

ORIGINAL ARTICLE

Can serum cytokine profile discriminate irritant-induced and allergen-induced symptoms? A cross-sectional study in workers mostly exposed to laboratory animals

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ABSTRACT

Background In workers exposed mostly to laboratory animals (LA), symptoms may be due to irritants or allergens. Correct aetiological diagnosis is important for health surveillance.

Objectives This study aims to test whether work-related (WR) allergen-induced symptoms are associated with a cytokine profile distinct from that due to irritants.

Methods In a cross-sectional study (n=114), WR respiratory and/or skin symptoms were assessed through a standardised clinical examination and sensitisation to rat and/or mouse allergen determined by serum immunoglobulin E. Serum cytokine concentrations were measured by multiplex assays. The predefined cytokine profiles 'sensitiser' (interleukin (IL)-4, IL-5, IL-13, eotaxin-1) and 'irritation' (IL-8, IL-17A, IL-17F, IL-22) were considered positive, when ≥ 3 concentrations exceeded the 95th percentile of the asymptomatic non-sensitised group. Results were examined by hierarchical clustering analyses (HCA) and multiple linear regression. Explorative analyses were carried out for nine additional cytokines. Exposure to allergens and endotoxin was assessed in a subpopulation.

Results The prevalence of the profile 'irritation' was comparable in 28 symptomatic non-sensitised workers and 71 asymptomatic non-sensitised workers. HCA showed that nearly all symptomatic non-sensitised workers were gathered in two subclusters, characterised by high IL-17A levels, but different IL-8 levels. Multiple linear regression identified drug consumption and current complaints as confounders. Sensitised subjects were too few (n=14) for testing the profile 'sensitiser'.

Conclusions In this unselected population of LA workers, the profile 'irritation' did not prove to be a valuable health surveillance tool. Low power precluded assessment of the profile 'sensitiser'. The increased IL-17A concentration may originate from irritative constituents of organic dust.

What this paper adds

- Data on the value of serum cytokine determinations in the prevention of laboratory animal allergy are limited.
- Cytokine profiles including several cytokine concentrations reflecting different aspects of a pathophysiological process may help identify the cause of symptoms.
- The usefulness of cytokine profiles for differentiating irritative from allergic respiratory or skin symptoms was examined in a population of laboratory animal workers.
- Results do not support the value of the cytokine profile 'irritation' for prevention in this population and low power precluded assessment of the profile 'sensitiser'.
- The recorded increase in serum concentrations of interleukin 17A may be due to components of organic dust.

urine, blood, saliva, dander or hair) are the cause of sensitisation and immunoglobulin E (IgE)-mediated conjunctivitis, rhinitis, asthma, urticaria and protein contact dermatitis.¹⁻³

In LA workers, a major problem in both health surveillance and tertiary prevention is the distinction between sensitiser-induced occupational asthma and irritant-induced work-related (WR) asthma-like symptoms.⁴ The quantitative importance of the problem is emphasised by a review of longitudinal studies in workers exposed to LA⁵ showing that the incidence of occupational asthma defined by WR chest symptoms was about 2–4 cases/100 person-years, whereas the incidence of allergic asthma defined by symptoms and positive prick tests was much lower (0.5–1.6 cases/100 person-years). Similar findings were reported for nasal symptoms.⁵ Consequences for the patient are quite different: redeployment to an area without sensitiser exposure with the risk of unfavourable socioeconomic consequences or improvement of the workplace to reduce exposure to irritants. Unfortunately, differential diagnosis is tricky because clinical history has a low predictive value,⁴ spirometry is non-specific

INTRODUCTION

Occupational allergy to laboratory animals (LA) is an occupational disease occurring in animal handlers, laboratory technicians and scientists working with LA. High molecular weight allergens from several sources of these animals (eg,



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Workplace

and specific IgE may indicate sensitisation without clinical significance.

Generally, high molecular weight allergens are associated with a T helper 2 (Th2) inflammation typically involving IgE, eosinophils, interleukin (IL)-4, IL-5, IL-13 and eotaxin.^{6–10} However, organic dust is a source of allergens and is made of microbial components capable of triggering a lower airway inflammation through the activation of Toll-like receptors by microbial constituents.^{11–13} The resulting irritant-induced asthma is characterised by sputum neutrophils where IL-8, IL-17A, IL-17F and possibly IL-22 and IL-1 β are likely to play a role.^{14–22} Besides the airway inflammatory reaction, this neutrophil asthma phenotype has been associated with systemic inflammation.²² Consequently, it may be hypothesised that characterising the serum cytokine pattern may point to the cause of the occupational asthma and help take the most appropriate measures in each individual asthmatic worker.

The primary purpose of this study was to test the hypothesis that the serum cytokine pattern from LA workers with symptoms but without specific sensitisation differs from that of symptomatic and sensitised workers and preferentially comprises increased serum concentrations of IL-8, IL-17A, IL-17F or IL-22. Secondary aims were to examine the diagnostic value of nine additional cytokines selected a posteriori and to assess consistency with previous publications.

METHODS

The study was conducted in the framework of an analysis of occupational risks among workers exposed to animals in university units (November 2012–January 2014). It was conducted according to the Declaration of Helsinki and approved by the ethics commission. The study purpose was explained at information meetings, workers received written information and all subjects gave written informed consent. The study consisted of three consecutive parts (figure 1).

Work with exposure to animals other than rodents was assumed to occur in too few subjects for allowing valid conclusions and/or to entail a lower sensitisation risk. Therefore, selection focused on workers exposed to rats and/or mice. As a lung–skin connection in occupational allergy has been suggested,^{23–25} animal bites were also considered in the selection procedure (figure 1).

Workers were categorised in four subgroups according to WR symptoms and specific sensitisation (non-symptomatic and non-sensitised (Sy–/Se–), symptomatic and sensitised (Sy+/Se+), symptomatic and non-sensitised (Sy+/Se–), non-symptomatic and sensitised (Sy–/Se+)). This categorisation was previously considered suitable for studying allergen-induced and irritant-induced WR respiratory and skin symptoms in LA workers.²⁶ According to this classification, symptomatic (Sy+) individuals were defined as those having ever had WR symptoms (subjects having any predefined symptom were considered asymptomatic when the symptom was not WR). The predefined symptoms systematically assessed in the clinical examination were irritation of conjunctiva, nose or throat, itchy, runny or stuffy nose, sneezing, common cold symptoms (anamnesic information pointing to allergic aetiology was additionally recorded), cough, wheeze, asthma attack and skin rash, itchy skin and other skin symptoms. WR symptoms were defined as those brought about by a specific work-task and without non-occupational cause. The occurrence of similar symptoms in coworkers was assessed as well to trace irritant-induced effects. The association between work and symptoms had to be at least probable as assessed by clinical history (possible was not included as WR).

Cases who were both WR and non-WR were considered as WR. Sensitised (Se+) means a specific IgE concentration to rat and/or mouse ≥ 0.35 kU/L.

Clinical and occupational history were recorded through a 60–90 min semistructured interview by trained physicians using a checklist and written instructions. The coding of the answers was reviewed by the study coordinator. The checklist for symptom ascertainment included questions about eye and/or nose or skin symptoms in the past year. Questions about cough, wheezing, asthma attack (past year) and previous history of asthma, eczema and skin allergy were taken from the Swiss Cohort Study on Air Pollution and Lung and Heart Disease (SAPALDIA) questionnaire.²⁷ Questions about previous allergic rhinitis and animal allergy were constructed by replacing the word ‘asthma’ in the SAPALDIA questionnaire with the terms ‘allergic rhinitis’ or ‘allergy to animals’. A history of allergic disease comprises (alone or in combination) allergic rhinitis, asthma, eczema or any other skin allergy and allergy to animals (mostly conjunctivitis). Recent symptoms were any symptom mentioned spontaneously by the patient and exclusively relating to the past 4 weeks. Four categories of drug consumption were considered (continuously taking drugs vs occasionally during the past month and any drug vs those likely to influence cytokine metabolism). Smoking was assessed by using questions proposed by the European Community of Steel and Coal (revision 1967). All diagnoses were made before knowing results of IgE, cytokine and airborne measurements.

Lifelong history of exposure to animals was reconstructed. Source of occupational exposure to animals was classified into five categories: Animal handler (caring for animals, changing and cleaning cages, changing litter, feeding and breeding); technicians (collecting blood/urine, surgery, biopsy, administering active ingredients, euthanasia); researchers (including trainees, students and any person conducting experimental work); various other tasks with exposure to animals (for example, veterinarians); no occupational exposure to animals. Kind of pets in childhood (<18 years) and adulthood was recorded (rat/mouse, rabbit/guinea pig/hamster, dog/cat, cow/horse/sheep/goat/swine, birds and other). As a decreasing effect of exposure to endotoxin with increasing duration of exposure (tolerance to endotoxin) has been described,¹³ the number of days at work since the last day on leave was recorded.

Spirometry was carried out with two Microlab 3500 spirometers (Cardinal Health UK 232, Kent, UK) calibrated daily with a 3 L syringe and every 2 years by the manufacturer’s representative. Forced expiratory volume in 1 s (FEV₁) and forced vital capacity (FVC) were recorded according to American Thoracic Society/European Respiratory Society (ATS/ERS) guidelines²⁸ and results assessed independently by two physicians. Predicted values of FVC and FEV₁ (FVC and FEV₁ per cent predicted) were calculated.²⁹

Measurements of serum IgE and airborne contaminants were carried out blinded in batches of coded samples.

Total IgE and specific IgE against mouse (e88, mouse epithelium, serum proteins and urine proteins) and rat (e87, rat epithelium, serum proteins and urine proteins) were measured by ImmunoCAP (Phadia, Uppsala, Sweden). Categories of total IgE were used to define atopy with cut-offs of <26 kU/L (probability of being non-atopic: 84%) and >100 kU/L (probability of atopy 78%). Concentrations of total IgE <2 kU/L were attributed a value of 1 kU/L.

Exposure assessment is described in detail elsewhere (see online supplementary file). Briefly summarised, exposure to airborne endotoxin and allergens was assessed by personal sampling on two half-days (71 half-day personal samples from a

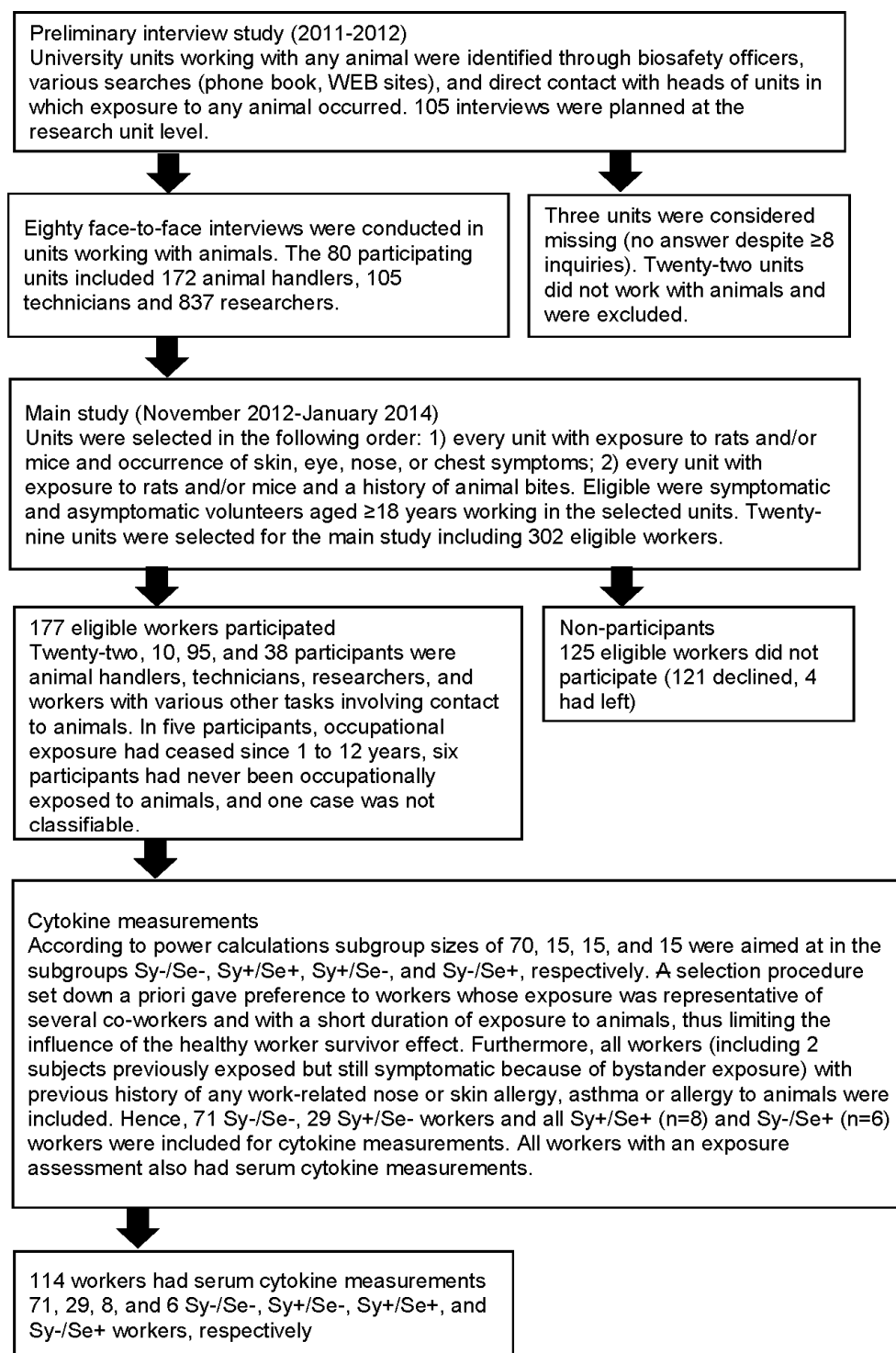


Figure 1 Selection of the population. Sy-/Se-, non-symptomatic and non-sensitised; Sy+/Se+, symptomatic and sensitised; Sy+/Se-, symptomatic and non-sensitised; Sy-/Se+, non-symptomatic and sensitised.

subpopulation of 41 workers). Peak exposure was defined as the highest of the morning and afternoon measurements. A 'cumulative exposure dose'²⁶ to endotoxins (duration of exposure in the current job multiplied by the mean measured daily endotoxin concentration in the current job group) was computed.

Endotoxins were analysed using a quantitative kinetic chromogenic Limulus Ameobocyte Lysate assay (Lonza Group, Visp, Switzerland) using *Escherichia coli* O55:B5 endotoxin (Lonza Group) as a calibration standard. Concentrations were expressed in units

of endotoxin (EU)/m³ air. Rat and mouse allergens were analysed using ELISA kits (Rat n 1 ELISA kit (EL-RN1) and Mus m 1 ELISA kit (EL-MM1); Indoor Biotechnologies, Warminster, UK) and results expressed in ng allergen/m³ air. Limits of detection (LODs) were 0.05 EU/filter, 0.097 and 0.024 ng/filter for endotoxin, rat allergen and mouse allergen, respectively. A concentration equal to half the LOD was attributed to samples below this limit, while two samples with very high concentrations (>25 ng/filter) were attributed a value of 25 ng/filter for statistical analysis.

Workplace

Blood sampling and lung function tests were carried out at the same time within 1 week of the clinical examination. Serum was kept at -80°C in 2 mL polypropylene tubes after progressive freezing in freezing containers (Nalgene Cryo 1°C ; $-1^{\circ}\text{C}/\text{min}$; No. 5100-0001) until analysis. Cytokine determinations were carried out with the Bio-Plex Pro cytokine, chemokine and growth factor 27-plex assays and the Bio-Plex workstation based on the Luminex technology (Bio-Rad Laboratories, Mississauga, Ontario, Canada). IL-17F, IL-22, IL-23 and IL-33 were measured separately using a four-plex assay from the same manufacturer. Concentrations below the LOD were attributed a value of LOD/2 (see online supplementary file). Two cytokine profiles were defined a priori. IL-4, IL-5, IL-13 and eotaxin-1 defined the 'sensitiser' profile, while IL-8, IL-17A, IL-17F and IL-22 defined the 'irritation' profile. In both cases, a profile was considered positive if the concentrations of ≥ 3 of the four cytokines exceeded the 95th percentile of the Sy-/Se- subgroup. A limited number of explorative analyses were conducted for additional inflammation-related cytokines (IL-1 β , IL-23, IL-12, IL-10, macrophage inflammatory protein (MIP) 1 α , MIP-1 β and tumour necrosis factor α (TNF- α)) and Th2-related cytokines (IL-9 and IL-33). IL-2, IL-6 and interferon γ (IFN- γ) were considered for comparisons with other studies.

Statistics

Additional detail on statistical analyses is provided online (see online supplementary file). Briefly, variable distributions were examined for normality, and non-parametric tests or logarithmic transformation was used when appropriate. Serum cytokine concentrations were used for performing hierarchical clustering analyses (HCA) generated with the TM4 Multiexperiment Viewer.³⁰ Multiple linear regression models were used to identify determinants of cytokine concentrations. On the basis of power calculations, a total population of at least 150 LA workers was aimed to have about 15 workers each in the Sy+/Se+, Sy+/Se- and Sy-/Se+ subgroups.

RESULTS

Gender and nationality did not differ statistically significantly between eligible but non-participating workers ($n=125$; figure 1) and participants with cytokine measurements ($n=114$), but the latter were younger (median (range) 30 (16–64) vs 37 (19–64)).

Although the whole participating population (figure 1) was larger than aimed at according to the power calculations, the subgroups Sy+/Se+ ($n=8$) and Sy-/Se+ ($n=6$) were much smaller than expected. Therefore, the two subgroups could only be considered in sensitivity analyses. In contrast, the Sy+/Se- subgroup was twice as large as expected ($n=29$ vs 15) and, thus, well above the power requirement. Hence, analyses had to be restricted to the question whether irritant-related symptoms were associated with the predefined cytokine irritation profile. As a result of the selection procedure (figure 1), the prevalence of WR symptoms was considerably higher in the Sy+/Se- than in the Sy-/Se- subgroup. However, the other variables of table 1 did not differ between the two subgroups in a statistically or clinically relevant way with the possible exception of the lifelong duration of exposure to animals.

Any allergic disease (rhinitis, asthma, eczema, other skin allergy or animal allergy) was recorded in 61 workers. A WR cause was clearly overrepresented in the Sy+/Se- subgroup despite no significant IgE to rat and/or mouse. Overall, asthma prevalence was 22%. Asthma (WR and/or non-WR) was confirmed by a doctor in 19/22 cases.

Table 1 Characteristics of the study population

	Group	
	Sy-/Se- ($n=71$)	Sy+/Se- ($n=29$)
Age (years)*	29.0 (16–46)	31.0 (22–58)
Sex (women)†	51 (71.8)	18 (62.1)
Education level		
High	55 (77.5)	20 (69.0)
Middle	15 (21.1)	8 (–)
Low	1 (–)	1 (–)
Nationality		
Switzerland	28 (39.4)	12 (41.4)
Germany	14 (19.7)	11 (37.9)
Italy	10 (14.1)	1 (–)
Other	19 (26.8)	5 (–)
Smoking		
Never	50 (70.4)	23 (79.3)
Former	8 (–)	4 (–)
Current	13 (18.3)	2 (–)
Pack-years (Cigarette smokers only) (n)	2.10 (0.05–14) ($n=21$)	0.25–15.0 ($n=6$)
BMI (kg/m^2)	22.3 (17.3–36.0)	23.3 (16.4–35.4)
FEV ₁ (per cent predicted)‡	103.1 (80.3–128.5)	103.1 (68.9–136.9)
FEV ₁ /FVC (%)‡	79.3 (63.7–94.9)	78.0 (58.2–90.1)
Health condition		
'Symptomatic'§		
No	23 (32.4)	0 (–)
Non-WR	48 (67.6)	0 (–)
WR	0 (–)	29 (100)
Allergic rhinitis		
No	49 (69.0)	11 (37.9)
Non-WR	22 (31.0)	8 (–)
WR	0 (0)	10 (34.5)
Any allergic disease		
No	36 (50.7)	3 (–)
Non-WR	35 (49.3)	2 (–)
WR	0 (0)	24 (82.8)
Asthma‡		
No	57 (80.3)	21 (72.4)
Non-WR	14 (19.7)	4 (–)
WR	0 (–)	4 (–)
Total IgE (kU/L)	28.1 (1.00–1104)	63.3 (1.00–1127)
Total IgE		
≤ 100	58 (81.7)	19 (65.5)
> 100	13 (18.3)	10 (34.5)
Lifelong occupational exposure to animals (years)	3.0 (0–9)	7.0 (0–37)
Exposure in last job (years)	2.0 (0–9)	2 (0–19)

*Median and range (one case without group assignment is missing).

†Number and per cent (one case without subgroup assignment is missing).

‡Results include only acceptable spirometric curves in Caucasians. For FEV₁ (FEV₁/FVC), subgroup size is 56 (53) and 28 (28) in Sy-/Se- and Sy+/Se-, respectively.

§Definition of 'symptomatic', see the Methods section. According to the classification scheme, subjects having any predefined symptom are considered asymptomatic when the symptom is not WR.

BMI, body mass index; FEV₁/FVC, forced expiratory volume in 1 s/forced vital capacity; IgE, immunoglobulin E; WR, work related.

One single worker from the Sy+/Se- subgroup had several cytokine concentrations greatly exceeding those found in the rest of the population (see online supplementary file) with a

Table 2 Serum cytokine concentrations (pg/mL) after excluding the outlier (n=99)

	Subgroup		Location shift,†95% CI	Significance level‡
	Sy-/Se- (n=71)	Sy+/Se- (n=28)		
	Median and range	Median and range		
Cytokines selected according to the a priori hypothesis				
'Allergy' profile				
IL-4	9.28 (4.52–14.44)	9.80 (6.88–13.40)	0.52 (-0.40 to 1.40)	ns
IL-5	3.84 (0.80–15.84)	4.72 (1.88–7.12)	1.08 (0.24 to 1.76)	**
IL-13	16.68 (3.20–58.52)	16.64 (10.40–28.20)	0.28 (-1.92 to 2.72)	ns
Eotaxin-1	46.00 (22.48–131.88)	39.38 (30.40–89.48)	–4.04 (-9.48 to 1.08)	ns
'Irritation' profile				
IL-8	19.28 (8.24–54.36)	22.78 (10.80–255.68)	–0.14 (-5.48 to 7.08)	ns
IL-17A	20.72 (6.80–213.56)	40.16 (4.80–163.92)	17.72 (7.68 to 27.52)	**
IL-17F	All concentrations less than the LOD			
IL-22	109/113 concentrations less than the LOD			
Cytokines selected for explorative analyses				
Cytokines related to the 'allergy' profile				
IL-9	33.68 (8.44–129.16)	24.90 (11.28–136.00)	–7.36 (-12.24 to 2.52)	**
IL-33	106/114 concentrations less than LOD			
Cytokines related to the 'irritation' profile				
IL-1β	1.84 (0.44–12.08)	2.32 (1.20–3.84)	0.40 (0.08 to 0.72)	*
TNF-α	26.88 (5.60–116.80)	24.24 (13.44–131.68)	–2.04 (-5.32 to 1.68)	ns
IL-23	84/114 concentrations less than the LOD. Moreover, 16 of the 30 concentrations greater than the LOD were the same (196.8 pg/mL)			
IL-12	66.76 (4.40–200.84)	41.08 (17.80–237.36)	–23.60 (-37.3 to –10.24)	**
IL-10	14.04 (3.60–269.00)	8.86 (4.36–41.36)	–4.52 (-7.00 to –1.56)	**
MIP-1α	4.84 (2.56–9.04)	4.10 (2.80–7.04)	–0.52 (-0.92 to –0.04)	*
MIP-1β	102.28 (43.24–224.56)	78.56 (33.88–239.32)	–21.04 (-38.2 to –5.64)	*
Cytokines selected for comparison with the literature				
IL-2	4.00 (4.00–265.64)	6.92 (3.60–39.48)	–0.40 (-0.40 to 2.92)	ns
IL-6	8.84 (2.52–84.16)	9.76 (2.96–46.64)	0.24 (-1.28 to 1.76)	ns
IFN-γ	229.88 (45.60–1534.64)	248.10 (56.68–563.12)	5.52 (-32.28 to 42.32)	ns

†Location shift: value of subgroup Sy+/Se- minus Sy-/Se-. 95% CI: 95% confidence limits of the difference between both groups.

‡Significance level of difference (Wilcoxon two-sample test): ns, not significant; *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.

IFN-γ, interferon gamma; LOD, limit of detection; MIP-1, macrophage inflammatory protein 1; TNF-α, tumour necrosis factor alpha.

major effect on ranges and residual distributions in the absence of any identifiable cause. She was excluded leaving 99 workers with cytokine measurements. Of note, inclusion of this single case could seriously affect residual distribution but had a limited effect on the regression coefficients (online supplementary file). 'Continuously taking any drug' was recorded in 52 subjects. 'Recent symptoms' were reported by 48 workers and attributed to a number of different causes, mostly a common cold (n=28). The prevalence of 'common cold' (28%–29%) was identical in the two subgroups. Exposure was mostly to more than one animal, but mice and rats were by far the most frequent species.

Concentrations of IL-17F, IL-22, IL-23 and IL-33 were all less than the LOD. The cytokines belonging to the profile irritation (IL-8, IL-17A, IL-17F and IL-22) did not show consistent trends. Indeed, the high occurrence of IL-17F and IL-22 concentrations less than the LOD precluded the assessment of the prevalence of the 'irritation' profile (table 2).

Comparison of serum cytokine concentrations between subgroups (table 2) suggested that the levels of the proinflammatory cytokines IL-17A and possibly IL-1β were significantly increased in the Sy+/Se- subgroup in comparison to Sy-/Se- workers. This effect was not observed for TNF-α or MIP-1α. IL-10, IL-12 and possibly MIP-1β concentrations were lower in the Sy+/Se- than in the Sy-/Se- subgroup. Finally, IL-17F, IL-22, IL-23 and IL-33 concentrations were mostly less than the LOD.

Serum cytokine concentrations were introduced into HCA to visualise potential subject clusters. IL-8 and IL-17A were chosen as irritation-related cytokines and IL-4, IL-5, IL-13 and IL-9 as allergy-related cytokines. Three distinct analyses were generated on subjects presenting with symptoms, with no symptom or with no sensitisation. Hierarchical tree for all symptomatic workers (see online supplementary figure part A) did not segregate sensitised subjects. However, results in the asymptomatic group clustered together five out of the six subjects from the Sy-/Se+ subgroup (see online supplementary figure part B). More interestingly, nearly all Sy+/Se- subjects among all not sensitised workers are gathered in two subclusters, characterised by high IL-17A levels, but differing in IL-8 levels, confirming the importance of irritation-related cytokine in this subgroup (see online supplementary figure part C).

In multiple linear regression models (table 3; see online supplementary tables S3–S5), the two master cytokines considered (IL-13, IL-1β) had the expected clear effect on Th-2 and inflammatory cytokines, respectively. Two potential confounding factors, recent complaints and drug consumption, were statistically significant in several analyses. The only fairly consistent association with symptoms and sensitisation was between cytokine concentration and subgroup Sy+/Se-. Age or body mass index were never significantly associated with any cytokine concentration in the best fit models. Specific IgE to rat and/

Workplace

Table 3 Multiple linear regression analyses: cytokines selected a priori after excluding the outlier

	IL-4	IL-5	IL-13	Eotaxin-1	IL-8	IL-17A
Sample size	n=99	n=99	n=98†	n=99	n=99	n=99
Concentrations less than the LOD	n=0	n=15	n=7	n=0	n=13	n=21
Partial regression coefficients and significance levels						
Intercept	0.643****	-0.343**	1.019****	1.710****	1.172***	1.008****
Subgroup (Sy+/Se-: code 1)	0.017 ns	0.131*	0.046 ns	-0.030 ns	0.030 ns	0.169*
Drug consumption (0=no; 1=yes)	NI	0.043 ns	0.092*	0.051 ns	NI	0.116 ns
Recent complaints (0=no; 1=yes)	NI	0.136**	0.105*	0.094***	NI	NI
Sex (0=male; 1=female)	NI	NI	NI	-0.091**	NI	NI
History of allergic disease (0=no; 1=yes)	NI	NI	NI	-0.080**	NI	NI
Number of days at work‡	NI	NI	0.035 ns	NI	NI	NI
IL-13 (pg/mL)	0.262****	0.602****	NR	NR	NR	NR
IL-1 (pg/mL)	NR	NR	NR	NR	0.449***	1.016****
Adjusted R ² ; Pr>F	0.39****	0.39****	0.12**	0.19***	0.12***	0.36****

*p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.

†n=98: One case with missing value for one independent variable was excluded.

‡Number of days at work since the last day on leave.

Statistically significant values are in bold typeface.

Serum cytokine concentrations were log transformed.

Drug consumption: regular consumption of any drug. Recent complaints, history of allergic disease and rationale for including IL-1 and IL-13, see the Methods section.

LOD, limit of detection; NI, variable not included in the model with the best fit statistics; NR, not relevant in this case (see the Methods section); ns, not significant; Pr>F, p value of an F-test, testing the null hypothesis that all model coefficients are zero.

or mouse, total IgE concentration, clinical history of allergic disease or asthma, smoking, tolerance to endotoxin and animal bites were mostly not retained in the models with the lowest Bayesian information criterion (BIC) value. Only 10 subjects had had no pet as a child making meaningful statistical analyses of pet exposure impossible. Overall, distribution of the residuals, concentrations less than the LOD and/or collinearity were often a problem.

All correlations between cytokines and FEV₁ (L/s), FEV₁ (per cent predicted) and FEV₁/FVC (in per cent) were non-significant with the exception of that between MIP-1 α and FEV₁/FVC ($r=0.25$, $p=0.02$; $n=80$). The effect of IL-5 and IL-17A on FEV₁ (L/s) and FEV₁/FVC was also examined in multiple linear regression models including the variables pack-years, time elapsed since smoking cessation and age, gender, height and Sy/Se subgroup as appropriate. As with correlation coefficients, no statistically significant association with cytokine concentration emerged.

Regarding exposure, rat allergen was greater than or equal to the LOD in four and six morning and afternoon samples, respectively, but in six samples concentrations were exactly equal to the LOD. Mouse allergen and endotoxin concentrations were greater than or equal to the LOD in 51.9% and 98.7% of the samples, respectively. Median (5th/95th percentile) peak mouse allergen and endotoxin concentrations were 0.30 (0.02–21.47) ng/m³ and 1.96 (0.31–109.96) EU/m³, respectively. Peak exposure to endotoxin was similar in the Sy-/Se- and Sy+/Se- subgroup ($p=0.09$) (online supplementary file). Overall, in multiple linear regression analyses, cumulative exposure dose to endotoxins was not a predictor of concentration of cytokines selected a priori ($p=0.02$ for IL-13 and $0.26 \leq p \leq 0.99$ for all other cytokines).

Several sensitivity analyses were conducted for cytokines selected a priori. First, the cytokines belonging to the profile 'sensitiser' (IL-4, IL-5, IL-13 and eotaxin-1) did not show consistent trends. Indeed, the 'sensitiser' profile was found in one subject

only (two if the case with greatly increased concentrations was not excluded). Moreover, levels of the Th2-related cytokine IL-5, but not IL-4, IL-13 and eotaxin-1, were also slightly increased in the Sy+/Se- compared with the Sy-/Se- subgroup, while serum IL-9 was decreased (table 2). Even if the Se+ subgroups were too small for drawing firm conclusions, it is striking that concentration ranges of IL-4, IL-5, IL-13 and eotaxin-1 were similar to those of the Sy-/Se- subgroup (see online supplementary table S2). After including the two Se+ subgroups, cytokines pointing to sensitisers (IL-4, IL-5, IL-13, eotaxin-1, IL-9, IL-33) were not increased in subgroups defined by specific sensitisation to rat and/or mouse, atopy or clinical history of allergic disease (details not shown). Second, including the worker with several very high cytokine concentrations or including the two small Se+ subgroups did not alter the results of the multiple regression analyses (see online supplementary tables S3–S5). Including the variable 'lifelong occupational exposure to animals' (table 1) had no effect either (details not shown). Third, as examination of residual plots suggested a distortion of residual distribution due to concentrations less than the LOD, an alternative definition of the LOD ($\text{LOD}/\sqrt{2}$) was used for IL-5, IL-8 and IL-17A. Indeed, >10% of the results were less than the LOD for these three cytokines. Residuals did not improve. Moreover, with respect to IL-8 and IL-17A results of multiple linear regression were not altered by using the highest LOD of the two plates (see online supplementary file) for the whole group. Fourth, even after including the two Se+ subgroups the p values of the correlations between cytokines and FEV₁ (L/s), FEV₁ (per cent predicted) and FEV₁/FVC (in per cent) were non-significant with the exception of that between IL-9 and FEV₁ (per cent predicted) ($r=-0.23$, $p=0.03$; $n=95$). In this population, the effect of IL-5 and IL-17A on FEV₁ (L/s) and FEV₁/FVC was re-examined in multiple linear regression models including the variables pack-years, time elapsed since smoking cessation and age, gender, height and Sy/Se subgroup as appropriate. As with correlation coefficients, no statistically significant association with cytokine concentration

Table 4 Effect of time of occurrence and work relatedness on cytokine concentrations in the reference group (single outlier excluded)

	Group Sy-/Se- according to categorisation scheme (n=71)* Median (range)	Group Sy-/Se- without any predefined symptom (n=23)† Median (range)
<i>Cytokines selected a priori</i>		
Profile 'allergy'		
IL-4	9.28 (4.52–14.44)	8.80 (5.12–12.80)
IL-5	3.84 (0.80–15.84)	3.04 (0.80–5.84)
IL-13	16.68 (3.20–58.52)	16.68 (3.20–25.40)
Eotaxine-1	46.00 (22.48–131.88)	44.52 (27.04–96.04)
Profile 'irritation'		
IL-8	19.28 (8.24–54.36)	19.28 (8.24–28.64)
IL-17A	20.72 (6.80–213.56)	20.72 (6.80–81.84)
<i>Cytokines selected for explorative analyses</i>		
Cytokine related to the profile 'allergy'		
IL-9	33.68 (8.44–129.16)	31.00 (8.44–95.60)
Cytokines related to the profile 'irritation'		
IL-1 β	1.84 (0.44–12.08)	1.76 (0.44–2.64)
TNF- α	26.88 (5.60–116.80)	26.88 (5.60–38.28)
IL-12	66.76 (4.40–200.84)	56.20 (4.40–105.52)
IL-10	14.04 (3.60–269.00)	10.28 (5.36–25.52)
MIP-1 α	4.84 (2.56–9.04)	4.84 (2.56–5.96)
MIP-1 β	102.28 (43.24–224.56)	100.12 (57.60–200.28)

*According to categorisation scheme, workers with predefined respiratory and/or skin symptoms were included in the Sy-/Se- group, if symptoms were non-work related.

Symptoms were previous and/or current symptoms.

†Without any predefined symptom, subjects having ever had any predefined symptom are excluded irrespective of time of occurrence and work relatedness.

emerged. Finally, the categorisation scheme²⁶ may lead to misclassifications with respect to symptoms and sensitisation. Indeed, 'symptomatic' was defined as having ever had symptoms that were WR and on a predefined list. Consequently, subjects having previously had a symptom non-relevant for current cytokine production may bias the classification. The multiple regression analyses including all four subgroups were, therefore, rerun after defining symptomatic (Sy+) individuals as having had symptoms during the past year instead of having ever had WR symptoms. Results did not show relevant changes. Furthermore, according to these classification criteria subjects currently having any predefined symptom(s) due to non-occupational exposure are considered asymptomatic as the symptoms are not WR, which may also distort comparisons if these symptoms alter cytokine production. However, excluding all subjects with predefined symptoms (previous, current, occupational and/or non-occupational) from the Sy-/Se- subgroup had only a minor effect on the reference ranges of cytokines and some medians remained unchanged (table 4).

DISCUSSION

In this cross-sectional study, 114 workers exposed mostly to LA were categorised according to WR symptoms and specific sensitisation to rat and/or mouse and 17 serum cytokine concentrations were measured. The cytokine profile 'irritation' was not associated with irritant-induced symptoms. However, IL-17A levels, a cytokine selected a priori, were consistently higher in the Sy+/Se- subgroup according to univariate comparisons, HCA and multiple linear regression analysis. IL-5 levels were less increased (table 2) and less consistent (HCA, multiple linear regression). Results for IL-1 β should be considered as suggestive only (exploratory analyses).

The number of Se+ workers was low precluding definitive conclusions. The reason for the low prevalence of Se+ workers is speculative. Although all eight Sy+/Se+ subjects from the

whole participating population (n=177) were included in the subpopulation with cytokine determination (figure 1), the prevalence of Sy+/Se+ workers was lower (4.5% vs 10%) than expected according to the power calculations. Likewise, the prevalence of Sy-/Se+ workers was much lower than expected (3.4% vs 11%–12%). We had initially included all university units working with any animal and found more than 1000 workers (figure 1), recruited 18% (177 instead of 150) more subjects than necessary according to the power calculations and preferentially selected the research units with a higher probability of symptoms (figure 1). A check showed that the procedure selecting workers for cytokine measurements had not fortuitously excluded symptomatic workers (details not shown). Overall, this suggests that the prevalence of sensitised workers was really lower than expected, which may have been due to chance. Alternatively, a declining prevalence may mirror declining sensitisation rates resulting from an exposure that was decreasing in the last years (replacement of rats by mice, introduction of individually ventilated cages).^{1 2} In this regard, it is striking that the airborne allergen concentrations were low and that very few cases of sensitisation have occurred in the last years (see online supplementary file). No previous information is available making objective comparisons impossible.

None of the two predefined cytokine profiles was associated with the assumed cause of symptoms (sensitisation or irritation). Misclassifications resulting from a flawed categorisation scheme may mask differences between groups but are not a likely explanation. Indeed, restricting the time period of symptom occurrence to the last year or using a completely symptom-free reference group did not alter the results. Second, in the Sy+/Se+ subgroup, the allergic disease was unambiguously confirmed by clinical history with the exception of one single case in whom a coincidence may explain the association of symptoms and sensitisation (see online supplementary file).

Workplace

Comparisons may have been partly underpowered owing to the small size of the Sy+/Se+ subgroup, but in contrast to IgE or FEV₁/FVC no systematic trend towards higher or lower values appeared. Furthermore, after lumping all sensitised subjects together no association appeared despite increased power. With respect to sensitisation, using the cut-off of >0.7 kU/L instead of ≥0.35 kU/L to increase test specificity^{31 32} did not change the composition of the Sy+/Se+ subgroup, while two of the six Sy-/Se+ workers were transferred to the Sy-/Se- subgroup. Altogether, misclassifications are, therefore, unlikely to have masked differences. Thus, this study does not support the use of a cytokine signature to diagnose irritation-induced symptoms in LA workers. Finally, it may be objected that no difference appeared because all workers were exposed to both allergens and irritants. However, the exposure effect, that is, symptoms and sensitisation, differed in a clinically very relevant way between subgroups, which may be assumed to cause different cytokine profiles.

HCA showed that nearly all Sy+/Se- subjects among all not sensitised workers were gathered in two subclusters, characterised by high IL-17A levels, but differing in IL-8 levels, which is in line with multiple linear regression results. The increased IL-17A concentration in the subgroup Sy+/Se- is reminiscent of the Th17 polarisation in mice exposed to dust extract or peptidoglycan.²⁰ Interestingly, a growing body of evidence supports a pathological role of IL-17A in asthma, especially regarding neutrophilic inflammation.¹⁵ Our observation suggests that this type of immune responses may preferentially occur in Sy+/Se- workers. The effect on IL-8 may also be explained by some component of organic dust.¹⁶ However, we cannot exclude that other agents (virus, bacteria, fungi, protozoa) were the cause of the increased IL-17A concentration. The nature of the causal agent remains unknown as components of organic dust were not measured and airborne infectious agents not sampled.³³

Several studies have examined cytokine secretion in LA workers. Two publications^{34 35} refer to the same cohort study of starting LA workers. In the first, to investigate whether production of cytokines was associated with the development of occupational sensitisation, IL-8 in nasal lavage fluid and in vitro IL-4, IL-6, IL-10, IL-12 and IFN-γ production (after non-specific stimulation of whole blood cultures) were measured at baseline. No difference was found between sensitised subjects and controls with the exception of higher IL-8 concentration in nasal lavage fluid from non-atopic workers. In the second publication, levels of IL-4, IL-6, IL-10, IL-12 and IFN-γ in supernatant of whole blood cultures were determined (unspecific stimulation). In addition, rat allergen-specific IL-4, IL-10 or IFN-γ production by peripheral blood mononuclear cells (PBMC) was assessed. There was a significant increase in rat-specific IL-4 producing cells between the last time point before and the first time point after sensitisation. Allergen-specific IL-10 and IFN-γ responses as well as non-specific cytokine responses showed no consistent differences between sensitised and non-sensitised subjects. Three other studies were cross-sectional. After unspecific stimulation, the proportion of peripheral T-helper cells producing intracellular IL-4, IL-13, IL-2 and IFN-γ was similar in symptomatic LA workers with and without sensitisation and controls.³⁶ IL-2, IL-4, IL-5, IL-10, TNF-α and IFN-γ concentrations were measured in supernatant of PBMC proliferation assays (stimulation with rat urinary protein) and IL-5, IFN-γ and ratios of IL-5/IFN-γ found to be increased in LA workers with a rat-specific sensitisation.³⁷ Finally, the ex vivo whole blood release of IL-8 and TNF-α tended to increase in response to exposure to workplace dust from animal facilities.³⁸

Altogether, these studies measured cytokine concentrations in supernatants of whole blood or PBMC cultures, with or without specific or unspecific stimulation, but, as in the present study, no cytokine profile proved easy to assess and helpful for routine surveillance of occupational diseases. Chance findings because of small study groups, outliers, concentrations less than LOD and confounding factors may also explain some associations. In this regard, little attention has been paid to potential confounders in the aforementioned studies. However, in the present study, two confounding factors were often statistically significant in several multiple regression analyses, that is, 'recent symptoms' and 'continuously taking drugs'. The effect of recent symptoms may have been due to the 28 workers with common cold. As only eight subjects were 'continuously taking drugs' capable of disturbing cytokine production further investigations were impossible.

This study has limitations. First, our findings are based on cross-sectional data, which does not allow for causal inference. In particular, changes in cytokine concentration in the course of time cannot be assessed. Second, a selection bias cannot be excluded. Indeed, because of data protection very little information is available for non-participants. However, demographic information about non-participants (gender and nationality) did not point to a selection bias and the small age difference is unlikely to be associated with different cytokine patterns. Third, explorative analyses with numerous multiple linear regression models were made whereby increasing the risk of type I error, and no good model could be fitted for some cytokines. Nominal p values must, therefore, be interpreted with great caution. Fourth, although endotoxin remains important, part of the inflammatory properties of organic dust are not due to endotoxin.^{13 33} A complete characterisation of all inflammatory agents would have been preferable but exposure measurements had to be limited to allergens and endotoxin for practical reasons.

In conclusion, in this large unselected working population exposed mainly to LA a typical profile of serum cytokine concentration associated with WR symptoms and/or sensitisation to LA could not be identified. Thus, it does not appear possible to use serum cytokine profiles for distinguishing sensitiser-induced from irritant-induced WR symptoms. Increased IL-17A levels may reflect exposure to organic dust.

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workers received written information and all subjects gave written informed consent.

Ethics approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. Research involving human participants: The study was conducted in the framework of an analysis of occupational risks of workers exposed to animals. It was conducted according to the Declaration of Helsinki and approved by the Ethics Commission (Kantonale Ethik-Kommission Zürich (KEK), reference number KEK-ZH-Nr. 2012-0142, Zurich (Switzerland), approval dated 15 June 2012).

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Can serum cytokine profile discriminate irritant-induced and allergen-induced symptoms? A cross-sectional study in workers mostly exposed to laboratory animals

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