

[SHORT COMMUNICATION]

THE ADJUVANT ACTIVITY OF PYRENE IN DIESEL EXHAUST ON IgE  
ANTIBODY PRODUCTION IN MICE

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In this communication, it is shown that pyrene has an adjuvant activity on IgE antibody production when mice are immunized by an intraperitoneal injection of ovalbumin (OA) or Japanese cedar pollen allergen (JCPA) with pyrene.

The effects of pyrene on IgE antibody production in mice were investigated to clarify the relation between pollen allergy and the adjuvant activity of the chemical compounds contained in diesel-exhaust particulates (DEP). In the first experiment, three groups of mice were immunized intraperitoneally six times at 2-week intervals with 1  $\mu$ g of OA alone, 1  $\mu$ g of OA plus 1 mg of pyrene, and 1  $\mu$ g of OA plus 1 mg of DEP, respectively. The IgE antibody responses to OA in mice immunized with OA plus pyrene or OA plus DEP were extremely enhanced as compared with those in mice immunized with OA alone, and the highest responses were observed in mice immunized with OA plus DEP. In the second experiment, mice were immunized with 10  $\mu$ g of JCPA alone or 10  $\mu$ g of JCPA plus 5 mg of pyrene in the same way. The IgE antibody responses to JCPA in mice immunized with JCPA plus pyrene were higher than those in mice immunized with JCPA alone. The intraperitoneal macrophages of the mice also clearly stimulated in vitro by pyrene on chemiluminescence assay. These results suggest that pyrene contained in DEP acts as an adjuvant in IgE antibody production when mice are immunized with antigens.

**Key words:** diesel exhaust — IgE antibody production — Japanese cedar pollen allergen — ovalbumin — pyrene

INTRODUCTION

Japanese cedar pollinosis was first found in the Nikko district of Tochigi Prefecture, Japan by Horiguchi and Saito<sup>1)</sup>. Ishizaki et al.<sup>2)</sup> reported that environmental factors appeared to influence the incidence of Japanese cedar pollinosis, i.e. the highest incidence was seen among residents living

alongside the intercity main road which is lined with older cedar trees and has heavy automobile traffic all day long in the Nikko district. They suggested that air pollution from automobile exhaust should be considered as an enhancing factor on the incidence of pollen allergy. Muranaka et al.<sup>3)</sup> reported that the IgE antibody responses to OA in mice immunized with an intraperitoneal injection of OA mixed with DEP were higher than those in animals immunized with OA alone. Approximately 70% to 90% of exhaust particles are emitted from diesel vehicles in Japan<sup>4,5)</sup>. We have reported that the neutral fraction extracted from DEP has an adjuvant activity on IgE antibody production in mice<sup>6)</sup>. Pyrene is abundant in the neutral fraction of DEP<sup>7)</sup>. Therefore, as the first

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**Abbreviations:** DEP diesel-exhaust particulates; ELISA enzyme-linked immunosorbent assay; HA hemagglutination; JCPA Japanese cedar pollen allergen; OA ovalbumin; PCA passive cutaneous anaphylaxis

step to make it clear that the chemical compounds in DEP would cause enhanced IgE antibody production, we investigated the adjuvant activity of pyrene on IgE antibody production in mice by intraperitoneal immunization with antigen-pyrene mixtures.

## MATERIALS AND METHODS

### *Animals*

BDF1 female mice, 6 to 7 weeks old, were obtained from Japan Charles River Co., Atsugi, Japan. Male Wistar rats weighing 200 to 300 g were also obtained from Japan Charles River Co. They were maintained in plastic cages and fed a standard chow diet.

### *Antigens*

OA (chicken egg, grade VII) was purchased from Sigma Chemical Co., MO, USA. JCPA was kindly provided by Dr. Yuichi Takahashi. After 80 ml of 0.125 M ammonium bicarbonate was added to 4 g of Japanese cedar pollen (*Cryptomeria japonica*), the crude JCPA was extracted by shaking the mixture for one hour at room temperature, followed by centrifugation for 10 min at  $1,100 \times g$ . The supernatant was used as the crude JACP. The protein contents of OA and the crude JCPA supernatant were determined with a micro bicinchoninic acid (BCA) protein assay reagent kit (Pierce Co., IL, USA).

### *Immunization schedule*

Six groups of mice, 5 in each, were immunized six times at 2-week intervals. In the first experiment, four groups of mice were administered intraperitoneally with 1  $\mu$ g of OA dissolved in physiological saline without adjuvant, 1  $\mu$ g of OA with 1 mg of pyrene (Aldrich Chemical Company, Inc. USA) dissolved in dimethyl sulfoxide (DMSO, Wako Pure Chemical Industries, Ltd., Osaka, Japan), 1  $\mu$ g of OA with 1 mg of DEP suspended in physiological saline and 1  $\mu$ g of OA with 1 mg of  $Al(OH)_3$  gel (alum), respectively. The animals in these groups were boosted 10 weeks after the final immunization with the same dose of OA alone. In the second experiment, two groups of mice were immunized with 10  $\mu$ g of JCPA alone or 10  $\mu$ g of

JCPA with 5 mg of pyrene in the same way. The animals in the two groups were boosted 7 weeks after the final immunization with the same dose of JCPA alone. The mice in each group were bled from the retroorbital plexus at 2-week intervals after the first immunization. Sera obtained from the mice in each group were stored at  $-80^\circ C$  until use. The optimum concentration of pyrene as an adjuvant was determined from the results of a preliminary test.

### *Measurement of antibodies*

IgE antibodies were measured by passive cutaneous anaphylaxis (PCA) assay<sup>8)</sup> in the Wistar rats. One tenth of a milliliter of serial twofold dilutions of the test sera was injected intracutaneously into the depilated dorsum of the rats. Forty-eight hours later, the rats were challenged intravenously with the antigen mixtures dissolved in 0.5 ml of physiological saline solution and 0.5 ml of 2% Evans blue dye (Wako Pure Chemical Industries, Ltd.). The doses of antigens used for the challenge were 1 mg for OA and 0.4 mg for JCPA. The PCA titer was expressed as the reciprocal number of the dilution giving a spot of 5 mm in diameter. The hemagglutination (HA) antibody was detected by the method of Boyden<sup>9)</sup>.

### *Chemiluminescence response of intraperitoneal macrophages by pyrene*

Intraperitoneal macrophages were harvested from the mice with about 10 ml of phosphate-buffered saline solution (PBS, pH 7.0). The isolated cells were sedimented by centrifugation at  $300 \times g$  for 15 min at  $4^\circ C$  and were washed twice with 5 ml of cold PBS. The washed cell pellet was resuspended in 2 ml of PBS. Cell counts were made with a Burkert-Turk's counting cell. The mixture of 0.1 ml of  $3 \times 10^9$  cells/ml, 0.79 ml of PBS and 0.1 ml of 330  $\mu$ g/ml luminol (Wako Pure Chemical Industries, Ltd.) dissolved in PBS were incubated in a chemiluminescence counting plastic tube. After incubation for 4 min at  $37^\circ C$ , 0.01 ml of various concentrations of pyrene dissolved in DMSO were immediately added. A chemiluminescence assay using Biolumat LB 9500 T (Berthold GmbH Co., Germany) was conducted in 1 ml of the reaction mixture for 20 min at  $37^\circ C$  after addition of the pyrene solution.

## RESULTS AND DISCUSSION

The anti-OA IgE antibody responses in the three groups of mice immunized six times by the administration of OA alone, OA plus pyrene and OA plus DEP, respectively, are presented in Fig. 1. The mean levels of the PCA titers in the mice immunized with OA alone were approximately 1:20 to 1:120 for 6 to 20 weeks after the first immunization with OA. As compared with the PCA titers in the mice immunized with OA alone, those in the mice immunized with OA plus pyrene were at the same level at 6 weeks, but the increased PCA titers were always observed for 8 to 20 weeks, especially at 16 weeks when the mean levels of the PCA titers increased to approximately 1:1100. In the mice immunized with OA plus DEP, the mean levels of PCA titers increased from 2 to 20 weeks and were always higher than those of the mice immunized with OA plus pyrene in this experiment; the levels especially increased to approximately 1:1600 at 16 weeks. The increased IgE antibody production in the mice immunized with both OA plus pyrene and OA plus DEP persisted for 10 weeks after the last immunization. These results suggest that pyrene and DEP have an obvious adjuvant activity on IgE antibody production in mice challenged repeatedly with mixtures of OA and pyrene or OA and DEP. The persistence of the enhanced IgE antibody responses after the last immunization might be associated with the retention of activated B cells participating in IgE antibody production in the mice in these groups. In the mice immunized with OA plus alum as a positive control, the mean levels of the PCA titers increased to approximately 1:2800 at 2 weeks and 1:4200 at 4 weeks, respectively, and the PCA titer levels were approximately 1:2500 to 1:4200 for 6 to 20 weeks.

The organic compounds in DEP were adsorbed on the surface of the carbonaceous core particles, and the proportions of deposits of DEP in the respiratory systems of large numbers of people living in urban areas were approximately 30% of the weight of DEP<sup>10)</sup>. A number of organic compounds have been identified in DEP, which contains many organic compounds apart from pyrene<sup>8)</sup>. The enhanced IgE antibody responses in the mice immunized with OA plus DEP might be

induced not only by the adjuvant activity of pyrene but also by the adjuvant activity of any other organic compounds contained in DEP. Moreover, the adsorption of OA to carbon particles might contribute to the increase in the IgE antibody responses. However, the PCA titers in the mice immunized intraperitoneally with 1  $\mu$ g of OA plus 1 mg carbon particles were less than 1:50, as reported in our previous study<sup>11)</sup>. Therefore, the highest IgE antibody responses, found in the mice immunized with OA plus DEP, might result mainly from the many organic compounds contained in DEP.

The anti-JCPA IgE antibody responses in the mice immunized six times by the administration of JCPA alone or JCPA plus pyrene are also presented in Fig. 1. In the mice immunized with JCPA plus pyrene, the increased PCA titers were observed from 8 to 16 weeks after immunization and the PCA titers were always higher than those in the mice immunized with JCPA alone. The anti-JCPA IgE antibody responses in the mice in each group persisted for 6 weeks after the last immunization. But the maximum mean levels of the PCA titers in the mice immunized with JCPA alone and JCPA plus pyrene were 1:40 and 1:160, respectively. The anti-JCPA IgE antibody responses in the mice immunized with JCPA plus pyrene were weak as compared with the anti-OA IgE antibody responses in the mice immunized with OA plus pyrene. BDF1 mice might be classified as high responders for OA and low responders for JCPA.

The mice received booster administrations of OA alone at 20 weeks or JCPA alone at 17 weeks. The mean levels of the PCA titers in the mice immunized with OA alone, OA plus pyrene and OA plus DEP increased to approximately 1:1000, 1:1300 and 1:1720 at 21 weeks, respectively. The mean levels of the PCA titers in the mice immunized with JCPA plus pyrene increased to approximately 1:130 at 18 weeks. On the other hand, the mean levels of the PCA titers in the mice immunized with JCPA alone remained unchanged at 18 weeks. The increased PCA titers in the mice after the booster administrations of allergen alone 7 or 10 weeks after the last immunization might indicate that those mice immunized previously with antigens possessed memory cells on IgE

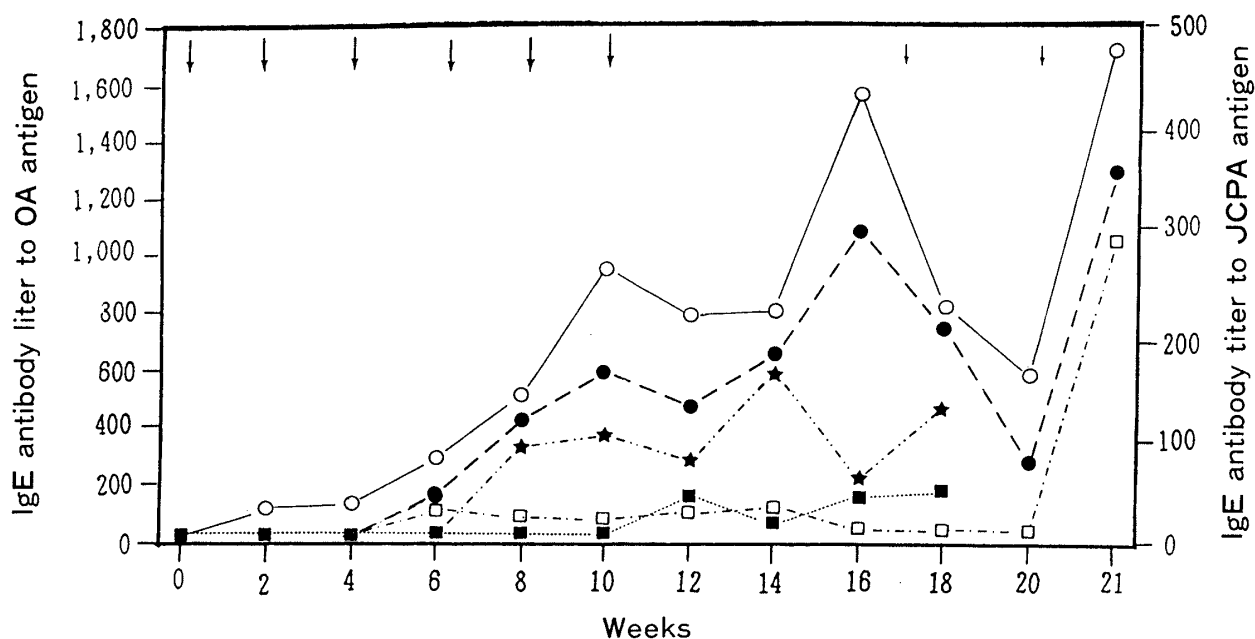


Fig. 1. Anti-OA and anti-JCPA IgE antibody responses in mice.

Mice were immunized with repeated intraperitoneal injections of 1  $\mu$ g of OA alone (□), 1  $\mu$ g of OA plus 1 mg of pyrene (●), 1  $\mu$ g of OA plus 1 mg DEP (○), 10  $\mu$ g of JCPA alone (■) and 10  $\mu$ g of JCPA plus 5 mg of pyrene (★). Arrow (↓) mean the times as immunization. Titration of IgE antibody to OA or JCPA was achieved by 48 hour-PCA. Small arrows mean the time as immunization in mice with the same dose of JCPA alone at 17 weeks or of OA alone at 20 weeks, respectively.

antibody production responding to the rechallenge with the same antigens.

The sera obtained from the mice immunized with the mixture of antigen (OA or JCPA) and pyrene were incubated for 4 hours at 56°C and the sera were used for PCA reaction. However, anti-OA or anti-JCPA IgE antibodies were not detectable in the sera. The skin-sensitizing activities of the IgE antibody found in the sera might be inactivated by heating. Moreover, the IgE antibody titers determined with a mouse IgE-ELISA kit (mouse IgE EIA kit, Yamasa Shoyu Co., Japan) were at much lower levels than those of non-heated sera from identical animals.

As compared with the IgE antibody responses in the mice immunized with mixtures of antigens and either pyrene or DEP, the HA antibody titers in the sera obtained at various periods from the mice immunized with OA plus pyrene, OA plus DEP and JCPA plus pyrene, respectively, were significantly low (1:10 to 1:320) in the mice immunized with OA, and 1:20 to 1:80 in the mice immunized with JCPA. Pyrene and DEP appeared to possess only weak adjuvant activity for the production of HA antibody.

As shown in Fig. 2-A, pyrene clearly stimulated the intraperitoneal macrophages of the mice. The highest peak of chemiluminescence intensity on macrophages stimulated by pyrene was observed on addition of 100  $\mu$ g of pyrene on the dose-response curve (Fig. 2-B); however, the chemiluminescence intensity on macrophages decreased upon addition of more than 100  $\mu$ g of pyrene. We have observed by microscopy that the intraperitoneal macrophages of the mice phagocytized pyrene and DEP promptly (unpublished data). It has been reported that interleukin-1 (IL-1) is secreted from stimulated macrophages<sup>12)</sup>, that the administration in vivo of IL-1 induces the increase of IL-4<sup>13)14)</sup>, and that increased IgE antibody synthesis is induced by IL-4 secreted from helper T cells<sup>15)–19)</sup>. On the basis of these facts, there is a possibility that macrophages might be stimulated and activated by pyrene and DEP as adjuvant materials as well as by antigen. In our next study, we intend to investigate whether pyrene induces the increased secretion of IL-1 from stimulated macrophages and then activates helper T cells, and whether any other organic compounds contained in DEP besides pyrene act as adjuvants in

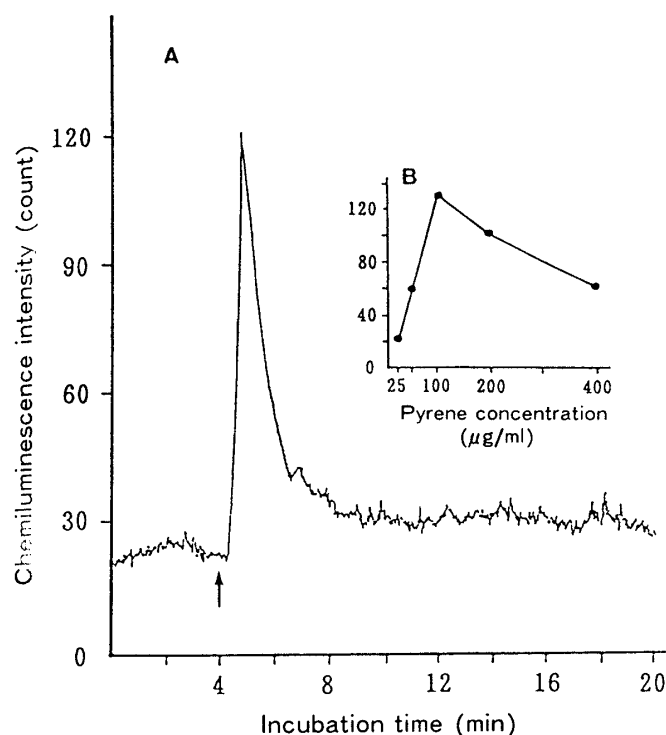


Fig. 2. Effect of pyrene on the chemiluminescence response of mouse intraperitoneal macrophages in vitro.

Arrow (†) indicates the an additional point of pyrene (Fig. 2-A). The chemiluminescence response of macrophages treated with the various concentrations of pyrene is shown in Fig. 2-B.

IgE antibody production when mice are immunized with antigens.

The prevalence rate of Japanese cedar pollinosis has increased strikingly in urban areas with air pollution<sup>20</sup>. Our present study indicates that pyrene and DEP have an adjuvant activity on IgE antibody production in mice, although the adjuvanticity of any other chemical compounds contained in DEP besides pyrene remains unknown. This suggests the possibility that DEP from diesel-powered cars may be one of the factors responsible for the high prevalence rate of pollinosis caused by Japanese cedar pollens in polluted districts, because the number of diesel-powered cars, which offer a substantial advantage in fuel economy over their gasoline-powered counterparts, has increased rapidly in Japan.

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## マウスの IgE 抗体産生におけるピレンのアジュバント活性

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ディーゼル排出粒子 (DEP) に含まれるピレンには、マウスが卵白アルブミン (OA) あるいは日本スギ花粉抗原 (JCPA) と共に感作された時、IgE 抗体産生におけるアジュバント作用があることを見出した。

花粉アレルギーと DEP に含まれる化学物質のアジュバント作用との関係を明らかにするため、マウスの IgE 抗体産生におけるピレンの影響を検討した。第一の実験では、マウスにそれぞれ 2 週間間隔で 1 $\mu$ g の OA 単純、1 $\mu$ gOA+1mg ピレンと 1 $\mu$ g OA+1mg DEP で 6 回腹腔内投与し、それぞれを感作した。OA+ピレンまたは OA+DEP で感作されたマウスにおける IgE 抗体産生は OA 単独で感作されたマウスと比べて著しく亢進した。また、OA+DEP で感作したマウスでは、より高い免疫応答が認められた。第二の実験では、マウスに 10 $\mu$ g JCPA 単独または 10 $\mu$ g JCPA+5mg ピレンを用いて同様の方法で感作した。JCPA+ピレンで感作したマウスの IgE 抗体産生は JCPA 単独で感作したマウスより高かった。また、マウスの腹腔マクロファージを試料としてピレンを添加し化学発光を測定した結果、マクロファージはピレンにより明らかに刺激された。これらの結果は、DEP 中に含まれるピレンは、OA や JCPA のような抗原に対する IgE 抗体産生においてアジュバント作用を有することを示している。