Clin Exp Immunol 1994; 95:333-336

# IL-4 production is increased in cigarette smokers

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*(Accepted for publication 21 October 1993)*

## SUMMARY

Cigarette smoking has been associated with both increases in serum levels of total IgE and an increased risk of developing allergic-like symptoms. IL-4 and interferon-gamma (IFN-$\gamma$) have reciprocal roles in the regulation of IgE synthesis, and as such prompted us to evaluate, in smokers, the production of these two cytokines. We demonstrate that phytohaemagglutinin (PHA)-induced IL-4 production by peripheral blood mononuclear cells (PBMC) of smokers ($n = 19$) is significantly higher than that of non-smokers ($n = 10$, $P < 0.005$). In addition, PBMC from heavy smokers, defined by the number of cigarettes smoked per day, produced significantly higher levels of IL-4 than those of light smokers. No difference between the groups was found for IFN-$\gamma$ production. Our data suggest an imbalance in cytokine production occurring in individuals who smoke. This imbalance, favouring IL-4 production, may be part of the mechanism responsible for the observed increases in serum IgE and allergic-like symptoms associated with cigarette smoking.

**Keywords** IL-4 interferon-gamma IgE smoking allergy

## INTRODUCTION

Reports over the past two decades have discussed a possible association between tobacco smoking and allergic disease. One hypothesis suggests that cigarette smoke increases respiratory mucosal permeability, which in turn leads to a possible increase in sensitization to aeroallergens [1]. Many reports have focused on parental smoking and the development of allergic-like symptoms in their children. For instance, Weitzman *et al.* [2] demonstrated earlier onset and higher rates of asthma amongst children whose mothers smoked. In another study, Magnusson [3] showed that newborn infants had higher levels of cord serum IgE and had a four-fold higher risk of developing atopic disease by the age of 18 months if their mother smoked compared with if she did not. Furthermore, in a study of 9-year-old children [4], boys whose parents smoked had both higher serum levels of total IgE and total eosinophil counts compared with boys of non-smoking parents.

Tobacco smoking has also been associated with increases in serum levels of total IgE in adults. In one study a group of smoking adults, as well as having higher serum levels, demonstrated a slower natural rate of decline of serum IgE with age [5]. Increases in serum IgE levels have also been confirmed in an animal model where rats have been exposed to tobacco smoke. In this study [6], it was demonstrated that rats exposed to tobacco smoke had higher levels of serum IgE compared with the unexposed controls.

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Since tobacco smoking appears to alter several immunological parameters associated with allergic disease, it is possible that the mechanism responsible may be related directly, or indirectly, to alterations of the cytokine network. This has prompted us to investigate, in smokers, the regulation of two important allergy-related cytokines, IL-4 and interferon-gamma (IFN-$\gamma$). We chose these two cytokines because of their demonstrated roles in the regulation of IgE production, with IL-4 being stimulatory and IFN-$\gamma$ being inhibitory. A demonstration of an altered balance in the production of these cytokines may suggest a mechanism for the observed association between individuals who smoke and elevated levels of serum IgE.

## SUBJECTS AND METHODS

### *Subjects*

Heparinized whole blood was collected from 19 volunteer employees of the Royal Melbourne Hospital who regularly smoke. The group consisted of 13 females and six males, with a mean age of 34 years (range 22–64 years). The reported average number of cigarettes smoked per day of each individual was recorded. Blood was also collected from a group of 10 non-smokers consisting of six females and four males, with a mean age of 32 years (range 25–52 years). Individuals from each group recorded a negative history of asthma, eczema and rhinitis.

### *Peripheral blood mononuclear cell isolation and mitogen induction of IL-4 and IFN-$\gamma$*

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized whole blood by Ficoll–Hypaque (Pharmacia Fine

Chemicals, Uppsala, Sweden) density gradient centrifugation. The cells were then resuspended at $10^6$/ml in RPMI 1640+5% autologous serum supplemented with glutamine (2 mM), penicillin (100 U/ml) and streptomycin (100 μg/ml). Replicates (200 μl) were then cultured at 37°C and 5% $CO_2$ in 96-well round-bottomed culture plates in the presence or absence of phytohaemagglutinin (PHA; 10 μg/ml) (Difco Laboratories, Detroit, MI). Cell-free supernatants were collected after 48 h, which had previously been found to be optimal for IL-4 production for both smokers and non-smokers.

*IL-4 assay*

IL-4 was determined by ELISA, which consisted of coating microtitre plates (Nunc, Roskilde, Denmark) with polyclonal anti-human IL-4 antibody (Schering-Plough, Bloomfield, NJ) diluted 1:20 000 in bicarbonate buffer pH 9·6. After the plates were washed with PBS/0·05% Tween, standards (recombinant IL-4, Schering-Plough) and cell-free supernatants were added. Bound IL-4 was then detected using monoclonal rat anti-human IL-4 (Schering-Plough) diluted 1:100, followed by anti-rat immunoglobulin–peroxidase conjugate (Dakopatts, Carpinteria, CA) diluted 1:1000. One hundred microlitre volumes were used throughout the procedure. All incubations were performed at room temperature for 2 h. Colour was then developed using tetramethylbenzidine-hydrogen peroxide (TMB). The reaction was stopped with 1 M phosphoric acid and absorbances read at 450 nm. No other known cytokine has been found to cross-react in the assay. The sensitivity of the assay was previously found to be 15 pg/ml.

*IFN-γ assay*

IFN-γ was measured as previously described [7]. Briefly, flat-bottomed microtitre plates were coated for 48 h with a polyclonal anti-human IFN-γ antibody (Genzyme, Boston, MA) diluted in bicarbonate buffer (pH 9·6). After the plates were washed, standards and cell-free supernatants were added and incubated at 4°C for 3 h, after which an anti-human IFN-γ MoAb (1 μg/ml; Genzyme) was added. Following incubation for 2 h at 4°C, rabbit anti-mouse immunoglobulin antibody coupled to peroxidase (Dakopatts) was added. Colour was developed using TMB. The reaction was stopped with phosphoric acid and absorbances read at 450 nm. The sensitivity of the assay is 6 pg/ml.

*IgE assay*

Serum total IgE was determined using a latex-enhanced immunonephelometric method which was performed on an automated nephelometer according to the manufacturer's instructions (Behring, Marburg, Germany). The standard provided by the manufacturer was calibrated against WHO reference material. The sensitivity of this assay was found to be 15 U/ml.

*Statistical analysis*

Statistical analysis of the results was performed using the Mann–Whitney unpaired *U*-test.

## RESULTS

*IL-4 production*

Figure 1 demonstrates the production of IL-4 by PBMC from smokers and non-smokers after stimulation with PHA for 48 h.

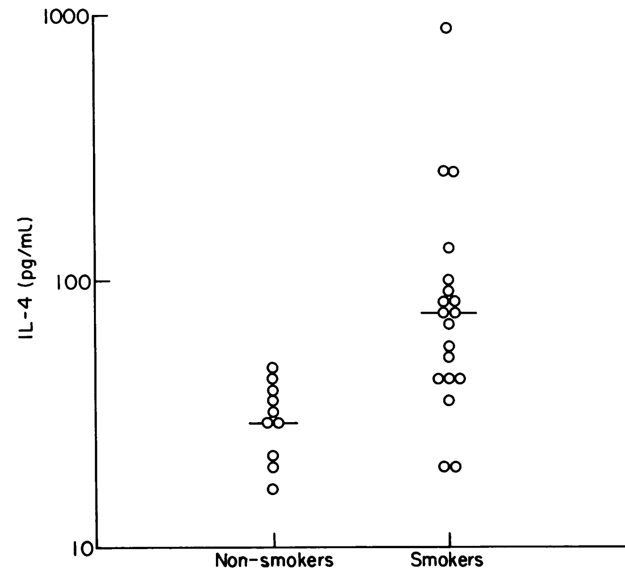


**Fig. 1.** Phytohaemagglutinin (PHA)-induced IL-4 production: smokers *versus* non-smokers. Peripheral blood mononuclear cells (PBMC) ($10^6$/ml) were cultured in the presence of PHA (10 μg/ml) for 48 h. The medians of each group are shown. ($P<0·005$.) Ninety-five per cent confidence limits: smokers 43–98 pg/ml; non-smokers 23–38 pg/ml.

It was noted that in the absence of PHA no spontaneous production of IL-4 was detected in cell cultures from either group (data not shown). As shown, PBMC from the smokers group produced significantly higher levels of IL-4 (mean = 130 pg/ml, median = 75 pg/ml) compared with the non-smokers (mean = 32 pg/ml, median = 31 pg/ml, $P<0·005$). Smokers were questioned as to the average number of cigarettes they smoked

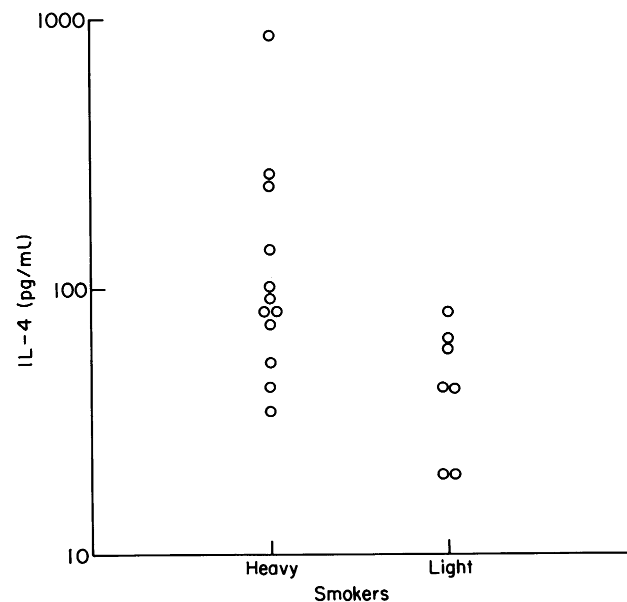


**Fig. 2.** Phytohaemagglutinin (PHA)-induced IL-4 production: smokers. Smokers were grouped according to the average number of cigarettes smoked per day (heavy = 20 or more cigarettes per day, light = less than 20 cigarettes per day). Peripheral blood mononuclear cells (PBMC) ($10^6$/ml) were cultured in the presence of PHA (10 μg/ml) for 48 h. ($P<0·02$.)

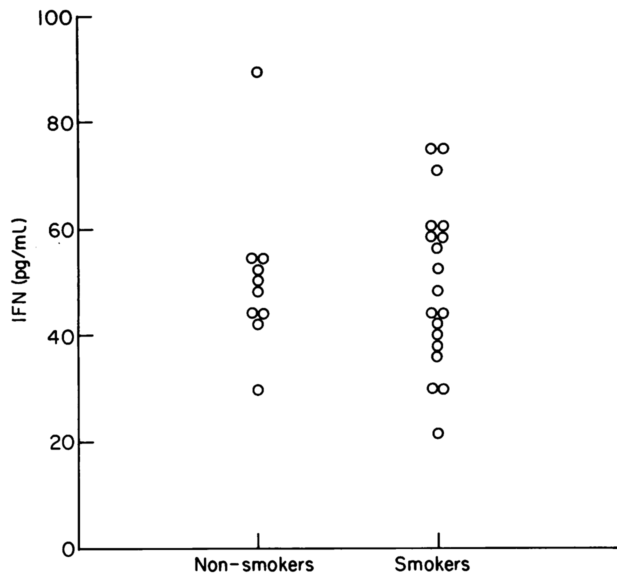


**Fig. 3.** Phytohaemagglutinin (PHA)-induced IFN-$\gamma$ production: smokers *versus* non-smokers. Peripheral blood mononuclear cells (PBMC) ($10^6$/ml) were cultured in the presence of PHA (10 $\mu$g/ml) for 48 h. ($P=0{\cdot}94$.)

per day. This was plotted against their PHA-induced IL-4 production. As seen in Fig. 2, IL-4 production tended to be higher in heavy smokers, defined as those who smoked 20 or more cigarettes per day, compared with light smokers, who smoked less than 20 per day ($P<0{\cdot}02$). To substantiate this finding an increased sample size of each group will be required. Also of interest was the dramatic fall in IL-4 production observed in two individuals from the smoking group who ceased smoking during the study period. In one, the IL-4 was found to be 252 pg/ml while smoking an average of 25 cigarettes per day. Eight months after ceasing to smoke, IL-4 production fell to 32 pg/ml. In the other subject, while smoking 20 cigarettes per day the IL-4 was 260 pg/ml, which fell to 95 pg/ml after only 28 days of non-smoking. We are currently recruiting more subjects who intend to cease smoking to validate this observation.

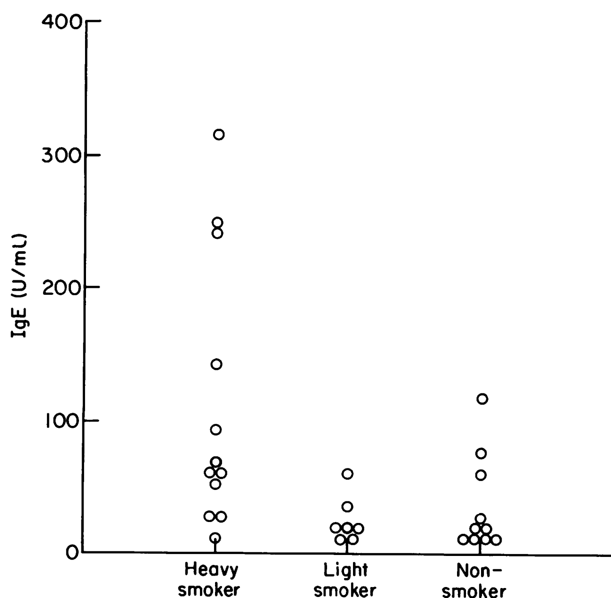


**Fig. 4.** Serum IgE levels. Heavy smokers (20 or more cigarettes per day), light smokers (less than 20 cigarettes per day) and non-smokers.

*IFN-$\gamma$ production*

Unlike IL-4, no difference was observed for IFN-$\gamma$ production between the two groups as demonstrated in Fig. 3 ($P=0{\cdot}94$). Also, no correlation could be found between IFN-$\gamma$ production and number of cigarettes smoked per day (data not shown) for the smoking group.

*Serum IgE*

Figure 4 demonstrates the serum levels of total IgE for each of the individuals tested. They are divided into three groups: light smoker, defined as those who smoke less than 20 cigarettes per day, heavy smokers, who smoke 20 or more cigarettes per day, and non-smokers. No statistical difference was found between the non-smoking and smoking groups. Differences were found, however, between heavy smokers and light smokers ($P<0{\cdot}02$) with geometric means being 72 and 19 U/ml respectively. Differences were also observed between heavy smokers and non-smokers (geometric mean = 29 U/ml), although the significance was weaker ($P<0{\cdot}05$).

## DISCUSSION

An increased risk of developing allergic-like symptoms, and higher than normal levels of serum IgE have been reported in individuals, and in children of individuals who smoke tobacco. In this study we have demonstrated a higher mitogen-induced production of IL-4 by PBMC of individuals who smoke compared with those who do not, whereas levels of IFN-$\gamma$ produced did not differ. Alterations in the production of these cytokines may in part explain the reported higher levels of total IgE in smokers, since IL-4 is stimulatory and IFN-$\gamma$ is inhibitory with respect to the synthesis of IgE. Although we did not find a difference in the serum levels of total IgE between the smoking and non-smoking groups, observable differences were noted between the heavy smokers, and both the light smokers and non-smokers. This observation was supported by our finding that the heavy smokers tended to have the highest mitogen-induced IL-4 production. With regard to specific IgE responses, there have been reports [8] of an increased incidence of positive skin and serological tests to occupational allergens amongst industrial workers who smoke. This may be the result of both increased respiratory mucosal permeability and a predominant IL-4 immunological response, leading to the induction of specific IgE and allergen sensitization.

It has been established in mice [9], and more recently in man [10], that T cell clones may be classified according to the cytokine profiles they produce upon activation. For instance, Th1 cells produce IL-2, IFN-$\gamma$ and tumour necrosis factor (TNF), whereas Th2 cells produce IL-4, IL-5, IL-6 and IL-10. These T cell phenotypes have been demonstrated in long-term clones, whereas *in vivo* it is more likely that intermediate phenotypes producing a range of cytokine profiles are found (e.g. Th0 shown to produce IL-2, IFN-$\gamma$ and IL-4). Our results suggest a shift toward the Th2 phenotype within individuals who smoke, since the ratio of IL-4/IFN-$\gamma$ production has increased. How this is achieved remains unclear. Several reports

[11,12], however, have shown that tobacco smoking is able to affect the expression of T cell surface antigens. Miller & Ginns [11] demonstrated a decrease in $CD4^+$ T cell numbers with a concomitant increase in $CD8^+$ T cells in the peripheral blood of heavy smokers. Furthermore, it was shown that 6 weeks after the cessation of smoking, individuals' $CD4^+/CD8^+$ ratio returned to normal. We have also demonstrated a similar finding in two individuals where a reduction in IL-4 production occurred after both had ceased to smoke.

If a shift in T cell phenotypes, or the cytokine profiles they produce upon activation, occurs in smokers, favouring Th2-like responses, it may then explain the mechanism for the observed association between cigarette smoking and the increased risk of allergic symptoms. For instance, IL-4 induces both the production of IgE [13] and the expression of CD23 [14], both of which are increased in allergic disease. IL-4 also plays a role in the growth and development of mast cells [15], which are central to the allergic reaction. IL-5, a cytokine also produced by Th2-like T cells, induces the growth, development and recruitment of eosinophils [16], cells critically involved in allergic asthma, and which have been shown to be increased in children whose mothers smoke [4].

It has recently been demonstrated that cross regulation occurs between individual T cell phenotypes [17]. IL-4 is required for the development of Th2-like cells, whereas IFN-$\gamma$ has been shown to inhibit their development. On the other hand, IFN-$\gamma$ is needed for Th1 development, whereas IL-4 acts as an inhibitor. If Th2-like IL-4 responses predominate in smokers, one may postulate the effect on T cell phenotypes in childhood, adult life and possibly *in utero* if the mother smokes during pregnancy. A predominance of Th2-like T cells may develop, which in turn respond to normally innocuous environmental antigens such as pollens, animal danders or dust mites by producing IL-4 rather than IFN-$\gamma$. The consequences of such Th2-like responses may then indirectly allow the development of symptoms of allergic disease.

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