

The environmental pollutant pyrene induces the production of IL-4

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Background: Epidemiologic studies and experiments with mouse models suggest that polyaromatic hydrocarbons contained in, among others, diesel exhaust particles can promote the development of allergy.

Objective: Because IL-4 organizes allergic responses *in vivo*, we have investigated whether pyrene, a major compound of diesel exhaust particles, can affect the production of IL-4.

Methods: IL-4 production by primary human T cells was assessed by ELISA and messenger RNA transcription was detected by Northern blotting. Activation of the IL-4 promoter was tested in reporter gene assays with transiently transfected cell lines.

Results: Pyrene induced transcription of IL-4 messenger RNA and expression of IL-4 protein in primary human T cells.

Pyrene, but not related polyaromatic hydrocarbons, enhanced basal transcription of the human and mouse IL-4 promoter.

Conclusion: Our results suggest that pyrene may promote allergic diseases by inducing the production of IL-4. (*J Allergy Clin Immunol* 2000;105:796-802.)

Key words: Pyrene, IL-4, allergy, polyaromatic hydrocarbons, environmental pollution, diesel exhaust particles

The prevalence of type 1 allergies in developed countries has increased in recent decades. Because the generation of type 1 allergies is determined by multiple genetic and environmental factors, several reasons may contribute to the observed rise in incidence.¹⁻⁴ Thus a lower rate of bacterial, viral, and parasitic infections in childhood may alter cytokine patterns during crucial periods in the development of the immune system. In addition, changes in diet, lifestyle, and behavior affect the availability of allergens, as well as of bystander substances that may act as allergy-promoting adjuvants. Proallergic adjuvant effects have been suggested for environmental pollutants, food additives, and other substances that do not have allergenic potential by themselves but may increase the likelihood that an allergic response is elicited by a potential allergen.

Abbreviations used

AH:	Aromatic hydrocarbon
Con A:	Concanavalin A
DEP:	Diesel exhaust particles
mRNA:	Messenger RNA
PAH:	Polyaromatic hydrocarbons
STAT-6:	Signal transducer and activator of transcription-6

Diesel exhaust particles (DEP) are a well-defined example of agents suspected of proallergic adjuvant activity.^{2,5} Studies in Japanese populations have shown that children living close to major highways were more likely than other children to have allergies against common pollen allergens.⁶ Because food allergies did not differ between these 2 groups, an airborne proallergic influence was suggested.

Polyaromatic hydrocarbons (PAH) containing 3 to 5 benzene rings are major chemical components of DEP. Studies with humans and mice showed that either total DEP, or PAH extracted from DEP, can enhance allergic responses toward specific allergens such as ovalbumin,⁷⁻⁹ Japanese cedar pollen,⁷ ragweed,¹⁰⁻¹² and house dust mite allergen.⁶ Both total DEP and PAH extracts significantly increase total and specific IgE and IgG1/IgG4 production. IgE synthesis was also enhanced by PAH extracted from DEP in purified human B cells stimulated with IL-4 plus anti-CD40.¹³

The analysis of specific PAH suggests that at least 2 different mechanisms can be responsible for the proallergic effects of PAH extracts. Phenanthrene up-regulates IgE production in an EBV-transformed human B-cell line by inducing higher levels of productive C ϵ transcripts, without influencing cytokine expression or the production of sterile I ϵ transcripts.¹⁴ In contrast, pyrene enhances in antigen-stimulated mice the production of IL-4, which in turn induces the production of sterile I ϵ transcripts.⁶ The first mechanism could enhance continuing allergic reactions, whereas the second may increase the predisposition for development of allergic sensitizations.

Studies with total DEP have revealed further evidence for an influence on cytokine expression. Intranasal challenge of healthy volunteers with DEP results in up-regulation of many cytokine messenger RNAs (mRNA), including IFN- γ , and without any particular skewing toward T_H1 or T_H2 cytokines.¹⁰ However, a later study showed that volunteers allergic to ragweed display on challenge with DEP in the presence of ragweed allergen-selective up-regulation of T_H2 cytokines and decreased expression of T_H1

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cytokines.¹¹ Mice treated with DEP in the presence of antigen also show enhanced T_H2 cytokine expression, but IFN- γ levels are unchanged or lowered.^{6,9,15} In summary, there is evidence for a T_H2 -promoting function of DEP in the presence of antigen, most likely by enhancing the production of IL-4. We have focused in this study on the PAH pyrene, an environmental pollutant contained in DEP and many other sources.

Pyrene could potentiate IL-4 effects by increasing its production or by enhancing IL-4-dependent signal transduction. We have therefore investigated the influence of pyrene on IL-4 mRNA production and protein expression. We also tested pyrene effects on human and mouse IL-4 promoters and the human IgE class switching promoter. The latter is regulated by IL-4 by the signal transducer and activator of transcription-6 (STAT-6) pathway, which also regulates several other important IL-4 target genes, including CD23, MHC II, and IL-4R α .¹⁶ Our data indicate that pyrene up-regulates the production of IL-4 but does not interfere with STAT-6-regulated gene expression.

METHODS

IL-4, concanavalin A, and PAH

Human IL-4, expressed in *Escherichia coli* and purified as described,¹⁷ was a gift from Dr W. Sebald, Würzburg, Germany. Concanavalin A (Con A) was obtained from Sigma (Deisenhofen, Germany). Pyrene, anthracene, phenanthrene, and fluoranthene, obtained from Sigma, were dissolved in cell culture medium at 2.5 mg/mL. The resulting mixture was sonicated with the microtip of a Branson Sonifier 250 (Heinemann, Schwäbisch-Gmünd, Germany) until a homogeneous suspension with small particles was obtained. For each assay this solution was freshly prepared and then diluted with cell culture medium to the final concentrations.

Cell culture

Human PBMCs were obtained from anonymous healthy donors (allergy status unknown) and prepared by density gradient centrifugation over Ficoll. Human PBMCs, human Jurkat T leukemic cells, murine EL4 thymoma cells, human Ramos Burkitt B lymphoma cells, murine Ba/F3 pro B lymphomas, and human HMC-1 mastocytoma cells were cultured in RPMI 1640 medium (Gibco Laboratories, Grand Island, NY) supplemented with 4% (EL4, PBMC) or 8% (all other cells) FCS (PAA Laboratories, Cölbe, Germany), 2 mmol/L L-glutamine, 100 U/mL penicillin, and 100 μ g/mL streptomycin at 37°C and 5% carbon dioxide.

The Ba/F3 cell line used was stably transfected with human IL-4R α and human γ c.¹⁸ For this cell line the culture medium was supplemented with 5% culture supernatant of murine IL-3-producing X63Ag8-653 BPV murine IL-3 cells,¹⁸ whereas all other cell lines were grown without additions.

To determine cell viability, cells were incubated with PAH and after 6 or 24 hours stained with trypan blue and counted. Viability was 95% or better.

IL-4 ELISA

To determine the effect of pyrene on IL-4 production, PBMCs were seeded at a final density of 2×10^6 cells per milliliter in cell culture medium supplemented with pyrene or Con A. After 48 and 72 hours, cell-free supernatants were taken and measured in duplicate with use of a human IL-4 ELISA kit (Amersham). The amount of secreted IL-4 was calculated with recombinant human IL-4 as standard. The sensitivity of the assay was 0.5 pg/mL.

RNA isolation and reverse transcription-PCR analysis of IL-4 mRNA

Total cellular RNA was isolated with use of the RNeasy kit (Qiagen, Hilden, Germany). One microgram of RNA was reverse transcribed after heat denaturation at 65°C for 3 minutes and cooling on ice. The reaction was carried out in 20 μ L of buffer for 45 minutes at 42°C and was stopped by heat inactivation for 5 minutes at 90°C. Five micrograms of complementary DNA was then PCR amplified with 12 U of Taq DNA polymerase in 45 μ L of buffer containing 10 μ mol/L of the human IL-4 primer (456 bp PCR product) and 2.5 μ mol/L of the human β -actin primer (661 bp PCR product) (Stratagene, Heidelberg, Germany). Thirty-five cycles were carried out, each cycle with a denaturation step at 95°C for 1 minute, an annealing step at 62°C for 1 minute, and an elongation step at 72°C for 1.5 minutes, except for the first cycle in which the denaturation and annealing steps were 5 minutes each. PCR products were analyzed on a 1.3% agarose gel.

Reporter gene constructs

The luciferase reporter gene construct containing DNA sequences from the human I ϵ promoter was obtained by cloning the *Bgl* I/*Bam* HI fragment of construct E2¹⁷ between the *Bgl* I/*Bam* HI sites of the Luc⁺ vector containing a TATA minimal promoter.¹⁹ The reporter gene construct with the multimerized STAT-6 binding site (5 $\times$ STAT-6: AGCTGATCCACTTCCCAAGAACAGAGATCCACTTCCCAAGAACAGAGATCCACTTCCCAAGAACAGAGATCCACTTCCCAAGAACAGAGATCCCG) was obtained by cloning a chemically synthesized *Hind* III/*Bam* HI oligonucleotide (Integra, Germany) between the *Hind* III/*Bam* HI sites of the Luc⁺ vector containing a TATA minimal promoter.¹⁹ Plasmids were analyzed by digestion with restriction endonucleases and DNA sequencing.

The reporter gene construct containing a fragment of the human IL-4 promoter is described by Li-Weber et al,²⁰ and the constructs containing fragments of the murine IL-4 promoter are described by Chuvpilo et al.²¹ Corresponding luciferase reporter gene constructs without enhancer sequences were used as controls.

Transient transfection, stimulation, and luciferase assay

Before transfection the cells were cultured in fresh medium for 24 hours. For electroporation 5 to 7×10^6 cells in 350 μ L of RPMI 1640 medium supplemented with 4% FCS (EL4, 2%) were mixed with 30 μ g of DNA in 50 μ L of the same medium. The transfection was performed by a single pulse (1200 μ F) from a Eurogentec Easyject pulser. Optimal voltages were determined for each cell line (230–270 V). Equal amounts of transfected cells were stimulated 30 minutes after transfection with the indicated substances for 6 hours. As a control, transfected cells were incubated with cell culture medium alone. Cell extracts were prepared with use of a luciferase assay kit (Promega, Mannheim, Germany) and relative light units were measured in a luminometer (Bertold, Bad Wildbad, Germany). Fold induction of luciferase activity was calculated compared with the unstimulated cells. The luciferase activity in the absence of pyrene was defined as 1 for each condition, and the fold induction over that level is indicated.

RESULTS

Pyrene enhances Con A-stimulated secretion of IL-4 from PBMCs

Pyrene alone did not induce detectable IL-4 production by PBMCs. However, Con A-stimulated IL-4 synthesis was observed, and simultaneous addition of pyrene increased Con A-stimulated IL-4 production in a con-

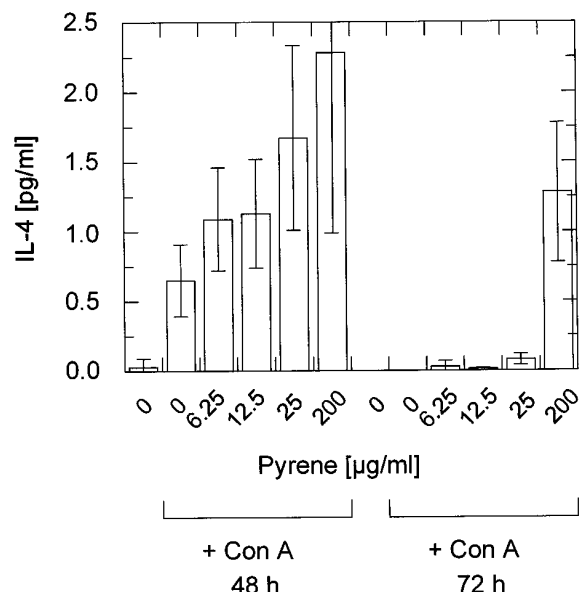


FIG 1. Pyrene induces secretion of IL-4 in peripheral T cells. Human PBMCs were incubated for 48 or 72 hours with indicated stimuli. Con A was applied at 2 µg/mL. Human IL-4 in supernatant was determined by ELISA. Each bar represents average value for 6 experiments performed with cells from different donors. All samples were measured in duplicate. Error bars indicate SEM.

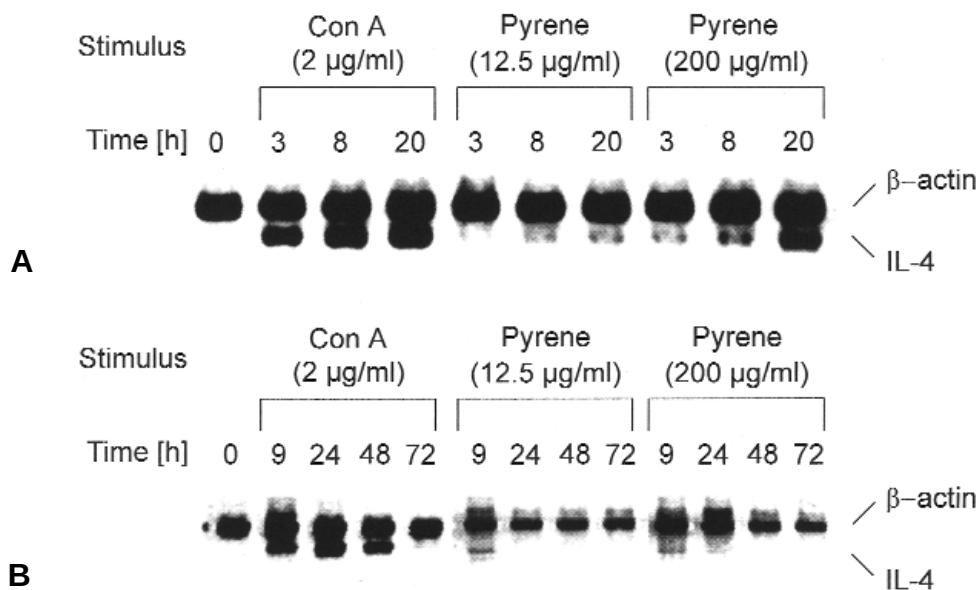


FIG 2. IL-4 mRNA expression on pyrene stimulation in peripheral T cells. PBMCs were stimulated in 2 separate experiments with Con A or pyrene for indicated times. RNA was isolated and subsequently subjected to reverse transcription-PCR analysis with use of primers specific for human IL-4 cDNA and for human β-actin cDNA as internal control.

centration-dependent manner (Fig 1). The highest pyrene concentration used, 200 µg/mL, led after 48 hours to a 3.4-fold increase in IL-4 compared with Con A treatment alone. After 72 hours no IL-4 was detectable with Con A alone, whereas costimulation with Con A and at least the highest pyrene concentration still induced a significant amount of IL-4.

Up-regulation of IL-4 mRNA transcription by pyrene

PBMCs were isolated from healthy donors, stimulated with Con A or pyrene as indicated, and analyzed for IL-4 mRNA transcription by reverse transcription-PCR. Treatment with either Con A or pyrene resulted in tran-

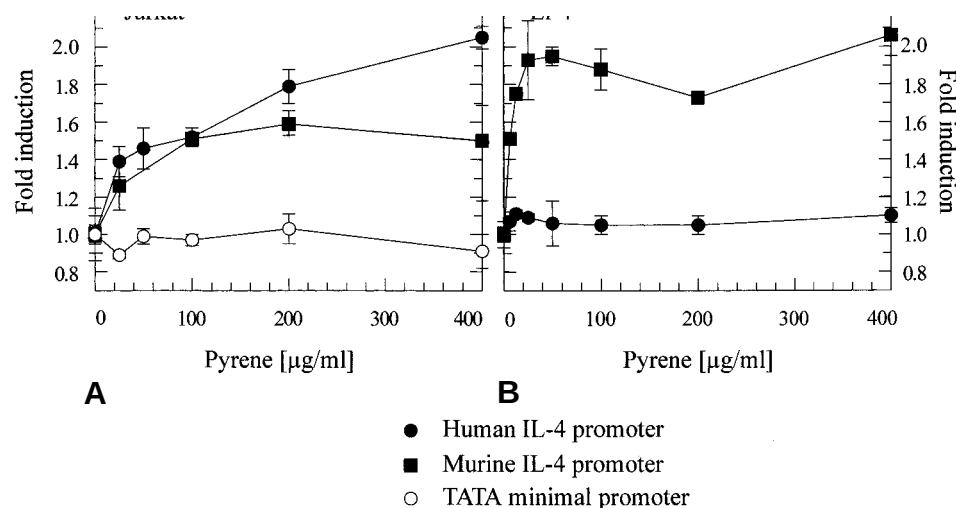


FIG 3. Pyrene activates the IL-4 promoter. Reporter gene constructs containing 270-bp fragment of murine or human IL-4 promoter or TATA minimal promoter alone were transiently transfected into human Jurkat (A) or murine El-4 (B) cells. Cells were stimulated for 6 hours with pyrene concentrations as indicated. Mean and SEM of typical experiment out of 3 to 8 performed in triplicate are given.

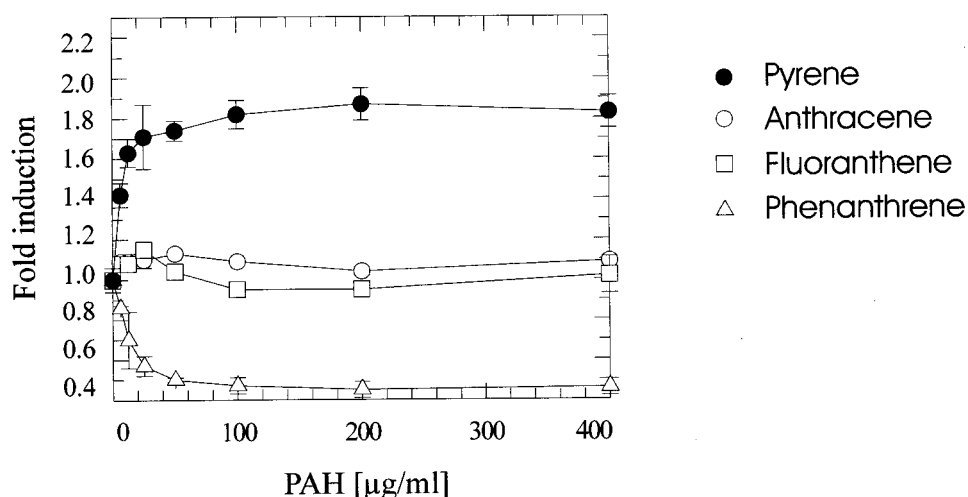


FIG 4. Different PAH have different effects on the human IL-4 promoter. The 270-bp human IL-4 promoter construct was stimulated in Jurkat cells with pyrene, anthracene, fluoranthene, or phenanthrene. Mean and SEM of a typical experiment performed in triplicate out of 2 to 8 experiments are given.

sient induction of IL-4 mRNA transcripts (Fig 2). Con A induced higher transcript levels compared with pyrene, and 200 µg/mL pyrene was more efficient than 12.5 µg/mL. Optimal stimulation times appeared to be variable for different donors (see Fig 2, A, vs Fig 2, B).

Pyrene specifically up-regulates basal IL-4 promoter activity

In the human T-cell line Jurkat, pyrene up-regulated basal transcription of the 270-bp human IL-4 promoter in a dose-dependent manner but did not influence transcription from a control reporter gene containing a TATA minimal promoter without an additional enhancer (Fig 3, A). A murine IL-4 promoter fragment was activated by pyrene

in Jurkat cells (Fig 3, A), whereas in the murine thymoma line El4 only the murine but not the human IL-4 promoter was activated by pyrene (Fig 3, B). Use of longer fragments of the murine IL-4 promoter (440 and 797 bp) or extending the stimulation time from 6 to 48 hours did not further increase the pyrene effect (not shown). Differences in stimulation time effects compared with Fig 2 may be the result of use of T-cell lines versus PBMCs.

Different PAH have different effects on the IL-4 promoter

To examine whether the effect of pyrene on the IL-4 promoter was a general feature of PAH, Jurkat cells were transiently transfected with the human (Fig 4) or murine

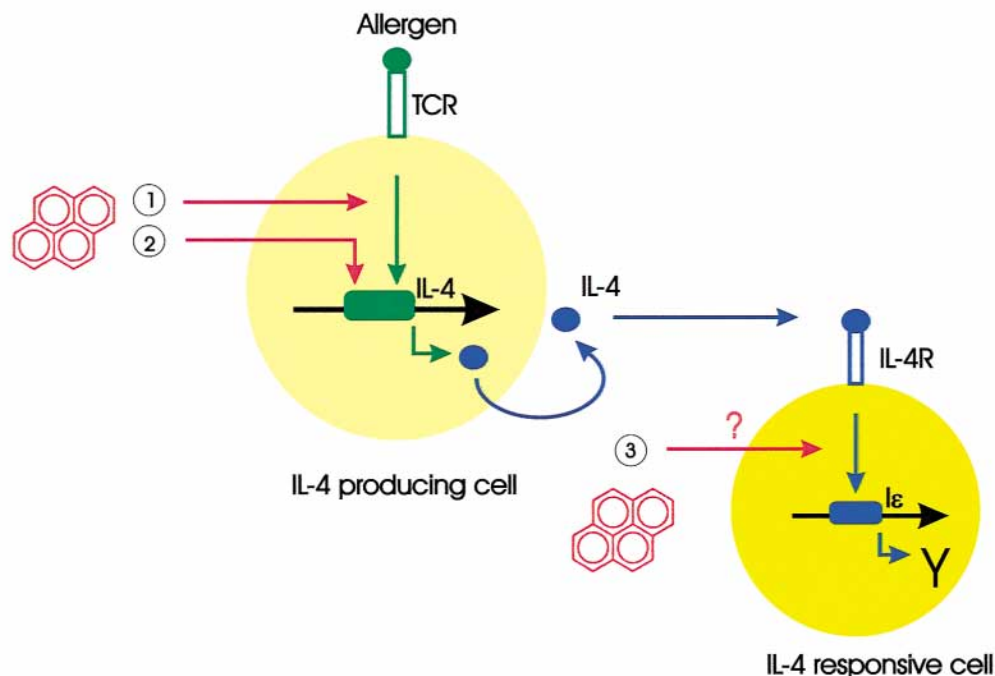


FIG 5. Possible mechanisms for proallergic effects of pyrene. 1, Synergistic enhancement of T-cell receptor (TCR) signaling. 2, Direct interaction with proteins leading to IL-4 promoter activation. 3, Synergistic enhancement of IL-4 cell receptor signaling.

(data not shown) 270-bp IL-4 promoter construct and stimulated for 6 hours with different PAH. Anthracene and fluoranthene did not influence the transcriptional activity of the human IL-4 promoter, whereas phenanthrene even reduced transcription.

Stimulation by pyrene is cell-type specific

Sources of IL-4 *in vivo* are exclusively T cells and FcεRI-positive cells such as mast cells, basophils, and eosinophils.²² Remarkably, we found activation of the IL-4 promoter by pyrene only in the T-cell line Jurkat, the murine thymoma line EL4, and the mast cell line HMC-1 but not in the B-cell line Ramos or the murine pro-B cell line Ba/F3 (not shown). The activity of pyrene on the IL-4 promoter may be restricted to cell types that synthesize IL-4 *in vivo*.

Pyrene does not interfere with STAT-6 activation

Because many studies indicate that DEP or associated PAH enhance IgE antibody production,⁶⁻¹² we examined the effect of pyrene on the IL-4-responsive element of the human IgE class switching promoter and on a multimerized STAT-6 binding site in human B cells.

Reporter gene constructs under the control of the IL-4-responsive region of the human Iε promoter or of an oligomerized STAT-6 binding site were not activated by pyrene in Ramos cells although transcription of these constructs was up-regulated by IL-4 stimulation 6.83 ± 0.41 -fold and 16.67 ± 0.85 -fold, respectively (not shown). Simultaneous stimulation with pyrene and IL-4

resulted in identical effects as found for IL-4 alone. These data suggest that pyrene has no effect on IL-4-induced STAT-6 activation and does not enhance IL-4-stimulated production of sterile Iε transcripts.

DISCUSSION

Particulate air pollutants, such as DEP, have been implicated as one of the reasons for the increased incidence of respiratory allergic diseases that has occurred over the last decades. Particle-associated PAH are the most likely candidates for such a proallergic effect. We have investigated the effect of PAH on IL-4 production and mRNA transcription and on the transcriptional activity of the IL-4 promoter.

IL-4 is crucial for the development of allergic diseases.²³ We found that pyrene induced IL-4 mRNA transcription and, on costimulation with Con A, IL-4 production in human PBMCs. IL-4 protein levels induced by pyrene alone may have been below the detection limit, whereas the more sensitive IL-4 mRNA reverse transcription-PCR clearly showed an activating effect of pyrene in the absence of other stimuli. Pyrene but not related PAH also enhanced basal transcription of the human and mouse IL-4 promoter. A fragment of 270 bp from the promoter was sufficient to respond to pyrene in T cells and mast cells, whereas B-cell lines were unresponsive. The activating effect of pyrene appears to be limited to cells types that can produce IL-4 *in vivo*.

In agreement with increased IL-4 production observed in our experiments, it has been reported that mice treated

with pyrene during sensitization toward the house dust mite antigen Der f 2 produced higher levels of specific IgE in vivo, and spleen lymphocytes derived from these mice secreted more IL-4 on antigen stimulation in vitro.⁶

The PAH concentrations applied were quite high, but because pyrene and the other PAH used are in cell culture medium mostly present in form of insoluble particles the biologically available concentrations were certainly much lower. It is similarly difficult to estimate how much inhaled particle-associated PAH will interact with cells and become biologically active.

Two other PAH, anthracene and fluoranthene, demonstrated no significant effect on the IL-4 promoter activity, whereas phenanthrene even inhibited basal IL-4 promoter activity. Other studies have also found that cytokine promoters are not stimulated by phenanthrene, β -naphthoflavone, and benzo(a)pyrene.^{24,25} The effect of pyrene is therefore surprisingly specific.

Because related compounds failed to show the same effect as pyrene, it is unlikely that the aromatic hydrocarbon (AH) receptor is involved. The AH receptor is present in many cell types, including lymphocytes and B-cell lines.¹³ Once PAH is bound to the cytoplasmic AH receptor, this complex is translocated to the nucleus where it induces the expression of multiple genes.¹³ Although all tested PAH can bind to the AH receptor, only pyrene activated the IL-4 promoter. In addition, sequence analysis of the IL-4 promoter revealed no putative binding site for the AH receptor. Takizawa et al²⁶ have recently reported that total DEP stimulate the promoter of the chemokine IL-8 by nuclear factor- κ B binding sites, so transcription factors different from the AH receptor can be influenced by PAH.²⁶

Three possible mechanisms for proallergic effects of pyrene are illustrated in Fig 5. Pyrene could enhance signals originating from the T-cell receptor, leading to higher IL-4 expression during antigen stimulation. Pyrene may also directly interact with signal pathways or transcription factors regulating the IL-4 promoter, which would induce IL-4 expression without requirement for T-cell receptor ligation. A third option is that pyrene could enhance the biologic activity of IL-4 by interacting with IL-4-regulated signal pathways, which would increase IL-4 effects without affecting cytokine levels. Our data indicate that the second alternative can occur because IL-4 mRNA transcripts were detected in PBMCs without T-cell receptor stimulation. Pyrene effects on the IL-4 promoter also did not require a costimulus. Because we could not detect IL-4 production in the absence of Con A, it remains possible that stimulation of the T-cell receptor is required for pyrene-enhanced protein production. We consider it unlikely that pyrene interferes with IL-4 signaling because we found no effect on the IL-4-regulated region of the *I ϵ* promoter in the presence or absence of IL-4.

In conclusion, increased IL-4 expression after exposure to pyrene may contribute to enhanced IgE class switching and T_H2 differentiation from naive T cells. Pyrene is present in many sources, including various foods, cigarette smoke, domestic heating, and industrial emissions. Our

continuous exposure to this substance may lead to increased IL-4 production and in this way it may promote the development of asthmatic and allergic diseases.

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