ORIGINAL ARTICLE EXPERIMENTAL ALLERGY AND IMMUNOLOGY

Dynamics in cytokine responses during the development of occupational sensitization to rats

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To cite this article: Krop EJM, van de Pol MA, Lutter R, Heederik DJJ, Aalberse RC, van der Zee JS. Dynamics in cytokine responses during the development of occupational sensitization to rats. *Allergy* 2010; **65**: 1227–1233.

Keywords
IgE; IL-4; laboratory animal allergy; occupational allergy; Th2 responses.

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Accepted for publication 15 December 2009

DOI:10.1111/j.1398-9995.2009.02323.x

Edited by: Hans-Uwe Simon

Abstract

Background: Occupational allergy forms an attractive model to study the development of allergic responses, as in some occupations it has a high incidence and develops quickly. In a cohort of starting laboratory animal workers, we previously found 20% sensitization to animal allergens within 2 years.

Methods: We compared cellular responses of incident laboratory animal workers who developed rat-specific sensitization (cases, $n = 18$) during 2 years of follow-up to control animal workers matched for atopic status but without sensitization after follow-up (controls, $n = 18$). Practically, this is a case–control study, nested within the cohort. Rat-specific IgE antibodies were measured in sera, and allergen-specific and nonspecific cytokine responses were measured in whole blood and in isolated peripheral blood mononuclear cells.

Results: Self-reported allergic symptoms were related to the presence of rat-specific IgE ($P \leq 0.01$). Cases developed a rat allergen–specific interleukin (IL)-4 response during sensitization, while controls did not show an increased IL-4 response (at visit D: 33 *vs* 5 IL-4 producing cells/10^6 cells, $P < 0.001$). The IL-4 response was related to the levels of rat-specific IgE in cases (visit D: rho = 0.706, $P < 0.001$). By contrast, allergen-specific IL-10 and interferon γ (IFNγ) responses as well as nonspecific cytokine responses were comparable between cases and controls.

Conclusion: This study is the first to show the development of an allergen-specific IL-4 response in adult human subjects during allergen-specific sensitization. This IL-4 response was quantitatively associated with the development of the specific IgE antibodies. Allergen-specific or nonspecific IL-10 and IFNγ responses showed no protective effect on the development of allergic sensitization.

It is generally accepted that a shift in the balance of T-helper (Th) cell populations toward a polarized Th2 subset is occurring in atopic disease. The Th2-derived cytokine interleukin (IL)-4 is increased in cell cultures of patients with established atopic disease (1–4) and triggers B cells to produce allergen-specific Immunoglobulin (Ig) E (5). IL-12 is suggested to be a crucial factor for Th1 cell polarization (6). The Th1 cell-derived cytokine interferon γ (IFNγ) was shown to reverse Th2 skewing *in vitro* (7), and it is decreased in atopics compared to controls (4, 8). IL-10, produced by monocytes as well as regulatory T cells, was also shown to be decreased in atopics (1, 8).

Allergic sensitization to environmental allergens most often occurs at young age making it difficult to study cellular immunologic events longitudinally. Cross-sectional and longitudinal studies of birth cohorts have provided insight into the significance of cytokine responses of allergic disease (1, 3, 4, 8). However, these results are often conflicting and differ with age and most studies had cross-sectional designs and focused on the difference between allergic individuals and controls. Therefore, changes in cellular responses and accompanying cytokine levels during the development of allergic sensitization remain largely unknown.

Laboratory animal allergy provides an attractive model to investigate changes in cytokine levels during the development of sensitization in a longitudinal design. It can be found in up to 30% of exposed workers (9–12). Occupational allergy appears to be very similar to sensitization to common environmental allergens with regard to symptoms and immunologic mechanisms and atopy is the major risk factor (13). The majority of sensitized workers develop sensitization within the first 2–3 years of exposure (10, 11).

We conducted a prospective cohort study on starting laboratory animal workers in the Netherlands (14). During a two-year follow-up, 18 laboratory animal workers developed sensitization to rats. The aim of the present study was to investigate in a nested case–control setting whether sensitization to rats was preceded or accompanied by changes in the levels of cytokines *in vitro*.

Materials and Methods

Study design

Our study population consisted of 36 selected apprentice laboratory animal workers from a previous described cohort of apprentice laboratory animal workers (14). In short, the cohort was followed for 2 years and development of animal-specific allergic sensitization was monitored. During the study, participants were seen four times: at the start of the study (visit A), after 4 months (visit B), 1 year (visit C) and 2 years (visit D). Blood was taken at each visit and participants completed a questionnaire based upon a previously described questionnaire on occupational allergy containing detailed questions concerning allergic symptoms, animal contact, and smoking (15).

In the present study nested within the cohort, we compared 18 workers who developed rat-specific sensitization during the 2-year follow-up (cases) to 18 workers (controls) matched by atopic status but without sensitization after the 2 years of follow-up. Development of rat-specific sensitization was determined by skin prick testing with rat allergens and development of rat-specific IgE as detected in serum. Cases developed a positive skin prick test, a positive IgE test or both. Cases and controls were matched for atopic status determined by the presence of specific IgE antibodies against common allergens.

Testing for atopy and sensitization

Rat urinary allergens, positive control (histamine 10 mg/ml) and negative control (ALK Abello, Nieuwegein, the Netherlands) were used for skin prick testing at visits A, C and D. Rat-specific tests were performed with previous described urine extracts (16) dissolved in negative control solutions. Skin prick tests were read after 15 min and were considered positive if the wheal diameter was at least 3 mm and redness was present. Additionally, the negative control had to have no erythema and a wheal diameter of maximal 1 mm.

Atopy was defined as having at least one positive IgE test for common allergens. Specific IgE antibodies against common allergens including house dust mite, grass pollen, tree pollen, cat dander, dog dander, guinea pig dander, rabbit dander and horse dander and the occupational allergen rat urine were determined by radioallergosorbent testing as previously described (17). For quantifying rat urine–specific IgE, the same urine extract was used as for skin prick testing. Results were expressed in International Units IgE per milliliter (IU/ml) (18). The detection limit was 0.15 IU/ml and levels of specific IgE above 0.35 IU/ml were considered positive.

Exposure to rat allergens

Exposure was assessed in the animal facilities and a personalized exposure to rat allergens was estimated as previously described (14). Cumulative exposure was obtained by multiplying the personalized exposure by the time exposed as reported in the questionnaires (12).

Whole blood culture (WBC)

Heparin-containing blood was diluted 10 times in Iscove's modified Dulbecco's medium (IMDM; BioWhittaker, Verviers, Belgium) supplemented with 0.1% FCS and 30 U/ml heparin (Leo Pharmaceutical products B.V., Weesp, the Netherlands). Blood was stimulated with *Staphylococcus aureus* Cowan I strain (SAC, 75 μg/ml; Calbiochem, Darmstadt, Germany) or lipopolysaccharide (LPS, 1 μg/ml; Sigma-Aldrich, St. Louis, MO, USA) both in the absence and in the presence of recombinant IFNγ (100 U/ml; U-Cytech, Utrecht, the Netherlands) at 37°C. Supernatant was harvested after 24 h and was stored at −20°C until analysis.

Levels of IL-4, IL6, IL-10, IL-12 and IFNγ in supernatant were determined with a Bioplex assay (Bio-Rad Laboratories, Hercules, CA, USA). The assay was performed according to manufacturer's protocol. IL-12 was measured in WBC with IFNγ, other cytokines were measured in the cultures without IFNγ.

Enzyme linked immunospot assay (ELISpot)

Cytokine production after allergen stimulation of peripheral blood mononuclear cells (PBMCs) with rat allergen was studied with ELISpot technique. PBMCs were isolated from heparin-containing blood samples using standard density gradient centrifugation techniques and subsequently cryopreserved.

For the assay, PBMCs were thawed, washed twice and diluted to a concentration of $4*10^5$ cells/ml in tissue culture medium (RPMI 1640; Gibco BRL, Life Technologies Ltd, Paisley, UK) in the absence or presence of rat urinary proteins (same material as used in skin prick testing and IgE assay, 42 μg/ml) or phytohaemagglutinin (PHA, 0.1 μg/ml, positive control) in round bottom tubes (Micronic, McMurray, PA, USA). Cells were preincubated at 37°C for 4 h. Multiscreen filter plates (Millipore, Billerica, MA, USA) were coated with IL-4-, IL-10- or IFNγ-specific antibodies (Mabtech, Nacka Strand, Sweden) as described in the manufacturer's protocol. Preincubated cells were subsequently

incubated on the coated microplates (IL-4: $2*10^5$ cells/well; IL-10: $5*10^4$ cells/well; IFNγ: $2*10^4$ cells/well) for 72 h. Plates were washed and cytokine production by PBMC was quantified by incubating the plates with biotinylated secondary antibodies according to the manufacturers protocol. Spots were counted using ELISPOT analysis software (A.EL.VIS GmbH, Hannover, Germany). All results were expressed as spots per 10^6 cells.

Statistical analysis

SPSS version 11.5 Statistics UK (Chicago, IL, USA) was used for statistical analysis. Antibody levels and cytokine levels from WBC were evaluated in terms of their log values. Values below the detection limit were allotted the value of half the detection limit. Differences between the cases and controls were tested with Student's *t*-test, ANOVA, chi-square or Mann–Whitney test depending on distribution. Parametric correlations between variables were expressed as Spearman correlation coefficients (rho). Relations between symptoms and other variables were determined by linear and multiple logistic regression. All reported *P*-values were two-tailed, and *P*-values of <0.05 were considered statistically significant.

Results

Study population

Characteristics of the cases and controls are in Table 1. No significant differences were found between cases and controls but controls tended to have worked for a longer time with rats while the cases tended to have higher exposure to rat allergens. One case was lost to follow up after he developed a rat-specific IgE response at visit B. One control did not donated blood at visit D and one case did not donate blood at visit B. Rat-specific skin prick test results increased in cases from 0 mm at visit A to 7 mm (range: 0–23 mm) at visit D ($P < 0.001$) and was significantly different from controls at visit C and D (both $P < 0.001$). Rat-specific IgE was <0.35 IU/ml for all participants at the start of the study. It increased over time in cases and was significantly higher in cases at visit B, C and D ($P > 0.01$). Results from rat-specific skin prick test and IgE measurements in serum correlated highly in the cases (visit C: rho = 0.688, $P = 0.002$; visit D: rho = 0.872, $P < 0.001$, Fig. 1). Twelve cases developed rat-specific IgE levels above 0.35 IU/ml.

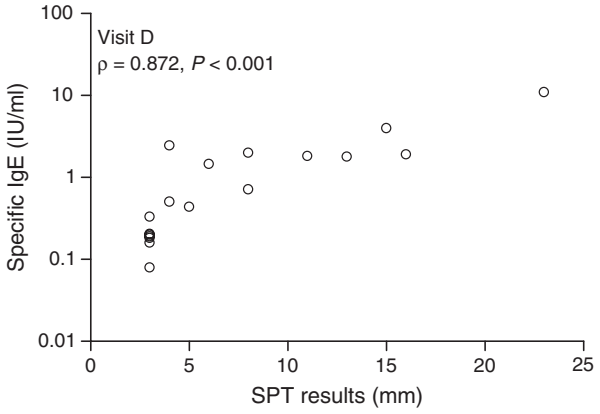

Figure 1 Correlation between rat-specific skin prick test (SPT) results and rat-specific IgE levels in cases at visit D.

Whole blood culture

Unstimulated WBC

During the first year of follow-up (visit B and C), IL-12 remained significantly lower for cases compared to controls (34 pg/ml *vs* 17 pg/ml at visit B, $P = 0.006$). After 2 years of follow-up, none of the cytokines measured showed significant differences between cases and controls in the unstimulated culture. We did find a decline in IL-10 between the last visit before development of sensitization and the first visit after sensitization in cases but this did not reach statistical significance (335–212 pg/ml, $P = 0.059$).

SAC- and LPS-stimulated WBC

Results from the stimulated WBC for all cytokines are in Table 2. In stimulations with SAC, IL-4 increased slightly but significantly in controls during follow-up (from 132 to 161 pg/ml, $P = 0.011$) and was higher in controls compared to cases at visit B, C and D ($P = 0.02$, $P = 0.03$, $P = 0.07$, respectively). None of the other cytokines showed ongoing

Table 1 Characteristics of the nested case–control study

	Controls ($n = 18$)	Cases ($n = 18$)	*P*
Male/female	8/10	8/10	1.000
Age (mean, years)	26	24	0.475
Atopy	15 (83%)	15 (83%)	1.000
Total IgE (IU/ml, GM, range)	59 (9–398)	103 (3–1288)	0.175
Exposure to rats in previous job (months, mean, range)	3.9 (0–24)	3.3 (0–15)	0.767
Exposure to rats in current job at visit A (months, mean, range)	7.3 (0–18)	4.1 (0–10)	0.074
Mean exposure to rat allergens/month (ng eq/m^3*hours/month, GM, range)	101 (1–11220)	526 (1–8913)	0.059
Cumulative rat exposure during follow-up (ng eq/m^3*hours, GM, range)	2914 (35–251189)	7530 (26–162181)	0.238

GM, Geometric mean; Ng eq/m^3, nanogram of equivalent animal urinary proteins per cubic meter.

Table 2 Cytokine responses in the whole blood culture (WBC) stimulated with *Staphylococcus aureus* Cowan I strain (SAC) or lipopolysaccharide (LPS)

Visit		A	B	C	D
SAC-stimulated WBC					
IL-4	Cases	131	125	125	118
	Controls	135	**165**	**167**	155
IL-6	Cases	23099	13095	22408	14441
	Controls	25055	**49454**	22331	33497
IL-10	Cases	246	256	248	251
	Controls	266	305	**313**	292
IL-12*	Cases	133	133	127	136
	Controls	191	80	161	171
IFNγ	Cases	2130	1960	2222	1499
	Controls	2794	2802	2163	1936
LPS-stimulated WBC					
IL-4	Cases	121	128	141	117
	Controls	141	135	157	135
IL-6	Cases	44249	53199	60298	46817
	Controls	45530	27650	49260	27714
IL-10	Cases	752	795	872	726
	Controls	544	550	598	606
IL-12*	Cases	328	243	154	234
	Controls	252	160	277	501
IFNγ	Cases	1499	1433	1344	1310
	Controls	2346	1561	1774	1579

Results are expressed in pg/ml as geometric means. Bold results are significantly higher ($P < 0.05$). IFNγ, interferon γ; IL, interleukin. *Cultured in the presence of IFNγ.

significant differences in production between cases and controls during follow-up.

Mean IL-10 levels after SAC stimulation during follow-up were negatively related to rat allergen–specific IgE production at the last visit ($r = -0.449$, $P = 0.006$) and to mean skin prick test (SPT) results during follow-up ($r = -0.357$, $P = 0.032$).

For LPS-stimulated WBC, we found no significant differences in cytokine production between cases and controls at any time point nor did we find changes in cytokine production over time.

Rat-specific stimulation of isolated mononuclear cells

To determine rat allergen–specific cytokine production by PBMC, ELISpots were performed. For IL-4, no significant differences were found in the number of IL-4 producing cells (spots) in the unstimulated cultures between cases and controls. For PHA stimulation (positive control), we found that cases had significantly more spots than controls only at visit C (1155 *vs* 835 spots/10^6 cells, $P = 0.02$). At the start of the study, there was no difference in rat-specific IL-4 spots between cases and controls, but during follow–up, the number of rat-specific IL-4 spots increased in cases and was significantly different from controls (B: $P = 0.03$; C: $P = 0.02$; D: $P < 0.001$, Fig. 2A). There was a significant increase in

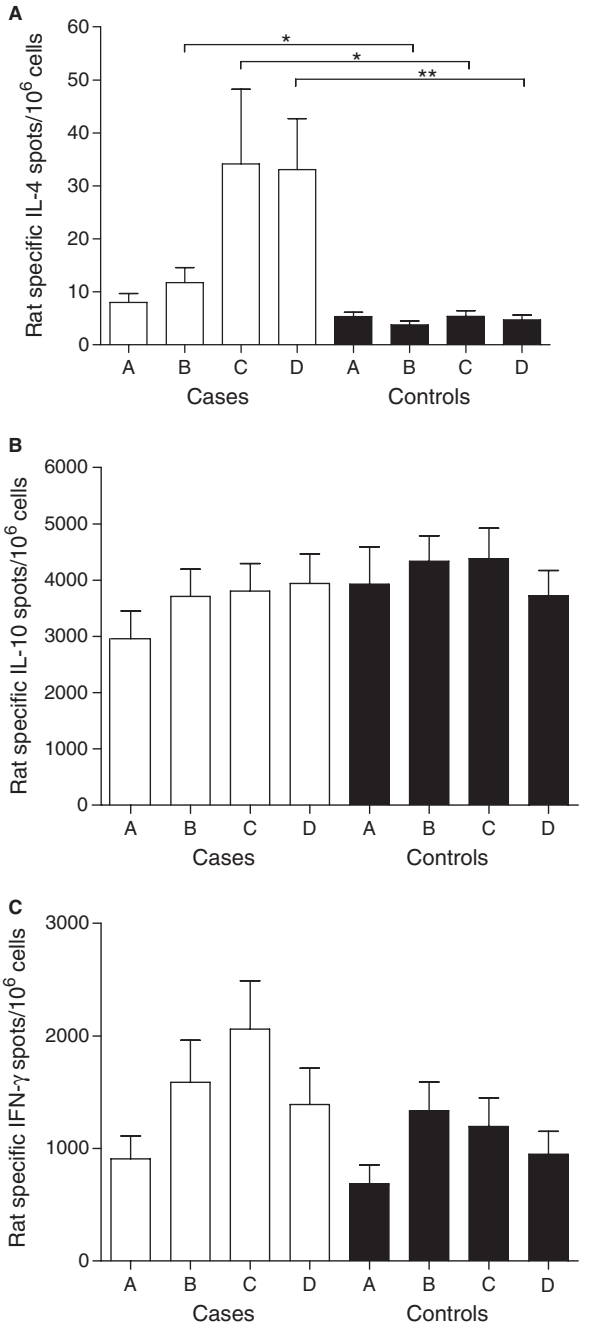

Figure 2 IL-4-, IL-10- and IFNγ-specific spots in ELISpot of cases and controls after stimulation with rat allergen. Visits are on the *x*-axes. (A) IL-4 spots in cases significantly increase during follow-up. At the first visit, there is no significant difference between cases and controls but after follow-up the difference in number of spots increases (*$P < 0.05$, **$P < 0.001$). (B) IL-10-specific spots after stimulation with rat allergen show no significant differences between cases and controls. IL-10 spots declined in controls between visits C and D ($P = 0.023$). (C) IFNγ-specific spots after stimulation with rat allergen are not significantly different between cases and controls. At the beginning of follow-up, there is a significant increase in IFNγ spots followed by a reduction in both cases and controls.

rat-specific IL-4 spots between the last time point before and the first time point after sensitization became apparent in cases (10 *vs* 35 spots/10^6 cells, $P = 0.03$). The number of rat-specific IL-4 spots correlated with the amount of rat-specific IgE of cases at visit B (rho: 0.480, $P = 0.004$), C (rho: 0.429, $P = 0.01$, Fig. 3) and D (rho: 0.706, $P < 0.001$, Fig. 3), but not at the start of the study or in controls.

No significant differences between cases and controls were found in IL-10 spots, neither in the unstimulated culture, nor after PHA stimulation or after rat-specific stimulation (Fig. 2B). No significant changes in IL-10 were found during follow-up in cases, but controls showed a small but significant decline in rat-specific IL-10 spots from visit C to D ($P = 0.02$, Fig. 2B). If results from the last visit before sensitization and the first with a positive allergy test were considered, we found a significant decrease in IL-10 spots in the unstimulated culture only ($P = 0.03$). IL-10 spots in unstimulated cultures, in PHA cultures or in rat-specific cultures showed no relationships with rat-specific IgE.

For IFNγ spots, no differences were found between cases and controls in the unstimulated culture or after stimulation with PHA or rat allergen. There was a significant increase in rat-specific IFNγ spots in cases (A–C: $P = 0.001$) and in controls (A–C: $P = 0.03$) in the first year of follow-up (Fig. 2C). After the first year of follow-up, the rat-specific IFNγ spots declined (cases: C–D: $P = 0.006$; controls: C–D: $P = 0.08$, Fig. 2C). No relationships were found between IFNγ spots and rat-specific IgE.

No relations between ELISpot results and cytokine responses in WBC were found.

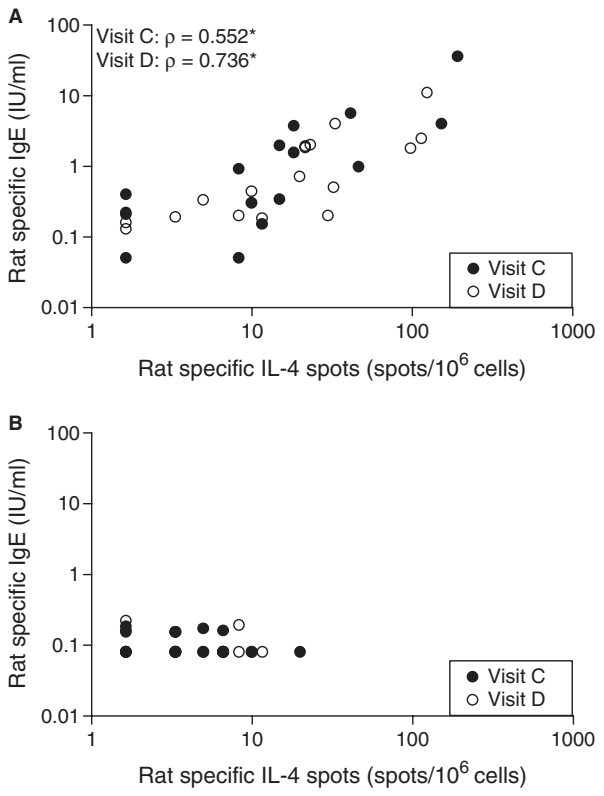

Figure 3 Relation between rat-specific IgE and rat-specific IL-4 spots in ELISpot in cases (A) and controls (B). There is a correlation between rat-specific IgE and rat-specific IL-4 spots at visit C (●) and at visit D (○) in cases but not in controls. *$P \leq 0.001$.

Self-reported allergic symptoms

Allergic symptoms were mainly reported by cases (Table 3). Reporting work-related allergic symptoms was related to SPT results at visit C ($P = 0.01$) and visit D ($P < 0.001$). Symptoms were also related to the level of IgE at visits B ($P = 0.002$), C ($P = 0.002$) and D ($P < 0.001$) but not to WBC results. At visit C and D, the number of IL-4 spots in the rat-specific ELISpot were related to symptoms ($P = 0.02$ and $P < 0.001$, respectively). In a multiple logistic regression model, specific IgE related to symptoms at both time points, but rat-specific IL-4 spots or SPT results did not contribute independently to the model.

Discussion

This cohort study on the development of occupational allergy provided a unique opportunity to study cellular responses during the development of allergic sensitization in a nested case–control setting. We showed the development of a rat allergen–specific IL-4 response that was quantitatively related to the development of rat-specific IgE antibodies. By contrast, allergen-specific IL-10 and IFNγ responses and nonspecific cytokine responses were similar for sensitized animal workers and nonsensitized workers.

In this nested case–control study, we matched controls by atopic status, the best known risk factor for the development

Table 3 Self-reported work-related allergic symptoms

Visit	Symptoms	Cases ($n = 18$)	Controls ($n = 18$)	P
A	Skin	0	0	ns
	Eyes	0	0	ns
	Nose	0	3	ns
	Asthma	0	0	ns
	Any	0	3	ns
B	Skin	2	0	ns
	Eyes	4	0	0.029
	Nose	5	0	0.013
	Asthma	2	0	ns
	Any	6	0	0.006
C	Skin	3	0	ns
	Eyes	7	0	0.002
	Nose	9	3	0.024
	Asthma	1	0	ns
	Any	11	3	0.004
D	Skin	4	0	0.033
	Eyes	5	1	ns
	Nose	6	2	ns
	Asthma	2	0	ns
	Any	9	2	0.010

of laboratory animal allergy (9–12, 14). Because of the matching of atopic status and the low percentage of nonatopics (17%) in this study, differences between atopics and nonatopics during sensitization could not be studied. The study focuses, therefore, on differences between atopics that became sensitized and nonsensitized atopics.

Because of the design of the study with only four sampling time points during 2 years of follow-up, we may have missed short-term changes in cytokine responses or antibody levels. We matched cases with negative controls at the end of follow-up. This maximized the contrast between cases and controls. However, it cannot be ruled out that prolonged follow-up would have resulted in the development of sensitization in some controls. This may have resulted in underestimation of differences between lifelong negative controls and sensitized individuals.

We found a negative correlation between SAC-induced IL-10 production and development of IgE. This cytokine produced by monocyte, regulatory T cells (19) and possibly by a subset of memory B cells (20) was suggested to interfere in production of IgE. However, we did not find a protective effect of rat allergen–induced IL-10. Possibly, the protective effect of IL-10 occurs in an allergen-independent manner. IL-10 also enhances the IgG4 production by B cells (21, 22). IgG4 antibodies may have a protective effect on IgE sensitization or development of allergic symptoms (23–25) However, we found no relation between IgG4 antibodies to rat allergens and IL-10 responses (data not shown). Likewise, there was no relation between symptoms and IL-10 levels in WBC or ELISpot.

Van der Pouw Kraan (6) showed that IL-12 levels in WBC were significantly lower in allergic patients compared to controls. We found no consistent differences in IL-4, IL-6, IL-10, IL-12 or IFNγ levels in WBC between cases and controls. However, matching of controls on atopic status may have reduced the differences reported for atopics and nonatopics.

It was suggested in literature that allergen-specific IL-10 or IFNγ responses could protect against the development of allergic sensitization (1, 4, 8). Our ELISpot results showed no significant difference in allergen-specific or nonspecific IFNγ responses between cases and controls. This suggests that there is no significant role for allergen-specific T-helper 1 cells in the protection against development of sensitization in the population studied. The same was found for IL-10 producing cells.

Our study is the first in which development of an allergen-specific Th2 response in human adults during sensitization was followed. Differences in allergen-specific IL-4 production between allergic patients and nonallergic controls were shown before (2, 26). However, from those studies, it remained unclear when these differences in IL-4 response developed in time. We showed a quantitative relation between the allergen-specific IL-4 response and development of IgE antibodies. This is in line with the assumption that an increased antigen-specific IL-4 response is the main factor responsible for the production of antigen-specific IgE antibodies. It was suggested in literature that IL-4 responses in nonallergic subjects could lead to IgG4 responses instead of IgE responses (22). However, we found no evidence for such a protective mechanism in our study.

In contrast to the rat allergen–induced IL-4 response, SAC-induced IL-4 responses in WBC were higher in controls than in cases. This indicates that the increased response in cases is highly allergen-specific. Self-reported allergic symptoms were related to allergen-specific IgE serum levels and to allergen-specific IL-4 spots. SPT appeared to be more sensitive compared to specific IgE tests in our study. However, IgE results showed the best relation to self-reported work-related allergic symptoms. Prolonged follow-up may have resulted in more IgE, more IL-4 spots and/or more symptoms in the cases that were only SPT after 2 years of follow-up.

A correlation between IL-4 production from WBC and ELISpot was lacking. Likewise, we found no relations between the other cytokines as detected in the ELISpot and in the WBC. In the WBC, we used nonspecific stimuli but cells were stimulated with occupational allergens in the ELISpot. The results from the WBC are therefore a reflection of the overall immune response, while the ELISpot results show the allergen-specific immune reaction. The fact that more cell types are present in whole blood than in isolated PBMC may have contributed to the different cytokine production pattern.

In conclusion, we showed that development of rat allergen–specific IL-4 responses in PBMCs of sensitized rat workers in a nested case–control study of laboratory rat workers was the main change in the cytokine production profile. This IL-4 response was quantitatively related to the development of an allergen-specific IgE response against rat allergens and to occupational allergic symptoms.

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