Expression of lymphocyte cell surface markers in workers exposed to different respiratory hazards: biomarkers of occupational respiratory disease?

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This study was designed to evaluate the potential of flow cytometry to measure biomarkers of airways inflammation in the peripheral blood of two cohorts of workers reporting work-related respiratory symptoms, who were exposed to different respiratory hazards. Nine bakers exposed to wheat flour and 10 glass bottle manufacturers exposed to a range of irritant chemicals were selected for study. Phenotypic and inducible cell surface markers were measured by flow cytometry. Results were compared with a control population of 58 volunteers reporting no respiratory problems. The bakers showed a significant increase above control values for cell types associated with inflammation; in particular CD3 + CD4+ cells ($p < 0.005$) and CD4 + CD25 + cells ($p < 0.01$). In contrast, the workers reporting work-related respiratory symptoms who were exposed to a range of irritant chemicals showed a different pattern of cell surface lymphocyte markers, with a significant decrease in the total T-cell population ($p < 0.05$). Comparison of results from a subset of smoking controls with the population of bakers (who were all heavy smokers) confirmed that the increase in CD3 + CD4+ cells and CD4 + CD25+ cells could not be ascribed to the effects of smoking alone. We have shown activation of helper T-cells in the peripheral blood of bakers reporting work-related respiratory symptoms consistent with the changes observed in mild to severe asthmatics. However, workers with similar symptoms who were exposed to irritant chemicals did not show this pattern of phenotypic or inducible cell surface markers, reflecting an absence of airways inflammation in these individuals. Our results suggest that flow cytometry may be of use as an objective test for detecting workers with airways inflammation to allow the identification of workers at risk of developing occupational asthma.

Keywords: *flow cytometry, asthma, occupational, allergens, irritants.*

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Introduction

In some individuals, workplace exposure to a variety of chemicals and proteins may result in the development of both upper and lower respiratory symptoms including runny nose and eyes, shortness of breath, wheeze, cough and chest tightness. If appropriate action is not taken quickly, and an affected worker remains exposed, these symptoms may develop into occupational asthma, which can significantly reduce their quality of life.

Respiratory questionnaires are often used to monitor individuals exposed to respiratory hazards, so that workers at risk of developing occupational asthma can be removed from exposure (Enarson *et al.* 1987, Stenton *et al.* 1993). However, for a variety of reasons questionnaires are not always answered correctly, resulting in the continued exposure of workers at risk of developing occupational asthma. For example, fear of enforced redundancy, or a change of job within the factory may cause 'at risk' workers to respond inappropriately (Gordon *et al.* 1997). Therefore, objective tests of airways inflammation and/or respiratory sensitization are required to help validate questionnaire data.

Specific IgE antibodies have been measured in workers exposed to a variety of respiratory sensitizers. However, whilst this approach has proved successful for many high molecular weight sensitizers, it is of limited use in workers exposed to many low molecular weight chemicals. For example, workers with no respiratory symptoms may also have specific IgE antibodies, as is the case with isocyanate-exposed workers (Butcher *et al.* 1993), or antibodies may be absent from workers who report symptoms, as is the case with glutaraldehyde-exposed workers (Curran *et al.* 1996). We have chosen to measure biomarkers of airway inflammation using flow cytometry.

Flow cytometry has been used for many years to identify, characterize and quantify immune cells in a number of disease states including leukaemia and HIV infection. Different cell types express a variety of phenotypic and inducible cell surface markers, which can be selectively labelled using monoclonal antibodies. Dual and triple staining with fluorochrome-labelled monoclonal antibodies allows the expression of multiple markers to be measured in a single sample, and from this information subpopulations of cells can be identified and quantified. This technique offers considerable advantages over other cellular tests since it is rapid, simple to perform and requires only a small sample of venous blood.

Flow cytometry has also been used to study the activation of inflammatory cells in the peripheral blood of patients with acute asthma (Corrigan *et al.* 1988), and has shown a significant increase in T-cell activation markers such as CD25 (the interleukin-2 receptor) compared with control subjects. Furthermore, these CD25 positive lymphocytes were exclusively of the CD4 positive (helper T-cell) phenotype. Whilst the involvement of the inflammatory response in the pathogenesis of idiopathic and work-related asthma seems clear, the role of inflammation in the production of symptoms following low molecular weight chemical exposure is less well documented. Some of the findings in atopic asthmatic patients

have been reproduced in patients with asthma induced by toluene diisocyanate, a known sensitizer and also a low molecular weight irritant compound (Finotto *et al.* 1991), and a review of the relationship between low molecular weight pollutants and asthma has emphasized the importance of the cell mediated response (Mapp *et al.* 1994).

Workplace exposure to toxic materials may cause changes in both the proportions of immunologically important cell types, and may increase their state of activation. For example, workers exposed to styrene showed a modified distribution of lymphocyte subsets, with a decrease in total T-lymphocytes and an increase in NK cells (Bergamaschi *et al.* 1995), and workers exposed to asbestos showed a decrease in peripheral blood natural killer cells (NK cells). Therefore, we have used flow cytometry to measure immunological changes in the peripheral blood of two groups of workers reporting similar work-related respiratory symptoms, but exposed to different respiratory hazards.

METHODS AND MATERIALS

Subjects

Three groups of workers were investigated in this study. A cohort of nine bakers exposed to wheat flour from a single bakery with either upper or lower respiratory symptoms related to work were recruited during clinical follow-up after administration of a standard workplace respiratory symptom questionnaire. A second group of workers exposed to a range of low molecular weight irritant chemicals were screened, and 10 workers reporting both upper and lower respiratory tract symptoms were identified. The 58 control subjects for this study were recruited from a local hospital. They reported no chronic inflammatory disease or recent respiratory symptoms, and each control subject performed spirometry. All volunteers provided informed consent, and local ethics committee approval was obtained for the study.

Clinical assessment

A respiratory physician (S. B. Gordon) examined each volunteer. Lung function was determined using a portable spirometer, and results were compared with predicted values obtained from standard tables (Cotes 1979). All workers in the study showed FEV1/FVC% less than predicted values, and all controls had values greater than 70%. Further clinical investigations were used to enable a diagnosis of respiratory disease to be made for each worker reporting respiratory symptoms.

Blood samples

Each volunteer provided a 5 ml venous blood sample for flow cytometric analysis, which was collected into EDTA containing Vacutainers™ (Becton Dickinson). All samples for flow cytometry were processed and analysed on the day of collection. In addition, each subject provided a 10 ml clotted sample for RAST analysis. Serum was separated, and stored at –20 °C until required.

Antibodies

The following labelled antibodies were used in the study to measure phenotypic and inducible cell surface markers. Fluorescein–isothiocyanate–conjugated mouse monoclonal anti-human CD3, CD8 and CD45 (Coulter Electronics, UK). Phycoerythrin-conjugated mouse monoclonal anti-human CD14, CD3 (Coulter Electronics, UK) and CD25 (Becton Dickinson). Energy-coupled dye-conjugated mouse monoclonal anti-human CD4 and CD19 (Coulter Electronics, UK). Appropriately labelled isotype control antibodies were purchased from both Coulter Electronics (UK) and Becton Dickinson.

Antibody staining

Aliquots (100 μl) of peripheral whole blood were added to tubes containing different combinations of labelled antibodies (shown in table 1). Tubes were gently mixed and left in the dark for 20 min. After this time the red cells were lysed and remaining cells were fixed using the Coulter Immunoprep system. Prior to analysis, 250 μl of Isoton II (Coulter Electronics, UK) was added to each tube to ensure good separation of lymphocytes from monocytes when visualized using forward and side scatter characteristics.

Flow cytometry

Lysed whole blood was analysed on a Coulter Epics XL flow cytometer equipped for four colour analysis. Prior to analysis the instrument was calibrated for optical alignment, and fluorescence intensity using Immunocheck and Immunobrite fluorescent microspheres (Coulter Electronics, UK). Fluorochrome-labelled cells were excited with a 488λ nm laser and FITC, PE and ECD emission spectra were identified with FL1, FL2 and FL3 channel detectors respectively, with suitable compensation based on single stained isotype control samples. Lymphocytes were identified by $CD14^{-}CD45^{+}$ backgating, and 5000–10000 events within this gate were analysed. The percentage of dual and triple labelled cells in each sample was calculated for each antibody combination used, relative to the appropriate control antibody.

RAST analysis

All sera were tested for specific IgE antibodies to common aero-allergens (cat fur, house dust mite, and mixed grass pollens), and the bakers' sera were also tested for specific IgE to wheat flour. Briefly, serum (200 μl of 1 : 4 dilution in PBS, pH 7.4) was incubated overnight at room temperature with the appropriate allergen disc. Discs were washed four times with 1.2 ml of 0.9% saline containing washing solution additive (Pharmacia, Uppsala, Sweden), and then 100 μl of ^{125}I-labelled rabbit anti-human IgE (Pharmacia, Uppsala, Sweden) was added. After an additional overnight incubation at room temperature, the discs were washed four times and bound ^{125}I was measured in a gamma counter (Canberra-Packard). All the assays were performed in duplicate. The results were expressed as a RAST percent binding which was defined as the percentage of total added counts per minute bound to the allergen disc incubated with the test serum, minus the percentage of added counts per minute bound to the allergen disc incubated with a negative control serum.

	Fluorochrome			
Cell type	FIT	CPE	ECD	Cells identified
Lymphocytes	CD45	CD14		Lymphocytes Monocytes
Lymphocytes	IgG1	IgG1	IgG1	(Control)
Lymphocytes	CD8	CD3	CD4	Helper T-cells Cytotoxic/suppressor T-cells
Lymphocytes	CD8	CD25	CD4	Activated helper T-cells Activated cytotoxic/suppressor T-cells
Lymphocytes	IgG1	IgG1	IgG2b	(Control)
Lymphocytes	CD3		CD19	T-cells B-cells

Table 1. Combinations of fluorochromes used to identify particular cell populations.

Age and gender	Exposure to flour	Years of exposure	URT symptoms[b]	LRT symptoms[b]	RAST % binding (atopy)	RAST % binding (wheat flour)[c]	Smoking	Clinical diagnosis
27 M	Minimal	12	N	Y	P (20.98)	P (2.69)	Y 16 years 10/day	Atopic asthma
49 F	Minimal Moderate	9 8	N	Y	P (2.2)	P (2.21)	Y 34 years 20/day	Occupational asthma and COAD
36 F	Heavy	3	N	Y	N (0.51)	N (0.92)	Y 18 years 10/day	Cough and irritancy
50 F	Moderate	19	Y	Y	N (0.50)	P (2.6)	Y 35 years 7/day	Allergic rhinitis
26 M	Moderate None	4 3	Y	Y	N (0.61)	P (1.37)	Ex 11 years 10/day	Occupational asthma
54 F	None Heavy	8 1	Y	Y	N (0.68)	P (6.83)	Y 35 years 20/day	Allergic rhinitis
51 F	Minimal None	5 11	N	Y	P (32.14)	P (1.64)	Y 34 years 20/day	Chronic bronchitis and flour allergy
54 F	Moderate	24	N	Y	N (0.55)	N (0.57)	Y 34 years 10/day	Late onset asthma
46 F	Moderate	8	Y	Y	P (6.66)	N (0.57)	Y 31 years 30/day	Atopic asthma

Table 2. Demographic and clinical details of symptomatic bakers studied.
[a] Heavy exposure: hand-making pizza bases. Moderate exposure: bake line, dusting baps, hot-cross buns. Minimal or none: wrapping, icing, sandwich factory.
[b] URT (upper respiratory symptoms) indicate eye, nose or throat complaint (Y = yes; N = no). LRT (lower respiratory symptoms) indicate cough, sputum, wheeze or dyspnoea (Y= yes; N = no).
[c] Corrected RAST percent binding of greater than 1% taken as positive (P = positive; N = negative).

Age and gender	Years at work	URT symptoms[a]	LRT symtoms[a]	Smoking (no./day and years)	RAST % binding (atopy)[b]	Clinical diagnosis
54 M	32	Y	Y	N	N (0.6)	Asthma? cause
39 F	6	Y	Y	Y (13 years 30/day)	N (0.6)	COPD
45 M	13	Y	Y	E (29 years 15/day)	N (0.7)	Normal
62 M	23	Y	Y	E (20 years 1/day)	N (0.6)	Normal
48 M	26	Y	Y	E (4 years 20/day)	N (0.7)	COPD
50 M	28	Y	Y	Y (36 years 20/day)	P (13.4)	Chronic bronchitis
51 M	26	Y	Y	Y (41 years 30/day)	N (0.6)	Chronic bronchitis
33 M	8	Y	Y	N	P (39.3)	Asthma
49 M	27	Y	Y	E (4 years 30/day)	N (0.7)	Chronic bronchitis
31 M	4	Y	Y	Y (10 years 20/day)	P (31.5)	COPD

Table 3. Demographic and clinical details of irritant-exposed workers studied.
[a] URT (upper respiratory symptoms) indicate eye, nose or throat complaint (Y = yes; N = no). LRT (lower respiratory symptoms) indicate cough, sputum, wheeze or dyspnoea (Y = yes; N = no).
[b] Corrected RAST percent binding of greater than 1% taken as positive value (P = positive; N = negative).

Age	Sex	Smoking history	*FEV1* % FVC	Atopy (RAST % binding)
Mean 33.7	Male 27	Present 17	Mean 85%	Mean 4.2
SD 10.0	Female 31	Ex-smoker 9	SD 0.055	SD 7.7
Range 16–57		Non-smoker 32	Range 71.5–98.7	Range 0.0–30.02

Table 4. Demographic details of control subjects (all had an FEV1 greater than 70% at baseline).

	Bakers		Matched smoking controls	
Age	Mean	42.7	Mean	39.1
	SD	10.8	SD	9.9
	Range	25–54	Range	28–52
Sex	M: 2	F: 7	M: 2	F: 7
Smoking status	Present smokers	8	Present smokers	6
	Ex-smokers	1	Ex-smokers	3
	Mean years smoking	27.5	Mean years smoking	19.0
	Mean number per day	15	Mean number per day	11.0

Table 5. Matching detail for bakers with subpopulation of smokers from the control group.

Marker	Bakers (n = 9), mean (SD)	Irritant-exposed workers (n = 10), mean (SD)	Controls (n = 58)	Bakers vs controls	Irritant exposed workers vs controls
CD3	75.62 (5.72)	63.6 (10.2)	72.92 (6.38)	NS	$p < 0.02$
CD3CD4	56.16 (8.06)	42.17 (10.7)	45.67 (6.8)	$p < 0.005$	NS
CD3CD8	18.44 (5.61)	20.2 (6.7)	23.98 (5.07)	$p < 0.02$	NS
CD4 : CD8	3.42 (1.52)	2.3 (1.0)	2.0 (0.71)	$p < 0.05$	NS
CD4CD25	1.04 (0.46)	0.6 (0.4)	0.51 (0.18)	$p < 0.01$	NS
CD19	14.4 (5.12)	10.8 (4.8)	13.22 (4.1)	NS	NS

Table 6. Percentage of gated lymphocytes (identified by CD14–CD45+ backgating) expressing phenotypic and inducible cell surface markers in a population of bakers reporting work-related respiratory symptoms and a population of irritant-exposed workers reporting work-related respiratory symptoms. Data are compared with a group of unexposed control workers describing no respiratory symptoms. Significant differences between groups were determined using Student's t-test.

Statistical analysis

Significant differences between groups were determined using Student's t-test. The c-stat statistical package was used for the calculations.

Results

Population characteristics

The demographic and clinical characteristics of the bakers, irritant-exposed workers and control population are shown in tables 2–4. The bakers showed an excess of cigarette smoking compared with the control group and therefore a subset of the control population was matched with the bakery workers for age, sex, smoking habit and atopy. The result of this matching is shown in table 5.

Flow cytometry

The bakers reporting work-related respiratory symptoms showed a significant increase in the percentage of lymphocytes identified as helper T-cells ($p < 0.005$), activated helper T-cells ($p < 0.01$) and in the ratio of helper T-cells to cytotoxic/suppressor T-cells ($p < 0.05$). There was also a significant reduction in cytotoxic/suppressor T-cells ($p < 0.02$). No significant differences were observed in either the total T-cells or B-cells. This pattern was not repeated for the irritant-exposed workers reporting work-related respiratory symptoms, who showed a significant decrease in total T-cells ($p < 0.02$), but no changes in CD4+ cells or activation state. These data are summarized in table 6.

Effect of smoking

The population of bakers were all found to be present or ex-smokers. Therefore, a comparison was made with a subgroup of the control population, matched (as far as possible) for smoking status, smoking years, age and sex. When compared with the matched population, the bakers still showed a significant increase in helper T-cells ($p < 0.05$) and activated helper T-cells ($p < 0.05$), see table 7.

Discussion

In this study we have used flow cytometry to measure differences in inflammatory cells in two populations of workers exposed to different respiratory hazards, but reporting similar upper and lower respiratory symptoms. Bakers reporting work-related respiratory symptoms showed an altered pattern of inflammatory cells which was consistent with results from acute severe asthmatics (Corrigan *et al.* 1988). However, workers reporting similar symptoms, but exposed to irritant chemicals did not show this pattern.

Bakers' asthma is a well described clinical entity caused by a high molecular weight allergen, often associated with specific IgE antibodies. Late phase reactions have been described after antigen challenge tests with flour, which suggests that a lymphocyte contribution to the process of bronchial inflammation is likely. This study has provided additional evidence of a role for T-lymphocytes in the aetiology of work-related respiratory symptoms in this condition, since these workers have increased levels of activated circulating helper T-lymphocytes. Small changes in symptom characteristics in atopic asthmatic subjects have resulted in detectable changes in lymphocyte markers (Walker *et al.* 1992, Robinson *et al.* 1993) and our findings are consistent with these reports in that similar patterns of activation were detected.

Studies on healthy workers exposed to flour dust have shown a decrease in CD4+, CD8+ and CD57+ lymphocytes (Kolopp-Sarda *et al.* 1995). Our findings also showed a decrease in CD8+ lymphocytes in workers with respiratory symptoms, but CD4+ lymphocytes were significantly increased. The original findings suggested an immunoregulatory role for these cell types, with the suggestion

Marker	Bakers (n = 9), mean (SD)	Matched controls (n = 9), mean (SD)	Bakers vs controls (t-test)
CD3	75.62 (5.72)	70.09 (7.61)	NS
CD3CD4	56.16 (8.06)	47.56 (7.79)	$p < 0.05$
CD3CD8	18.44 (5.61)	22.58 (7.79)	NS
CD4: CD8	3.42 (1.52)	2.34 (0.85)	NS
CD4CD25	1.04 (0.46)	0.6 (0.41)	$p < 0.05$
CD19	14.4 (5.12)	15.2 (5.19)	NS

Table 7. Percentage of gated lymphocytes (identified by CD14–CD45+ backgating) expressing phenotypic and inducible cell surface markers in a population of bakers reporting work-related respiratory symptoms compared with a matched smoking population taken from the control group. Significant differences between groups were determined

that this helped to maintain immunological homeostasis during flour exposure. Our results would indicate that there is very fine balance between CD4+ and CD8+ lymphocytes, and a shift in this balance resulting in increased CD4+ cells is associated with respiratory symptoms. The population of bakers investigated in this study was too small to enable correlations to be made between the cellular markers of inflammation and the presence or absence of specific IgE antibodies.

No relationship has yet been demonstrated between irritant exposure and the incidence of asthma (Wardlaw 1993, Tattersfield 1996). Results from the cohort of workers exposed to a range of irritant chemicals who reported work-related respiratory symptoms provided additional evidence for this observation, in that they did not show the same pattern of inflammatory cells in peripheral blood as atopic asthmatics, or the cohort of bakers with respiratory symptoms.

These preliminary studies indicate that the identification of inflammatory cells in peripheral blood using flow cytometry may prove to be a useful tool for the biological monitoring of workers exposed to respiratory hazards, and may help to distinguish workers with occupational asthma, from those suffering from related occupational respiratory diseases.

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