

Combined Diesel Exhaust Particulate and Ragweed Allergen Challenge Markedly Enhances Human In Vivo Nasal Ragweed-Specific IgE and Skews Cytokine Production to a T Helper Cell 2-Type Pattern¹

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We have previously shown that in vivo nasal challenge with diesel exhaust particles (DEP) induces both quantitative and qualitative changes in local IgE production and stimulates generalized local cytokine production. We have now investigated the combined effects of intranasal challenge with DEP plus ragweed allergen on local humoral immune responses. We collected nasal lavages from ragweed sensitized subjects at different times after nasal challenge. As compared with challenge with ragweed alone, challenge with both DEP and ragweed induced markedly higher ragweed-specific IgE but not total IgE levels or IgE-secreting cell numbers. Total and specific IgG4 levels also were enhanced, while total IgG levels were not. Synergy was also observed between the DEP and ragweed in altering the profile of ϵ mRNAs generated by alternative splicing, mRNAs that code for different expressed IgE proteins. Intranasal challenge with ragweed alone induced inconsistent and low levels of mucosal cytokine mRNAs. In contrast, challenge with both ragweed plus DEP resulted in decreased expression for Th1-type cytokines (IFN- γ and IL-2) but elevated expression of mRNA for other cytokines (IL-4, -5, IL-6, IL-10, IL-13). This synergy between DEP and natural allergen exposure is suggested as a key feature in increasing allergen-induced respiratory allergic disease. *The Journal of Immunology*, 1997, 158: 2406–2413.

Previous studies from our laboratory have directly addressed the effects of diesel exhaust particle aggregates (DEP)³ and their associated polyaromatic hydrocarbons (DEP-PAH) on the human allergic response (1–3). This has been undertaken to establish the molecular basis for observations suggesting a linkage between increasing pollution and allergic illness. Many epidemiologic studies have linked atmospheric pollutants with the increased incidence and morbidity of allergic airway disease observed in recent times (4–8). Employing in vitro and in vivo models, we have extended these studies by investigating the mechanisms by which pollutants, such as aromatic hydrocarbons, interact with allergen exposure.

We have shown that DEP-PAH and phenanthrene, (the major organic constituent of DEP), can enhance IgE production in vitro and preferentially up-regulate production of certain mRNAs cod-

ing for distinct isoforms of the ϵ -chain (1). We have also demonstrated that DEP can induce a heightened total IgE (and IgG4) response in the human upper airway in vivo following intra-nasal challenge associated with an increase in the number of local IgE-secreting cells. Furthermore, following provocation with DEP, the ϵ mRNA isoform pattern observed was altered toward a more differentiated pattern. Thus we had shown that DEP alters IgE production both quantitatively and qualitatively.

In vivo nasal challenge with DEP increased cytokine production in an apparently nonspecific manner (3). Before challenge, only IFN- γ , IL-2 and IL-13 mRNA could be detected in subject's nasal lavage cells. After challenge the levels of all cytokine mRNA increased; the cells producing readily detectable mRNA for IL-2, -4, -5, -6, -10, and -13 and IFN- γ . This broad increase in cytokine production was not simply the result of an increase in T cells recovered in the lavage fluid. This increase in nasal cytokine expression following exposure to DEP can be predicted to contribute to enhanced local IgE production.

In this study we extend our previous findings by investigating the interaction of DEP on allergen-driven responses in vivo in allergic human subjects. We show that DEP will enhance the allergen-specific IgE response but not the total IgE response in ragweed-challenged subjects sensitive to ragweed. We also show a possible mechanism for this result by demonstrating that while intranasal challenge with ragweed alone induces low level and inconsistent cytokine mRNA production, in the nasal mucosa of ragweed-sensitive subjects, challenge with ragweed plus DEP will result in decreased or unchanged expression of mRNA for Th1-type cytokines but in increased expression for Th2-type and other cytokines.

Materials and Methods

Subjects

Thirteen nonsmoking volunteers (6 men and 7 women) age 21 to 49 years old were recruited in Los Angeles, CA. All had been shown to have a

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³ Abbreviations used in this paper: DEP, diesel exhaust particles; PAH-DEP, polyaromatic hydrocarbons of diesel exhaust particles; AU, allergen units.

positive intradermal skin test to short ragweed and an allergy history consistent with allergic rhinitis. While 8 of the 13 subjects showed positive skin tests for other allergens, all were asymptomatic and none complained of symptoms during the course of the study. The subjects did not take any medication for the 3 days before or at any time during the study. None of the volunteers had any known extensive or extraordinary exposure to pollutants. All subjects were asked to fill out and record symptom score cards 2 days prior to and throughout the study period. The Ag used in the challenge studies, short ragweed, is not present in the Los Angeles area and the cross-reacting Western ragweed is a minor allergen. This provided us with the opportunity to use subjects who had been sensitized to short ragweed during prior habitation in a different locale but who were not naturally exposed to it during the duration of the study. This provided for very low levels of ragweed IgE in nasal lavages before challenge. All studies were approved by the Human Subject Protection Committee of the University of California at Los Angeles.

By definition, all subjects in this study who were challenged intranasally with the ragweed allergen *Amb a 1* displayed immediate allergic symptoms such as sneezing, runny nose and ocular itching. Additionally, seven of the subjects reported symptoms consistent with late phase reactions (such as recurrent congestion and rhinorrhea) some 5 to 12 h later. Challenge with both DEP and ragweed allergen did not result in a significant increase in the symptom severity scores as compared with challenge with ragweed alone (data not shown).

DEP and ragweed nasal provocation and lavage

Nasal washes and provocation challenges were performed as previously described (1). Briefly, 5 ml of normal saline was delivered into each nostril of the subjects and after 10 s, the wash fluid was collected. The initial wash was discarded. The subjects then performed four subsequent nasal washes at 10-min intervals with each wash being collected into a separate tube. The tubes were centrifuged at $350 \times g$ for 10 min at 4°C , and the aqueous supernatants separated from the cell pellets. When cells were needed for filter spot-ELISA or for RNA extraction, the number of nasal washes was increased to eight. The number of cells recovered remained reasonably constant for each wash but the percentage of epithelial cells decreased with repeated washing, resulting in an increase in mononuclear cell numbers. Ten minutes after the last wash, subjects were challenged by spraying the nose with increasing doses of short ragweed *Amb a 1*, starting at 10 allergen units (AU) and increasing in 10-fold steps until symptoms (sneezing/irritation) were apparent. No subject received $>10,000$ AU or <100 AU. Nasal washes were performed, as above, on subjects 18 h after challenge (day 1), and on days 4 and 8. After a minimum of 60 days, subjects underwent a combined challenge with the same dose of *Amb a 1* previously given as well as 0.3 mg of DEP in 200 μl saline. Again, additional nasal washes were performed 1, 4, and 8 days after the combined challenge. To ensure that there was no priming effect, a control group was challenged with ragweed alone 60 days after the initial challenge with ragweed. No significant priming effect was observed; results were similar at the two times in this group (data not shown).

DEP were obtained from a light duty diesel passenger car and processed as previously described (1, 2). The time period of 18 h was chosen because maximal expression for cytokines was observed between 18 and 24 h (data not shown), as has previously been reported (9, 10).

Ig, Ab, and albumin determination

The levels of IgE, IgG, IgG4, and IgA in the supernatants of cultured cells were measured by isotype specific ELISAs as previously described with minor modifications (1, 11, 12). For the determination of albumin levels, an similar ELISA procedure was used as previously described (1). All samples were run in duplicate and repeated if there was $>5\%$ variation between the duplicates. Ragweed-specific IgE, IgG, and IgG4 were determined using the same procedure as for total Ig isotypes except that an amplification system previously described was used with minor modifications (11). The plates were cultured were coated with 10 $\mu\text{g}/\text{ml}$ *Amb a 1* (Sigma Chemical Co., St. Louis, MO) and alkaline phosphatase-labeled anti-IgE, anti-IgG, or anti IgG4 (Tago, Burlingame, CA) at 1/3000 were used for detection.

Measurement of IgE- and IgA-secreting cells

IgE and IgA secreting B cells were enumerated using a filter spot-ELISA as previously reported (1, 13, 14). The sensitivity of the IgE spot assay was determined by using serial dilutions of 2C4/F3 IgE producing cells (15); direct counts of these cells were compared with the number of spots detected and used to construct a standard curve. The assay was shown to be specific because no plaques were detected when using myeloma cells producing other Ig isotypes. Similarly, IgA-producing myeloma cells (GM1056) (16) were used to construct a standard curve for the IgA filter

spot assay. Positive reactions were recognized as circular granulated foci with diameters greater than 0.05 mm. Samples were run in quintuplicate and serially diluted.

Reverse transcription-PCR assay for cytokines and ϵ mRNA splice variant expression

Total cellular RNA from the cells recovered from nasal washes was isolated and then reverse transcribed using Moloney murine leukemia virus (MMLV) reverse transcriptase (Bethesda Research Laboratories, Gaithersburg, MD) as previously described (12). Cytokine mRNAs were measured using the same primers and conditions as previously described (3). The PCR strategies were based on that reported by Yamamura et al. (17) that allowed comparison of cytokine mRNA in different samples. This PCR strategy had been demonstrated with the same primers and conditions to be quantitative throughout a broad range of cDNA concentrations by the use of cytokine cDNA containing plasmids. The amounts of cDNA were normalized to yield equivalent PCR products of β -actin; a "housekeeping gene" constitutively expressed by cells and a direct measure of the amount of cells from which RNA was extracted. The adjusted PCR product was then electrophoresed on 1.5% agarose gels and visualized by ethidium bromide staining. PCR results were confirmed by transfer of the electrophoresed PCR products to nylon membranes (Hybond-N membrane, Amersham Corp., Arlington Heights, IL), and hybridization with ^{32}P -radiolabeled oligonucleotide probes complementary to internal sequences of the PCR products as previously reported (1, 12, 18). Comparisons of the amounts of the PCR products between the samples were performed by comparing the intensity of the resultant bands. The autoradiograph was scanned on an Apple One 24-bit Scanner at 300 dpi and converted into a TIFF uncompressed format and the resultant pattern analyzed using the Scan Analysis program from Biosoft, (Cambridge, U.K.) which delineates peaks and integrates the area under each peak. To ensure the validity of this strategy, reverse transcription-PCR were performed for each cytokine using different starting concentrations of cDNA. As previously noted, this procedure resulted in a log-linear relationship between the starting concentration of cDNA and the resultant band intensity (17). The results were expressed as densitometry units.

The pre-mRNA from the single human functional ϵ heavy chain gene can be alternatively spliced to produce at least five different ϵ mRNA splice variants (18, 19). We have previously developed two modified PCR techniques to compare the relative amounts of different isoforms of ϵ mRNA produced (1, 20). The first PCR, termed PCR1, amplified the three ϵ mRNA isoforms CH4-M1'-M2, CH4-M2' and CH4-M2'' (19) and the housekeeping gene β -actin. The second PCR, termed PCR2' was used to amplify two other ϵ mRNA splices: the CH4-S form (the classic secreted variant), the alternative spliced CH4'-CH5 form, and β -actin mRNA. The resultant PCR products were visualized on an 8% acrylamide gel and quantitated by densitometry as previously described (1). All densitometry values for ϵ isoforms were normalized to β -actin expression. All samples were run in duplicate.

Results

Challenge effect on total Ig levels

As shown in Figure 1A, IgE levels found in nasal washes performed either one (mean = 3.79 ± 2.02 ng/ml) or four days (mean = 6.22 ± 2.48 ng/ml) after challenge with ragweed allergen were significantly higher ($p < 0.001$, paired t test for both time points) than prechallenge levels (mean = 0.74 ± 0.53 ng/ml). Similar results were seen when the subjects were challenged with both ragweed plus DEP (Fig. 1B). IgE levels were significantly elevated at day 1 (mean = 3.82 ± 2.98 ng/ml) and day 4 (mean = 4.92 ± 2.10 ng/ml) ($p < 0.001$, paired t test for both time points) compared with baseline values (mean = 0.93 ± 0.57 ng/ml). There was no significant difference in total IgE levels at any time between challenge with ragweed or ragweed plus DEP.

Total IgG levels in nasal lavage fluids showed no significant increase over baseline levels at any of the time points studied regardless of whether challenge was performed with ragweed or ragweed plus DEP (Table I). Similarly, no change in total IgA levels was detected following either challenge regime (Table I). In contrast, Table I shows that while nasal challenge with ragweed alone did not increase lavage fluid total IgG4 levels significantly, challenge with ragweed plus DEP led to significantly higher levels at

FIGURE 1. The effect of ragweed (A) or ragweed plus DEP (B) on total IgE levels in nasal washes following intranasal challenge. Subjects were challenged intranasally with up to 1000 U/ml *Amb a 1* or both *Amb a 1* and 0.3 mg DEP (see *Materials and Methods*). The mean level of total IgE in four nasal washes performed immediately before challenge or 1, 4, and 8 days after challenge were measured by ELISA. The results for all 13 subjects are shown. All subjects had increased total IgE in response to ragweed and this was not further increased in the presence of DEP.

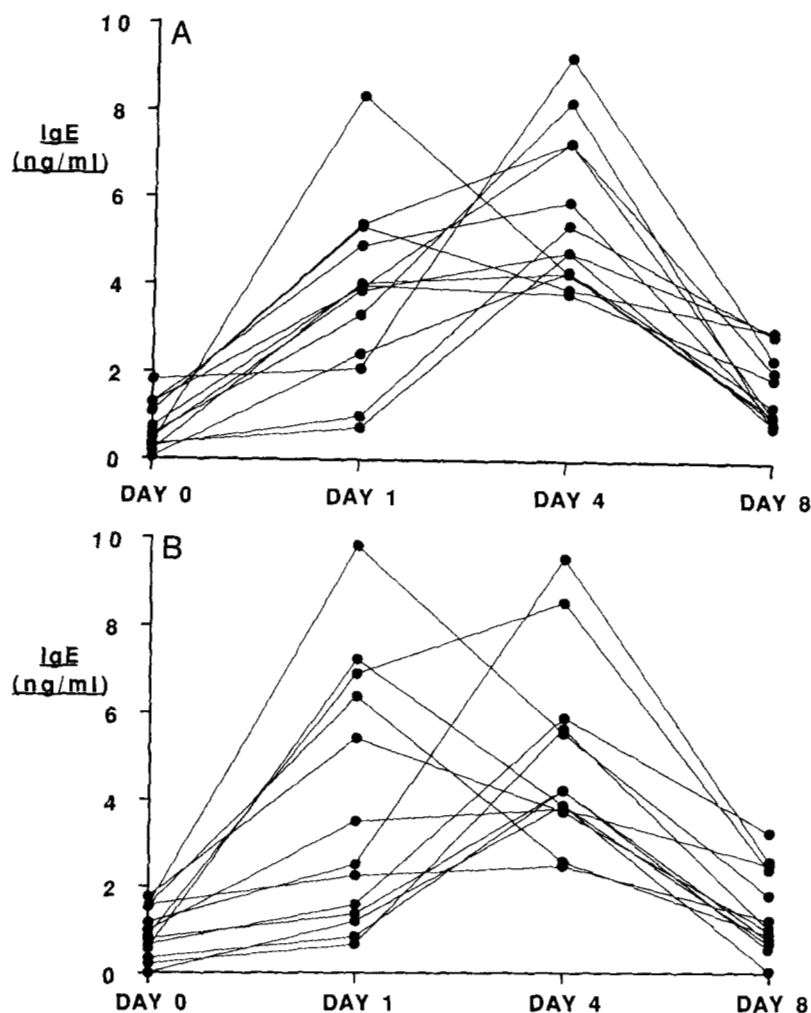


Table I. The effect of ragweed or ragweed plus DEP on immunoglobulin levels in nasal washes over time^a

Day	IgG (ng/ml)		IgA (ng/ml)		IgG4 (ng/ml)	
	RW	RW + DEP	RW	RW + DEP	RW	RW + DEP
0	4963 (3291)	4073 (3899)	5051 (1865)	6509 (1858)	8.33 (1.87)	12.51 (12.36)
1	8667 (6823)	6499 (2609)	4882 (1822)	6018 (1484)	14.16 (8.66)	23.75 (8.45)
4	8001 (5464)	4319 (3895)	4861 (1381)	9212 (1598)	10.70 (4.84)	42.48 (24.25)
8	8192 (4556)	4588 (4462)	5443 (965)	6172 (1554)	13.9 (8.51)	47.80 (16.99)

^a The levels of IgG, IgA, and IgG4 in nasal washes performed immediately before challenge (day 0) with up to 1000 U/ml *Amb a 1* or both *Amb a 1* and 0.3 mg DEP and 1, 4, and 8 days after. The mean levels of the 13 subjects is shown. Values in parentheses are 1 SD. There was no significant change compared to baseline levels in total IgG or total IgA at any time point ($p > 0.05$, paired t test for both Ig at day 4 and 8), while the total IgG4 was increased by ragweed plus DEP at day 4 and 8 ($p < 0.01$, paired t test for both timepoints).

both day 4 and day 8 compared with day 0 ($p < 0.01$, paired t test for both). Mean IgG4 levels after ragweed plus DEP challenge at day 4 were 42.4 ± 24.2 ng/ml compared with 10.7 ± 4.84 ng/ml when subjects were challenged with ragweed alone. This was statistically significant ($p < 0.01$, t test). Again, at day 8, ragweed plus DEP challenge resulted in significantly more IgG4 than challenge with ragweed alone (mean = 47.8 ± 16.9 compared with 13.9 ± 8.5 ng/ml, $p < 0.01$, t test).

IgE- and IgA-secreting cells

The effect of ragweed plus DEP on total Ig responses was confirmed by using filter spot-ELISA to determine the number of IgE- and IgA-secreting cells present in nasal washes following challenge with ragweed alone or with ragweed plus DEP. Table II

shows that the effect of ragweed was isotype-specific; that is, while challenge with either ragweed or ragweed plus DEP caused an increase in the number of IgE-secreting cells detected at day 4 compared with baseline levels ($p < 0.01$, paired t test for both), the number of IgA-secreting cells remained constant ($p > 0.05$, paired t test for both).

Challenge effect on allergen-specific Ig levels

In contrast to the results seen for total Ig production, DEP enhanced the ragweed-driven allergen-specific IgE response (Fig. 2A). Challenge with ragweed alone caused a significant increase in ragweed-specific IgE at days 1, 4, and 8. However, these responses were dwarfed by the levels detected in nasal lavage fluids after

Table II. Ragweed and DEP induce IgE-secreting cells *in vivo* but do not show an additive or synergistic effect

Challenge Protocol	Number of Ig-Producing Cells/10 ⁶ Nasal Lavage Cells ^a	
	IgE-secreting cells	IgA-secreting cells
Preragweed	0.254 ± 0.24	0.656 ± 0.47
Postragweed	12.44 ± 7.48	1.064 ± 0.32
Preragweed + DEP	0.326 ± 0.39	0.864 ± 0.70
Postragweed	14.20 ± 6.19	1.196 ± 0.38

^a Total cells recovered from nasal lavages before or 4 days after challenge with up to 1000 U of Amb a 1 or 0.3 mg DEP plus Amb a 1 assayed for IgE or IgA cells by filter spot assay. The spots were counted and the number of Ig-secreting cells present in the population calculated by comparison to a standard curve. All results shown are the mean ± 1 SD of Ig-secreting cells/10⁶ cells for 13 subjects.

challenge with both ragweed plus DEP. On day 1 mean ragweed-specific IgE levels were 6 times higher after ragweed plus DEP challenge compared with ragweed challenge alone (mean = 62.7 ± 55.1 compared with 10.2 ± 10.2 U/ml) and by day 4 they were 16 times higher; at this time point the highest level recorded after ragweed challenge was 12 U/ml, while after ragweed plus DEP challenge levels reached up to 180 U/ml. These results resulted in highly significant differences between the two challenge protocols ($p < 0.005$ and $p < 0.001$ for day 1 and day 4 respectively, t test). By day 8, ragweed-specific IgE levels after DEP plus ragweed challenge declined but were still significantly higher than after challenge with ragweed alone ($p < 0.05$, t test).

In contrast to allergen-specific IgE production, the ragweed-specific IgG levels (Fig. 2B) measured in nasal washes were the same after challenge with ragweed alone or with both ragweed and DEP ($p > 0.05$, t test). As was seen for total IgG4 levels, following ragweed-challenge, ragweed-specific IgG4 did not change (Fig. 2C). However, combined ragweed plus DEP resulted in clearly elevated levels at both day 4 (mean = 48.1 ± 22.4 U/ml) and day 8 (mean = 69.0 ± 21.7 U/ml) compared with prechallenge levels (mean = 0.63 ± 0.46 U/ml).

Ragweed plus DEP challenge selects against Th1-type cytokines

We had previously shown that *in vivo* nasal challenge with DEP in humans induce a broad increase in mRNA levels for all cytokines (3). In the present study we investigated the effect of ragweed or ragweed plus DEP on cytokine mRNA in nasal lavage cells recovered 18 h after challenge. Table III shows the amount of mRNA detected for a panel of cytokines and illustrates that ragweed plus DEP challenge altered cytokine mRNA production into a Th2-like pattern. As reported previously (3), cells recovered from nasal lavages, performed before either challenge regime, made detectable amounts of mRNA for several cytokines. At these times IFN- γ was present in all but 1 of the 13 samples, while IL-10 mRNA was detected in none of the samples. After challenge with ragweed alone, there was no significant increase or decrease in the amounts of mRNAs detected for any of the cytokines measured.

In contrast to the minor cytokine differences observed after challenge with ragweed alone, challenge with DEP plus ragweed resulted in major changes in nearly all cytokine mRNAs tested (Table III). Following challenge, mRNA levels for the Th2-type cytokines IL-4, IL-5, IL-6, and for IL-10 and IL-13 were significantly increased compared with prechallenge levels. In contrast, levels for the sentinel Th1-like cytokine IFN- γ were significantly decreased. Interestingly, there was no significant difference be-

tween the levels of IL-2 mRNA before or after challenge with allergen plus DEP.

Epsilon mRNA isoform ratios are altered by ragweed and ragweed plus DEP

We have previously showed that DEP challenge can alter the local qualitative IgE response by altering the absolute and relative levels of alternatively spliced ϵ mRNAs encoding distinctive IgE proteins (1, 21). The total amount of ϵ mRNA (the sum of all splice variants detected) obtained four days following ragweed challenge increased 42 ± 5-fold, while following ragweed plus DEP challenge it increased 52.8 ± 15-fold compared with prechallenge levels (Fig. 3). This is in accord with the total IgE protein levels and the numbers of IgE spot forming cells.

Ragweed challenge alone induced a shift in the relative amounts of the ϵ mRNAs generally similar to that observed after challenge with DEP alone (1). This change involved the preferential increase in four variants (CH4-M1'-M2; CH4-M2'; CH4-M2'', and CH4-S) in which mRNA levels were up to 51.4 times higher than prechallenge levels, compared with the expression of CH4'-CH5, which increased 17.4 ± 1.4-fold above prechallenge levels. The disparity between the CH4'-CH5 splice and the other variants was even more pronounced after challenge with ragweed plus DEP than after ragweed alone ($p < 0.01$, paired t test). Combined ragweed plus DEP challenge increased expression of the CH4'-CH5 splice variant 4.9-fold, but increased expression of the other four variants by up to 64-fold above prechallenge levels.

Discussion

We had previously provided direct evidence that DEP exposure *in vivo* can enhance IgE production and increase production of a host of cytokines in a non-Th1, non-Th2-type fashion. In the present study we show that in ragweed-allergic subjects, 18 h after simultaneous challenge with ragweed allergen plus DEP there is a decrease in mRNA expression for Th1-type cytokines, but an increase in Th2-type and other cytokines. Four days after challenge, allergen-specific IgE and IgG4 production were markedly increased. These data suggest that exposure to DEP, at levels that are comparable to normal exposure, may synergize with allergen-induced stimulation in IgE Ab production. This could lead to increased IgE production in already sensitized persons and thereby increase the likelihood of clinical respiratory disease. Additionally, recent data suggests that Ags may trigger mast cells release of cytokines in nonsensitized subjects via their properties as lipases or proteases (22). Under these circumstances, DEP exposure may synergize with allergens in nonsensitized subjects to induce a state of primary sensitization characterized by Ag specific IgE Ab production with subsequent development of respiratory allergic disease.

Many studies have provided indirect evidence of an increased incidence (prevalence) of asthma and atopy linked to airborne pollution (4–7, 23, 24). Not only do ambient levels of pollution correlate with acute respiratory disease, but perhaps more importantly, long term epidemiologic studies suggest that the prevalence of atopy and asthma is related to pollution and urbanization (5, 24). However, some recent studies have demonstrated a lower prevalence of allergies in Eastern rather than Western Europe despite higher pollution levels (25). However, as shown by Björkstén and others (26), lifestyle is also a critical factor in these studies. Thus, allergic disease are generally lower in countries that were under communist rule than in democratic countries for reasons unknown (possibly health care, diet, accommodation, etc.). However, when comparisons are made within these countries, where lifestyles are

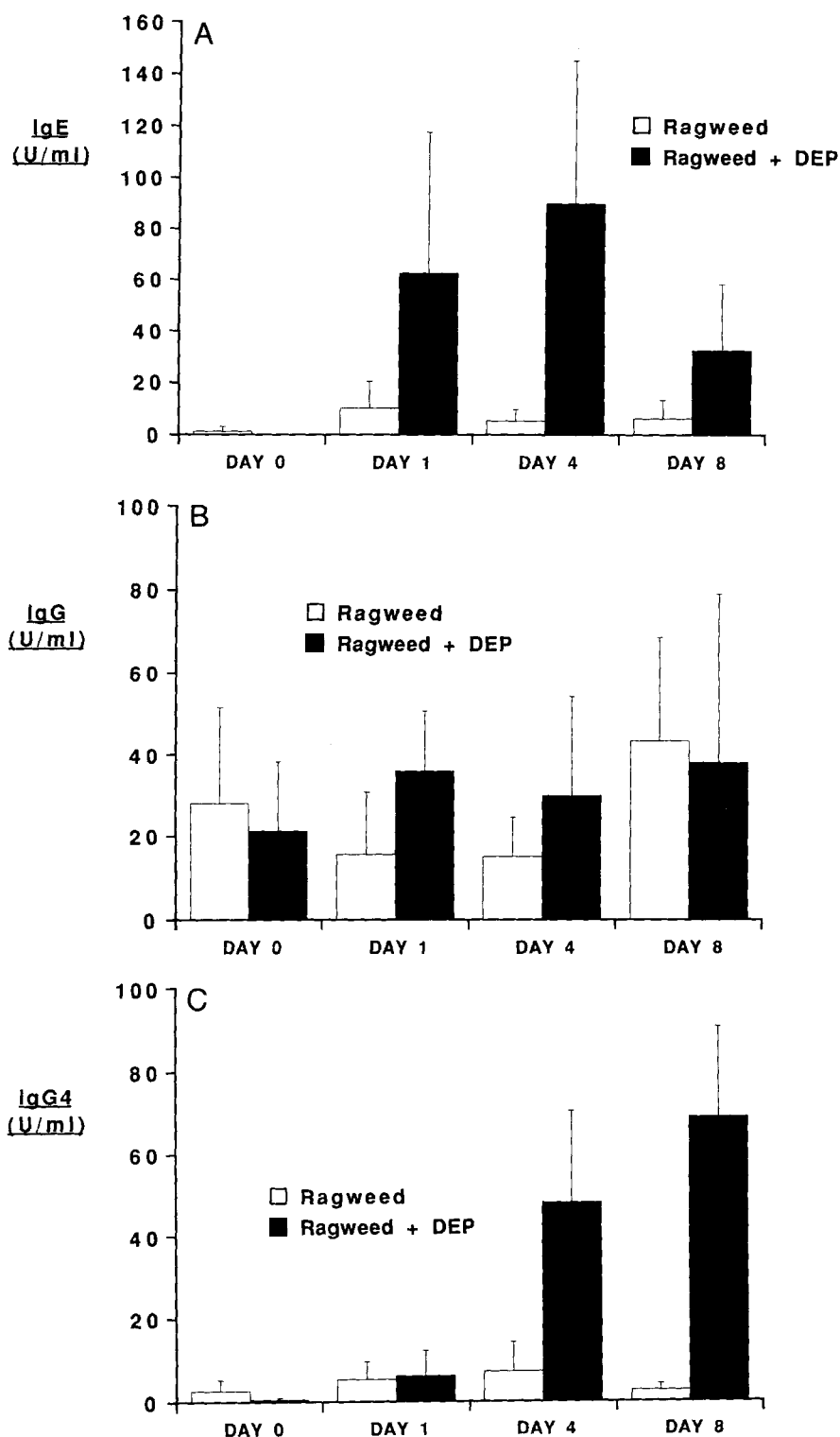


FIGURE 2. The effect of ragweed or ragweed plus DEP on Ag-specific Ig levels in nasal washes over time is compared. Ragweed-sensitive subjects were challenged intranasally with up to 1000 U/ml *Amb a 1* or both *Amb a 1* and 0.3 mg DEP (see *Materials and Methods*). The levels of ragweed-specific IgE (A), IgG (B), and IgG4 (C) in nasal washes performed immediately before challenge or 1, 4, and 8 days after challenge were measured by ELISA. The mean levels of the 13 subjects $\pm$ 1 SD is shown. The day 0 values represent the baseline unchallenged levels. The mean levels of the 13 subjects $\pm$ 1 SD is shown. There was significantly more ragweed specific IgE produced in response to challenge with DEP and ragweed than to ragweed challenge alone ($p < 0.005$ and $p < 0.001$ for day 1 and day 4 respectively, t test).

similar, there is a clear increase in allergic disease between polluted and nonpolluted areas.

An important contributor to airborne pollution is diesel exhaust emissions (27, 28). Studying DEP as a prototype anthropogenic pollutant has the advantages that the levels of DEP can be quantitated, the sources of DEP are readily identified, the occurrence of DEP can be traced relatively easily in the historic record and DEP and the compounds they contain are amenable to laboratory use with rodents and humans. Miyamoto and colleagues performed

initial in vivo studies in mice that suggested that DEP may have a preferential enhancing effect on IgE Ab production (29–31). We have previously reported that there is an increase in total IgE and IgG4 but not IgA, IgM, IgG or IgG3 in human nasal lavages 4 days after in vivo challenge with DEP alone (1).

In the present study we extended these previous findings to examine the Ag-specific Ig responses and alterations in cytokine mRNAs following combined allergen plus DEP challenge. Ragweed-specific IgE was 16 times higher following ragweed plus

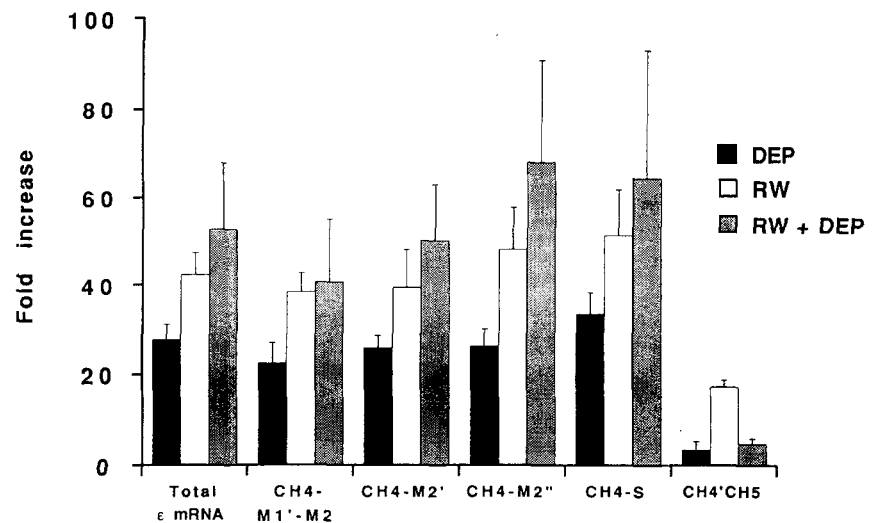
Table III. Cytokine mRNA production in lavage cells after challenge with ragweed alone or ragweed plus DEP^a

Cytokine	Cytokine mRNA Production (mean densitometry units) Following			
	Prechallenge 1	Ragweed	Prechallenge 2	Ragweed + DEP
IL-2	4,413 ± 3,916	1,988 ± 997	2,483 ± 2,156	8,125 ± 3,657
IL-4	2,984 ± 2,910	4,291 ± 3,109	3,124 ± 2,842	25,126 ± 6,123*
IL-5	3,918 ± 3,820	5,910 ± 4,864	4,986 ± 2,131	41,356 ± 7,123*
IL-6	2,987 ± 1,789	1,245 ± 2,366	5,123 ± 4,123	35,421 ± 8,945*
IL-10	<200	<200	<200	23,511 ± 8,875*
IL-13	7,983 ± 4,512	12,341 ± 5,423	9,281 ± 6,514	24,351 ± 9,456†
IFN-γ	12,934 ± 4,445	10,326 ± 4,459	10,235 ± 4,441	2,412 ± 2,123†

^a RT-PCR was performed on mRNA from cells recovered from nasal lavages immediately before and 18 h after in vivo challenge with either *Amb a 1* (up to 1000 U/ml) or *Amb a 1* plus 0.3 mg of DEP. The same 13 subjects were used for each experiment. The amount of PCR product was determined by the intensity of the bands following Southern blotting as measured by densitometry (see *Materials and Methods*). Results are the mean densitometry units for the 13 subjects ± 1 SD.

* $p < 0.01$ vs prechallenge; † $p < 0.05$ vs prechallenge (Mann-Whitney *U* test).

FIGURE 3. Ragweed plus DEP enhance the absolute levels and alter the relative levels (pattern) of the epsilon mRNA isoforms. This figure shows the relative enhancement (as fold increase) for each of the epsilon mRNAs noted as induced by either ragweed or DEP or ragweed plus DEP. The amount of mRNA detected for each epsilon isoform from cells recovered in nasal washes performed before and 4 days after challenge was measured and standardized relative to the amount of β -actin present. The results shown are the mean and SD of 13 subjects. The total epsilon mRNA levels (the sum of all splice variants detected) went up nearly 30-fold, as did mRNA for all of the splice variants except the CH4'-CH5 form. The preferential effect on increasing the isoforms except CH4'-CH5 was most marked after challenge with ragweed plus DEP.



DEP challenge compared with challenge with ragweed alone, while the total IgE amounts were equivalent. Thus, while DEP alone can enhance total IgE (1), DEP in the presence of allergen specifically enhanced Ag-specific IgE. In contrast, both total and Ag-specific IgG4 levels were boosted by the presence of DEP. These seemingly conflicting data strongly suggest that mucosal ragweed IgE and IgG4 are under similar but not identical control mechanisms. This concept is supported by previous studies showing differential effects of immunotherapy on nasal ragweed IgE vs ragweed IgG4 (32) as well as studies showing that switching to human IgE does not necessarily proceed through IgG4 (33).

The mechanism for the IgE Ag-specific effect demonstrated is not known at present. It may be that DEP acts preferentially on cells previously activated by ragweed or that DEP stimulation directly or indirectly allows ragweed-specific cells to respond to Ag. DEP in the absence of allergen may act directly or indirectly on preexisting populations of IgE committed memory B cells; a "non-specific" IgE response would then be generated as there is no one allergen-specific memory cell population that dominates. Distinct from either the direct or indirect effects of DEP, the presence of ragweed Ag induces the expansion of ragweed-specific memory cells in sensitive subjects via cross linking the membrane Ig. DEP may act on these Ag-stimulated cells and expand them further, resulting in a marked Ag-specific IgE response.

In this study, subjects were challenged with 0.3 mg of DEP. While this dose may seem high, it should be noted that exposure to such doses can occur from standing at a bus stop when a bus starts up or being in a freeway tunnel for 10 min or more (4).

Furthermore, due to the fact that most (95%) DEP are smaller than 10 μ m, they are trapped in the upper and lower airways and the time taken for them to be cleared has been estimated to be from between 6 and 24 h (28). Thus, actual exposure is more accurately the accumulative dose of at least 6 h. The amount of allergen used in this study was high but was chosen as it was the smallest amount that would elicit an immediate response.

DEP alone can act in vivo to drive or potentiate cytokine production at the human upper respiratory mucosal surface (3). Previously, we have shown that in vivo exposure to DEP resulted in an increase in cytokine mRNA expression in cells recovered following nasal lavage that was neither of a Th1- or Th2-type. This enhanced production of mRNA was observed for a broad array of the cytokines and the increase for one of the cytokines (IL-4) was confirmed at the protein level.

In this study, cytokine mRNA levels were measured in cells from nasal washes as cytokine protein levels from nasal lavage or mucosa cells are normally below the levels of detection for most assay systems (34). Previous studies have shown that in experimental allergen-induced rhinitis there is an increase in IL-4, IL-6 and especially IL-5 gene expression (10, 35, 36) or secretion (36–38). One distinction between those studies and that presented here is that most did not to measure expression of IFN- γ , while those that did used in situ hybridization on biopsies of the nasal inferior turbinate (24, 10), which provided nonquantitative data. In this study the effect of ragweed on increased cytokine mRNA expression was small, statistically nonsignificant, and far less than we have observed with DEP alone (3). However, the combination of

ragweed plus DEP notably enhanced the Th2-type effect and nearly all subjects had detectable levels of mRNA for all the Th2-type cytokines tested (IL-4, IL-5, IL-6) as well as for IL-10 and IL-13 (cytokines made by Th2 cells but also by other Th subsets and non-T cells). Additionally, while ragweed challenge alone resulted in virtually no change in IFN- γ gene expression, challenge with ragweed plus DEP had a pronounced inhibitory effect.

Our results are in agreement with the finding that in mice following intratracheal instillation of DEP plus Ag, IL-4 production in mediastinal lymph node cells was enhanced compared with that in mice given Ag alone (39). Additionally, the same group recently showed IFN- γ production in cervical lymph node cells was 3 times lower following instillation of DEP plus Ag markedly compared with Ag alone (40). However, in both these studies, the effect of DEP alone was not reported.

Although DEP plus ragweed results in a Th2-type response, DEP alone induces a non-Th1-, non-Th2-specific pattern of cytokine production and likely provides a stimulus that is nondirecting and serves to augment cellular profiles that have already initiated (2). It seems clear that allergen exposure induces activation of Th2 cells (41). One explanation of the results presented here is that ragweed provides the orientation toward the Th2-like pattern and DEP amplifies this response. Allergen exposure may result in an early release of IL-4, which is thought to be critical to the development of Th2 responses and can inhibit the development of Th1-type effector cells (42). Following this allergen-specific activation, it is possible that the ability of DEP to provide a potent stimulus to these Th2 cells for further differentiation/proliferation could result in the heightened Th2-type cytokine response observed. The source for this early burst of IL-4 is not known and may be T cells, mast cells, NK cells, basophils (42), or even eosinophils (43). Studies are currently underway to test this hypothesis. It is of interest to note that following challenge with ragweed plus DEP in patients not sensitive to ragweed, the cytokine profile was identical with that seen with DEP alone and there was no decrease in IFN- γ expression or ragweed-specific IgE detected (our unpublished observations).

The cytokines generated after DEP and ragweed challenge are of obvious importance to the development of an IgE response in general and to allergic airway disease. Many cell types central to atopy are influenced by these cytokines, for example IL-4, IL-5, IL-6, IL-10, and IL-13 all can act on B cells (44); differentiation/proliferation and infiltration of eosinophils can be induced by IL-5 and IL-10 can induce T cell proliferation (44). Additionally, increased expression of IL-10 following DEP challenge may be responsible for the change in pattern of observed ϵ mRNAs encoding the family of ϵ proteins. Alternative 3' RNA splicing in humans results in generation of multiple ϵ mRNA coding for distinct IgE isoforms from the single active ϵ gene (18, 19). This ϵ mRNA isoform pattern is not fixed and differs between normal and some atopic patients. IL-10 can change the pattern from that of a normal to that of an atopic patient (20), a pattern characterized by the marked relative decrease in one of the forms (CH4'-5). Challenge with either ragweed or DEP alone somewhat preferentially selected against this form, but once again, after challenge with DEP plus allergen this effect was accentuated. This synergy resulted in a pattern reminiscent of that seen in diseases associated with highly elevated IgE levels such as atopy or parasite infestation, presumably due to the increased levels of IL-10.

In summary, in potentially allergic subjects, natural exposure to allergen alone may result in low or nondetectable cytokine and IgE levels. In sensitized but clinically well subjects, allergen exposure may result in nondetectable to low cytokine expression and IgE production. However, simultaneous natural exposure to DEP may

lead to markedly boosted localized Th2 responses and direct effects on mucosal B cells. This could result in initiation or enhanced production of Ag specific IgE leading to allergic sensitization or boosting and subsequently increase the likelihood of symptomatic respiratory allergic disease.

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