

Enhanced human IgE production results from exposure to the aromatic hydrocarbons from diesel exhaust: Direct effects on B-cell IgE production

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Epidemiologic and experimental studies suggest that air pollution, and particularly diesel exhaust particles (DEPs) may play a role in the increasing prevalence and severity of airway allergic disease. We show that the extract of polyaromatic hydrocarbons (PAHs) from DEPs (PAH-DEP) enhances human IgE production from purified B cells. Interleukin-4 plus CD40 monoclonal antibody-stimulated IgE production was enhanced 20% to 360% by the addition of PAH-DEP over a period of 10 to 14 days. This effect was increased when PAH-DEP was added 2 to 5 days after cultures were initiated. PAH-DEP itself did not induce IgE production or synergize with interleukin-4 alone to induce IgE from purified B cells, suggesting that it was enhancing ongoing IgE production rather than inducing germline transcription or isotype switching. The prototype nonmetabolized aromatic hydrocarbon 2,3,7,8 tetrachlorodibenzo-p-dioxin, which functions solely through activation of the cytosolic aromatic hydrocarbon receptor complex, also increased IgE production. Additionally, the pattern of mRNAs coding for distinct isoforms of the ϵ chain was altered by PAH-DEP, and B-cell expression of the low-affinity IgE receptor was upregulated by PAH-DEP. Enhanced IgE production in the human airway, resulting from exposure to PAH-DEP, may be an important factor in the increase in airway allergic disease. (J ALLERGY CLIN IMMUNOL 1995; 95:103-115.)

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Allergic diseases of the upper and lower airways represent a major worldwide health issue. These diseases have been increasing in incidence and severity throughout this century and undergoing a

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Abbreviations used

AH:	Aromatic hydrocarbon
DEPs:	Diesel exhaust particles
mAb:	Monoclonal antibody
PAHs:	Polyaromatic hydrocarbons
PAH-DEP:	Extract of polyaromatic hydrocarbons from diesel exhaust particles
PBMCs:	Peripheral blood mononuclear cells
PCR:	Polymerase chain reaction
TCDD:	2,3,7,8 Tetrachlorodibenzo-p-dioxin

more recently publicized upturn. The hallmark of these disorders is increased IgE production. Epidemiologic studies have demonstrated that increasing atmospheric pollution may play an important role in the allergic response,¹⁻⁴ as a result of both the decrease in air quality occurring over time and regional increases in pollution. Thus the change in the quality of the air we breathe and

the effects related to airborne pollutants may be a factor in the increasing worldwide incidence, prevalence, and severity of allergic airway disease.⁵⁻⁷

Environmental materials such as polyaromatic hydrocarbons (PAHs) from diesel exhaust are of particular interest. Murine *in vitro* and *in vivo* studies demonstrating a direct effect of diesel exhaust particles (DEPs) on IgE antibody production have been performed by several investigators.⁸⁻¹⁰ Intraperitoneal injection of DEPs increased the IgE response to ovalbumin and Japanese cedar pollen.^{8, 10} Even more compelling, intranasal inoculation of DEPs along with antigen markedly enhanced the IgE response without increasing the hemagglutination titer (IgG response) to the same degree.^{9, 10}

At the same time, there have been major efforts to define the mechanism or mechanisms by which PAHs may alter cell growth and differentiation because of their known potential to be immunosuppressive and to induce mutation and carcinogenesis.^{11, 12} Studies of the effects of aromatic hydrocarbons (AHs) in animals have shown that they can be suppressive to both T-cell and B-cell compartments.^{13, 14} However, most of these studies were concerned with the potential suppressive effects of PAHs and were therefore designed to identify inhibitory rather than enhancing effects. More recent studies showed that the prototype aromatic hydrocarbon 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) was able to enhance immunoglobulin responses (along with B-cell activation and certain kinase activities), if given after induction of a humoral response *in vivo*.¹⁵⁻¹⁷ When such alterations of immune cell function have been reported, there has been little ability to connect the observed phenomena and the ever expanding information about the mechanisms by which cell function is altered. Thus although PAHs clearly alter immune function, earlier studies were not designed to pursue the more subtle issues of immune regulation and the quality of the resulting immune response that might alter or enhance IgE responses.

We now show that the organic extract of PAHs from DEPs (PAH-DEP), as well as the prototype AH molecule TCDD, enhance human IgE production by interleukin (IL)-4 plus CD40-stimulated purified human B cells and peripheral blood mononuclear cells (PBMCs). The mechanism by which this occurs is increasing IgE production from cells that are already committed to IgE synthesis.

METHODS

Cell preparation and culture conditions

PBMCs were obtained from healthy donors between the ages of 21 and 48 years. Tonsil cells were obtained from specimens removed at surgery, and single cell suspensions were prepared. Mononuclear cells were obtained by density-gradient centrifugation with Lymphoprep (Nycomed Pharma, Oslo, Norway; 1077 kg/m³). For complete T cell removal, two cycles of 2-aminoethylisothiuronium bromide hydrobromide (Sigma Chemical Co., St. Louis, Mo.) treated sheep red cell rosette formation and depletion were performed. The T-cell depleted populations of PBMCs and tonsil cells consisted of approximately 50% and greater than 95% CD19⁺ cells, respectively. Adherent cells were then removed from the non-T-cell fractions by adherence to plastic in the presence of serum resulting in B-cell fractions from blood and tonsil that were generally greater than 95% B cells. These procedures have been reported in detail previously.¹⁸

Cells were cultured in 96-well round-bottomed microtiter culture plates (Nunc, Roskilde, Denmark) in RPMI 1640 (M.A. Bioproducts, Walkersville, Md.) supplemented with 10% fetal calf serum (Hyclone, Laboratories, Logan, Utah), 2 mmol/L glutamine, 50 U/ml penicillin, and 50 µg/ml streptomycin (complete RPMI 1640). PBMCs and B cells were seeded at 2×10^5 and 2×10^5 cells in 0.2 ml complete RPMI per well, respectively. Four or five replications were done at each condition in every experiment. Cultures were maintained at 37°C in a 5% CO₂ atmosphere for 10 to 14 days or as indicated, at which time the supernatants were harvested for immunoglobulin measurement.

Immunoglobulin measurements

The levels of various immunoglobulin isotypes (IgE, IgG, and IgM) in the supernatants of cultured cells were measured by isotype-specific ELISAs, as previously described, with minor modifications.^{19, 20} These procedures were modified by deleting the use of enhancing reagent on occasion but were otherwise identical to the reported methods.

Flow cytometric analysis of cell-surface molecules

The methods used to quantitate subsets of various cells before and after culture by two- and three-color flow cytometric analysis have been described previously.²¹ In these studies conjugated monoclonal antibody (mAb) directed against CD19 (Leu 16; Becton Dickinson Immunocytometry Systems, Mountain View, Calif.) was used as a pan B cell marker. For determination of B cell size, the forward scatter of CD19⁺, CD3/CD14⁻ cells was determined on freshly obtained samples. Wide gating was used to avoid excluding larger B cells.²² The CD23 antigen was detected with a fluorescein isothiocyanate-labeled mAb-CD23 (AMAC,

Inc., Westbrook, Maine). The flow cytometric data were acquired on a FACScan (Becton Dickinson, San Jose, Calif.) and analyzed with LYSIS II software (Becton Dickinson).

Reagents

Human purified recombinant IL-4 was a kind gift of Dr. Gene Medlock of Amgen Biologicals (Thousand Oaks, Calif.). The mAb against CD40 (G28.5) was kindly provided by Dr. E. Clark (University of Washington, Seattle, Wash.).

DEPs were obtained by collecting the exhaust from a light-duty diesel passenger car into a cyclone impactor equipped with a dilution tunnel constant volume sampler system as described by Muranaka et al.⁸ The DEPs were collected on high-capacity glass fiber filters. The particulate DEP consists of large-chain aggregates of DEP in which the individual particles are less than 1 μ m in diameter. The DEP-containing filters were stored in glass containers under nitrogen gas to preserve all of the PAHs. To prepare PAH-DEP, a high-capacity filter was extracted in dichloromethane (Aldrich Chemical Co. Milwaukee, Wis.) because this was shown to be highly efficient in recovering PAH-DEP.²³ Furthermore, dichloromethane has been used as a human anesthetic and thus can be used in vivo in human beings and animals, as well as for in vitro studies. We extracted 134 mg of PAH-DEP from a filter with 330 mg of total DEPs. The resulting extract was adjusted to a stock concentration of 2 mg of organic material per milliliter in dichloromethane and stored at 4° C. Working concentrations of PAHs were diluted in 2% acetone (Fisher Scientific Co., Pittsburgh, Pa.) plus complete RPMI.

TCDD was the kind gift of Dr. Oliver Hankinson (UCLA School of Medicine, Los Angeles, Calif.) and was prepared as described previously.²⁴

Analysis of mRNA coding for ϵ proteins

Alternate splicing of the single ϵ heavy-chain gene results in different mRNA isoforms.^{25, 26} The procedure for comparisons of mRNA levels for different ϵ isoforms with reverse transcription and amplification by polymerase chain reaction (PCR) has been detailed elsewhere.²⁷ Briefly, total cellular RNA was isolated from B cells (1 to 5 $\times 10^6$ cells/sample), which had been cultured with IL-4, CD40, and different concentrations of PAH-DEP as described above. The RNA was reverse-transcribed with Moloney murine leukemia virus reverse transcriptase (Bethesda Research Laboratories, Gaithersburg, Md.) as previously described.²⁵ Different concentrations of the reverse-transcriptase product were added to a reaction mixture that contained 1 U "Perfect Match" (Stratagene Ltd, La Jolla, Calif.), 10% dimethyl sulfoxide, 50 mmol/L KCl, 20 mmol/L Tris-HCl (pH 8.4), 2.1 mmol/L MgCl₂, 0.1% (wt/vol) gelatin, 0.2 mmol/L deoxyribonucleoside triphosphate, 2.0 U Taq polymerase (Promega, Madison, Wis.), 30 pmol/L of each of the upstream and downstream oligonucleotide

primers for ϵ isoforms, 25 pmol/L 5' and 3' primers for β -actin, and 1 $\times 10^6$ of each of the downstream primers, which had previously been end-labeled with phosphorous 32. The sequences of the 5' and 3' primers pairs respectively were: GGCCATCGATAGGCAGCGAGC-CCCTCACAGACCG and GGCCGTCGACGACG-GGGATGCCAGGCAGTTGGATC for ϵ -mRNA and GTGGGGCGCCCCAGGCACCA and CTCCTTAAT-GTCACGCACGATTTC for β -actin. The mixture was then amplified by PCR on a preheated Perkin Elmer thermal cycler (Perkin-Elmer Corp., Norwalk, Conn.) for 25 cycles. Each cycle consisted of denaturation at 94° C for 1 minute, followed by 1 minute at 67° C and then 1.5 minutes at 72° C. The reaction mixture and cycle times were chosen after extensive titration of each to ensure equal amplification of all three ϵ isoforms. Aliquots of 20 μ L of each PCR product were run on an 8% polyacrylamide gel and dried; the gel was then autoradiographed with Kodak XAR-5 film (Eastman Kodak Co., Rochester, N.Y.). In place of a densitometer, the autoradiograph was scanned on an Apple One Scanner (Apple Computer, Inc., Cupertino, Calif.) converted into TIFF uncompressed format, and the resultant pattern was analyzed with the Scan Analysis program from Biosoft (Cambridge, England), which delineates peaks and integrates the area under each peak.

To ensure the validity of this PCR strategy, three cDNA plasmids, each containing one of the different mRNA isoforms, were titrated and subjected to the same procedure. There was a log-linear correlation between the number of starting copies of the plasmid and the PCR yield over a broad range. Those samples in which the PCR yield was not in this range were discarded. The PCR yield of each sample was determined by the intensity of the appropriate band (corresponding to the three isoforms) on comparison with the intensity of the β -actin band and were therefore expressed as a value of β -actin mRNA.

RESULTS

PAH-DEP enhances IgE production induced by IL-4/CD40

We tested the ability of PAH-DEP to induce human IgE production. When added alone over a dose range of 0.005 to 500 ng/ml, PAH did not induce de novo IgE production from either PBMCs or purified human B cells. Similarly, PAH-DEP was unable to induce IgE production from PBMCs or purified human B cells in the presence of CD40 mAb. When purified B cells were treated with IL-4 to induce ϵ germline transcription,²⁸ addition of PAH-DEP still did not lead to the appearance of IgE protein in the supernatants when cultured over a 21-day time course (data not shown).

In contrast, the addition of PAH-DEP over a dose range of 1 to 50 ng/ml enhanced IgE produc-

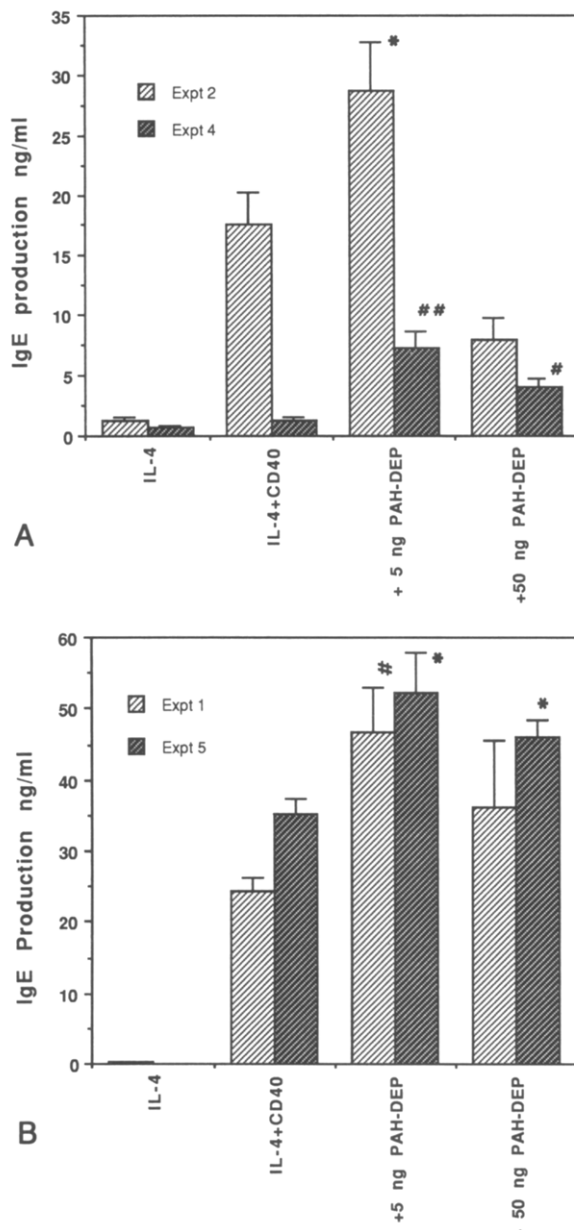


FIG. 1. Enhancing effect of PAH-DEP on IL-4 plus CD40 driven IgE production. In each panel (**A** and **B**), the light and dark bars represent individual experiments. **A**, PBMCs (2×10^5 cells/0.2 ml) were cultured for 14 days in the presence of 200 U/ml of IL-4, IL-4 plus 0.1 μ g/ml of CD40, and 5 or 50 ng/ml of PAH-DEP. The mean IgE $\pm$ 1 SD of four replications of cultures for two separate experiments are shown. Neither medium alone nor CD40 alone induced IgE production (data not shown). **B**, Purified blood B cells (2×10^4 cells/0.2 ml) were cultured for 14 days in the presence of 200 U/ml of IL-4, 0.1 μ g/ml of CD40, and 5 or 50 ng/ml of PAH-DEP. The mean IgE $\pm$ 1 SD of four replications of cultures for two experiments are shown. Neither medium alone nor CD40 alone induced IgE production (data not shown). * $p < 0.05$ compared with IL-4 plus CD40 only. # $p < 0.05$ compared with IL-4 plus CD40 only. ** $p < 0.01$ compared with IL-4 plus CD40 only.

tion in cultures of PBMCs or purified B cells that were simultaneously stimulated with IL-4 plus CD40 mAb (Fig. 1, *A* and *B*). PAH-DEP, at doses of 1 to 50 ng/ml, when added simultaneously with IL-4 and CD40, enhanced IgE production from 31% to 180% in the experiments with purified B cells, whereas IgE production with PBMCs was increased up to 395% by the addition of PAH-DEP at the initiation of the culture. This enhancing effect was observed with cells derived from both nonallergic and allergic individuals. Although we occasionally noted a small increase in IgE in cultures treated with vehicle only, the enhancement observed was primarily due to the PAH-DEP as shown by comparison of vehicle alone with DEP-PAH cultures (Fig. 2). In two of 11 experiments PAH-DEP failed to enhance IgE production by B cells stimulated with IL-4/CD40 in the optimal concentration of IL-4 (200 U/ml) and CD40 mAb (0.1 μ g/ml).

Time course of IgE production in IL-4/CD40-stimulated B cells

We next determined whether PAH-DEP was increasing IgE production in culture by altering the time course of IgE appearance compared with IL-4 plus anti-CD40-driven IgE production. IgE synthesis was determined in B cell culture over a 21-day time course. As with IL-4 plus CD40 stimulation, in the cultures containing PAH-DEP, IgE was first detected in the supernatant on day 10 of culture, with IgE concentrations peaking on day 14 and little change in levels thereafter up to day 21 (Fig. 3). Thus a change in the kinetics of IgE appearance in cultures with PAH-DEP did not account for the increased IgE production observed on days 10 to 14.

Delayed addition of PAH-DEP increased its enhancement of IgE production

The effects on IgE production of altering the timing of addition of PAH-DEP to cells were determined because data in other systems suggested that early addition of PAHs may inhibit cell responses, whereas later addition may be stimulatory.¹² Indeed, delaying addition of PAH-DEP until 2 to 5 days after the initiation of cultures further increased the ability of PAH-DEP to stimulate IgE production as compared with addition on day 0 (Fig. 4). In four experiments with purified blood B cells stimulated with 0.1 ng of PAH-DEP on day 2, the increase in IgE ranged from 75% to 334%, with a mean increase of 217%. Similar results with 25% to 58% enhancement were ob-

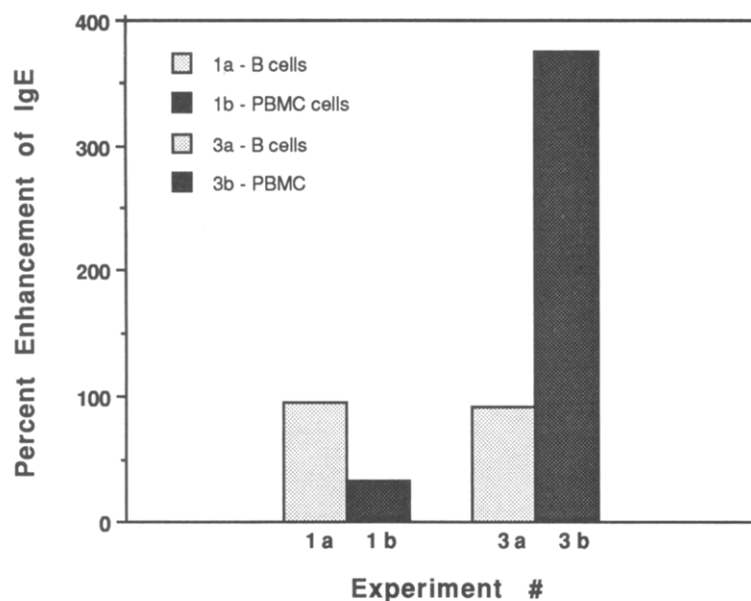


FIG. 2. Enhancing effect of PAH-DEP at 5 ng/ml on IgE production compared with the effect of vehicle (dichloromethane and acetone) alone in the presence of IL-4 (200 U/ml) plus CD40 (0.1 μ g/ml). The values displayed represent the mean of four replicated cultures from two separate experiments (1 and 3). Cultures 1a and 3a (indicated by the light stippled bars) were performed with purified B cells, whereas cultures 1b and 3b (indicated by the dark stippled bars) were performed with PBMCs. The results are expressed as a percent of vehicle-treated cells calculated as follows: % Enhancement effect = $(\text{IgE}_{\text{PAH}} - \text{IgE}_{\text{V}}) / \text{IgE}_{\text{C}}$ where IgE_{PAH} is the amount of IgE in the presence of PAH-DEP, IgE_{V} is the amount of IgE in the presence of vehicle at the same concentrations as with PAH-DEP, and IgE_{C} is the amount of IgE induced by IL-4 and CD40 alone. Net values of IgE ($\text{IgE}_{\text{PAH}} - \text{IgE}_{\text{V}}$) were 23.2, 17.2, 1.4, and 4.5 ng/ml for experiments 1a, 1b, 3a, and 3b, respectively.

tained with the delayed addition of PAH-DEP to IL-4 plus CD40-stimulated PBMCs. In contrast, when PAH-DEP was added to purified B cells 2 days before stimulation with IL-4 plus CD40, there was inhibition of IgE production, which varied from 58% to 66%.

IgE production was consistently enhanced when addition of PAH-DEP was delayed until 2 to 5 days after cultures were stimulated with IL-4 plus CD40 as compared with when PAH-DEP was added at the initiation of the cultures. Day 5 addition induced greater IgE production than day 2 addition in four of six experiments. Addition on day 8 was generally without effect or was inhibitory.

PAH-DEP shows enhancement of B cells stimulated with suboptimal concentrations of IL-4

The effect of PAH-DEP added to B cells stimulated with concentrations of IL-4 ranging from 50 to 200 U/ml was tested to determine whether PAH-DEP induced greater IgE enhancement when the cultures were stimulated by suboptimal

concentrations of IL-4. In a number of experiments, IgE production from B cells stimulated with 50 U/ml and IL-4 CD40 showed a greater percent increase with PAH-DEP, (i.e., 97%) than in cultures with optimal amounts (100 to 200 IU) of IL-4 plus PAH-DEP, (i.e., 19%). However, the absolute amount of IgE at suboptimal concentrations of IL-4 plus PAH-DEP never exceeded the amount with optimal amounts of IL-4. Thus the greater percent increase with suboptimal IL-4 actually relates to the fact that the absolute amount of IgE in suboptimal IL-4 plus CD40 cultures was lower. As with optimal concentrations of IL-4, delayed addition of DEPs to nonoptimal levels of IL-4 (50 U) plus CD40 further increased PAH-DEP's IgE-enhancing ability (Fig. 5).

There was no induction of IgE production by PAH-DEP in cultures of suboptimal doses of IL-4 plus CD40 that in and of themselves did not produce IgE. Thus at 0.01 μ g/ml of CD40, no IgE production was observed with 50 to 200 units of IL-4, and addition of PAH-DEP did not induce the appearance of IgE in these cultures.

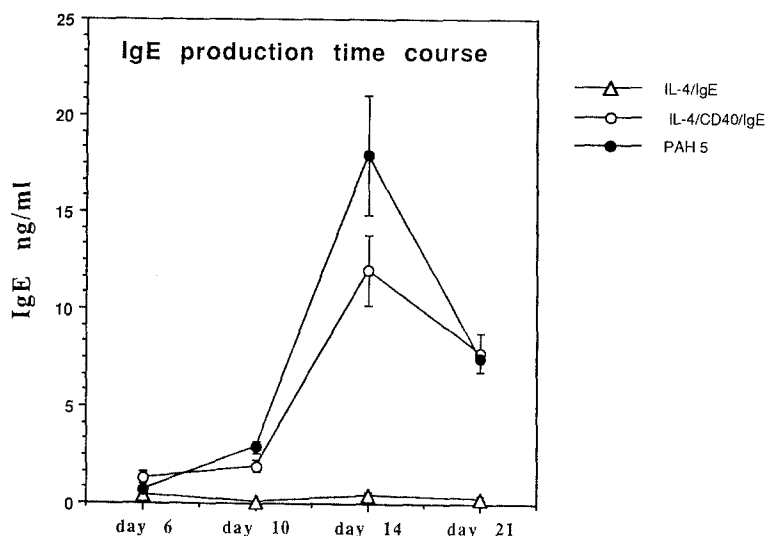


FIG. 3. Kinetics of IgE production by purified B cells in the presence of IL-4 plus CD40 in the presence or absence of PAH-DEP. Purified B cells ($2 \times 10^4/0.2$ ml) were cultured with IL-4 (200 U/ml), IL-4 plus CD40 mAb (0.1 μ g/ml) (open circles) or IL-4 plus CD40 plus PAH-DEP at 5 ng/ml (filled circles). Supernatants were harvested on days 6, 10, 14, and 21 and stored at -20° C. IgE in all of the samples was assayed simultaneously by ELISA. All cultures were done in four replications. No IgE production was observed with IL-4 alone, and no enhanced IgE synthesis was seen with 0.5 ng/ml of PAH-DEP (data not shown).

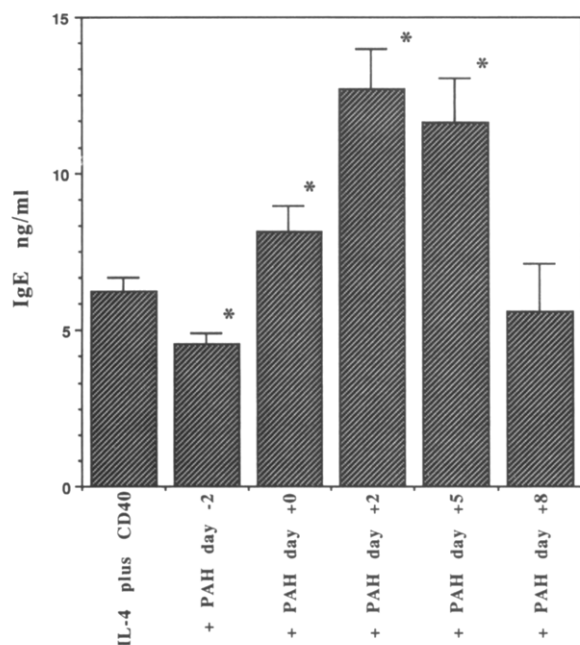


FIG. 4. Delayed addition further increases the IgE-enhancing effect of PAH-DEP. Purified B cells (2×10^4 cells/0.2 ml) stimulated with IL-4 (200 U/ml) plus CD40 mAb (0.1 μ g/ml) had PAH-DEP (1 ng/ml) added before (day -2), simultaneous with (day 0), or after (days 2, 5, and 8) stimulation with IL-4 plus CD40. IgE production was determined on day 14. Values represent the mean and 1 SD of five replicated cultures. * $p < 0.001$.

PAH-DEP selectively enhances IgE production induced by IL-4 and CD40

Whether PAH-DEP altered the production of immunoglobulin isotypes other than IgE after IL-4 plus CD40 mAb stimulation was assessed. The effects of PAH-DEP on the multiple isotypes in CD40 plus IL-4-stimulated cultures are shown in Table I. Only enhancement of IgE was seen with cultures of both PBMCs and B cells when PAH-DEP was added along with IL-4 plus CD40. The marked decrease in IgM levels caused by IL-4 alone or IL-4 plus CD40 has been previously observed,²⁰ and this was not altered or reversed by PAH-DEP treatment. It should be noted that we have not detected increased IgG₄ production in cultures of IL-4 plus CD40 with the cells and mAbs we use.²⁰

TCDD increases IgE production in a fashion similar to DEP-PAH

Because the extracted PAH-DEP is a complex mixture of compounds, we tested the ability of TCDD, a prototype AH, to alter IgE production. In three of three experiments when TCDD was added to IL-4 plus CD40-stimulated B cells on day +2, there was an increase in the IgE synthesis observed. This increase occurred consistently at

10^{-4} mol/L TCDD, and the percent increase at this concentration of TCDD for the three experiments was 57%, 60%, and 67%.

PAH-DEP increases B-cell CD23+ display in the presence of IL-4 plus anti-CD40

CD23, the low-affinity receptor for IgE, appears to play a role in IgE production.²⁹ B cells' expression of CD23 is increased by IL-4.³⁰ Because PAH-DEP was able to synergize with suboptimal and optimal concentrations of IL-4, we attempted to determine whether PAH-DEP was able to increase CD23 display on B cells. Cells were cultured under various conditions including stimulation with IL-4 plus CD40 $\pm$ PAH-DEP. On days 3 to 10, the CD19+ cells' display of CD23 and CD21 was measured.

On day 3, IL-4 increased CD19+ cells' display of CD23 from 15% (culture with medium alone) to 64%. Addition of PAH-DEP with IL-4 resulted in no further increase in the B-cell display of CD23 (66%+) or intensity of CD23 fluorescence. Cells treated with IL-4 plus CD40 showed a decrease in intensity of CD23 display compared with IL-4 alone from a mean channel of 225 to 127 but no change in the percent positive (65%) cells. Addition of PAH-DEP (IL-4+CD40+PAH-DEP) increased the intensity of CD23 display to a mean channel of 160, and 71% of the cells were positive. There was no change in the display of CD21 on the CD19-bearing cells under any of these conditions (data not shown).

On day 10, there was a clear increase in both the percent of CD23+ cells and the intensity of CD23 brightness in the cultures when PAH-DEP was added to IL-4 plus CD40. (Fig. 6). This increase in CD23 display was not due to nonspecific fluorescence related to the presence of the PAH-DEP because cells cultured with PAH-DEP alone did not show increased fluorescence (data not shown). IL-4 plus CD40 cultures showed 49% of the B cells to display CD23 with a mean fluorescence intensity of 65. This was decreased from the cultures with IL-4 alone in which 64% of B cells were CD23+ with a mean fluorescence intensity of 86. Addition of PAH-DEP to IL-4 plus CD40 more than reversed the CD40-driven decrease in CD23 and increased the B-cell CD23 display to 77%, and the mean fluorescence intensity of the CD23+ B cells increased to 134. Essentially identical results were obtained with the addition of PAH-DEP on day +2 (data not shown). In the experiment shown in Fig. 6, IgE production in day 10 supernatants from cultures stimulated with IL-4 plus CD40 plus

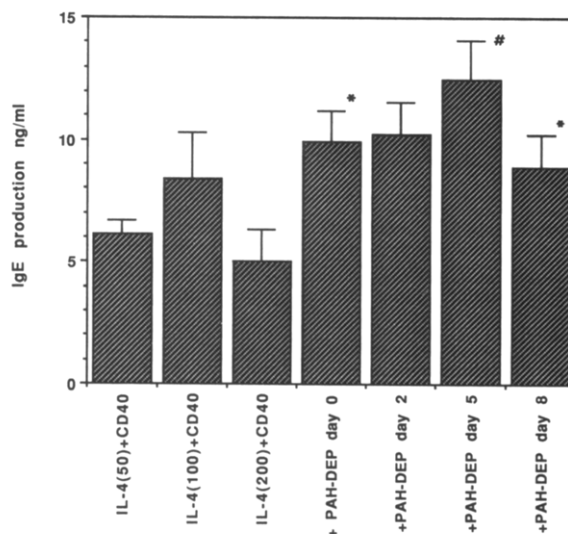


FIG. 5. Delayed addition of PAH-DEP increases its enhancement of IgE synthesis by B cells stimulated with a suboptimal dose of IL-4. B cells (0.2×10^4 cells/well) were cultured in the presence of IL-4 and CD40. At a suboptimal dose of IL-4 (50 U/ml) as shown, the effect of the timing of PAH-DEP addition was determined. PAH-DEP (5 ng/ml) or an equal amount of plain medium was added on days 0, 2, 5, and 8; culture supernatants were assayed for IgE on day 14. Wells with plain medium added had no change in IgE production compared with the control cultures. IL-4 alone at 50 to 200 U/ml did not induce IgE production. * $p < 0.05$ compared with cells treated with 50 U of IL-4. # $p < 0.01$ compared with cells treated with 50 U of IL-4.

PAH-DEP was 4.8 ng/ml, whereas 1.1 ng/ml of IgE was detected in supernatants from cultures stimulated with IL-4 plus CD40.

PAH-DEP alters the pattern of mRNA coding for ϵ proteins

Alternate 3' splicing of the ϵ heavy-chain gene results in different isoforms for ϵ mRNA.^{24,25} A modified PCR procedure was used to determine the production and relative ratios of three different previously reported ϵ isoforms. The different cDNA bands obtained from a PCR are shown in an autoradiograph in the top portion of Fig. 7. The exact level of each ϵ mRNA isoform was particular to the individual and somewhat variable among individuals after IL-4 plus CD40 stimulation of human B cells for 10 days. However, the pattern of the relative expression of these mRNAs was similar among individuals.²⁷ The highest molecular weight (first) band represents an ϵ mRNA coding for the novel dominant membrane form designated CH4-M1'-M2'. The next heaviest band is the β -actin control, a "housekeeping" gene for which production by cells is generally constant. The PCR

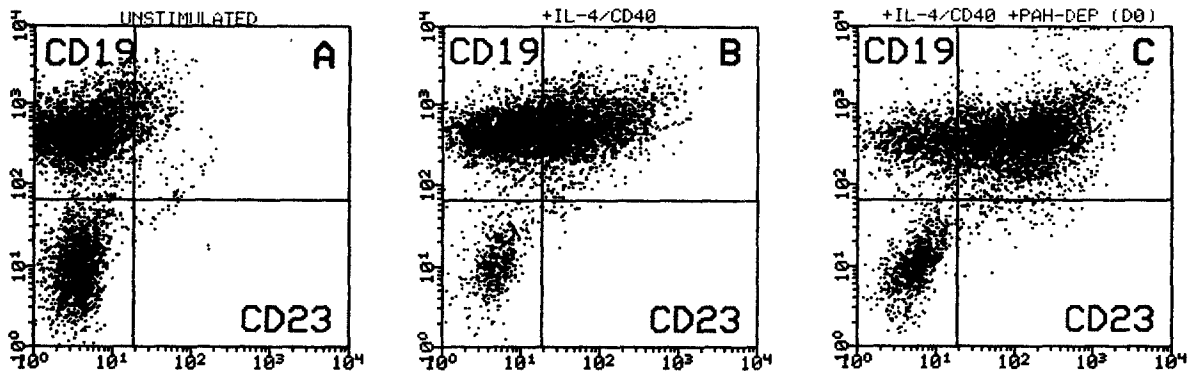


FIG. 6. Display of CD23 on B cells stimulated with IL-4 plus CD40 is enhanced by PAH-DEP. Tonsil B cells (1×10^6 cells in 2 ml) were stimulated with IL-4 (200 U/ml) and CD40 (0.1 μ g/ml) and cultured for 10 days in the presence or absence of PAH-DEP (50 ng/ml). The cells were stained for determination of their expression of CD19 (Leu-19, phycoerythrin-labeled) and CD23 (fluorescein-labeled). **Panel A** shows cells cultured with medium alone: 8% of the CD19+ cells are CD23+, and the mean channel fluorescence of the positive cells is 35. **Panel B** shows cells cultured with IL-4 plus CD40: 49% of the CD19+ cells are CD23+, and the mean channel fluorescence of the CD23+ cells is 65. **Panel C** shows cells cultured with IL-4 plus CD40 plus PAH-DEP: 77% of the CD19+ cells are CD23+, and the mean channel fluorescence of the positive cells is 134.

TABLE I. Effect of PAH-DEP on IgG, IgM, and IgE production by IL-4- and CD40-stimulated cultures

Experiment No.	Stimuli	Ig synthesis (ng/ml)		
		IgG	IgM	IgE
1a	Medium	408	401	<0.2
1a	IL-4	658	300	0.2
1a	IL-4 + CD40	432	5	24.3
1a	IL-4 + CD40 + PAH-DEP 5 ng	329	29	46.8
1a	IL-4 + CD40 + PAH-DEP 50 ng	387	16	36.2
3	Medium	347	134	1.0
3	IL-4	331	44	1.1
3	IL-4 + CD40	217	5	24.3
3	IL-4 + CD40 + PAH-DEP 5 ng	357	10	20.3
3	IL-4 + CD40 + PAH-DEP 50 ng	292	19	34.8
1b	Medium	2034	2136	1.4
1b	IL-4	1403	102	13.7
1b	IL-4 + CD40	371	ND	44.0
1b	IL-4 + CD40 + PAH-DEP 5 ng	778	ND	56.9
1b	IL-4 + CD40 + PAH-DEP 50 ng	709	ND	47.1
4	Medium	1892	1070	0.3
4	IL-4	2010	201	3.2
4	IL-4 + CD40	1257	125	5.2
4	IL-4 + CD40 + PAH-DEP 5 ng	965	89	3.3
4	IL-4 + CD40 + PAH-DEP 50 ng	890	23	6.1

Purified B cells (experiments 1a and 3) or PBMCs (Experiments 1b and 4) were cultured in media alone or stimulated with IL-4 (200 U/ml), IL-4 plus CD40 (0.1 μ g/ml), or IL-4 plus CD40 and PAH-DEP (5 to 50 ng/ml). Culture supernatants were assayed for immunoglobulin on day 14.

ND, Not determined.

product derived from the mRNA coding for the next ϵ isoform has been designated CH4-M2' and encodes a novel secreted ϵ chain protein that is 134 amino acids longer than the classic ϵ secreted form.

The third and most abundant ϵ mRNA product observed encodes for an ϵ isoform represented by the smallest ϵ isoform, a novel secreted form designated CH4-M2'', which when translated

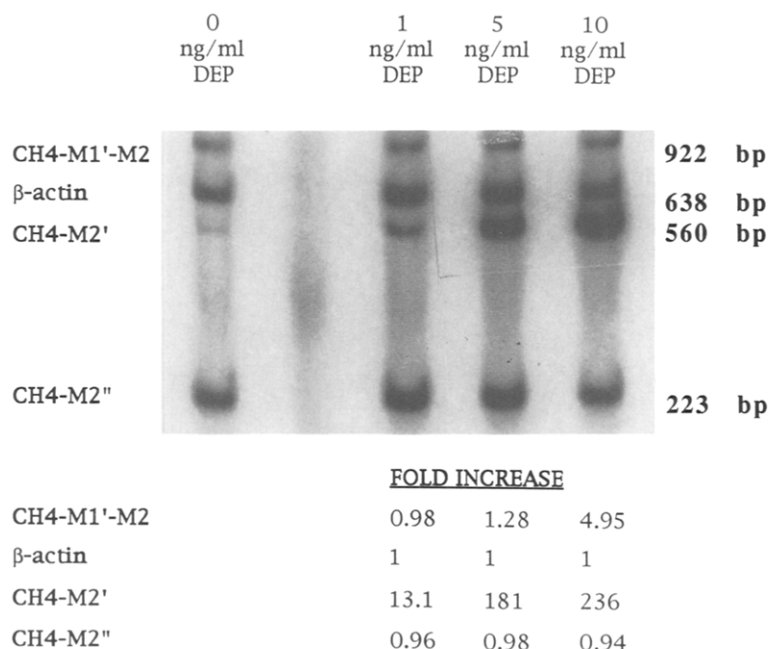


FIG. 7. Change in the pattern of ϵ mRNAs after culture with PAH-DEP. Purified B cells were cultured with IL-4 (100 U/ml) plus CD40 mAb (0.1 μ g/ml) alone or with increasing concentrations of PAH-DEP for 14 days. RNA was then extracted, and the relative amounts of β -actin compared with the various ϵ mRNAs were quantified as described. The top of the figure shows an autoradiograph of the three ϵ mRNA isoforms and β -actin PCR products as labeled on the left. The first lane, 1, shows the ϵ mRNA pattern from cells cultured in the absence of PAH-DEP. The three lanes on the right show the ϵ mRNA patterns from cells cultured with 1 ng/ml, 5 ng/ml, and 10 ng/ml of PAH-DEP, respectively. The size of the amplified fragments in base pairs is shown on the right. The table at the bottom of the figure gives the change (as fold increase) in mRNA levels produced by B cells cultured with the different PAH-DEP doses as compared with the control cells to which no PAH-DEP was added (*lane 1*). There is a dramatic increase in the amount of mRNA representing the CH4-M2'' splice variant. All samples were normalized for the amount of β -actin present.

would differ from the classic secreted ϵ chain by 8 C-terminal amino acids and would be a total of 6 amino acids larger.

The addition of PAH-DEP to the culture led to a shift in the ϵ mRNA isoform pattern. This shift was dose-dependent on the amount of PAH-DEP present. The most striking difference was the increased production of ϵ mRNA for CH4-M2'. In the example shown in Fig. 7, the presence of 1 ng/ml of PAH-DEP resulted in a 13-fold increase in the level of mRNA for this isoform compared with cultures to which no PAH-DEP was added. PAH-DEP at 10 ng/ml enhanced production of the CH4-M2' mRNA isoform by more than 200-fold. In marked contrast, the two other ϵ mRNA isoforms studied varied only slightly. Even at the highest concentration of PAH-DEP, there was only a fivefold increase in CH4-M1'-M2' mRNA levels and no significant increase in the levels of CH4-M2'' mRNA.

DISCUSSION

In this report we demonstrate that PAH-DEP enhances the production of IgE from human B cells. Production of IgE from human cells involves a number of distinct steps: (1) induction of ϵ germline transcription, (2) DNA recombination (isotype switching), (3) RNA transcription, (4) RNA splicing, and (5) translation of the mature mRNA into protein.^{20, 28, 31, 32} PAH-DEP-enhanced IgE production was not attributable to induction of germline transcription or isotype switching. PAH-DEP was unable to provide for IgE synthesis either alone in the presence of IL-4-induced germline transcription or in conjunction with CD40. However, once IgE production was accomplished, PAH-DEP was capable of increasing the amount of IgE produced. The increase in IgE observed with PAH-DEP could not simply be attributed to a change in the time course of IgE appearance in the cultures. Thus PAH-DEP

must exert its effect on total IgE synthesis by providing for greater ϵ RNA transcription of translation. This interpretation is in accord with the known ability of PAHs to alter transcription of a number genes via the cytoplasmic Ah receptor complex.^{33,34} Although in our studies we found that PAH-DEP had a specific enhancing effect on IgE (Table I), this may well result from the fact that we used an immunoglobulin induction system that itself specifically induces IgE. Thus it will not be surprising that PAH-DEP may augment other immunoglobulin isotypes under different stimulation conditions.

PAH-DEP had a qualitative effect on IgE production as well. Thus in addition to altering the levels of IgE, it also altered the pattern of ϵ mRNA isoforms observed. Alternate 3' splicing of the only active ϵ heavy-chain gene in human beings results in distinct ϵ mRNA coding for distinct IgE isoforms.^{25,26} Using a reverse-transcription PCR technique, which measures the relative amounts of three of these novel ϵ mRNAs, we observed that PAH-DEP favors the production of a novel secreted ϵ form, designated CH4-M2', which is produced by a splice that omits the transmembrane segment and therefore encodes for a protein that is 134 amino acids longer than the "classic" ϵ secreted form. Because PAH-DEP does not induce isotype switching, these results suggest that the production of the different ϵ mRNA may be regulated by factors active at later stages in IgE production. How PAH-DEP modulates the isoform pattern remains to be determined. One possibility is that it acts by the nuclear transporter/DNA-binding protein Ah complex,³⁵ binding to xerobiotic response elements either in the ϵ gene itself or in genes that code for splice regulatory proteins. The role of these different isoforms has yet to be determined, although we have shown that they are they present in vivo where DEPs can also alter their relative production. Current studies are aimed at determining the importance of each particular isoform in expression of immediate hypersensitivity.

Because PAH-DEP is a complex mixture of organic compounds, we used TCDD, a polychlorinated bicyclic AH, to demonstrate that the enhancing effect observed was attributable to AHs rather than some other contaminating substance. These experiments with TCDD were revealing in another way. AHs are highly hydrophobic and directly enter cells where they are known to mediate their effects by binding to the cytoplasmic Ah receptor complex, which consists of at least a PAH ligand-binding protein and a nuclear transporter/DNA-

binding protein.^{35,36} The Ah receptor complex is widespread in tissues and has been identified in fresh human tonsil lymphocytes,³⁷ as well as in human B-cell lines.³⁸ Once AH is bound to the Ah receptor, this complex is translocated to the nucleus where it induces several enzyme systems, prominent among which is the P450IA1 cytochrome system, which includes an aryl hydrocarbon hydroxylase and src-like tyrosine protein kinases. The aryl hydroxylase activity of P450IA1 in turn metabolizes the PAHs. In the process of this metabolism, several epoxide intermediates are generated; these are electrophilic intermediates known to be mutagenic for DNA and thereby secondarily alter gene functions. The effects of PAH-DEP on IgE theoretically result from such secondary effects. However, the effects of Ah activation alone can be dissected from that of the secondary electrophilic metabolites by using TCDD because TCDD activates the Ah receptor, but it is not metabolized by the P450IA1-dependent aryl hydrocarbon hydroxylase activity. Thus the observed effect on IgE production must result from the direct activity of the Ah receptor on nuclear processes.

Several investigators performed pioneering in vitro and in vivo studies with mice, demonstrating a direct effect of DEPs on IgE antibody production.⁸⁻¹⁰ Intraperitoneal injection of DEPs increased the IgE response to ovalbumin and Japanese cedar pollen. Intranasal inoculation of DEPs along with antigen primarily enhanced the antigen-specific IgE response. This result contrasted with the adjuvant effect of alum or *Bordetella pertussis*, which increased the IgG but not the IgE response. The investigators suggested that DEPs might enhance IgE responses primarily via the particulate nature of DEPs, providing for antigen adsorption and retention in the host. Our in vitro studies with purified B cells provide evidence for an additional explanation: PAHs contained in DEPs have a direct biochemical effect on B-cell production of IgE.

Because of the known potential for AHs, such as TCDD and benzo[a]pyrene, to be immunosuppressive and to induce mutations and carcinogenesis, major efforts have been made to define the mechanism or mechanisms by which AHs may alter cell growth and differentiation.³⁹ Initially, studies were concerned with the potential carcinogenic or inhibitory effects of PAHs, so they were not designed to evaluate enhancing effects on differentiative cell functions. Studies directed at immunosuppressive effects of TCDD and related

compounds in animals have shown that such compounds can be "suppressive" to both T-cell and B-cell compartments.^{13, 14} In adult mice TCDD inhibits B-cell IgM and IgG responses, primarily by a direct effect on B cells. TCDD and the prototype PAH benzo[a]pyrene must be present before stimulation of B cells; that is, they are suppressive on G₀ but not G₁/S cells, and suppression is associated with the increase of specific B-cell tyrosine protein kinases.^{16, 40} PAHs can also alter expression of important growth/differentiation molecules such as the epidermal growth factor receptor⁴¹ and the genes for IL-1 β and plasminogen activator inhibitor-2.⁴² However, these studies focused on suppression were generally designed to determine inhibitory effects (i.e., the effects of compounds on already optimal immune responses were tested).

Recent studies show that TCDD and PAHs are capable of enhancing immunoglobulin responses (along with B-cell activation and certain kinase activities) if given after induction of a humoral response *in vivo*.¹⁵⁻¹⁷ Thus when TCDD was added to B cells after they were stimulated to G₁/S by α or IL-4 immunoglobulin, an opposite tyrosine protein kinase effect was observed compared with that seen with B-cell suppression.⁴⁰ Such evidence led us to assess the effects of delayed addition of PAH-DEP to IL-4 + CD40-stimulated B-cell cultures. As predicted, addition of PAH-DEP between 2 and 5 days after the initiation of the culture provided for optimal increase in IgE production. In contrast, the presence of PAH-DEP before the cells were stimulated by IL-4 + CD40 led to inhibition. These results also suggest that PAH-DEP's mechanism of action is the alteration of transcriptional programs already activated in the cells. One such system we studied was the effect of PAH-DEP on the expression of CD23, the low-affinity FcER (FcER-II), on B cells. As noted, although PAH-DEP alone had no effect on CD23 expression, it was able to dramatically increase CD23 expression on B cells after 10 days in culture in combination with IL-4 and CD40.

The fact that PAH-DEP was able to increase IgE production in both optimally and suboptimally stimulated human B cells is probably important because it suggests that *in vivo*, these compounds may boost a wide range of allergic responses. Indeed, we have undertaken studies of the *in vivo* nasal effects of DEPs on IgE production in human beings. These studies have also shown that DEPs can enhance IgE production of the human upper respiratory mucosa.²⁷ Overall, our results showing that PAH-DEP can directly enhance human B-cell

production suggest that exposure to such compounds augments human allergic antibody responses. Epidemiologic data, discussed in the introduction, point to a strong association between industrialization and the occurrence of allergic disease.¹⁻⁷ We propose that the ubiquitous occurrence of DEPs has played an important role in increased expression of airway allergic disease, observed this past century, by directly enhancing the production of IgE antibodies in allergic individuals. This could help to account for the increased prevalence of airway allergic disease, because although the percent of potentially atopic persons and level of airborne allergens may not have changed dramatically in the past century, the IgE adjuvant effect of PAH-DEP will have helped induce clinical allergy in a large segment of the potentially allergic population.

Extensive studies have characterized the nature of the PAHs present in diesel exhaust.⁴³⁻⁴⁶ This comprises a long list of hydrocarbons, several of which are prominent because of their occurrence in tobacco smoke as well. Tobacco smoke is associated with an increase in IgE levels *in vivo* in human beings⁴⁷ and inhaled smoke, particularly "side-stream smoke," is high in the prototype PAH benzo[a]pyrene. If specific chemicals in PAH-DEP are ultimately shown to be particularly potent in enhancing IgE, production of these compounds by automotive exhaust may be decreased because fuel composition can profoundly alter the nature of the PAH-DEP generated.⁴⁸

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