



Inhaled chemicals may enhance allergic airway inflammation in ovalbumin-sensitised mice

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Abstract

Occupational allergy and asthma is a challenging issue in the developing countries. Chemicals inhaled in the workplaces may act not only as allergens but also as immune response modifiers, contributing to asthma exacerbation.

In this study, we tested the adjuvant effect of 20 ppm chloroform, 10 ppm 1,1-dichloroethylene, and 100 ppm styrene in mice. Female BALB/c mice were sensitised to ovalbumin (OVA) without using alum. During the OVA-sensitisation period, these mice were exposed by inhalation to the chemicals studied for 6 h/day for four consecutive days. After two OVA-intratracheal challenges, a mild Th2 immune response was observed in the OVA-exposed groups. This response was characterised by a mild increase in serum specific IgE level, in local Th2 cytokine production, and in lung inflammatory reaction.

Exposure to styrene or chloroform alone slightly increased Th2 cytokine production by lung-draining lymph node cells cultured with concanavaline A, except for the IL-4 level in the chloroform exposure group, which decreased. On the other hand, exposure to 1,1-dichloroethylene alone markedly increased the Th2 cytokine levels compared to those observed in the groups exposed to OVA alone.

In the combined OVA + chemical-treated groups, styrene potentiated IL-4, -5 and -13 production efficiently (approximately two, four and three times higher, respectively), resulting in an increase in the total IgE levels and inflammatory reaction. On the other hand, the enhanced IgE levels and the exacerbation of the inflammatory response by 1,1-dichloroethylene or chloroform were associated with only minor changes in local cytokine levels.

These findings suggest that exposure to chemicals through inhalation may aggravate the allergic lung inflammation. And this, depending on the chemical exposure conditions, may result from the synergistic effect of chemicals and allergen on local Th2 cytokine production.

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1. Introduction

Data in the literature show that the prevalence of allergic diseases, including asthma, has increased in recent decades in industrialized countries (Howardth, 1998;

Holgate, 1999; Beasley et al., 2000). There is a growing consensus that the increasing trends in allergic disease may be work-related (Arif et al., 2002), because in workplaces the environmental risk factors like chemicals may act not only as allergens but also as immune response modifiers (adjuvants).

Allergic asthma is a chronic inflammatory disease of the airways. There is accumulating evidence that T helper (Th1/Th2) lymphocytes help orchestrate allergen-

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induced inflammation of the airways through the production of specific cytokines (Kay, 2001; Moss et al., 2004). Th2 cells, which produce IL-4 (enhancing IgE synthesis), IL-5 (increasing eosinophil growth and differentiation), and IL-13 (increasing mucus production and inducing airway hyperreactivity), control and amplify the allergic inflammatory response (Coffman et al., 1993; Kuperman et al., 2002; Foster et al., 1997). On the other hand, Th1 cells, which produce IL-12 (increasing IFN- γ production) and IFN- γ (suppressing differentiation of naive T cells to Th2 subtypes and local recruitment of eosinophils) are capable of counteracting Th2 responses and vice versa (Gavett et al., 1995; Tang et al., 2001; Lack et al., 1996).

In mouse asthma models, mice are sensitised with ovalbumin (OVA) and exposed to repeated OVA challenges. In these models, a Th2-mediated allergic response is observed, characterised by eosinophilia, mucus overproduction, and increased IgE level (Funabashi et al., 2004; Yamauchi et al., 2002). Several studies have used these OVA mouse models to study the adjuvant activity of dust, ozone, ambient particulate matter, and ultrafine carbon black and diesel exhaust particles (DEPs) (Steerenberg et al., 2004; De Haar et al., 2005; Funabashi et al., 2004). DEPs have been shown to be capable of promoting Th2 cytokine responses directly by stimulating production of inflammatory cytokines through an oxidant-dependent pathway and indirectly by interfering with cytokine-signalling pathways that produce IFN- γ , which is important for down-regulating Th2 response (Finkelman et al., 2004). However, to our knowledge, few studies have been conducted on the impact of chemical-induced adjuvant activity on Th2 response. In the workplace, inhaled chemicals, even at low doses, may alter the integrity and barrier function of respiratory epithelium and modify the profiles of its micro environmental cytokines, including IFN- γ , TNF- α , IL-1 β , which are involved in airway inflammation. Reisinger et al. (2005) have identified IFN- γ as a factor that can reduce the barrier function of respiratory epithelial cells and thus considerably enhance the penetration of allergens.

The respiratory tract is routinely exposed to a variety of potentially allergic substances. The presentation of their derivative epitopes to lymphocytes T, a key event in adaptive immune response, takes place in the lung-associated lymph nodes (Banchereau and Steinman, 1998; Lambrecht et al., 2000). Little is known about the pathomechanism of the adjuvant effect of environmental chemicals. In a previous study, we showed that pulmonary exposure to certain chemicals, namely styrene, 1,1-dichloroethylene, chloroform and carbon tetrachlo-

ride, increased the IFN- γ release in the lung-associated lymph nodes as well as the numbers of IgM producing B cells against sheep red blood cells (SRBCs) (Ban et al., 2003). These chemicals may therefore promote sensitisation through an adjuvant effect, i.e. by increasing antigen-presenting activity. If an astmatogen like OVA is used in place of SRBCs, the increased antigen-presenting function may be one of the factors contributing to the aggravated allergic response, which is characterised by an increase in IgE production, eosinophilic inflammation, and airway hyperreactivity.

Based of these previous results, the aim of this study was to determine whether pulmonary exposure to three chemicals (chloroform, 1,1-dichloroethylene and styrene) might result in an additive Th2 response in OVA-sensitised mice. To do this, mice were sensitised to OVA without the aid of alum. This model allows not too strong a Th2 response to be obtained in order to appropriately assess a potentially enhanced Th2 effect of the chemicals studied. The Th2 response was evaluated by measuring the production of interleukin (IL)-4, IL-5, IL-13, and interferon (IFN)- γ by the lung-associated lymph node cells and the serum levels of total and specific IgE, and by evaluating the inflammatory reaction in the lungs.

2. Materials and methods

2.1. Materials

Styrene and 1,1-dichloroethylene were purchased from Merck (Darmstadt, Germany), and chloroform from Prolabo (Fontenay S/Bois, France). The purity of the chemicals was >99%. Ovalbumin (Grade V) was supplied by Sigma (St. Quentin Fallavier, France). IFN- γ , IL-4 and -13 DuoSet were purchased from R&D Systems and IL-5 Opt EIA Sets and mouse IgE from BD Biosciences. Diff-Quik was obtained from Dade Behring AG, Switzerland.

2.2. Mice

Female, 8–10-week-old BALB/c mice were obtained from Charles River (Italy). The animals were housed in an environmentally controlled animal facility maintained at 22 °C with a 12 h/12 h-light/dark cycle. They were provided with food and water ad libitum throughout the experiment, except during the exposure periods in the inhalation chambers for chemical intoxication.

2.3. Experimental design

2.3.1. Pilot study

We developed the OVA mouse model in which aluminium hydroxide was not used to obtain a mild Th2 response. Its experimental design, where the mice received intra-

dermally 0.1% OVA in phosphate buffered saline (PBS) (25 μ l/shaved flank) at day 0, and intratracheally 0.01% in PBS (100 μ l/mouse) at day 7, 20 and 24, was based on the results of IgE in sera, cellular influx in alveolar spaces, Th2 cytokine levels and lung histology. Different concentrations of the chemicals studied were tested with this OVA mouse model. The chemical concentrations tested with this OVA mouse model have already been used in a previous study on mice where they were found to cause an increase in the number of antibody-forming cells in lung-associated lymph nodes (Ban et al., 2003).

2.3.2. Main study

In the main study, the chemical concentrations (20 ppm for chloroform [CF], 10 ppm for 1,1-dichloroethylene [DCE] and 100 ppm for styrene [SR]) as well as the measured end-points for allergic response were selected according to the significant results recorded in the pilot study. These chemical concentrations are not too far from the threshold limit values (TLV-TWA) recommended by American Conference of Governmental Industrial Hygienists for occupational exposure, approximately two-fold higher for CF and DCE and five-fold higher for SR.

In each experiment, a total of 48 mice were divided into four experimental groups (12 mice/group): control air + PBS group, chemical + PBS group, air + OVA group, and chemical + OVA group. For each chemical studied, two separate experiments were run in the same conditions to study the adjuvant effect of the chemicals on lung inflammation response and on IgE levels in OVA-sensitised mice. However, the adjuvant effect of the chemicals on cytokine response was investigated in one experiment only for each chemical.

2.3.2.1. Chemical exposure during the OVA sensitisation process. At day 0, the mice from the air + OVA and chemical + OVA groups were injected intradermally into both flanks (each 25 μ l) with 0.1% OVA in PBS; the mice from the air + PBS and chemical + PBS groups received PBS.

From days 3 to 6, the mice from the chemical + PBS and chemical + OVA groups were exposed to one of the chemical compounds studied for four consecutive days (6 h/day) in the inhalation chambers, whereas the mice from the air + PBS and air + OVA groups were exposed to filtered air.

At day 7, the mice from the air + OVA and chemical + OVA groups received intratracheally 100 μ l/animal of 0.01% OVA in PBS, whereas the mice from air + PBS and chemical + PBS groups received PBS.

2.3.2.2. OVA challenges. The mice from the air + OVA and chemical + OVA groups were challenged intratracheally, at days 20 and 24, with 0.01% OVA in PBS (100 μ l/animal), whereas the mice from the air + PBS and chemical + PBS groups received PBS.

2.3.2.3. Animal exposure. For inhalation exposure, the chemical + PBS and chemical + OVA group animals were placed

in inhalation chambers (temperature $23 \pm 2^\circ\text{C}$, humidity $55 \pm 5\%$) with dynamic and adjustable laminar air flow (6–20 m^3/h). Chemical vapours were generated by passing an additional air-flow rate through the fritted disk of a heated bubbler containing the chemicals studied. The concentrations of these chemicals were monitored continuously with a gas chromatograph (Shimadzu model GC14 and Perkin-Elmer XL autosystem) equipped with a flame ionisation detector and an automatic gas-sampling valve. For CF, DCE, and SR, the column lengths were 2, 4 and 4 m, and the temperature was maintained at 85, 75 and 100°C , respectively. The exposure levels were determined twice during each 6 h exposure period by collecting samples of the atmosphere through glass tubes packed with activated charcoal. For CF and SR, the samples were then desorbed with carbon disulfide and analysed using ethyl benzene and *o*-xylene as internal standards. For DCE, the samples were desorbed with ethanol and analysed using carbon tetrachloride as internal standard. The air + PBS and air + OVA groups were exposed to filtered air in the same conditions.

2.4. Sample collection

Sample collection was performed under intraperitoneal pentobarbital anesthesia (60 mg/kg body weight).

The lung-associated lymph nodes (three lymph nodes/animal) were removed and pooled (six animals/pool) at day 25. The blood sampling through cardiac punctures, the bronchoalveolar lavages (BAL) and lastly the left lobe lung removal were carried out at day 27.

2.5. Lung-associated lymph node cell culture

As mentioned above, the lung-associated lymph nodes were pooled. Single cell suspensions were prepared by gently mashing the lymph nodes through a sterile 70 μm cell strainer (Falcon) in cold RPMI-1640 medium (Gibco) supplemented with 10% fetal calf serum (Biosys), 2 mM L-glutamine, 100 U/ml penicillin and 100 $\mu\text{g}/\text{ml}$ streptomycin (Gibco). After washing by centrifugation, the cell number was adjusted to 2×10^6 cells/ml, and 5×10^5 cells/well (250 μ l) were seeded on plastic 96-well plate with concanavaline A (3 $\mu\text{g}/\text{ml}$) and cultured for 24, 48 and 72 h at 37°C in a controlled atmosphere: 14% oxygen, 7% carbon dioxide and balanced nitrogen. The culture supernatants were collected and stored at -80°C until use for the assessment of cytokine levels. Representative results from 72 h cultures are presented in this paper.

2.6. ELISA for total serum IgE

Briefly, 96-well microimmunoplates (NUNC) were incubated with a 2 $\mu\text{g}/\text{ml}$ rat monoclonal anti-mouse IgE antibody overnight at 4°C , washed four times with 0.1-M phosphate-buffered saline (PBS) containing 0.05% Tween-20, and incubated successively with (1) 200 μ l per well of PBS containing 10% fetal calf serum (blocking buffer), (2) 100 μ l per well of mouse serum diluted 1:25 in PBS/fetal calf serum or mouse

anti-trinitrophenol IgE standard, and (3) 100 μ l per well of rat anti-mouse IgE antibody labelled with peroxidase. After each incubation, which was carried out at room temperature, the plates were washed five times. To reveal peroxidase activity, substrate buffer containing tetramethyl benzidine and H_2O_2 (Sigma) was added to each well and the reaction was terminated 30 min later by adding H_2SO_4 (2N). Absorbance was measured at 450 nm by a micro plate reader (Lab System, Multiskan, MS). The level of total serum IgE (ng/ml) was calculated.

2.7. ELISA for OVA-specific serum IgE

The technique to assess specific IgE was similar to that employed for total IgE, but the mouse anti-IgE was replaced by 5% OVA solution and the specific IgE levels were expressed as optical density values.

2.8. ELISA for culture supernatant cytokines

IL-4, IL-5, IL-13 and IFN- γ in culture supernatants of pooled lymph nodes were measured by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions. The cytokine levels (pg/ml) were expressed as the means $\pm$ S.D. of two or three repeated measurements.

2.9. BAL fluid analysis

After cardiac puncturing, BAL fluid collection was performed by cannulating the trachea and lavaging the lung four times with 0.3 ml of sterile PBS. The total fluid recovered from each mouse was about 1 ml. For the differential cell counts, 100 μ l ($\sim 6 \times 10^4$ cells) of the suspended cells were spun (cytospin) on microscope slides, air-dried and Diff-Quik stained. From each slide, at least 200 cells were differentiated by means of conventional morphologic criteria.

2.10. Lung histology

Two slides per mouse were prepared by sectioning the left lung lobe (6 μ m) along the main stem bronchus. One cryostat section was stained by means of the periodic acid-Schiff method to identify airway mucous (goblet) cells and the other was stained using the "Diff-Quik" method to highlight the

influx of inflammatory cells around the airways. The relative degree of severity of inflammatory cell influx and proliferative change was semi-quantitatively assessed by a score from 0 to 4. For cell influx around the airways and vessels: score 0 (no inflammatory cell); score 1 (with a few inflammatory cells); score 2 (with scattered aggregates of inflammatory cells); score 3 (with thin layer of inflammatory cells surrounding the airways and vessels); score 4 (with a thick layer inflammatory cells surrounding the airways and vessels). For goblet cell proliferation: score 0 (positive goblet cells $\leq 5\%$); score 1 (positive goblet cells $\leq 20\%$); score 2 (positive goblet cells $\leq 35\%$); score 3 (positive goblet cells $\leq 50\%$); score 4 (positive goblet cells $> 50\%$). The results were expressed as the means of the numerical scores.

3. Data analysis

One-way analysis of variance (ANOVA) followed by multiple group comparison (Duncan's post hoc test) was used to analyse the total and specific IgE, eosinophil, and lymphocyte data. When the variance was heterogeneous, the data were log-transformed. For the histological scores, the data were analysed by Kruskal–Wallis non-parametric ANOVA, with post hoc testing by the distribution free multiple-comparison test. Significance was set at ($p < 0.05$).

The data for cytokines were not statistically analysed as the cytokine levels were assessed in the supernatants of pooled lymph nodes (not individually analysed).

4. Results

4.1. OVA-sensitised mouse model of asthma

The OVA-sensitised model of asthma of all six experiments of the present study shows a mild Th2 response to OVA, which was observed in the lungs, in the lung-draining lymph nodes, and in the blood.

The Th1 and Th2 cytokine levels in the lung-draining lymph nodes of OVA-sensitised mice were analysed in three of the six experiments. The results are shown in Tables 1–3. Exposure to OVA provoked a marked

Table 1

Effect of inhaling 20 ppm chloroform (CF) on the cytokine levels induced by ovalbumin (OVA)

Group	IL-4 (pg/ml)	IL-5 (pg/ml)	IL-13 (pg/ml)	IFN- γ (pg/ml)
PBS + air	41.8 $\pm$ 2.6	87.2 $\pm$ 0.3	134.7 $\pm$ 9.0	723 $\pm$ 14
PBS + CF	25.4 $\pm$ 0.4	208.9 $\pm$ 4.6	178.1 $\pm$ 1.8	1013 $\pm$ 10
OVA + air	52.6 $\pm$ 0.2	309.2 $\pm$ 3.3	356.3 $\pm$ 8.1	1214 $\pm$ 67
OVA + CF	33.5 $\pm$ 0	471.6 $\pm$ 1.6	479.0 $\pm$ 0.9	1050 $\pm$ 9

Two identical experiments were carried out at different times, but the levels of cytokine production were only assessed in experiment 2. At day 25, the lung-associated lymph nodes were removed and pooled to obtain a sufficient number of cells. Single cell suspensions were cultured in the presence of concanavaline A (a final concentration of 3 μ g/ml). Cytokine levels (pg/ml) were assessed by ELISA in the 72 h supernatants of the cell cultures. The data are presented as the mean $\pm$ S.D. of two to three repeated measurements.

Table 2

Effect of inhaling 10 ppm 1,1-dichloroethylene (DCE) on the cytokine levels induced by ovalbumin (OVA)

Group	IL-4 (pg/ml)	IL-5 (pg/ml)	IL-13 (pg/ml)	IFN- γ (pg/ml)
PBS + air	32.3 $\pm$ 0	79.2 $\pm$ 0	133.0 $\pm$ 2.0	1097 $\pm$ 31
PBS + DCE	198.5 $\pm$ 4	360.8 $\pm$ 19.4	610.4 $\pm$ 39.7	1853 $\pm$ 31
OVA + air	101.4 $\pm$ 3.6	262.6 $\pm$ 13.9	519.7 $\pm$ 7.1	1640 $\pm$ 43
OVA + DCE	103.6 $\pm$ 1.3	244.5 $\pm$ 7.1	367.0 $\pm$ 9.2	1253 $\pm$ 48

Two identical experiments were carried out at different times, but the levels of cytokine production were only assessed in experiment 2. At day 25, the lung-associated lymph nodes were removed and pooled to obtain enough cell numbers. Single cell suspensions were cultured in the presence of concanavaline A (a final concentration of 3 μ g/ml). Cytokine levels (pg/ml) were assessed by ELISA in the 72 h supernatants of the cell cultures. The data are presented as the mean $\pm$ S.D. of two to three repeated measurements.

increase in IL-5 (up to 3.5-fold the control levels) and IL-13 (up to 3.9-fold) in the three experiments, whereas a marked increase in IL-4 levels was observed only in one experiment (Table 2). A Th1 cytokine IFN- γ increase was also observed in OVA-sensitised mice in the three experiments. Their levels were up to 1.7-fold higher than those of the air + PBS control mice.

The IgE levels, the eosinophil and lymphocyte number, and the inflammatory scores were analysed twice (experiments 1 and 2) for each chemical. Figs. 1a, 4a, and 7a show the serum total IgE. In the air + OVA groups, the total IgE levels tended to increase in three of the six experiments, but this increase was statistically significant in only one experiment (Fig. 4a, experiment 2). With regard to OVA-specific IgE (Figs. 1b, 4b and 7b), in the six experiments, the levels augmented significantly in the OVA-sensitised groups compared with those of the corresponding controls. Figs. 2a, 5a, and 8a show the eosinophil percentages in the BAL fluids. In all six experiments, exposure to OVA provoked a significant influx of eosinophils in the alveolar spaces (up to 7.5% of the total number of cells), whereas the influx of lymphocytes observed in four of the six experiments was not significant (Figs. 2b and 5b). Figs. 3a, 6a, and 9a show the scores of lung inflammatory cell infiltration. Compared with the values of the air + PBS control mice, the inflammatory scores were significantly higher in the mice sensitised with only OVA (three of the six experiments). These higher inflamma-

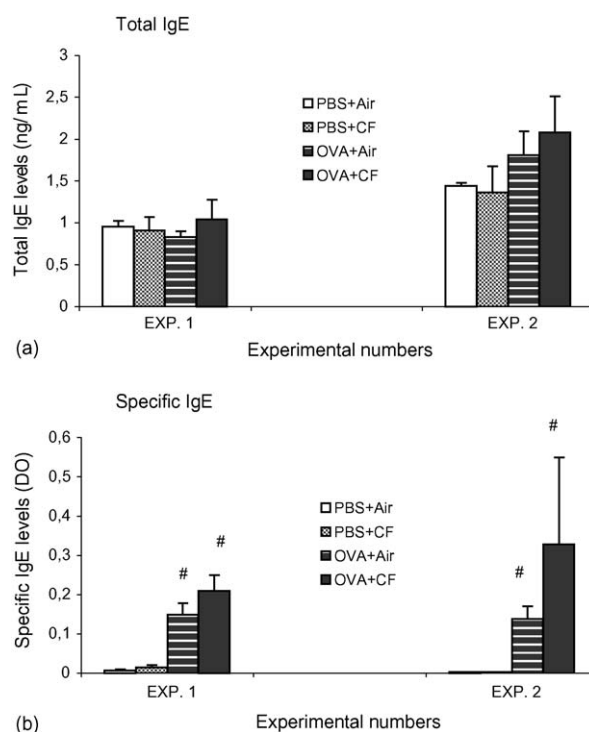


Fig. 1. Effect of inhaling 20 ppm chloroform (CF) on IgE production. (a and b) The total and specific IgE levels, respectively. Two identical experiments were carried out at different times: at day 27, blood was obtained by cardiac punctures and the IgE levels were assessed by means of ELISA. The results are expressed as the mean $\pm$ S.E. of five to six animals. #Significantly different from the control group (air + PBS), $p < 0.05$.

Table 3

Effect of inhaling 100 ppm styrene (SR) on the cytokine levels induced by ovalbumin (OVA)

Group	IL-4 (pg/ml)	IL-5 (pg/ml)	IL-13 (pg/ml)	IFN- γ (pg/ml)
PBS + air	47.5 $\pm$ 0	83.9 $\pm$ 4.6	79.8 $\pm$ 1.3	562 $\pm$ 16
PBS + SR	62.8 $\pm$ 4.2	119.4 $\pm$ 7.7	85.2 $\pm$ 1.3	722 $\pm$ 1
OVA + air	51.20 $\pm$ 5.3	178.4 $\pm$ 12.3	125.6 $\pm$ 0	732 $\pm$ 6
OVA + SR	108.3 $\pm$ 24.3	710.5 $\pm$ 2.5	342.9 $\pm$ 15.2	565 $\pm$ 1

Two identical experiments were carried out at different times, but the levels of cytokine production were only assessed in experiment 2. At day 25, the lung-associated lymph nodes were removed and pooled to obtain enough cell numbers. Single cell suspensions were cultured in the presence of concanavaline A (a final concentration of 3 μ g/ml). Cytokine levels (pg/ml) were assessed by ELISA in the 72 h supernatants of the cell cultures. The data are presented as the mean $\pm$ S.D. of two to three repeated measurements.

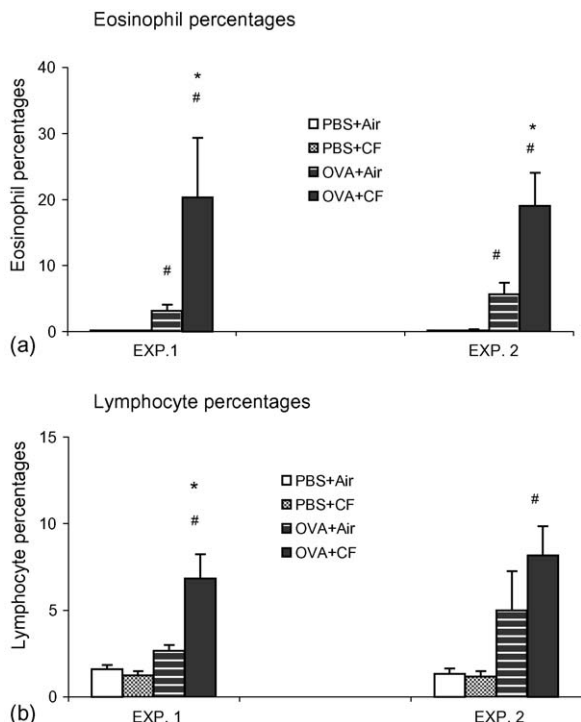


Fig. 2. Effect of inhaling 20 ppm chloroform (CF) on cell composition in the BAL fluid. (a and b) The percentages of eosinophil and lymphocyte, respectively. Two identical experiments were carried out at different times: at day 27, BALF were obtained by lung lavages with 0.3 ml PBS repeated four times. Each cell type was determined after a cell count on cytocentrifugation slides stained with Diff-Quik. The results are expressed as the mean $\pm$ S.E. of five to six animals. #Significantly different from the control group (air + PBS), $p < 0.05$. *Significantly different from the sensitised group (air + OVA), $p < 0.05$.

tory scores were related to the high score of mucus-engorged goblet cells in the airway epithelium. In four of the six experiments, the hyperplastic goblet cell scores were significantly higher than those of the air + PBS control mice (Figs. 3b, 6b, and 9b).

4.2. The CF and OVA-combined treatment

4.2.1. Effect on cytokine production (Table 1)

Exposure to CF alone, 20 ppm for four consecutive days, 6 h/day) decreased IL-4 production slightly, but increased IL-5 and IFN- γ production. In the CF + OVA-combined treatment groups, CF decreased the IL-4 and IFN- γ level slightly, but increased the IL-5 and -13 level induced by OVA (+53% for IL-5 and +34% for IL-13).

4.2.2. Effect on total and specific IgE production (Fig. 1a and b)

Exposure to CF alone raised neither the total nor the OVA-specific IgE. In the CF + OVA-combined treatment

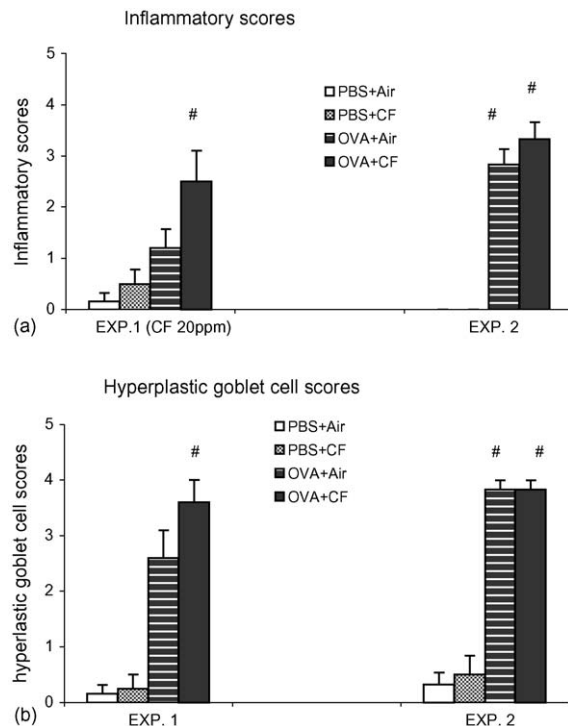


Fig. 3. Effect of inhaling 20 ppm chloroform (CF) on inflammation scores. (a and b) The scores of inflammatory cell influx and of goblet cell proliferation, respectively. Two identical experiments were carried out at different times: at day 27, after lavage, the left lung was removed and frozen. The severity of lung inflammation was determined on cryostat lung sections according to the criteria described in Section 2. The score results are expressed as the mean $\pm$ S.E. of five to six animals. #Significantly different from the control group (air + PBS), $p < 0.05$.

groups, CF did not modify the total IgE level. However, it increased the OVA-specific IgE level; +42% in experiment 1 and +138% in experiment 2 compared with that of the corresponding air + OVA groups.

4.2.3. Effect on eosinophil and lymphocyte influx (Fig. 2a and b)

Exposure to CF alone had no effect on inflammatory cell influx into the alveolar spaces. In the CF + OVA-combined treatment groups, CF significantly augmented the number of eosinophils (in both experiments 1 and 2) and lymphocytes (in experiment 2) in the BAL fluids compared with that of the corresponding air + OVA groups.

4.2.4. Effect on lung inflammation (Fig. 3a and b)

Exposure (of mice) to CF alone did not significantly increase the inflammatory scores compared with those of the corresponding air + PBS control groups. In the CF + OVA-combined treatment groups, CF tended to increase the inflammatory and hyperplastic goblet scores

(experiment 1) compared with those of the corresponding air + OVA groups.

4.3. The DCE and OVA-combined treatment

4.3.1. Effect on cytokine production (Table 2)

Exposure to DCE alone, 10 ppm for four consecutive days, 6 h/day, provoked a marked increase in IL-4, -5, and -13 levels, which were 6, 4.5, and 4.5 times those of the air/PBS control mice, respectively. In the DCE + OVA-combined treatment groups, DCE had no synergic effect on the production of these cytokines. Furthermore, in the case of IL-4, IL-13 and IFN- γ , DCE decreased rather than increased their production.

4.3.2. Effect on total and OVA-specific IgE production (Fig. 4a and b)

Exposure to DCE alone raised neither the total nor the OVA-specific IgE. In the DCE + OVA-combined treatment groups, DCE tended to increase the total and specific IgE levels induced by OVA (experiment 1) compared with those of the corresponding air + OVA groups (Fig. 4).

4.3.3. Effect on eosinophil and lymphocyte influx (Fig. 5a and b)

Exposure to DCE alone had no effect on inflammatory cell influx into the alveolar spaces. In the DCE + OVA-combined treatment groups, DCE strongly potentiated the influx of eosinophils and lymphocytes (experiment 1), but there was no significant score differences compared with those of the corresponding air + OVA groups (Fig. 5).

4.3.4. Effect on lung inflammation (Fig. 6a and b)

Exposure (of mice) to DCE alone raised neither the inflammatory nor the hyperplastic goblet scores. In the combined DCE + OVA treatment groups, DCE potentiated the inflammatory (experiment 2) and the hyperplastic goblet cell scores (in both experiments). However, there was no significant score differences compared with those of the corresponding air + OVA groups (Fig. 6).

4.4. The SR and OV-combined treatment

4.4.1. Effect on cytokine production (Table 3)

Exposure (of mice) to SR alone, 100 ppm for four consecutive days, 6 h/day, provoked a slight increase in IL4, IL-5, IL-13, and IFN- γ levels compared with those of the corresponding air + PBS control groups. In the SR + OVA-combined treatment groups, SR had a synergic effect on the Th2 cytokine production induced by

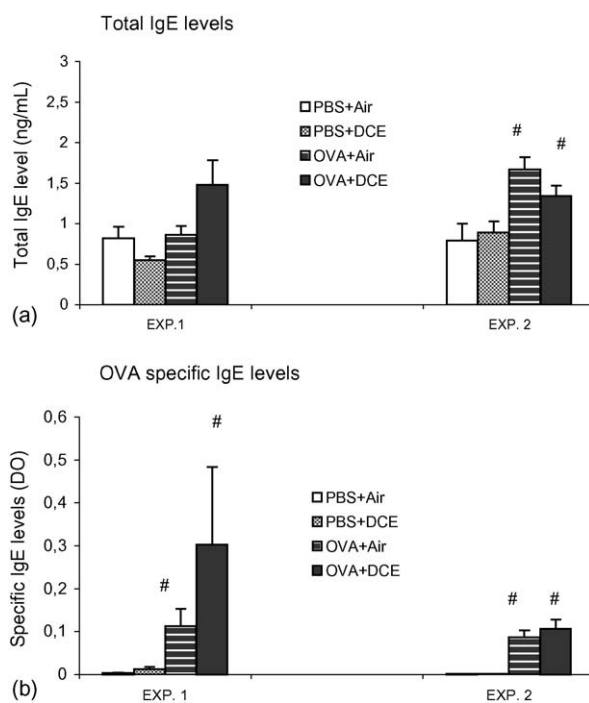


Fig. 4. Effect of inhaling 10 ppm 1,1-dichloroethylene (DCE) on IgE production. (a and b) The total and specific IgE levels, respectively. Two identical experiments were carried out at different times: at day 27, blood was obtained by cardiac punctures and the IgE levels were assessed by means of ELISA. The results are expressed as the mean $\pm$ S.E. of five to six animals. #Significantly different from the control group (air + PBS), $p < 0.05$.

OVA. The levels of IL-4, -5 and -13 were approximately two, four, and three times those observed in the corresponding air + OVA groups, respectively. However, SR decreased rather than increased the Th1 cytokine IFN- γ .

4.4.2. Effect on total and specific IgE production (Fig. 7a and b)

Exposure to SR alone did neither raise total nor OVA-specific IgE. In the SR + OVA-combined treatment groups, SR significantly potentiated the total IgE levels (both experiments) compared with those of the corresponding air + OVA groups. SR also potentiated the increase in OVA-specific IgE levels in the two experiments, but the differences were not statistically significant (Fig. 7).

4.4.3. Effect on eosinophil and lymphocyte influx (Fig. 8a and b)

Exposure to SR alone had no effect on inflammatory cell influx into the alveolar spaces. In the SR + OVA-combined treatment groups, SR non-significantly potentiated the influx of eosinophils (both experiments 1 and 2)

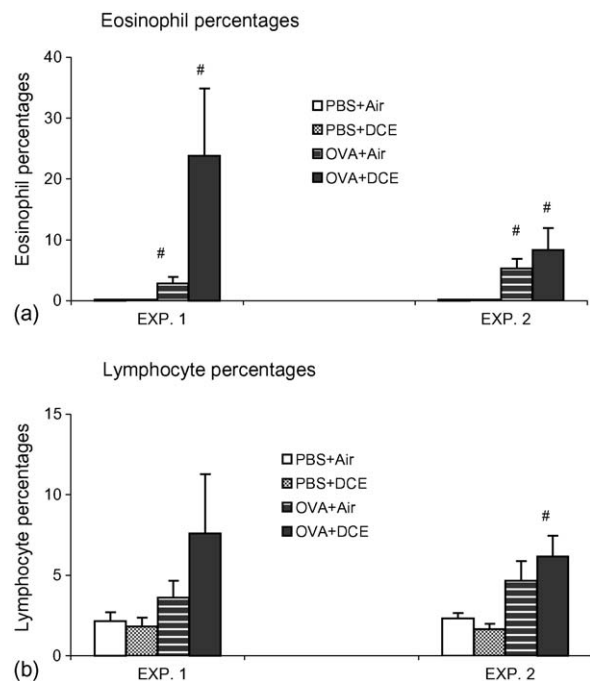


Fig. 5. Effect of 10 ppm 1,1-dichloroethylene (DCE) on cell composition in the BALF. (a and b) The eosinophil and lymphocyte percentages, respectively. Two identical experiments were carried out at different times: at day 27, BAL fluids were obtained by four times lung lavages with 0.3 ml PBS. Each cell type was determined after a cell count on cytocentrifugation slides stained with Diff-Quik. The results are expressed as the mean $\pm$ S.E. of five to six animals. #Significantly different from the control group (air + PBS), $p < 0.05$.

and lymphocytes (experiment 1) in the BAL fluids compared with that of the corresponding air + OVA groups Fig. 8.

4.4.4. Effect on lung inflammation (Fig. 9a and b)

Exposure to SR alone increased the inflammatory scores very slightly compared with those of the corresponding air + PBS control groups. In the SR + OVA-combined treatment groups, SR potentiated the increased scores of inflammatory infiltrate and goblet cell hyperplasia in both experiments. However, these scores were not significantly different from those of the corresponding air + OVA groups Fig. 9.

5. Discussion

Occupational allergy and asthma is a challenging issue in the developing countries. Several mouse models of allergic asthma have been developed to enhance the understanding of the immunological mechanisms underlying the pathogenicity of these diseases, including local cytokine production, IgE regulation, Th1/Th2

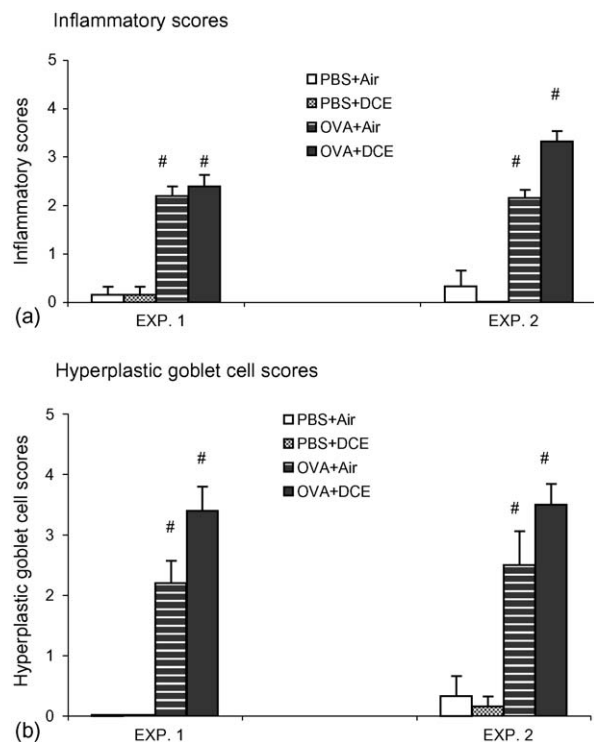


Fig. 6. Effect of 10 ppm 1,1-dichloroethylene (DCE) on the inflammation scores. (a and b) The scores of inflammatory cell influx and of goblet cell proliferation, respectively. Two identical experiments were carried out at different times: at day 27, after lavage, the left lung was removed and frozen. The severity of lung inflammation was determined on cryostat lung sections according to the criteria described in Section 2. The score results are expressed as the mean $\pm$ S.E. of five to six animals. #Significantly different from the control group (air + PBS), $p < 0.05$.

balance, eosinophilic airway inflammation and mucus production. In the present work, we used an OVA-mouse model to study the potential adjuvant effect of inhaling certain industrial chemicals, namely chloroform, 1,1-dichloroethylene and styrene, on Th2 response. Our mouse model has identified characteristics of mild Th2 response such as a moderate increase in OVA-specific IgE in the sera, in inflammatory cell influx in the lungs, in goblet cell proliferation, and in local IL-5, IL-13 production. We did not use a model employing alum in order to obtain an allergic response that could be increased or decreased by a combined treatment with the chemicals studied. The exacerbated Th2 markers were therefore able to be appropriately evaluated.

In workplaces airborne materials enter the host via inhalation. The body's primary response to inhaled chemicals takes place in the target epithelium of the respiratory tract, and involves several mechanisms such as generation of cytokines by the epithelial cells (Carter et

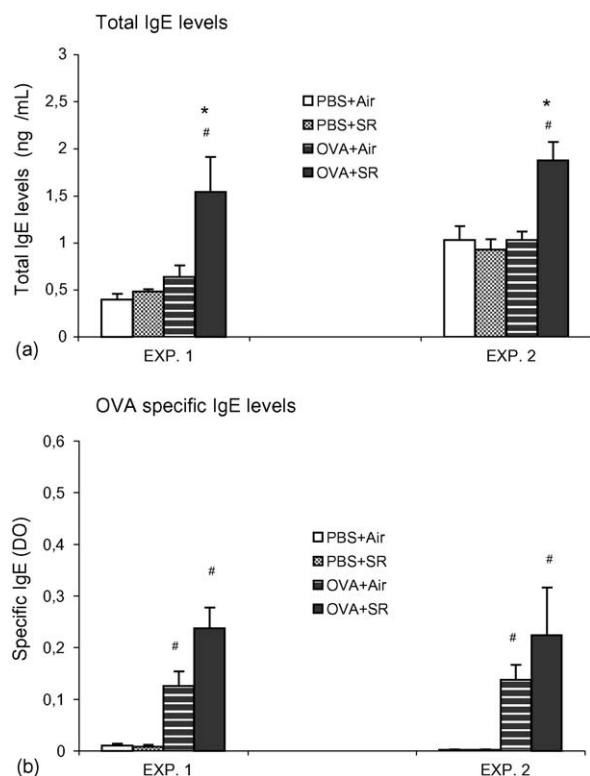


Fig. 7. Effect of inhaling 100 ppm styrene (SR) on IgE production. (a and b) The total and specific IgE levels, respectively. Two identical experiments were carried out at different times: at day 27, blood was obtained by cardiac punctures and the IgE levels were assessed by means of ELISA. The results are expressed as the mean $\pm$ S.E. of five to six animals. #Significantly different from (air + PBS) control group $p < 0.05$. *Significantly different from (air + OVA) sensitised group $p < 0.05$.

al., 1997), DC cell migration to the lung-draining lymph nodes for antigen presentation to T lymphocytes (Ban et al., 1997; Lambrecht et al., 2000), and mast cell degranulation. The DCs playing a crucial role in the generation of all adaptive T cells are important in the development of airway inflammation in response to antigens (Kapsenberg, 2003; Lambrecht et al., 1998). The results of our previous study showed a higher number of plaque forming cells in the lung-draining lymph nodes of mice exposed to CF, DCE and SR. This increase was explained by an enhanced role of antigen-presenting DCs. In the present study using the OVA mouse model, these chemicals administered to mice during the OVA-sensitisation period exacerbated respiratory allergy. This may result from an adjuvant effect of the chemicals on the Th2 response of OVA-sensitised mice. This finding appears to suggest that inhaling chemicals in the workplace, even at non-immunotoxic concentration, may have an effect on the balance of the local Th1/Th2 immune response.

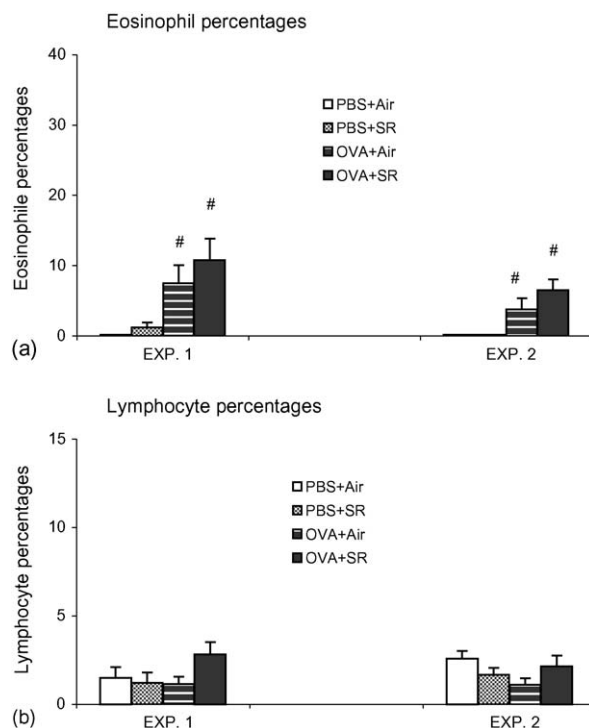


Fig. 8. Effect of inhaling 100 ppm styrene (SR) on cell composition in the BAL fluid. (a and b) The eosinophil and lymphocyte percentages, respectively. Two identical experiments were carried out at different times: at day 27, BALF were obtained by four times lung lavages with 0.3 ml PBS. Each cell type was determined after a cell count on cytocentrifugation slides stained with Diff-Quik. The results were expressed as the mean $\pm$ S.E. of five to six animals.

When shifted in the Th2 direction, the deviated adaptive immune response may be partly responsible for the initiation and exacerbation of allergic disease.

Exposure to 100 ppm styrene for 6 h/day for four consecutive days increased both the Th1 and Th2 cytokines very slightly. There was a synergic effect of SR + OVA on Th2 cytokine production. A 2-, 4-, and three-fold increase in IL-4, -5 and -13 was observed in combined SR and OVA-treated mice, respectively. In the combined SR + OVA treatment, there was a positive relationship between the increase in Th2 cytokine production in the lung-draining lymph nodes (IL-4, -5 and -13), the increase in serum IgE levels, and the exacerbation of the inflammatory reaction, characterised by the higher number of goblet cells and eosinophils in the BAL fluid and the respiratory tracts. IL-4 has a major role in B cell activation and isotype switching, particularly in IgE production (Coffman et al., 1993), IL-13 seems to be critical for mucus production, and IL-5 plays a pivotal role in inducing eosinophilic inflammation (Kuperman et al., 2002; Foster et al., 2002). Furthermore, the increased Th2

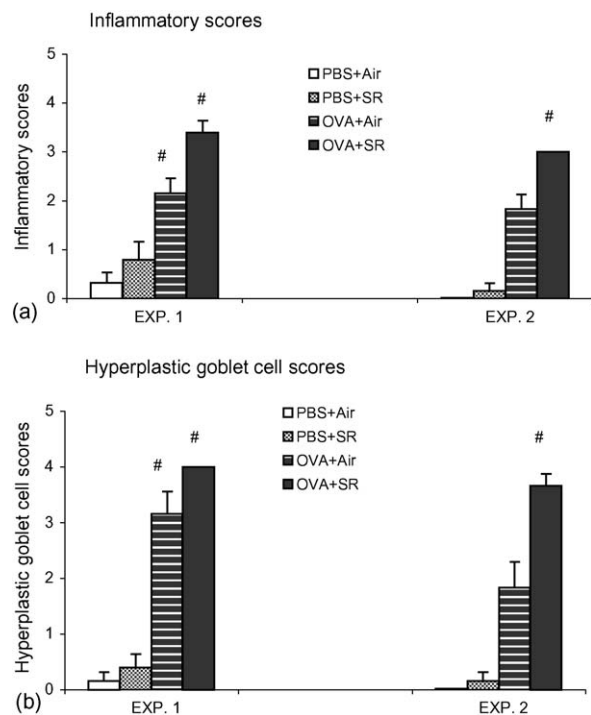


Fig. 9. Effect of inhaling 100 ppm styrene (SR) on the inflammation scores. (a and b) The scores of inflammatory cell influx and of goblet cell proliferation, respectively. Two identical experiments were carried out at different times: at day 27 after lavage, left lung was removed and frozen. The severity of lung inflammation was determined on cryostat lung sections according to the criteria described in Section 2. The score results were expressed as the mean $\pm$ S.E. of five to six animals. # Significantly different from the control group (air + PBS), $p < 0.05$.

cytokine production induced by SR in OVA-sensitised mice was concomitant with a decrease in Th1 cytokine IFN- γ production, indicating that the production of IFN- γ is important for down-regulating the Th2 response (Nakamura et al., 1997).

Unlike styrene, the chloroform exposure decreased the basal production of IL-4 and inhibited the increase in IL-4 production induced by OVA. This difference, a lower rather than a higher IL-4 level, may partly explain why there was no augmentation in total IgE levels in the combined OVA- and CF-treated groups in two identical experiments. With regard to IL-5, although its level was higher in the CF-alone group than that in the SR-alone group, CF potentiated IL-5 production less strongly in the CF/OVA-sensitised mice compared with the SR/OVA-sensitised mice. However, the significant increase in the number of eosinophils in CF + OVA-sensitised mice was more marked than that in SR/OVA-sensitised mice (20% versus 10%). IL-5 may not be the only cytokine involved in the development, the recruitment, the activation and the survival

of eosinophils in CF + OVA-sensitised mice. A number of CC chemokines, including RANTES and eotaxin, have been identified with causing eosinophil chemotaxis (Alam et al., 1993; Palframan et al., 1998).

Concerning DCE exposure, this, rather surprisingly, enhanced both Th1 and Th2 local lymph node cytokines in the DCE-alone group. Their levels were higher than those observed in the OVA-alone group. However, in the DCE/OVA-sensitised mice, an additive effect on cytokine production was not observed. A possible explanation might be the induction of a regulatory mechanism contributing to local homeostasis in the lung to avoid a severe inflammatory reaction. Alveolar macrophages have been found to do so, at least partly. By secreting anti-inflammatory mediators such as prostaglandin E₂, transforming growth factor- β , IL-10 and nitric oxide, they play a key role in damping immune responses (Peters-Golden, 2004; Laskin et al., 2001). Although a synergistic effect of DCE and OVA on cytokine production did not occur, DCE potentiated the production of IgE, the influx of inflammatory cells, and goblet cell hyperplasia in the DCE + OVA-sensitised mice.

In summary, exposing mice to 20 ppm CF, 10 ppm DCE or 100 ppm SR inhalation during the sensitisation period exacerbated the inflammatory reaction after OVA challenges. This exacerbation was characterised by the higher number of inflammatory and goblet cells in the lungs, and the increased levels of total and/or specific IgE in the blood. Exposure to the chemicals alone had an effect on cytokine production by the lung-draining lymph node cells. The data of these local cytokine levels may improve our understanding of the cellular and molecular mechanism involved in the exacerbation of inflammatory reaction in humans. Chemicals like SR, which cause a slight increase locally in Th2 cytokine levels, may have a synergic effect on allergen-inducing Th2 cytokine production, whereas others like DCE, which cause a strong increase, may have no effect.

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References

- Alam, R., Stafford, S., Forsythe, P., Grant, J.A., 1993. RANTES is a chemotactic and activating factor for eosinophils. *J. Immunol.* 150, 3442–3447.
- Arif, A.A., Whitehead, L.W., Delclos, G.L., Tortolero, S.R., Lee, E.S., 1988–94. Prevalence and risk factors of work related asthma by industry among United States workers: data from the third national

- health and nutrition examination survey. *Occup. Environ. Med.* 59, 505–511.
- Ban, M., Hettich, D., Bonnet, P., 2003. Effect of inhaled industrial chemicals on systemic and local immune response. *Toxicology* 184, 41–50.
- Ban, M., Hettich, D., Goutet, M., Bonnet, P., 1997. TDI inhalation in guinea-pigs involves migration of dendritic cells. *Toxicol. Lett.* 93, 185–194.
- Banchereau, J., Steinman, R.M., 1998. Dendritic cells and the control of immunity. *Nature* 392, 245–252.
- Beasley, R., Crane, J., Lai, C.K., Pearce, N., 2000. Prevalence and aetiology of asthma. *J. Allergy Clin. Immunol.* 105, 466–472.
- Coffman, R.L., Lebman, D.A., Rothman, P., 1993. Mechanism and regulation of immunoglobulin isotype switching. *Adv. Immunol.* 54, 229–270.
- Carter, J.D., Ghio, A.J., Samet, J.M., Devlin, R.B., 1997. Cytokine production by human airway epithelial cells after exposure to an air pollution particle is metal-dependent. *Toxicol. Appl. Pharmacol.* 146, 180–188.
- De Haar, C., Hassing, I., Bol, M., Bleumink, R., Pieters, R., 2005. Ultra-fine carbon black particles cause early airway inflammation and have adjuvant activity in a mouse allergic airway disease model. *Toxicol. Sci.* 87, 409–418.
- Finkelman, F.D., Yang, M., Orekhova, T., Clyne, E., Bernstein, J., Whitekus, M., Diaz-Sanchez, D., 2004. Diesel exhaust particles inhibit Interferon- γ production by inhibiting cytokine effects on NK and NKT cells. *J. Immunol.* 172, 3808–3838.
- Foster, P.S., Hogan, S.P., Matthaei, K.I., Young, I.G., 1997. Interleukin-4 and interleukin-5 as targets for the inhibition of eosinophilic inflammation and allergic airways hyperreactivity. *Mem. Inst. Oswaldo Cruz* 92, 55–61.
- Foster, P.S., Hogan, S.P., Yang, M., Mattes, J., Young, I.G., Matthaei, K.I., Kumar, R.K., Mahalingam, S., Webb, D.C., 2002. Interleukin-5 and eosinophils as therapeutic targets for asthma. *Trends Mol. Med.* 8, 162–167.
- Funabashi, H., Shima, M., Kuwaki, T., Hiroshima, K., Kuriyama, T., 2004. Effects of repeated ozone exposure on pulmonary function and bronchial responsiveness in mice sensitised with ovalbumin. *Toxicology* 204, 75–83.
- Gavett, S.H., O'Hearn, D.J., Li, X., Huang, S.K., Finkelman, F.D., Wills-Karp, M., 1995. IL-12 inhibits antigen-induced airway hyperresponsiveness, inflammation, and Th-2 cytokine expression in mice. *J. Exp. Med.* 182, 1527–1536.
- Holgate, S.T., 1999. The epidemic of allergy and asthma. *Nature* 402, B2–B4.
- Howardth, P.H., 1998. Is allergy increasing? Early life influences. *Clin. Exp. Allergy* 28 (Suppl.), 2–7.
- Kapsenberg, M.L., 2003. Dendritic-cell control of pathogen-driven T-cell polarization. *Nat. Rev. Immunol.* 3, 984–993.
- Kay, A.B., 2001. Allergy and allergic diseases. First of two parts. *N. Engl. J. Med.* 344, 30–37.
- Kuperman, D.A., Huang, X., Koth, L.L., Chang, G.H., Dolganov, G.M., Zhu, Z., Elias, J.A., Sheppard, D., Erle, D.J., 2002. Direct effects of interleukin-13 on epithelial cells cause airway hyperreactivity and mucus overproduction in asthma. *Nat. Med.* 8, 885–889.
- Lambrecht, B.N., Salomon, B., Klatzmann, D., Pauwels, R.A., 1998. Dendritic cells are required for the development of chronic eosinophilic airway inflammation in response to inhaled antigen in sensitised mice. *J. Immunol.* 160, 4090–4097.
- Lack, G., Bradley, K.L., Hamelmann, E., Renz, H., Loader, J., Leung, D.Y., Larsen, G., Gelfand, E.W., 1996. Nebulized IFN- γ inhibits the development of secondary allergic responses in mice. *J. Immunol.* 157, 1432–1439.
- Lambrecht, B.N., De Veerman, M., Coyle, A.J., Gutierrez-Ramos, J., Thielemans, K., Pauwels, R.A., 2000. Dendritic cells induce Th2 responses to inhaled antigen leading to eosinophilic airway inflammation. *J. Clin. Invest.* 106, 551–559.
- Laskin, D.L., Weinberger, B., Laskin, J.D., 2001. Functional heterogeneity in liver and lung macrophages. *J. Leukocyte Biol.* 70, 163–170.
- Moss, R.B., Moll, T., El-Kalay, M., Kohne, C., Soo Hoo, W., Encinas, J., Carlo, D.J., 2004. Th1/Th-2 cells in inflammatory disease states: therapeutic implications. *Expert Opin. Biol. Ther.* 4, 1887–1896.
- Nakamura, T., Lee, R.K., Nam, S.Y., Podack, E.R., Bottomly, K., Flavell, R.A., 1997. Roles of IL-4 and IFN- γ in stabilizing the T helper cell type 1 and 2 phenotype. *J. Immunol.* 158, 2648–2653.
- Palframan, R.T., Collins, P.D., Williams, T.J., Rankin, S.M., 1998. Eotaxin induces a rapid release of eosinophils and their progenitors from the bone marrow. *Blood* 91, 2240–2248.
- Peters-Golden, M., 2004. The alveolar macrophage: the forgotten cell in asthma. *Am. J. Respir. Cell. Mol. Biol.* 31, 3–7.
- Reisinger, J., Triendl, A., Kuchler, E., Bohle, B., Krauth, M.T., Rauter, I., Valent, P., Koenig, F., Valenta, R., Niederberger, V., 2005. IFN- γ -enhanced allergen penetration across respiratory epithelium augments allergic inflammation. *J. Allergy Clin. Immunol.* 115, 973–981.
- Steenberg, P.A., Withagen, C.E., van Dalen, W.J., Dormans, J.A.M.A., Cassee, F.R., Heisterkamp, S.H., van Loveren, H., 2004. Adjuvant activity of ambient particulate matter of different sites, sizes and seasons in a respiratory allergy mouse model. *Toxicol. Appl. Pharmacol.* 200, 186–200.
- Tang, C., Inman, M.D., van Rooijen, N., Yang, P., Shen, H., Matsumoto, K., O'Byrne, P.M., 2001. Th type 1-stimulating activity of lung macrophages inhibits Th2-mediated allergic airway inflammation by an IFN- γ -dependent mechanism. *J. Immunol.* 166, 1471–1478.
- Yamauchi, T., Shima, M., Kuwaki, T., Ando, M., Ohmichi, M., Fukuda, Y., Adachi, M., 2002. Acute effects of ozone exposure on lung function in mice sensitised to ovalbumin. *Toxicology* 172, 69–78.