

# Cytokine Synthesis in Occupational Allergy to Caddisflies in Hydroelectric Plant Workers

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## Key Words

Caddisfly · Allergy · Cytokine synthesis · IFN- $\gamma$  · IL-5 · IL-13

## Abstract

**Background:** Workers in hydroelectric plants appear to be readily sensitized to caddisfly allergens. This sensitization probably occurs de novo from occupational exposure. In some workers, sensitization occurs on a non-atopic background. Cytokine synthesis of IFN- $\gamma$ , IL-5 and IL-13 in atopic and non-atopic caddisfly-allergic workers was examined to determine if responses were similar or different.

**Methods:** Peripheral blood mononuclear cells were isolated from atopic caddisfly-allergic workers, non-atopic caddisfly-allergic workers and non-atopic caddisfly-exposed but non-allergic workers. Stimulation with caddisfly antigens was carried out and synthesis of IFN- $\gamma$ , IL-5 and IL-13 was determined by sandwich ELISA. **Results:** Both caddisfly-allergic and non-allergic subjects responded to stimulation with caddisfly extract. The response in non-atopic caddisfly-non-allergic subjects was TH1 predominant, while that in atopic caddisfly-allergic subjects was TH2 predominant. The response in non-atopic caddisfly-allergic subjects was between that of the atopic caddisfly-allergic workers and the non-atopic caddisfly-non-allergic workers and the trend was to a TH2 response. Work-related symptoms were similarly intermediate between the atopic caddisfly-allergic and non-atopic caddisfly-non-

allergic group. Differences were significant for IFN- $\gamma$ /IL-5 ratios but not IFN- $\gamma$ /IL-13 ratios for atopic and non-atopic caddisfly-allergic individuals, compared to non-atopic caddisfly-non-allergic workers. However, a linear relationship existed between IFN- $\gamma$  synthesis and IL-5 and IL-13 synthesis in non-atopic caddisfly-allergic workers but not in atopic caddisfly-allergic subjects. **Conclusions:** Caddisfly allergy in hydroelectric workers may be a useful model for the development of allergy to a previously unencountered allergen, and points to some interesting differences between atopic and non-atopic subjects who become sensitized to environmental allergens.

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When a cross-sectional survey was conducted in hydroelectric powerplant workers in which the workforce is exposed to large numbers of caddisflies, up to 50% of the participants reported work-related eye, nose and sinus symptoms, as well as wheezing [1]. Sixty-one percent of the workers studied were epicutaneous skin prick test positive to an extract of the local caddisfly, while 39% had positive reactions to a commercial caddisfly extract. Ninety-three percent of the commercial extract-positive workers reported three or more work-related symptoms, while only 64% of the local caddisfly extract-positive workers had three or more work-related symptoms. There was a significant correlation between levels of exposure and sensitization to the commercial extract. Subsequent

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investigations at two other hydroelectric plants have demonstrated reduced levels of exposure to caddisfly and lower rates of sensitization to the commercial extract, 20.8 and 13.8%, respectively. The high rate of sensitization suggests that caddisfly antigens are potent allergens, when present in high concentrations, that will induce sensitization in a large proportion of exposed individuals, including normally non-atopic subjects. Sensitization to the commercial caddisfly extract was seen in workers with a longer duration of exposure to the caddisflies. This model might therefore be useful for examining the development of occupational respiratory allergy *de novo* in atopic and non-atopic individuals.

We studied a group of hydroelectric plant workers who were atopic or non-atopic as defined by skin test reactivity to common inhalant allergens, and who had positive or negative epicutaneous skin prick tests to a commercial caddisfly extract, and determined the *in vitro* cytokine synthesis of IFN- $\gamma$ , IL-5 and IL-13, in response to caddisfly extract.

Both atopic and non-atopic caddisfly-allergic subjects tended to produce more TH2 cytokines than non-atopic workers who were not caddisfly-allergic, but the TH2 phenotype predominance was significantly greater in atopic caddisfly-allergic subjects. Thus a potent inhaled allergen can induce allergy in atopics and non-atopics but the non-atopic caddisfly-allergic subjects produce a reduced TH2 cytokine response compared to atopic caddisfly-allergic workers. The model may be useful for studying the development of allergy in atopic and non-atopic backgrounds.

## Materials and Methods

### Patient Populations

Hydroelectric plant workers who volunteered for the study were from hydroelectric plants in Manitoba where caddisflies were prevalent, and had been skin tested with a commercial caddisfly whole body extract (Hollister-Stier Toronto, Canada) by epicutaneous skin prick test. The commercial extract was used because reactivity to it correlated best with clinical symptoms [1], and because the extract produced from local caddisflies contained a high level of LPS that could not be removed without losing a number of the higher molecular weight protein bands present initially on SDS-gel electrophoresis. Skin test controls used were histamine phosphate 1% and isotonic saline diluent. Participants were also skin tested by skin prick tests with common inhalant allergens: mixed tree pollens, mixed grass pollens, mixed weed pollens, molds, cat dander and house dust mites *Dermatophagoides farinae* and *Dermatophagoides pteronyssinus* as well as shrimp. Reactions were measured by outlining the wheal with a pen and transferring this to paper, where the surface area in square millimetres was then determined. A reaction >9 mm<sup>2</sup> greater than saline control was defined as being positive. Atopy was considered to

be present when an individual reacted positively to one or more common inhalant allergens, as defined previously by Cookson et al. [2], a definition used in many subsequent studies.

Skin test reactivity or sensitization is not necessarily indicative of allergy. Based upon our previous clinical investigation of hydroelectric workers, it was considered that a subject had work-related symptoms if he or she were skin test positive and reported 3 or more symptoms related to exposure to caddisfly [1]. Workers' characteristics in terms of skin test reactivity to caddisfly extract, atopic status, work-related symptoms and years in the workplace are shown in table 1.

### Cell Culture

Whole blood (20 ml) was collected into 1 ml of 2.7% EDTA. Peripheral blood mononuclear cells (PBMC) were obtained by centrifugation on Histopaque-1077 (Sigma, St. Louis, Mo., USA) within 30 min of the blood being collected. Cells were isolated from the interface and washed 3 times in saline, counted and viability was determined by trypan blue dye exclusion. Cells were frozen immediately in liquid nitrogen. Frozen samples in which the viability exceeded 90% were used subsequently for activation with caddisfly extract. PBMC from the thawed samples were cultured at a final concentration of  $2.5 \times 10^6$ /ml for antigen stimulation in 96-well U-bottom plates (Nunc, Naperville, Ill., USA) in RPMI 1640 supplemented with 10% FCS, 10 mM *L*-glutamine,  $2 \times 10^5$  M 2-ME, and antibiotic-antimycotic (Life Technologies, Burlington, Canada). For each subject, a minimum of three wells were set up for each condition. Cells were cultured in the absence of a stimulus and with commercial caddisfly extract of varying concentrations and for varying periods of time, up to 6 days, to determine optimal conditions. Subsequently, PBMC were stimulated with caddisfly aqueous extract at 1:200 and 1:2,000 for 4 days and 6 days to assess cytokine production. The caddisfly extract was shown to contain <15 EU/ml LPS by the LAL test (BioWhittaker, Walkersville, Md., USA). Complete medium with fetal calf serum contained similar levels of LPS.

### Cytokine Assays

**IL-13.** A sandwich ELISA using purified monoclonal rat anti-human IL-13 as the capture reagent and a purified, biotinylated polyclonal anti-human IL-13 (Pharmingen, Mississauga, Canada) in combination with streptavidin-alkaline phosphatase (Jackson ImmunoResearch, Westgrove, Pa., USA) was used. Internal IL-13 standards of specific activity  $10^6$  units/mg (Pharmingen) were included on each plate. The lower limit of detection was ~8 pg/ml.

**IL-5.** A sandwich ELISA was also used for IL-5 detection with a purified anti-human IL-5 Mab (TRFK4, Pharmingen) for antigen capture and biotinylated JE21-dA10 (Pharmingen). IL-5 levels in each sample were calculated against a standard (Pharmingen) set in each plate. Assay sensitivity was ~8 pg/ml.

**IFN- $\gamma$ .** IFN- $\gamma$  was determined in ELISA using MAGS obtained from Pharmingen. Each assay was conducted using internal standards of an IFN- $\gamma$ -containing PHA-stimulated PBMC supernatant, calibrated against human IFN- $\gamma$  reference reagent Ag 23-901-530 (1 National Institutes of Health unit = 115 pg).

### Statistical Analysis

Median values for each group are shown in a box-whisker plot. Statistical significance was evaluated by the Mann-Whitney rank sum test and also by the *t* test after log transformation to produce a normal distribution and equal variance. Regression analysis was performed using Sigmastat (SPSS, Chicago, Ill., USA).

**Table 1.** Subject characteristics of hydroelectric workers

| Subject | Caddisfly +/- skin test | Atopic +/- | Work-related symptoms <sup>a</sup> | Years in workplace |
|---------|-------------------------|------------|------------------------------------|--------------------|
| 1       | +                       | -          | 0                                  | 36                 |
| 2       | +                       | -          | 3                                  | 9                  |
| 3       | +                       | -          | 2                                  | 2                  |
| 4       | +                       | -          | 4                                  | 5                  |
| 5       | +                       | -          | 0                                  | 16                 |
| 6       | +                       | -          | 4                                  | 18                 |
| 7       | +                       | +          | 4                                  | 8                  |
| 8       | +                       | +          | 3                                  | 7                  |
| 9       | +                       | +          | 4                                  | 30                 |
| 10      | +                       | +          | 0                                  | 5                  |
| 11      | +                       | +          | 3                                  | 10                 |
| 12      | +                       | +          | 4                                  | 19                 |
| 13      | -                       | -          | 0                                  | 15                 |
| 14      | -                       | -          | 0                                  | 4                  |
| 15      | -                       | -          | 0                                  | 4                  |
| 16      | -                       | -          | 2                                  | 26                 |
| 17      | -                       | -          | 0                                  | 4                  |
| 18      | -                       | +          | 2                                  | 6                  |
| 19      | -                       | +          | 0                                  | 1                  |
| 20      | -                       | +          | 5                                  | 23                 |

<sup>a</sup> 3 or more work-related symptoms in caddisfly skin test-positive subjects were considered significant.

## Results

### Patient Data

The exposure to caddisfly in the studied hydroelectric workers was approximated by visual assessment of levels of exposure, and by known duration of exposure. At site 1 on the Winnipeg River, where heavy exposure occurred 11/28 subjects (39%) tested were allergic to commercial caddisfly extract and 100% of these were atopic. The heavy exposure related to poor cleaning of the site, where caddisfly debris was present in heaps in the work environment. At site 2, also on the Winnipeg River, where caddisfly exposure was intermediate, 20.8% (10/48) were skin test positive and 4/6 atopics (66.6%) were sensitized. Therefore 6/42 (14.3%) of non-atopics were sensitized. At site 3, at Grand Rapids, where exposure was low, 4/29 (13.8%) were sensitized, and this included only 2/9 (22.2%) of atopics and 2/20 (10%) of non-atopics. The risk of sensitization to caddisfly in these workers at three different sites varied in atopics from 22 to 100% and in non-atopics from 10 to 14.3%.

Contact was made by letter and by telephone with workers from the hydroelectric plants and 20 patients

**Table 2.** Median values and statistical differences in ratios of IFN- $\gamma$ :IL-5 secreted on day 4 and 6 in atopic and non-atopic caddisfly-allergic subjects and non-atopic caddisfly-non-allergic subjects

| Worker groups               | Stimulation period, days | Median IFN- $\gamma$ /IL-5 ratio | p values       |
|-----------------------------|--------------------------|----------------------------------|----------------|
| Atopic positive (n = 6)     | 4<br>6                   | 1.025<br>0.85                    | 0.007<br>0.032 |
| Non-atopic positive (n = 6) | 4<br>6                   | 3.25<br>0.93                     | 0.059<br>0.027 |
| Non-atopic negative (n = 5) | 4<br>6                   | 14.3<br>4.0                      |                |

p values determined by Mann-Whitney rank sum test in comparison to the non-atopic caddisfly-non-allergic group.

agreed to participate. Of these, 6 atopics were caddisfly allergic, as were 6 non-atopic individuals. There were 5 non-atopic subjects who were not caddisfly allergic and only 3 atopics who were negative to skin testing with the caddisfly commercial extract. The latter 3 individuals were left out of the cytokine analysis. Only 2 of the atopic subjects had positive skin prick tests to shrimp allergen, which has been shown to cross-react with caddisfly antigen [3]. Of the workers analyzed, only subjects in the caddisfly-allergic group had work-related symptoms (table 1) and such symptoms were more frequent in the atopic group than in the non-atopic group ( $\chi^2 = 24.98$ ,  $p < 0.001$ ). There was one atopic caddisfly-non-allergic subject who complained of >3 work-related symptoms. In this selected group of workers from three separate hydroelectric sites, a relationship between duration of exposure and sensitization was not apparent ( $p = 0.471$ ).

### Cytokine Assays

The dose-response curves for IFN- $\gamma$ , IL-5 and IL-13 are shown in figure 1a, b and c, which includes data from 4 subjects, 2 atopic and 2 non-atopic with positive skin tests to caddisfly extract (workers 2, 3, 7, 12). There was optimal cytokine synthesis and secretion using 50  $\mu$ l/ml of a 1:200 dilution of 5% caddisfly extract (w/v) at day 4 or 6 and these conditions were used for the analysis. No statistically significant differences were found between the three groups in terms of the absolute levels of cytokine produced, which may be a reflection of the small number of subjects studied and the multiple allergenicity of the caddisfly extract. Levels of IFN- $\gamma$  and IL-5 synthesis were greater in all groups than the levels of IL-13 produced (data not shown). Non-atopic caddisfly skin test-negative

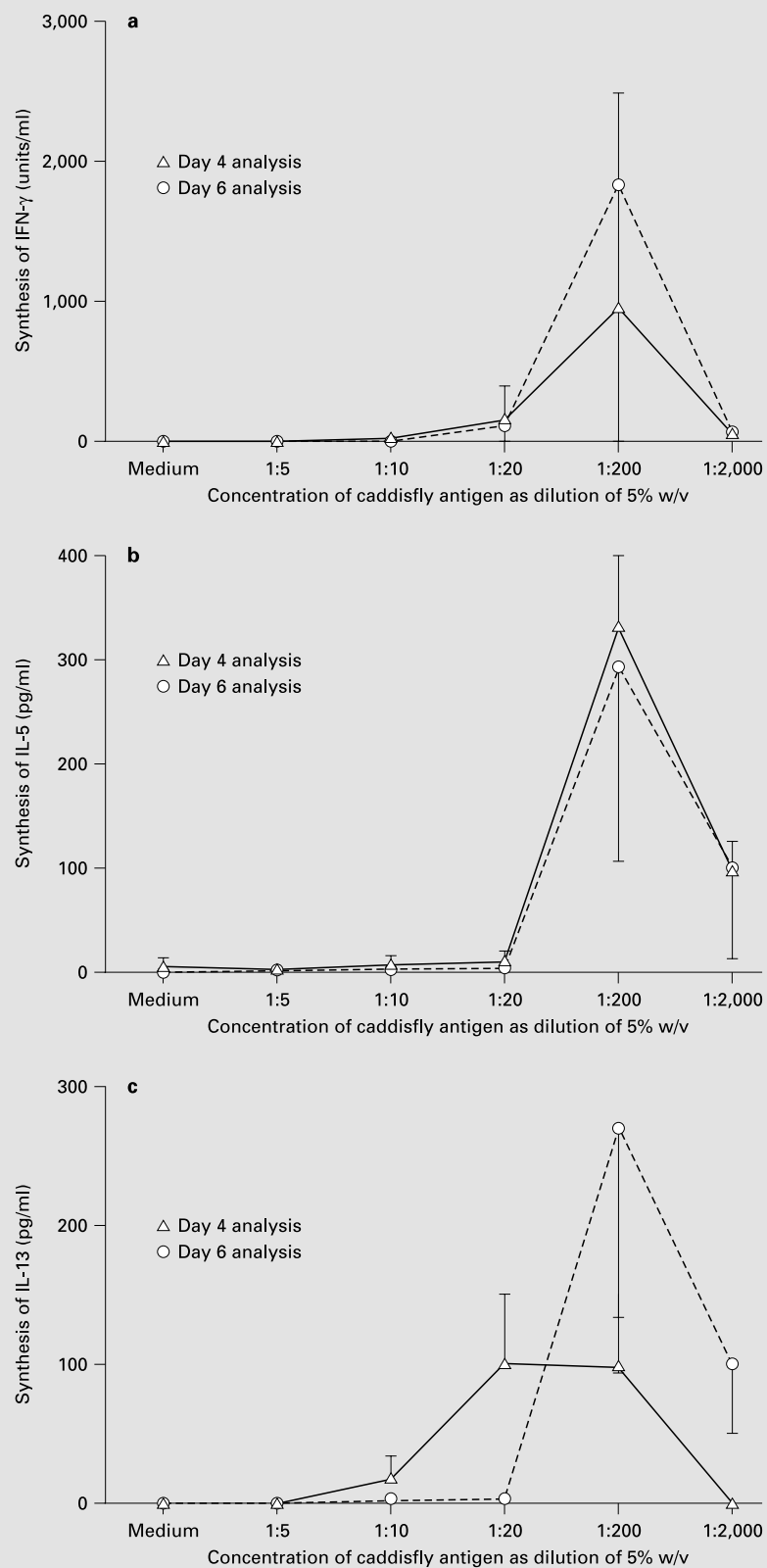
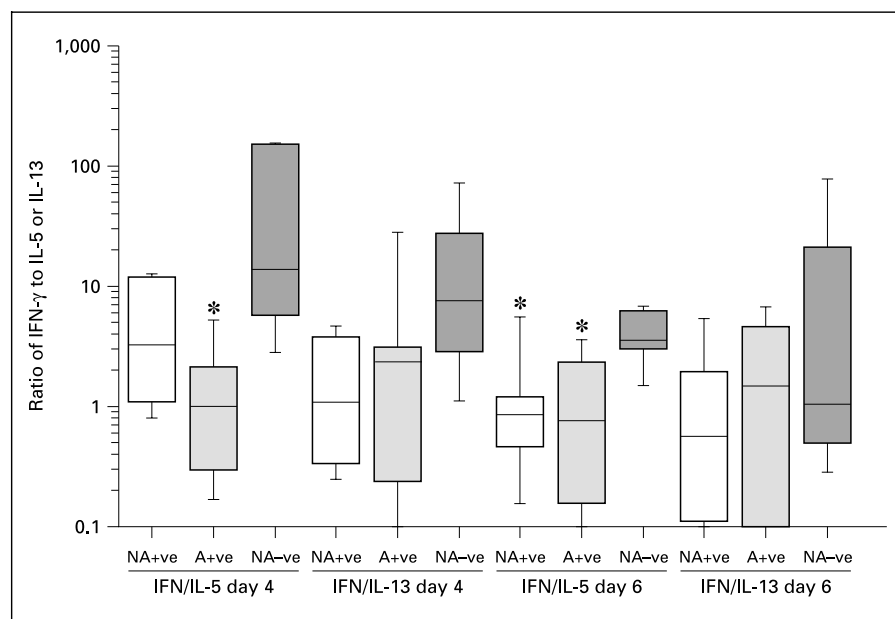


**Fig. 1.** Dose-response curves for IFN- $\gamma$  (**a**), IL-5 (**b**), and IL-13 (**c**) synthesis in vitro in response to caddisfly extract. Mean  $\pm$  SD for 4 workers is shown.

**Fig. 2.** Box-whisker plots for IFN- $\gamma$ /IL-5 and IFN- $\gamma$ /IL-13 ratios found in vitro culture of PBMCs stimulated with caddisfly antigen in non-atopic caddisfly-allergic workers (NA+ve), atopic caddisfly-allergic workers (A+ve) and non-atopic caddisfly-non-allergic workers (NA-ve). Significant differences compared to non-atopic non-allergic subjects: \*  $p < 0.05$ .



subjects secreted significantly higher levels of IFN- $\gamma$  than caddisfly-allergic subjects, whether atopic or non-atopic (table 2).

The ratios of IFN- $\gamma$  to IL-5 and to IL-13 were compared for the three groups on day 4 and on day 6 (fig. 2). There was a significant difference between the IFN- $\gamma$ /IL-5 ratio for atopic caddisfly-allergic subjects and non-atopic caddisfly-allergics when compared to non-atopic caddisfly-non-allergic subjects on day 6 ( $p = 0.03$ ) and for atopic caddisfly-allergics and non-atopic caddisfly-non-allergic subjects on day 4 ( $p = 0.009$ ). The caddisfly-allergic subjects did not differ significantly in terms of IFN- $\gamma$ /IL-5 ratios, although there was a trend towards higher levels of IFN- $\gamma$  production in the non-atopic group on day 4, but this did not reach statistical significance ( $p = 0.091$ ). No significant differences were found between the groups for IFN- $\gamma$ /IL-13 on day 4 ( $p = 0.454$ ), for IFN- $\gamma$ /IL-5 on day 6 ( $p = 0.276$ ) and for IFN- $\gamma$ /IL-13 on day 6 ( $p = 0.971$ ).

When the relationships between IFN- $\gamma$  production, IL-5 production and IL-13 production were examined, IFN- $\gamma$  production in atopic caddisfly-allergic subjects was not related to IL-5 or IL-13 production ( $R = 0.5$ , adjusted  $R^2 = -0.117$ ,  $p = 0.554$ , power = 0.2) but a relationship did exist between these parameters for non-atopic caddisfly-allergic subjects ( $R = 0.980$ , adjusted  $R^2 = 0.935$ ,  $p = 0.008$ , power = 0.979). Therefore the synthesis of IFN- $\gamma$  could be predicted from a linear combination of IL-5 and IL-13 production in non-atopic caddisfly-allergic subjects, but not in atopic caddisfly-allergic individuals (fig. 3).

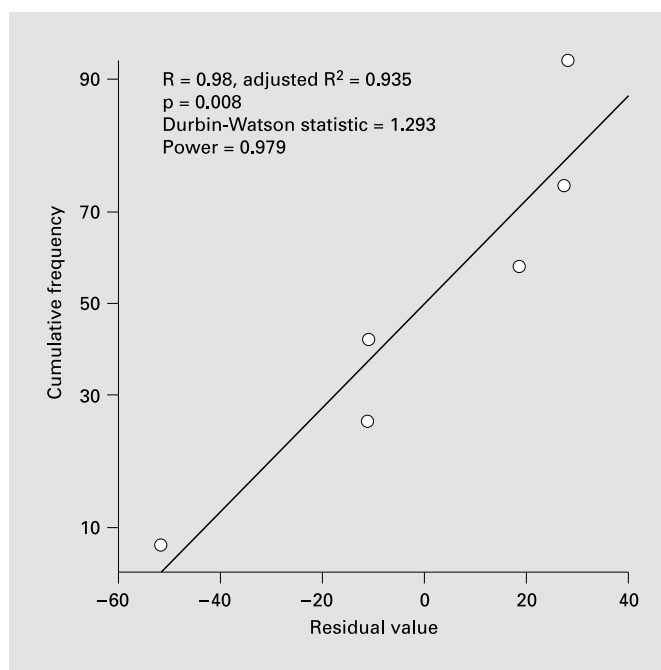


**Fig. 3.** Probability plot for linear regression analysis of IFN- $\gamma$  as the dependent variable and IL-5 and IL-13 as independent variables for non-atopic caddisfly-allergic workers. IFN- $\gamma = 3.915 + (0.76 \times \text{IL-5}) + (0.443 \times \text{IL-13})$ .

## Discussion

There is considerable difficulty in studying the development of allergic responses to antigens that are not present in the general environment, and in most circumstances, such allergens are occupational in origin. While the immunological atopic milieu is assumed to predispose to the development of de novo allergic responses, little is known about the occurrence of allergy in what would be considered to be a non-atopic milieu. In the case of caddisfly allergy, allergic responses to these insects were first reported in 1929 [4] and sensitization to them is common in Japan, where 53.6% of asthmatic patients (30/56) compared to 8.7% (2/23) of non-asthmatic patients had positive skin tests with an extract of caddisfly wing [5]. Kagen et al. [6] reported that 40% of workers exposed in the workplace had allergic rhinitis or symptoms of asthma aggravated by exposure to caddisflies. In a previous study [1], we showed that approximately 50% of hydroelectric workers participating in a study reported work-related eye, nose or sinus symptoms and wheezing, and working in locations with greater exposure to caddisflies was significantly associated with work-related symptoms. Thirty-nine percent had positive skin test responses to a commercial caddisfly antigen and the relative risk for skin test reactivity in caddisfly-exposed workers was 4.12 compared to non-exposed workers. There was a significant correlation between work-related symptoms and skin test reactivity to caddisfly commercial extract (RR = 9.09, CI = 1.4–58.9).

It is of considerable interest that caddisfly allergy can occur in a small but significant proportion of individuals who would be considered to be non-atopic since they fail to show significant skin test reactivity to common inhalant allergens [2]. While it is understandable that atopic subjects exposed to caddisfly allergen would become sensitized, it is probably a reflection of the allergenicity and/or levels of exposure that non-atopic workers also become caddisfly allergic.

It has been shown that IgE antibodies to caddisfly antigens may also cross-react with antigens from shrimp and other crustacea [3]. The possibility therefore exists that sensitization could develop from exposure to the latter, rather than caddisflies in the workplace. However in our previous study there was a relationship between sensitization and level of exposure, which would be unlikely to be seen if individuals were presensitized prior to beginning work in the hydroelectric plant. In addition only 2 subjects (No. 8 and 9) in this study were shrimp allergic on skin testing and caddisfly allergic and both were symp-

tomatic when exposed to caddisfly dust, developing upper and sometimes lower respiratory tract symptoms.

We examined the cytokine responses of atopic and non-atopic hydroelectric workers, when the PBMCs were stimulated in vitro with a commercial caddisfly extract. Three groups of subjects were analyzed, those who were atopic and caddisfly allergic, those who were non-atopic, i.e. did not show skin test reactivity to common inhalant allergens but were caddisfly allergic, and those who were exposed to caddisflies but were not allergic to either these or other common inhalant allergens. The synthesis of IFN- $\gamma$  was used as a measure of TH1 responses, while IL-5 and IL-13 were used as indicators of a TH2 response. In vitro antigen-stimulated IL-4 synthesis is difficult to measure in human peripheral blood lymphocyte cultures especially in vitro in non-atopic subjects [7]. There was evidence for a greater production of IFN- $\gamma$  in response to the caddisfly extract in non-atopic caddisfly-non-allergic subjects, compared to caddisfly-allergic workers, whether atopic or non-atopic. No statistically significant differences were found however, either for IFN- $\gamma$  synthesis or IL-5 and IL-13 synthesis. However when the ratios of IFN- $\gamma$  to IL-5 or IL-13 were examined, atopic caddisfly-allergic workers had statistically significant lower IFN- $\gamma$ :IL-5 ratios than caddisfly non-allergic but exposed subjects. Non-atopic caddisfly-allergic individuals also tended towards a TH2-predominant profile, and in this group the difference from the non-atopic caddisfly-non-allergic group was also statistically significant at day 6, just failing to reach significance on day 4. But in this group levels of IFN- $\gamma$  synthesized on day 4 were higher than those of the atopic group, although not significantly so. It would appear therefore that a TH2-predominant response occurs in individuals who are caddisfly allergic, whether this develops on an atopic background or not, but the TH2 response in non-atopic caddisfly-allergic subjects tends to be not as weighted towards TH2 as that seen in atopic caddisfly-allergic subjects. In support of this finding, no relationship could be shown between IFN- $\gamma$  and IL-5 or IL-13 production in atopic caddisfly-allergic workers, whereas a significant relationship was found between these cytokines for non-atopic caddisfly-allergic subjects. As shown previously for other allergens [8], lymphocytes from non-atopic non-allergic subjects also recognize caddisfly allergens in vitro but produce a TH1-predominant response.

The cytokine results may reflect the multiple allergenicity of the caddisfly extract, with some antigens inducing a TH2 response and others inducing a TH1 response. In non-atopic caddisfly-allergic subjects there could be a

greater recognition of TH2-specific allergens than in non-atopic caddisfly-non-allergic workers, but this may be less than occurs in atopic caddisfly-allergic subjects. Alternatively, it might be suggested that in the non-atopic subjects who develop caddisfly allergy, the response occurs in a TH0 helper cell population. It has been shown that TH0 clones are more readily established in the presence of IL-12, which is likely to be in the milieu of a non-atopic subject, but such clones primarily produce IL-4 and IFN- $\gamma$  in man, and may not provide help for IgE synthesis [9, 10]. In addition, TH0 clones rarely produce IL-5 with IFN- $\gamma$ , but synthesize IL-4 with the latter cytokine [8]. So perhaps in the current situation, the T cell response occurs to multiple antigens in the caddisfly extract, some of which stimulate IL-4 and IL-5 and others that synthe-

size IFN- $\gamma$ . The relationship between IL-5 synthesis and IFN- $\gamma$  synthesis in the non-atopic caddisfly-allergic individuals is therefore intriguing. Whatever the explanation, the model of caddisfly allergy may be a useful one in which to study the development of allergy in both atopic and non-atopic individuals responding *de novo* to an allergen.

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