

In vitro effects of the pyrethroid S-bioallethrin on lymphocytes and basophils from atopic and nonatopic subjects

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**F. Diel¹, M. Detscher¹, B. Schock¹,
M. Ennis²**

¹Department of Nutrition, University of Applied Sciences Fulda, Fulda, Germany; ²Department of Clinical Biochemistry, Institute of Clinical Science, Queen's University of Belfast, Belfast, UK

Synthetic pyrethroids are increasingly used as insecticides and marketed as having relatively low human toxicity. The aim of this study was to examine the *in vitro* effects of the synthetic pyrethroid S-bioallethrin on human blood lymphocytes and basophils in atopic individuals and nonatopic control subjects. S-bioallethrin caused inhibition of lymphocyte proliferation after a 72-h culture period in a concentration-dependent manner. The inhibition of the lymphocyte proliferation by S-bioallethrin at the concentration 6.5 μ M correlated well with the total serum IgE values ($r = -0.89$, $P < 0.001$). Samples from atopic subjects were more sensitive to this inhibition than those from nonatopic volunteers. The regulatory interleukin-4/interferon-gamma (IL-4/IFN- γ) balance showed a significant difference between atopic and nonatopic subjects after a short-term culture period (24 h) in the presence of the same concentration range of S-bioallethrin ($P < 0.001$). Additionally, IFN- γ secretion was consistently lower in cells from the atopic donors.

Furthermore, S-bioallethrin induced histamine release from human basophils in a concentration-dependent manner. Although the effect was small compared to histamine liberators such as *N*-formyl-Met-Leu-Phe and anti-IgE, the response to S-bioallethrin was significantly different in atopic donors from nonatopic ($P = 0.0431$). These findings are the first demonstration of the immunotoxicologic properties of the synthetic pyrethroid S-bioallethrin by this combined *in vitro* approach with human lymphocytes and basophils. Further studies will investigate the responses of lymphocytes from patients who are sensitive to these agents.

Key words: histamine liberation; interferon- γ ; interleukin-4; lymphocytes; pyrethroids

Professor Dr Friedhelm Diel
Department of Nutrition
University of Applied Sciences Fulda
Marquardstrasse 35
D-36039 Fulda
Germany

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Synthetic pyrethroids are increasingly used as insecticides and marketed as having relatively low human toxicity. "Natural pyrethroids" are derived from the chrysanthemum flower. Chrysanthemum flower harvesters suffer from a special form of occupational allergy, namely, chrysanthemum pickers' allergy (1), and extracts of chrysanthemum flowers cause anaphylactic and anaphylactoid reactions (2, 3). The synthetic bioallethrin (S-bioallethrin, Esbiol) is very similar to the main compound in chrysanthemum flower extracts, pyrethrum (Fig. 1). However, the synthetic pyrethroids, such as cismethrin, bioresmethrin, and deltamethrin, show a weak allergenic potency in animal experiments, and it has been suggested that covalent binding of the reactive pyrethroids to liver

proteins can create new haptens and potent antigens (4, 5). Pyrethroids also cause contact allergies. The natural pyrethroids pyrethrosin and pyrethrin II are both potent contact allergens (6), whereas pyrethrin I, cinerin I and II, jasmolin I and II, and synthetic ester molecules such as allethrin, chrysanthemin, and pyrethrol exhibit a very weak contact allergic response.

Some pyrethroids also possess immunotoxicologic (7) as well as immunosuppressive and mitogen-inhibiting effects; for example, allethrin (10^{-6} M) inhibits proliferation of concanavalin A-stimulated splenic lymphocyte cultures (8). Permethrin also exhibits concentration-dependent effects on human lymphocytes *in vitro* (9). Furthermore, immunosuppression in various models has been described

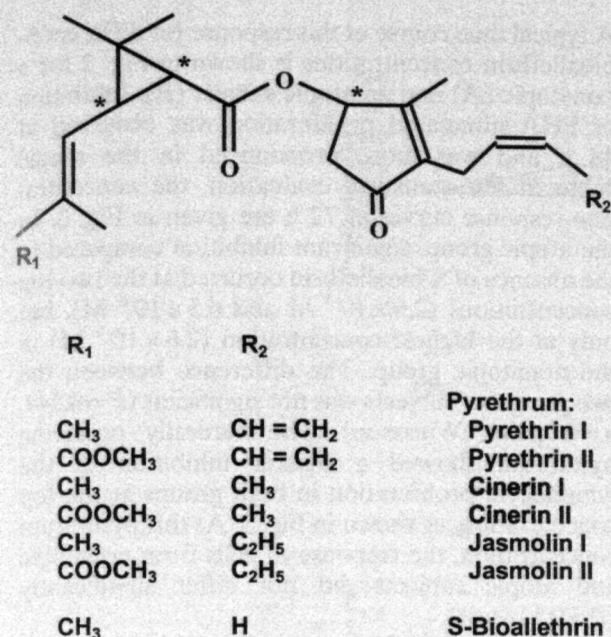


Fig. 1. Structure of pyrethrum and S-bioallethrin (molecular weight: 302.5). *Asymmetric carbon.

with deltamethrin, cypermethrin, and permethrin (10–12).

The role of cytokines in pyrethroid-induced reactions of the human immune system is not known, nor are the possible cytokine interactions. As the Th2-derived interleukin-4 (IL-4) stimulates B-cell activation as well as pathologic IgE secretion, and the Th1-derived interferon-gamma (IFN- γ) causes antagonistic responses, these cytokines may be suitable markers for the immunotoxicologic effects of pyrethroids (13–16). In addition, pyrethroids reduce the lipid packing order of the cell-membrane bilayers (17). Thus, these agents may also directly cause mediator release from basophils/mast cells (18).

Therefore, this study aimed to examine the *in vitro* effects of the synthetic pyrethroid S-bioallethrin on human lymphocytes and basophils from atopic individuals and nonatopic controls.

Material and methods

Volunteers

The atopic patients ($n=17$, three male), median age 25 years (range 15–39 years), presented with a history of allergy (19). The symptoms were as follows (only the main symptom is listed): atopic dermatitis ($n=10$), asthma ($n=4$), and hay fever ($n=3$). The nonatopic controls ($n=12$, three male), median age 29 years (range 21–50 years), had no history of allergy and a total IgE of <100 IU/l.

Total serum IgE was measured in all subjects with a commercially available assay (PRIST, Pharmacia Upjohn, Germany).

Materials and sample preparation

Reagents: Histopaque® 1077, RPMI 1640, L-glutamine, penicillin/streptomycin, fetal calf serum (FCS), PHA (phytohemagglutinin), MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium-bromide), DMSO (dimethylsulfoxide), FMLP (*N*-formyl-Met-Leu-Phe) (all SIGMA, Deisenhofen, Germany)

PBS: NaCl (149.5 mM), KCl (2.96 mM), $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ (7.98 mM), K_2HPO_4 (1.62 mM), pH 7.2; Tyrode's buffer: NaCl (154 mM), glucose (5.6 mM), HEPES (10 mM), KCl (2.7 mM), MgCl_2 (0.5 mM), CaCl_2 (1 mM), pH 7.3 (all Merck, Darmstadt, Germany)

S-bioallethrin: stock solution 2.6×10^{-4} M in DMSO/ H_2O (maximum final DMSO concentration was $<1\%$, which had no detectable effect on lymphocytes [8]) (Ehrenstorfer, Augsburg, Germany)

anti-IgE (rabbit-antihuman, DAKO A/S, Denmark)

OPT (*o*-phthaldialdehyde) (Fluka Buchs, Switzerland).

For cell proliferation and cytokine production, peripheral EDTA blood (20 ml) was withdrawn from the volunteers. The mononuclear cells (PBMNCs) were separated by gradient density centrifugation with Histopaque 1077. Cells were cultured in supplemented RPMI 1640 (2 mM L-glutamine, 100 IU/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin, and 10% heat-inactivated FCS) and incubated at 37°C in a humidified atmosphere of 5% CO_2 in air (20).

Lymphocyte cultures

PBMNCs (2×10^5) were cultured as described above for 72 h in 96-well flat-bottomed plates in the presence of T-cell mitogen PHA (5 $\mu\text{g}/\text{ml}$). Four hours after plating, the pyrethroid S-bioallethrin was added to give the following final concentrations: 2.6×10^{-5} M, 6.5×10^{-6} M, 1.6×10^{-6} M, and 4×10^{-7} M. This period was found to be the most effective in preliminary studies (data not shown).

Cell viability was studied by the Trypan blue exclusion test and was $>95\%$ at the beginning of the culture period.

MTT test and cytokine measurements

The MTT test (21) was used to examine cell proliferation. Lymphocyte cultures were per-

formed as described above. The cells achieved exponential proliferation rate after 2 days in culture, and culture periods up to 72 h were examined. At the end of the designated culture period, MTT (20 μ l of 5 mg/ml) was added and incubated with the cells for 4 h at 37°C. The supernatants were removed after centrifugation (200 g, 10 min, 4°C). The formazan salt crystals in the wells were dissolved in DMSO (100 μ l). Absorbences were measured by an ELISA plate reader, and the differences in absorbance at 540 and 620 nm were calculated.

For measurement of the effect of pyrethroids on cytokine production by Th1 and Th2 lymphocytes, cells were cultured as described above. Supernatants of the cell cultures were collected after 4, 24, 48, and 72 h and stored at -30°C before assay. IL-4 and IFN- γ were measured by commercial ELISA kits (DPC Biermann, Germany).

Histamine liberation test (HLT)

Blood (30 ml) was drawn by venipuncture, anticoagulated with heparin, and mixed with dextran 70 (5 ml Lomodex 70) and glucose (150 mg). After sedimentation for 90 min, the supernatant was collected. The cells were obtained by centrifugation (200 g, 7 min 4°C) and washed twice in Tyrode's buffer. The basophils were preincubated at 37°C for 10 min before challenge. S-bioallethrin was added at the same concentration range as described above. FMLP (10^{-6} M) and anti-IgE (1/3000-fold dilution) were used as positive controls. The reaction was stopped after 45 min by adding 0.5 ml ice-cold Tyrode's buffer, and the samples were centrifuged (200 g, 7 min, 4°C). The supernatants and cell portions were stored at -20°C, and histamine was assayed by a fluorometric method after HPLC separation, as described recently (22, 23).

Statistical calculations

The results of the MTT test were expressed as mean extinctions of triplicate cultures $\pm$ SEM. All other experiments were performed in duplicate. Statistical comparisons were performed by the Mann-Whitney U test and the Wilcoxon test as appropriate. Correlation coefficients were calculated by Pearson's regression analysis. $P < 0.05$ was considered statistically significant.

Results

Cytotoxicity of S-bioallethrin

S-bioallethrin caused inhibition of PHA-stimulated proliferation in a concentration-dependent manner.

A typical time course of this response for different S-bioallethrin concentrations is shown in Fig. 2 for a nonatopic (A) and an atopic subject (B). Inhibition of PHA-stimulated proliferation was observed at 48 h and was more pronounced in the atopic subjects. For statistical evaluation, the concentration-response curves at 72 h are given in Fig. 3. In the atopic group, significant inhibition compared to the absence of S-bioallethrin occurred at the two top concentrations (2.6×10^{-5} M and 6.5×10^{-6} M), but only at the highest concentration (2.6×10^{-5} M) in the nonatopic group. The difference between the two groups of subjects was not significant ($P = 0.144$, $n = 6$ pairs, Wilcoxon). The naturally occurring pyrethrum showed a weaker inhibition of the lymphocyte proliferation in both groups at the top concentration, as shown in Fig. 3. At this pyrethrum concentration, the response of cells from nonatopic and atopic subjects did not differ significantly ($P > 0.1$, t -test).

The percent inhibition of cell proliferation after 72-h culture time in the presence of 6.5 μ M S-bioallethrin correlated significantly with total serum IgE levels of the atopic subjects ($r = -0.89$, $P < 0.001$, $n = 17$) (Fig. 4).

Cytokines

The ELISA methods used were suitable to measure cytokines in both groups. The detection limit for IL-4 was 2 pg/ml (95% confidence), and for IFN- γ 90 pg/ml (95% confidence).

In both normal and atopic subjects, IL-4 secretion was lowest after 72 h in culture (Fig. 5). IL-4 secretion was consistently higher in atopics. This achieved statistical significance at the 4- and 48-h culture periods ($P < 0.05$). In the presence of 6.5 μ M S-bioallethrin, IL-4 secretion was also higher at the 24-h culture period with cells from atopic subjects than nonatopics. However, S-bioallethrin reduced IL-4 secretion in both groups of subjects.

In contrast, IFN- γ release increased with time using cells from both normal and atopic subjects in the presence or absence of 6.5 μ M S-bioallethrin (Fig. 6). The atopic state tended to reduce the release of IFN- γ . S-bioallethrin also reduced IFN- γ secretion, but this did not achieve statistical significance.

IL-4/IFN- γ -ratio – an allergotoxicologic index?

The IL-4/IFN- γ ratios over the 3-day culture periods are shown in Fig. 7. In cells from nonatopic subjects stimulated with PHA, these ratios decreased with increasing culture time (Fig. 7A). The difference in the IL-4/IFN- γ ratio in the

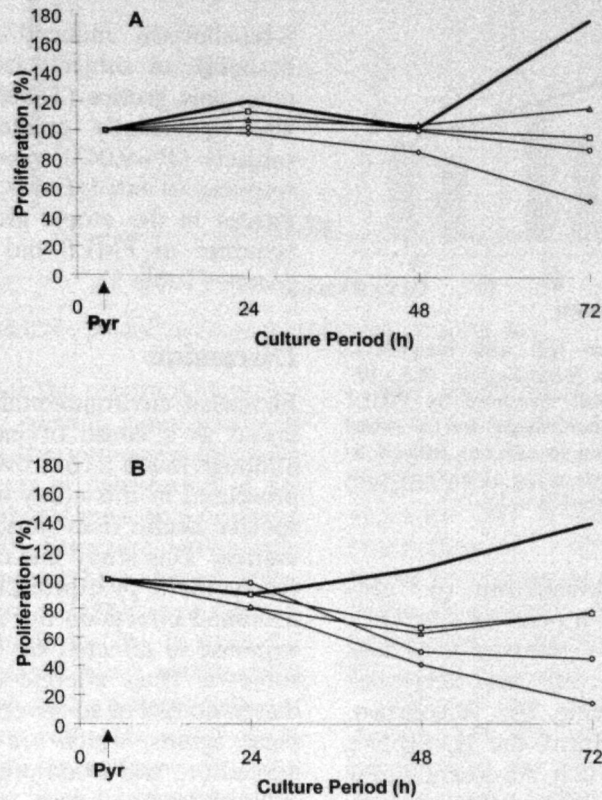


Fig. 2. PHA-stimulated lymphocyte proliferation (MTT test) in presence of different S-bioallethrin concentrations (μM : 0 —, 0.4 Δ , 1.6 $\square$, 6.5 $\circ$, 26 $\diamond$). Lymphocytes were cultured from two 20-year-old volunteers (matched controls) without (A) and with atopic history (B). S-bioallethrin was added 4 h after plating. 100% = 0.30 ± 0.04 extinction of MTT-color reagent measured as described in Material and methods section: *MTT test and cytokine measurements*. (Pyr: pyrethroids added).

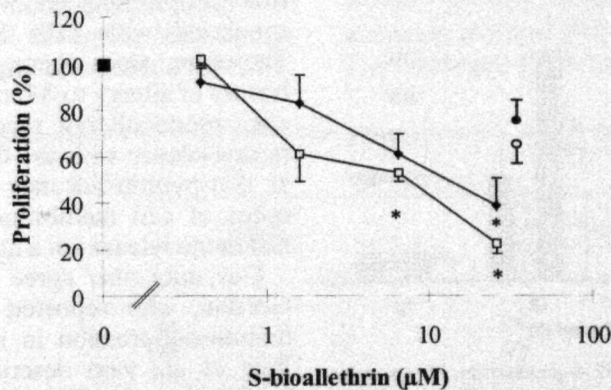


Fig. 3. Effect of S-bioallethrin on lymphocyte proliferation with cells cultured for 72 h from normal ($\blacklozenge$) and atopic donors ($\square$). Data are expressed as percentage of value obtained in absence of S-bioallethrin, which had been normalized to 100% ($\blacksquare$). 100% value was 0.515 ± 0.062 for normals and 0.529 ± 0.055 for atopics. Points are shown as mean $\pm$ SEM for $n=12$ (normals, 100% value), $n=6$ (normals, S-bioallethrin) and $n=15$ (atopics, 100% value), and $n=9$ (atopics, S-bioallethrin). Data are also shown for effect of pyrethrum at 72 h (normals $\bullet$ SEM, $n=6$; atopics $\circ$ SEM, $n=6$). *Proliferation significantly different from proliferation in absence of S-bioallethrin ($P < 0.05$).

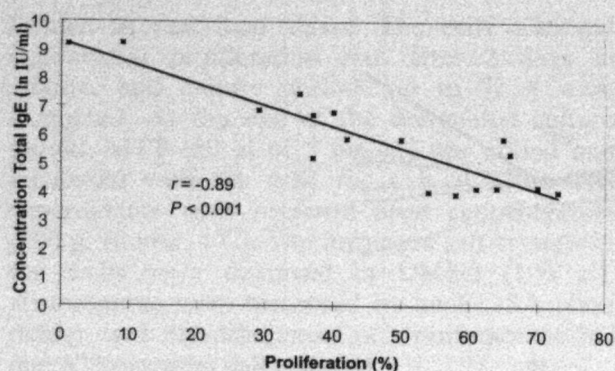


Fig. 4. Correlation of total serum IgE with lymphocyte proliferation after incubation with S-bioallethrin (6.5×10^{-6} M) ($n=17$). Total serum IgE was measured by PRIST (Pharmacia Upjohn). Proliferation (percentage) was calculated after 72-h culture period in relation to samples without S-bioallethrin. All individual values are mean of triplicates for each sample ($r=-0.89$; Pearson, regression to ln).

presence and absence of S-bioallethrin ($6.5 \mu\text{M}$) was significant only at the 24-h point ($P<0.01$).

A similar pattern could be observed with cells from atopic individuals. The ratio also decreased with increasing culture time (Fig. 7B). In contrast, in the presence of S-bioallethrin, the IL-4/IFN- γ balance increased after up to 48 h. As described for the nonatopic subjects, the effect of this pharmacotoxicologic relevant amount of the pyrethroid was significant only at 24 h ($P<0.01$). Atopics and nonatopics had a significantly different IL-4/IFN- γ ratio in the presence of S-bioallethrin at 24 h ($P<0.001$).

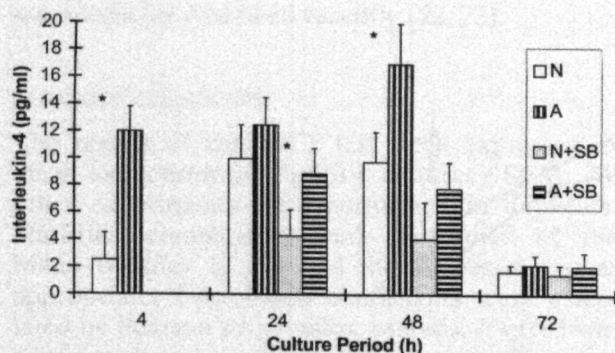


Fig. 5. Effect of culture time on IL-4 production. N: normals ($n=12$, SEM), A: atopics ($n=14$, SEM), N+SB: normals in presence of $6.5 \mu\text{M}$ S-bioallethrin ($n=6$, SEM), A+SB: atopics in presence of $6.5 \mu\text{M}$ S-bioallethrin ($n=8$, SEM). IL-4 was determined in duplicates with ELISA from DPC Biermann (Bad Nauheim, Germany). *Cytokine concentrations significantly different between atopics and nonatopics ($P<0.05$, t -test).

Histamine liberation

S-bioallethrin induced histamine release from basophils of subjects from both the atopic and nonatopic groups (Table 1). Overall, this release was significantly greater in cells from atopic subjects ($P=0.0431$, $n=6$ pairs, Wilcoxon). The response to anti-IgE was, as expected, significantly greater in the atopic group ($P<0.0001$), but the response to FMLP did not differ between the groups (Table 1).

Discussion

Increased environmental pollution has been proposed as a cause of increasing allergic disease, although this is a controversial issue (24). The data presented in this study may help to evaluate the specific health risks caused by pyrethroid contamination. This study has clearly demonstrated that the synthetic pyrethroid S-bioallethrin (Fig. 1) has profound effects on the immunologic system. The response is affected by the atopic history of the subjects. Thus, allergic individuals may have an increased risk of an adverse event after exposure to these agents, which are commonly employed in agriculture and wood preservation, and as insecticides within the home.

The MTT test is widely used as a marker for proliferation studies (25, 26). S-bioallethrin and the natural pyrethrum reduced lymphocyte proliferation with cells from both nonatopic and atopic subjects. For S-bioallethrin, this was observed at lower concentrations in the atopic patients than in the nonatopic subjects (Fig. 3). Furthermore, at these concentration ranges, basophil degranulation may occur as demonstrated with the histamine-liberation tests (Table 1). The sensitivity of the cells from atopic subjects was significantly increased compared with cells from nonatopic subjects. Therefore, since these subjects did not have a history of allergy to S-bioallethrin, pyrethroids may elicit pseudoallergic reactions. This observation is in accordance with the findings of Moya-Quiles et al. that pyrethroids may interfere with the packing order of cell membranes and hence may cause histamine release via a nonspecific mechanism (27).

Our data also agree with those of Stelzer & Gordon, who reported that pyrethroids caused immunosuppression in rodents (8), and those of Puig et al., who described inhibition of human lymphocyte proliferation rates with $>24 \mu\text{M}$ cypermethrin and $>25.5 \mu\text{M}$ fenvalerate (28). Our studies employed lower concentrations.

The evaluation of an "immunotoxicologic profile" of deltamethrin led to the suggestion that most of the pyrethroids cause immunosuppression (10).

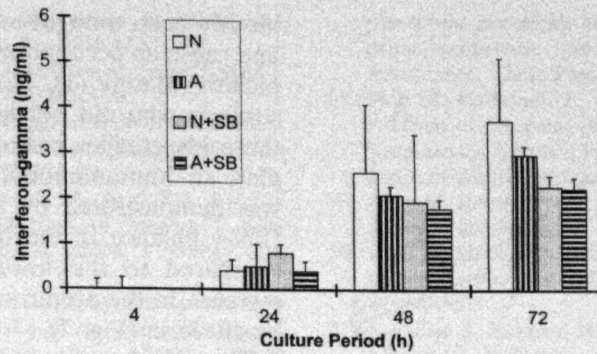


Fig. 6. Effect of culture time on IFN- γ production (experimental details as in Fig. 5).

It is as yet unclear whether the reported cytotoxic effects are based on the specific genotoxicity of pyrethroids which causes the immunosuppression (28). The mutagenic effects of pyrethroids at the relevant gene loci of cytokines must also be taken into consideration (29, 30). Structure-activity studies demonstrate that cypermethrin, deltamethrin, and fenpropathrin possess a higher genotoxicity than fenvalerate and permethrin (31). In the same study, the use of a cytokinesis block proliferation index (CBPI) is proposed, in agreement with our suggestion of a cytokine ratio (IL-4/IFN- γ) to calculate the special immunotoxicity of pyrethroids. In contrast, recent work has demonstrated that the genotoxicity of fenpropathrin is relatively low (32) and that the lowest effect level of new synthetic pyrethroids such as supermethrin is relatively high (range 10^{-5} M) in studies using porcine and bovine peripheral blood lymphocytes (33). However, synergistic effects may aggravate immunotoxicity and allergotoxicity (34). Pre- and neonatally DDT-exposed mice showed an increased response to pyrethroids (35), and pre-natal exposure modulates rat natural killer (NK) cell function (36). Furthermore, the interactions of pyrethroids with neuronal receptors may be an important regulator of immunomodulation (37, 38).

The mechanisms by which agents such as pyrethroids modulate allergic reactions are related to the lipophilic character of the effective molecule (hapten). The solubility of, say, permethrin in water (1.5×10^{-5} M) is much lower than that of S-bioallethrin (4.7×10^{-4} M). Thus, permethrin may better be integrated into the lipophilic lymphatic tissue. In contrast, S-bioallethrin may be better transported in the blood system and therefore find the target cells more rapidly.

Although the results of the MTT test clearly demonstrate the immunotoxicologic potency of pyrethroids, the IL-4/IFN- γ ratio measurements may provide a higher sensitivity with respect to the functional ability of the lymphatic system. IL-4 and IFN- γ production is widely investigated and may explain pathologic B-cell activation and hyper-IgE secretion. It has been suggested that basophils are the major IL-4-producing cell type, not PBMCs, as used in our experiments (39). Van Neerven et al. found that the antagonistic responses of IL-4 and IFN- γ function at different levels of T-cell development. Thus, the influence of insecticidal pollutants on these pathomechanisms may be of special interest, as well reflected by the IL-4/IFN- γ ratio screening method (40). In this study, the exclusive T-cell mitogen PHA has been used. Unfortunately,

Table 1. Histamine release induced by S-bioallethrin, FMLP, and anti-IgE from basophils obtained from normal ($n=6$) and atopic ($n=6$) donors

		Histamine release (%)				
		Normal		Atopic		
	Concentration (μ M)	Mean	$\pm$ SEM	Mean	$\pm$ SEM	<i>P</i>
S-bioallethrin	0	4.8	1.2	5.6	1.3	Two-tailed 0.0431*
	0.4	7.9	1.5	8.0	1.5	
	1.6	7.2	1.6	7.8	2.4	
	6.5	8.1	1.4	10.2	2.5	
	26	7.5	1.5	10.0	2.3	
Anti-IgE	1/3000 diluted	12	5	71	12	<0.0001**
FMLP	1	61	9	66	15	NS

*Wilcoxon matched-pairs signed-ranks test; **Student's *t*-test.

NS: not significant.

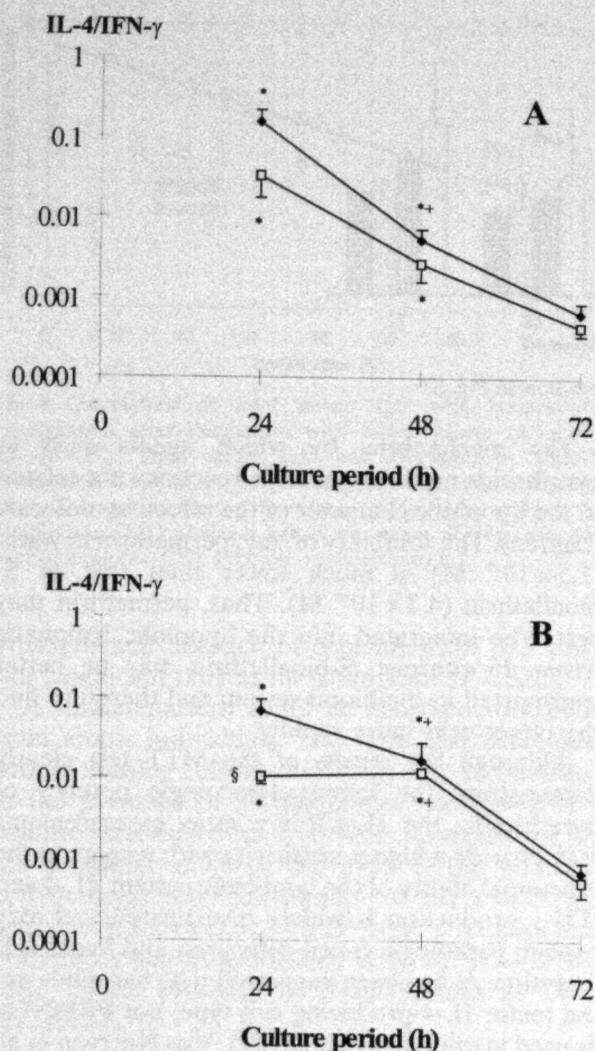


Fig. 7. Effect of S-bioallethrin (6.5×10^{-6} M) on IL-4/IFN- γ ratios from lymphocytes of normal (A) and atopic (B) donors. Closed symbols represent in absence and open symbols in presence of S-bioallethrin. Data are given as mean $\pm$ SEM for $n=12$ (normal donors, absence of S-bioallethrin) and $n=14$ (atopic donors, presence of S-bioallethrin) and $n=14$ (atopic donors, absence of S-bioallethrin) experiments. *Ratios significantly different from 72-h ratio ($P<0.02$); +ratios significantly different from 24-h ratio ($P<0.02$). §Significance between nonatopics (A) and atopics (B) at 24-h value in presence of S-bioallethrin was calculated to be $P<0.001$ (t -test).

suitable test systems for immunotoxicologic assessments of pyrethroids are not available. Commercial ELISA techniques are not sensitive enough to determine IL-4 and IFN- γ in human blood samples. Therefore, lymphocyte cultures are necessary to obtain sufficiently high cytokine concentrations.

The correlation between S-bioallethrin-induced inhibition of lymphocyte proliferation and circulating IgE (Fig. 4) clearly demonstrates the higher sensitivity to pyrethroid pollutants in more severe atopy. Natural sources of pyrethrum have pre-

viously been thought to possess allergenic potency and cause an occupational disease, chrysanthemum pickers' allergy (1). However, our data on atopic subjects who did not have a history of allergy to these insecticides indicate that S-bioallethrin may play an immunomodulatory role. A modulation was demonstrated by the reduction in the IL-4/IFN- γ balance in the normal subjects after 24 h, compared to the lower decrease, and even an increase in the atopic subjects after addition of S-bioallethrin (Fig. 7).

These findings are the first demonstration of the immunotoxicologic properties of the synthetic pyrethroid S-bioallethrin by this combined *in vitro* approach with human lymphocytes and basophils. Further studies will investigate the responses of lymphocytes of patients who are sensitive to these agents.

Acknowledgments

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