

Th1 and Th2 Cytokines and IgE Levels in Identical Twins with Varying Levels of Cigarette Consumption

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Accepted: June 7, 2004

Some have suggested that tobacco smoke may skew the immune system toward a Th2 pattern, however the effects of genetics or childhood exposures could explain these results. We compared PMBC supernatant or serum Th1 (INF- γ) and Th2 (IL-4, IL-5, and IL-13) cytokine and IgE levels in members of 45 pairs of nonasthmatic monozygotic twins with varying levels of current cigarette consumption to determine if smoking was associated with Th1/Th2 function after accounting for genetic factors. A statistically significant dose-response was observed between levels of smoking and IL-13 ($p = 0.05$). Mean IL-13 level among heavy smokers (20+ cigarettes/day) was 146% higher than that among nonsmokers (+26.2 pg/mL; $p = 0.04$). The mean IL-5 level among heavy smokers was 166% higher than that among light (<20 cigarettes/day) smokers (+3.4 pg/mL; $p = 0.03$). No statistically significant differences in INF- γ , IL-4, or IgE levels were observed. Smoking appears to be associated with increased levels of IL-13.

KEY WORDS: Smoking; interleukin-13; interleukin-5; interferon- γ ; twins.

INTRODUCTION

Tobacco smoking is associated with a number of non-malignant respiratory diseases, including asthma, chronic

bronchitis, and chronic obstructive pulmonary disease. Analytic studies have generally shown a positive association between active, current smoking, and exacerbation of asthma (1, 2) and a dose-response relationship between the amount smoked and the level of asthma mortality (3). The mechanism for these effects has not been fully elucidated. Bronchial inflammation and hyperreactivity is increased in both current and past smokers (4), alveolar permeability is increased in rats (5) and humans (6) exposed to tobacco smoke, and smokers have increased exposure to inflammation-promoting oxygen radicals thought to be involved in pathogenesis. In addition, mainstream and secondhand smoke may modulate immune responses by a direct effect on the cells that produce the Th2 cytokines involved in asthma and allergy, including interleukin-4 (IL-4), interleukin-5 (IL-5), and interleukin-13 (IL-13) (7). Evidence for this mechanism is supported by the findings of higher IL-4 levels in smokers compared to nonsmokers (8), and higher IL-13 levels in cord blood mononuclear cells from infants exposed to smoke in utero compared to those not exposed (9). However, these studies did not account for potentially confounding effects of age, gender, inherited factors, and ethnicity on cytokine levels and tobacco use.

Identical twins with different smoking exposures provide a set of subjects matched with respect to age, gender, genotype, and early childhood exposures. We tested the hypothesis that tobacco smoke exposure is correlated with Th2 (IL-4, IL-5, and IL-13) cytokine and IgE levels, and inversely correlated with Th1 (interferon- γ [INF- γ]) cytokine levels, in a sample of nonasthmatic identical (monozygotic) twin pairs with varying levels of tobacco smoke exposure, in order to account for the genetic and demographic factors that might influence cytokine production.

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METHODS

The study was approved by the Institutional Review Board of the Keck School of Medicine of the University of Southern California and all participating subjects provided signed informed consents. Eligible subjects were twins from a population-based registry of California-born twins (10), if either twin ever smoked OR one twin was currently living with a smoker. Those over 69, or those with chronic respiratory disease, cancer, autoimmune disease, chronic infections, asthma, eczema, or with a temporary condition affecting cytokine activity (pregnancy, acute febrile illness, recent use of antibiotics, non-steroidal anti-inflammatory medications or steroids) were excluded.

One hundred and thirty twin pairs were identified from the twin registry directory based on reported smoking histories. After a brief interview, members of 91 twin pairs, ages 39–69, met the eligibility requirements, and agreed to participate (70% of 130 pairs contacted). At least one member of 16 pairs (12%) was ineligible on the basis of illness or medication use, and at least one member of 23 pairs (18%) refused.

Blood specimens from the members of each participating pair were simultaneously collected and processed. Peripheral blood mononuclear cells were separated using ficoll gradient. Cells (2 million/mL) were cultured in RPMI with 10% fetal calf serum media for 24 h with 10 μ L/mL of Phytohemagglutinin (PHA); supernatant was collected and frozen at -70°C . Supernatant levels of IL-4, IL-5, and IL-13 were determined by ELISA (R & D Systems (Minneapolis, MN) (11), according to manufacturer's instructions. Supernatant was exhausted so INF- γ was measured from serum by ELISA (BD Biosciences, San Diego, CA). Total serum IgE was measured by ELISA as previously described (12). The CV (%) for all assays was below 10%. Samples from both members of each pair were run on the same plate. Numbers vary slightly by assay when a particular set of samples was exhausted.

Current cigarette consumption was collected in ordinal categories of 5 cigarettes per day (1–10, etc). A lifetime nonsmoker (hereafter referred to as a nonsmoker) was defined as a person who smoked less than 100 cigarettes in their lifetime. The 91 participating twin pairs were distributed with respect to smoking history as follows: former smoker + current smoker (26 pairs); former smoker + nonsmoker (20 pairs); nonsmoker + current smoker (18 pairs); current smoker + current smoker, with different levels of cigarette consumption (17 pairs); current smoker + current smoker, with the same levels of cigarette consumption (6 pairs); nonsmoker living with a smoker + non-smoker not living with a smoker (4 pairs).

Serum cotinine levels were determined using standard gas chromatography (MedTox, St Paul, MN), to validate reports of nonsmoking. Because of the short half-life of cotinine, the test is not useful for validation of reported current smoking, since it would identify as nonsmokers persons who had not smoked within 24 h of the test. None of the twins who identified themselves as nonsmokers tested positive for cotinine (>25 ng/mL). However, 11% of twins who called themselves former smokers had a level consistent with current smoking, therefore we lacked confidence in self-reports of former smokers. We thus excluded all pairs that included a former smoker and limited the analysis to current smokers and nonsmokers (90 individual monozygotic twins from 45 twin pairs).

For some analyses, current cigarette consumption was collapsed into three major classifications: nonsmoker (less than 100 lifetime cigarettes), light smoker (currently <20 cigarettes per day), and heavy smoker (currently 20 or more cigarettes per day).

All analyses were performed with the Statistical Analysis System (SAS) version 8.1 (SAS Institute Inc, Cary, NC). Since the distributions of Th2 cytokine and IgE measures were skewed, transformations were applied to compute geometric means (logarithm, natural logarithm; 8th root transformation; 4th root transformation for IgE, IL-4, IL-5, and IL-13, respectively). We employed analysis of covariance (ANCOVA; GLM procedure in SAS), including terms for gender and age (as a continuous variable) in the model. We also included a term for twin pair in the model (i.e. considering each pair as a separate stratum) to account for genetic factors and nonindependence of the measurements (13). To test for linear trend, cigarette consumption level was considered as a continuous variable.

Because these Th2 cytokines are reciprocally inhibited by INF- γ , we examined levels of this Th1 cytokine as well. Since a large proportion of the subjects had levels falling below the detectable sensitivity of the test (<3 pg/mL), we chose to examine INF- γ levels dichotomously as undetectable vs detectable. We used logistic regression, conditioning on twin pair, to estimate odds ratios and 95% confidence limits.

To determine the baseline effects of gender and age on outcome measures, values were compared among nonsmokers using Spearman rank correlation coefficients.

To assess correlations between immune measures, one twin was randomly selected from each pair and Spearman rank correlation coefficients and *p* values were calculated. Since the INF- γ levels were not assessed as continuous outcomes, they were not included in the correlation between immune measures.

All hypothesis testing assumed a 0.05 significance level and 2-sided alternative hypotheses.

Table I. Demographic Characteristics of 90 individual Monozygotic California Twins (Both Members of 45 Twin Pairs)

	N (%)
Gender	
Male	26 (29%)
Female	64 (71%)
Age	
39–44	30 (33%)
45–53	32 (36%)
54–69	28 (31%)
Smoking Status (Cigarettes/Day)	
Never smoker	26 (29%)
<20	35 (39%)
20+	29 (32%)

RESULTS

Demographic characteristics of individual twins are shown in Table I. Over twice as many female as male twins participated, but individuals were evenly distributed across age and smoking status groups. Among nonsmokers, there was no association between gender and either cytokine or IgE levels (data not shown), but age, modeled as a continuous variable, was correlated with IL-4 ($r = 0.67$, $p < 0.01$), IL-5 ($r = 0.36$, $p < 0.01$) and IL-13 ($r = 0.22$, $p = 0.03$) levels, but not with INF- γ level ($r = 0.14$, $p = 0.50$) or IgE level ($r = -0.11$, $p = 0.92$).

Differences in mean cytokine and IgE levels by current cigarette consumption level, adjusted for the variance attributable to gender, age, and twin pair, are shown in Table II. The mean IL-13 level was 146% higher among heavy smokers than among nonsmokers ($p = 0.04$). The mean IL-13 level in light current smokers was also higher than that observed in nonsmokers, but the difference was not statistically significant. Little difference in IL-13 levels was observed between heavy and light current smokers.

The mean IL-5 level of heavy current smokers was 166% higher than that of light current smokers ($p = 0.03$). IL-5 levels were also higher in heavy smokers compared to nonsmokers, but the difference was not statistically significant. No significant differences in IL-4 or total serum IgE levels by cigarette consumption level were observed.

A significant dose-response relationship between the three levels of current cigarette use and IL-13 secretion was observed (ANCOVA test for linear trend $p = 0.05$), with the heavy current smokers having the highest IL-13 level. Heavy current smokers had the highest, and the nonsmokers had the lowest, levels of IL-4 and IL-5, but the test of trend was not significant for either cytokine ($p = 0.06$ and 0.08 for IL-4 and IL-5, respectively). IgE serum levels did not show a dose-response effect ($p = 0.56$).

Table II. Differences in the Geometric Mean^a Th2 Cytokine (pg/mL) and IgE levels (ng/mL) of Lifetime Nonsmokers, Light Smokers (Less Than 20 Cigarettes Per Day) and Heavy Smokers (20 or More Cigarettes Per Day); ($n = 90$ Individuals, Both Members of 45 Monozygotic Twin Pairs.)

Smoking level comparisons	Difference in means ^a (pg/mL)	Percent difference	P-value ^b
IL-4^c			
Light smokers-Nonsmokers	+0.1	13%	0.78
Heavy smokers-Nonsmokers	+0.6	122%	0.08
Heavy Smokers-Light smokers	+0.5	96%	0.11
IL-5^d			
Light smokers-Nonsmokers	-0.6	-21%	0.57
Heavy smokers ^e -Nonsmokers	+2.9	110%	0.11
Heavy Smokers ^e -Light smokers	+3.4	166%	0.03
IL-13^e			
Light smokers-Nonsmokers	+16.8	94%	0.08
Heavy smokers-Nonsmokers	+26.2	146%	0.04
Heavy Smokers-Light smokers	+9.3	27%	0.52
IgE^f			
Light smokers-Nonsmokers	+29.0	49%	0.24
Heavy smokers-Nonsmokers	+15.8	27%	0.56
Heavy Smokers-Light smokers	-13.2	-15%	0.68

^aGeometric mean difference from transformed cytokine levels, adjusted for twin pair, gender, and age.

^bP-value obtained using ANCOVA (SAS GLM procedure) including terms for twin pair, gender, and age included in the model.

^cIL-4 ($n = 26$ nonsmokers, 35 light smokers, and 29 heavy smokers).

^dIL-5 ($n = 26$ nonsmokers, 33 light smokers, and 29 heavy smokers).

^eIL-13 ($n = 26$ nonsmokers, 35 light smokers, and 29 heavy smokers).

^fIgE ($n = 24$ nonsmokers, 29 light smokers, and 25 heavy smokers).

Twins who smoked more were less likely to have a detectable INF- γ level compared to their identical twin who smoked less (Table III), suggesting that INF- γ levels are inversely correlated with smoking level (although the effect was not statistically significant).

IL-4, IL-5, and IL-13 supernatant levels were significantly correlated with each other, with correlation coefficients ranging from 0.4 to 0.6 (Table IV), but none were correlated with serum IgE levels.

Table III. Odds Ratio (OR) and 95% Confidence Interval (CI) for Risk of Detectable vs Undetectable Serum INF- γ Level (pg/mL) by Smoking Level in 90 Monozygotic Twins (45 Pairs)

	Serum INF- γ Level (pg/mL)			
	Undetectable	Detectable ^a	Odds Ratio ^b	95% CI ^b
Smoke level				
Never	10	14	1.00	—
0–19 cigs/day	18	11	0.84	0.27–2.56
20+ cigs/day	18	7	0.54	0.15–1.96
Trend				$p = 0.36$
Total	46	32		

^aDetectable INF- $\gamma \geq 3.0$ pg/mL.

^bOdds ratio, 95% confidence interval obtained using conditional logistic regression, with subjects matched by twin pair.

Table IV. Intra-Individual Correlation of Th2 Cytokine and IgE Levels in Randomly Selected Twins (One from Each Twin Pair, $n = 45$ Individuals)

	IL-5		IL-13		IgE	
	r^a	p	r^a	p	r^a	p
IL-4	0.60	<.0001	0.61	<.0001	-0.06	0.70
IL-5	—	—	0.40	0.0064	-0.09	0.57
IL-13	—	—	—	—	-0.07	0.67

^aSpearman rank correlation coefficient.

DISCUSSION

Among genetically identical twins, smoking appears to be associated with a small increase in production of IL-13, a Th2 cytokine with a central role in the etiology and pathogenesis of asthma and allergy. The difference is not compatible with a chance effect, and we believe it is biologically meaningful because the twins are matched on factors, which might otherwise introduce "noise" and artificially inflate differences between groups. Further, when compared directly to each other using a Wilcoxon signed rank test for pairs, the IL-13 level was significantly higher in the smoker than in his or her nonsmoking twin ($p = 0.02$).

We also observed higher mean IL-5 levels in heavy smokers compared to nonsmokers and light smokers (although only the comparison with light smokers was statistically significant), but no dose-response effect by level of smoking.

Previous studies investigating smoking and cytokines have reported contradictory results. Byron *et al.* found increased mean levels of IL-4 among smokers compared to nonsmokers, and increased mean IgE levels in heavy compared to light smokers (8), but no difference in INF- γ between the groups. Noakes *et al.* recently demonstrated that infants born to mothers who smoke had higher IL-13 and IL-5 levels (in cord blood mononuclear cell supernatant) compared to infants born to nonsmoking mothers (9). However, another group found lower Th2 (IL-4, IL-5, and IL-6) and Th1 (INF- γ) cytokine levels among pregnant women who smoked compared to pregnant nonsmokers (14).

Hagiwara demonstrated a deficit of Th1 secreting cells (cells secreting INF- γ and IL-2) in the airways of smokers compared to nonsmokers (15). Fischer and colleagues found that nicotine and cotinine applied directly to PBMC cultures stimulated increased secretion of IL-4 but not IgE (16). In contrast, Le Cam *et al.* reported that nicotine had no effect on IL-4 or INF- γ production on PBMC cell cultures from nonallergic and allergic subjects (17). Some of these discrepancies may be due to the use of disparate

populations and a failure to account for cytokine variation by genotype.

Genetic control of secretion has been demonstrated for IL-13 (18), IL-4, and IgE (19). We have examined the effects of smoking on cytokines while adjusting for the age, gender, and genotype by using identical twins, thereby eliminating the confounding effect of these factors and reducing the effect of early life exposure.

Some have reported an increase in total IgE level in smokers compared to nonsmokers (20, 21). Given the increased Th2 cytokine levels observed in smokers in this study, we might have expected a concomitant increase in IgE levels. However, heritable factors may be more important than environmental factors in determining IgE levels. Smoking did not account for the variation in IgE levels among randomly sampled people from 20 to 44 years old (22) and total IgE levels remained stable over a 5-year period in spite of changes in smoking habits (23). Previous twin studies report that 80% of the variance in IgE levels in neonates (24) and 60% of that in adult twins (25) is explained by inheritance. Further, IgE within MZ twins reared apart and those reared together have been observed to be very similar (26). All of these studies suggest a substantial genetic influence on IgE levels.

The most consistent finding in this study is the association between IL-13 and smoking, supporting the findings of Byron *et al.* (8) and Noakes *et al.* (9). The action of IL-13 has yet to be fully elucidated but that cytokine seems to have the potential to affect allergic inflammation at multiple levels. Like IL-4, IL-13 can regulate μ to ϵ isotype class switching, although not as potently (27). IL-13 suppresses Th1 cytokine production in monocytes, particularly INF- γ and IL-12, and inhibits the production of NO by macrophages which ultimately downregulates their effector function. These suppressor actions may be important in the early allergic response, blocking inhibition by INF- γ , which in turn blocks the Th2 response. IL-13 can induce adhesion molecules; regulate recruitment, homing, and activation of inflammatory cells; induce subepithelial fibrosis and mucus hypersecretion; and may even induce airway hyperresponsiveness by directly inducing alterations in smooth muscle function, all actions that promote an allergic response independent of IgE (27).

The mechanism by which tobacco smoke alters cytokine production to increase IL-13 and other cytokines is as yet unknown. Inhalation of environmental tobacco smoke has been shown to enhance secondary responses (28) and allergic sensitization (29) in mouse models. Other pollutants such as diesel exhaust that share many of the same characteristics and chemicals as tobacco smoke have been shown to skew the immune system to a Th2 profile in both human and animal *in vivo* and *in vitro* models. In

those models, diesel exhaust seems to act by inducing oxidative stress and promoting inflammation (30). GM-CSF may be a key player; in humans, eosinophils and possibly other cells produce functional IL-13 in response to GM-CSF (31).

In murine models, IL-13 is a both necessary and a sufficient determinant of allergic asthma (28). Individuals with asthma (32) have significantly higher IL-13 levels than healthy controls, and smokers have an increased risk of asthma, possibly via unregulated IL-13 levels. More importantly, there are public health consequences if secondhand smoke exposure can mimic mainstream smoke and enhance IL-13 proliferation. Increased production of IL-13 by *in vitro* stimulated T cells is a marker of newborns at high risk for subsequent development of atopic disease (33).

It is possible that the true explanation for our observation is a decrease in INF- γ production rather than an increase in Th2 cytokines, since INF- γ and these Th2 cytokines are mutually inhibitory. Although decreased levels of INF- γ have been found in pregnant smokers compared to pregnant nonsmokers (14), others have found no difference in INF- γ levels between smokers and nonsmokers (8) or in those exposed or unexposed to secondhand smoke (9). Our evidence suggested that smokers had lower INF- γ levels compared to their nonsmoking twins, however the relationship was weaker (i.e. not statistically significant) than that observed for IL-13. Genetic regulation of INF- γ protein levels has not been well-delineated (34), and INF- γ polymorphisms have not generally been associated with increased risk of asthma and allergy (34), whereas IL-13 cytokine polymorphisms have been strongly linked (35). It is thus much more likely that the action of tobacco smoke is related to IL-13 than INF- γ .

We found higher IL-5 levels in heavy smokers compared to both nonsmokers and light smokers, but unlike IL-13, we found no dose-response effect. This pattern suggests a different threshold effect of tobacco smoke on IL-5 levels such that only heavy smoking produces an increase.

IL-5 has rarely been evaluated in the context of smoking, and there is even less information on a dose-response effect. Although one study reported that IL-5 levels in neonates of smoking mothers were positively correlated with numbers of cigarettes their mothers smoked, the same study showed no statistically significant difference in IL-5 levels when smoking mothers were grouped together (9), supporting some type of threshold effect. This is in contrast to the pattern observed for IL-13, which, in our study, was higher in both light and heavy smokers compared to nonsmokers, in a dose-response manner. Studies are underway to elucidate the exact mechanisms responsible for

the difference in IL-5 and IL-13 cytokine responses to tobacco smoke.

CONCLUSIONS

In summary, when genotype, age, and gender are accounted for, smoking appears to be associated with increased capacity to secrete IL-13, and possibly IL-5. This study supports earlier observations (8, 9) and suggests that tobacco smoke may exacerbate or even lead to asthma through "priming" of immune cells toward a Th2 phenotype, possibly through an IgE independent pathway.

ACKNOWLEDGMENTS

This study was conducted with support from the Tobacco-Related Disease Research Program, grant numbers 7RT-0134H, 8RT-0107H, and 6RT-0354H. The authors wish to thank Susan Gundell, RN, Nicole J. Stroud, Shabneet Brar, MD, Maya Mahue, PhD, Joseph House, Alicia M. Nelson, and all of the participating twins.

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