the anti-JCPA IgE antibody responses to repeated injections of 1 µg or 10 µg of JCPA without adjuvant or mixed with alum or DEP. Mice immunized with JCPA plus DEP developed persistent IgE antibody responses after the second or fourth booster injection. However, the response was

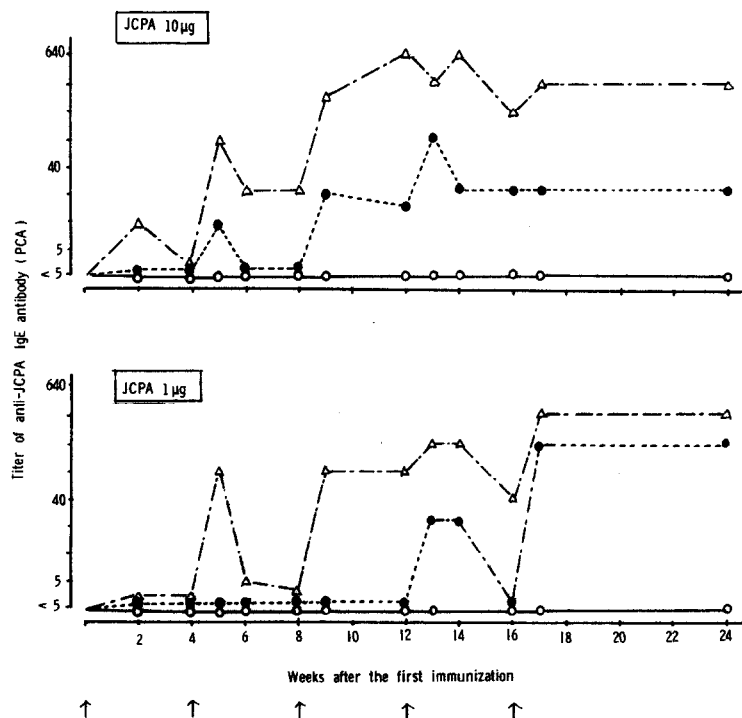


FIG. 4. Anti-JCPA IgE antibody responses in mice immunized with repeated intraperitoneal injections of JCPA alone (○), JCPA plus 2 mg of $\text{Al}(\text{OH})_3$ (△), or JCPA plus 2 mg of DEP (●). Doses of JCPA injected in each were 10 μg (upper abscissa) or 1 μg (lower abscissa). Time of immunizations (↑).

not observed in mice immunized with JCPA alone throughout the period of 24 weeks even with four booster immunizations. The difference in the kinetics of the responses between mice immunized with JCPA alone and JCPA plus DEP was statistically significant ($p < 0.01$). The magnitude of the responses in mice immunized with JCPA plus DEP was, however, less than that in mice immunized with the same doses of JCPA plus alum ($p < 0.01$).

Amount of antigens adsorbed to DEP

Protein concentration of the supernatant obtained after stirring the solution of OA mixed with DEP was 66.2% of that after stirring the solution without DEP. It was calculated that 33.8% (approximately 169 μg) of OA was adsorbed to DEP when the mixture of 500 μg of OA and 10 mg of DEP in 1.5 ml of BBS was stirred for 1 minute.

Maximal dilutions of supernatant I to evoke wheal-and-flare skin reaction in four sensitive subjects were 1:10, 1:10², 1:10², and 1:10, whereas those dilutions of supernatant II were 1:10⁵, 1:10⁵, 1:10⁵, and 1:10⁶. Thus, it was demonstrated that the allergenic activity of the JCPA solution (10 μg of JCPA per 1.5 ml of BBS) decreased at least to one thousandth by

stirring the solution together with 10 mg of DEP for 1 minute.

DISCUSSION

The immune response to an antigen depends in part on the way the antigen is presented. Intraperitoneal injection of antigen mixed with alum adjuvant is commonly used to generate IgE antibodies in mice. In contrast to immunization with antigen alone, this adjuvant favors production of persisting high titers of IgE antibodies. Although the mechanism by which alum enhances IgE antibody production is poorly understood, the capacity of the alum to adsorb antigenic proteins is considered important.¹⁴

Humans are constantly inhaling a variety of airborne antigens into the airway. Despite their immunogenicity, these substances usually do not trigger production of significant IgE antibodies.

The Japanese cedar (*Cryptomeria japonica*) indigenous to Japan is a needle-leaved tree that belong to the toxodiaceae family and the *Cryptomeria* genus. It has been demonstrated that the tree has flourished in Japan at least for 1,000,000 years. Large numbers of people have undoubtedly been exposed to the pollen throughout the history of Japan. Nevertheless, there

had been no report of either a definite or suspected case of AR caused by Japanese cedar pollen until 1964 when Horiuchi and Saito¹⁵ reported such cases for the first time. Before 1950 ARP was such a rare disease in Japan that it was doubted that it ever existed in the country.¹⁶ Even in those days, however, it was recognized that Japanese in the United States could be afflicted with ARP. In a survey of Japanese people (1118 American-born, 16 Hawaiian-born, and 803 Japanese-born) residing in suburban and rural territories of southern California, Hara¹⁷ reported in 1934 that 3.5% of the subjects suffered from ARP. A difference in the genetic predisposition of Japanese people residing in Japan and the United States is an unlikely cause of this phenomenon. Collectively, these findings suggest that important factors responsible for the enhanced production of IgE antibodies and pollinosis have been introduced in the Japanese environment in the last three decades.

In a previous study the authors examined the adjuvant activity of an activated carbon, an adsorbent in wide use, to enhance the production of IgE antibody to penicillin in mice.¹⁸ In the course of the study, it became apparent that carbonaceous particles in black smoke emitted from diesel cars have the capacity to adsorb proteins.¹⁹

Diesel exhaust consists of carbonaceous particulates and trace amounts of metals in addition to gaseous compounds such as nitrogen oxides (NOx), sulfur oxides (SOx), hydrocarbons (HC), and carbon monoxide (CO).⁶

DEP have a carbon particle as the core, which has probably formed during combustion of light fuel oil. Approximately 15% of the weight of DEP consists of the soluble organics; in fact, a number of hydrocarbon compounds including some mutagenic substances has been identified.²⁰ Other components of exhaust gas are also adsorbed on the surface of DEP that have a large surface area.²⁰ A large part of DEP is <1 μ m in diameter and is easily kept buoyant in the atmosphere for a long period of time to enable that a certain fraction of it can be carried deep into lungs when it is inhaled.²¹ DEP function as carriers of organic and inorganic substances produced by diesel cars.

Our present study indicates that DEP have an adjuvant activity for IgE antibody production in mice, although they are less potent than alum. The capacity of DEP to adsorb proteins or to carry organic or gaseous components of diesel exhaust may be involved in the mechanism of its adjuvant activity.

Laboratory and clinical data so far accumulated have demonstrated that DEP, once they are inhaled, mostly adhere to the mucosal membranes of the upper airway or are carried into the deep region of the lung

where they are phagocytized by alveolar macrophages. It has been demonstrated that some of the alveolar macrophages containing DEP infiltrate into the perialveolar region or enter into lymphatic vessels to be processed by lymphatic tissues.⁷⁻¹²

The mechanism by which IgE antibody is produced in response to pollen antigen remains unknown. It is possible that allergenic substances are readily released from pollen after contact with mucus on the surface of the nasal or upper respiratory mucosa. The allergenic substances thus released may be combined with DEP on the mucosal membrane. Meanwhile, Busse et al.²³ have demonstrated that during the ragweed season, there were airborne particles <5 μ m in diameter that carry antigens capable of reacting with ragweed reagins. Habenicht et al.²⁴ also reported that the naturally occurring mists in a ragweed season, although they are free of native pollen particulates, may yet carry allergens of clinical significance. The investigations to examine the adjuvant activity of DEP when they are administered with antigen via the nostril in mice are in progress.

The regulations for discharge of DEP from diesel cars have been lenient in Japan. A striking increase in the number of diesel cars recently in Japan may correspond to the increase of the concentration of DEP in the environmental atmosphere of urban districts in Japan, although accurate data have not yet been reported. One cannot exclude the possibility, therefore, that DEP may have some relation to the associated changes in the number of diesel cars and ARP in Japan.

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