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Role of Antigen-Induced Cytokine Release in Atopic Pruritus

Abstract

In order to further evaluate the role of cytokines in the induction of atopic pruritus, leukocytes from 10 atopic eczema patients or 10 nonallergic controls were stimulated *in vitro* with mite or birch pollen antigen for 1 and 4 days. Subjects were prick-tested with the supernatants, and whealing and itching were evaluated 20 and 60 min later. The supernatants were also examined for the contents of GM-CSF, IL-2, IL-6 and IL-8 by ELISA and TNF α . Two hours prior to testing, the antihistamine cetirizine (20 mg) or a placebo tablet were given to the patients according to a randomized, double-blind study protocol. After pricking with antigen-stimulated leukocyte supernatants, 6 of 10 patients but no controls reacted mostly at 20 min with whealing and/or pruritus. In the cetirizine-treated group, no decrease in these skin reactions was seen compared to placebo. Analysis for cytokines showed increased levels of IL-8 in allergen-stimulated samples, with no correlation to the induction of itching or whealing by these supernatants. IL-6 levels were low and variable, and GM-CSF, IL-2 and TNF α levels were always below standard values. These data show that leukocytes selectively release IL-8 in response to *in vitro* antigen stimulation. They furthermore provide additional support for the concept that as yet to be identified products play a role in atopic pruritus.

Key Words

Antihistamines
Atopic eczema
Cytokines
Pruritus

Introduction

Atopic eczema is a frequent, severely pruritic disease that responds well to corticosteroids, but only poorly to antihistamines [1]. Itching is the most vexing problem for patients with atopic eczema, but the exact mediator inducing this symptomatology remains uncertain. While pruritus can be induced by a number of mediators other than histamine, including mast cell proteases and neurotransmitters [2], cytokines are increasingly implicated as possible causative agents. Evidence for this is based on the response of atopic itching to corticosteroids and cyclosporin, on the pronounced involvement of cytokine-producing leukocytes in atopic inflammation, and on the induction of itching by se-

lected cytokines like GM-CSF and interleukin-2 (IL-2) during human clinical studies [3–5].

In order to further explore the possible role of cytokines in the pathogenesis of atopic pruritus, we have previously performed a pilot study which provided some evidence that supernatants of mitogen-stimulated peripheral blood mononuclear cells of atopic eczema patients, and less so of controls, induced whealing and itching in the respective individuals [6]. Since allergens rather than mitogens are the natural stimulants for the inflammatory reactions in atopic eczema, we have designed the current pilot study by selecting atopic eczema patients with mite and/or pollen allergy, stimulating their blood cells with birch pollen or mite antigen *in vitro*, and examining the supernatants for whealing

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and itching reactions in skin tests. In order to rule out that the skin test reactions might have occurred secondarily to stimulating mast cell histamine release agents in the test supernatants, patients were pretreated with a potent H1 blocker or placebo in a randomized, double-blind fashion prior to skin testing. Furthermore, the supernatants were examined for the release of various cytokines in order to correlate cytokine secretion with skin test reactions.

Materials and Methods

Patients and Study Design

This pilot study was conducted in a double-blind, randomized, placebo-controlled, crossover design after approval by the local ethical committee. Ten atopic eczema patients, with the disease being defined according to the criteria of Hanifin and Rajka [7], and 10 healthy non-atopic controls were recruited and consented to participate. They had to be between 18 and 50 years of age with a positive (atopics) or a negative (controls) prick test to mite or tree pollen allergens and a normal skin reactivity to histamine (1 mg/ml of a histamine solution with a positive prick test reaction requiring a size of at least 4 mm in diameter at 10 min). Exclusion criteria were lactation, pregnancy, concomitant chronic or acute diseases, psychological disorders, drug addiction, alcoholism, cardiovascular, renal or liver diseases and known allergy to cetirizine, other piperazines, lactose, cellulose, or corn starch. Patients who had taken dexchlorpheniramine during the previous 3 days, cetirizine or mequitazine during the previous 4 days, hydroxyzine, terfenadine, and loratadine during the previous week, ketotifen, NSAIDs and any other treatment during the previous 2 weeks, systemic corticosteroids during the previous month and astemizole during the previous 6 weeks were not eligible.

Treatment

Each subject was seen 3 times. At the first visit, subjects were evaluated clinically, a histamine skin test was performed to make sure that the patients were not taking antihistamines, and skin tests with house-dust mite and mixed tree pollen (Allergopharma, Reinbek, Germany) were done to identify specific allergies. If patients and controls were eligible, 10 ml of heparinized blood were taken from each subject and stimulated *in vitro* for 1 and 4 days with mite and birch antigens (Bencard, Munich, Germany). Supernatants were stored at -20°C until testing (see below).

At visit 2, at least 1 week after visit 1, a prick test with histamine was done on controls and patients who then took either 20 mg of cetirizine p.o. or placebo under the physician's supervision in a randomized, double-blind fashion. Two hours later, prick tests were performed on each subject with his own stimulated and unstimulated cell supernatants. At visit 3, at least 1 week after visit 2, a prick test with histamine was done again on controls and patients, and they then received the alternative drug or placebo, followed by prick tests as described for visit 2. Since cetirizine at only half the dose used is very effective in suppressing histamine-induced whealing [8] and since the test physician would have been unblinded if a histamine prick test was done during skin testing with the supernatants, this positive test was omitted.

Antigen Extracts

Lyophilized antigen extracts (Bencard) of housedust mite (*Dermaphagoides pteronyssinus* No. 3115) or birch pollen (No. 3114) were dissolved in PBS (GIBCO RBL, Berlin, Germany) at a final concentration of 1 mg/ml, dialyzed (dialysis tubes; Sigma, Munich, Germany) against PBS overnight and sterilized through a 20- μm Millipore filter (Sarstedt, Nümbrecht, Germany). The antigen solutions were then aliquoted and stored at -20°C until use.

Leukocyte Stimulation

In pilot experiments, we had noted that cytokine secretion occurred more reliably with whole heparinized blood than with Ficoll-separated mononuclear cells. For the present experiments, 1 ml of whole heparinized blood diluted with 9 ml of RPMI 1640 (Biochrom, Berlin, Germany) supplemented with 2 mM glutamine was therefore taken for *in vitro* stimulation in the presence of 10 $\mu\text{g}/\text{ml}$ mite or birch antigen or control medium only. Stimulation was allowed to proceed for 24 and 96 h at 37°C , 5% CO_2 . Medium and antigen without added blood were incubated during the same period as a control. Supernatants were harvested after centrifugation at 2,500 g for 10 min and stored in aliquots at -20°C .

Cytokine Analysis

IL-2, IL-6, IL-8 and GM-CSF contents of the supernatants were measured by ELISA (IL-8 Quantikine, RD Systems, Boston, Mass., USA; GM-CSF and IL-2, Immuno TEC, Hamburg, Germany) according to the instructions of the manufacturers. IL-6 were determined by sandwich enzyme-linked immunosorbent assays, and TNF α by an *in vitro* assay, as described before [9, 10].

Skin Prick Tests

The cell supernatants of each subject were applied to the left and right lower arm, 2 h after either cetirizine or placebo had been given. Test sites were evaluated at 20 min and 1 h. Whealing was scored on a scale from 0 to 3 (0 = none, 1 = weak, 4-mm wheal; 2 = moderate, >4-mm wheal; 3 = strong, >4-mm wheal with pseudopods), and itching was assessed on a scale from 0 to 3 (0 = none; 1 = barely discernible; 2 = moderate; 3 = intense). Statistical evaluation was done on a descriptive basis since the low subject number did not allow significance testing.

Results

Table 1 summarizes the basic data of the 10 patients and controls. As can be seen, the eczema patient and the control groups were closely matched for age and weight, except for the distribution of males and females. Eight of the patients and none of the controls had an additional history of allergic rhinitis and asthma, and 3 patients had a positive prick test to pollen, 3 to mite, and 4 to both pollen and mite. The severity of the atopic eczema at the time of testing was marked to severe (3–4 $\times$) in only 2 patients and mild (++) in another 2, with the remaining patients having only minor (+) eczema or only dry skin.

Tables 2 and 3 summarize the skin prick test reactions to cell supernatants in the patients with atopic eczema and the

Table 1. Basic data of patients and controls

	Patients	Controls
Age, years	31.8±7.39	33.1±6.38
Sex (m/f)	6/4	3/7
Weight, kg	65.4±5.80	61.1±10.57
Allergic rhinitis	6	0
Asthma	2	0
Positive prick test		
Pollen	3	0
Mite	3	0
Pollen and mite	4	0
Severity of AD		
—	2	0
+	4	0
++	2	0
+++	1	0
++++	1	0

AD = Atopic dermatitis.

Table 2. Number of patients with positive prick test reaction to 0-, 24- and 96-hour culture supernatants in cetirizine- or placebo-treated atopic dermatitis patients

Duration of culture	Stimulus	Prick reaction after cetirizine				Prick reaction after placebo			
		20 min		60 min		20 min		60 min	
		W	Pr	W	Pr	W	Pr	W	Pr
0 h	mite antigen	0	0	0	0	0	0	0	0
	birch antigen	0	0	0	0	0	0	0	0
	medium C	0	0	0	0	0	0	0	0
24 h	mite antigen	2	0	0	0	3 ^b	2 ^b	1	0
	birch antigen	1	0	0	0	0	0	1	0
	medium C	0	0	0	0	0	0	0	0
96 h	mite antigen	2 ^a	2 ^a	0	1	3 ^b	3 ^b	1	0
	birch antigen	1	1	0	0	0	0	0	0
	medium C	0	0	0	0	0	0	0	0

W = Whealing; Pr = pruritus.

^a One patient with both pruritus and whealing.^b Two patients with both pruritus and whealing.**Table 3.** Total prick test score of patients or controls after treatment with cetirizine or placebo, developing whealing (W) or pruritus (Pr) in response to birch or mite antigen-stimulated cell supernatants or control medium

	Patients				Controls			
	20 min		60 min		20 min		60 min	
	W	Pr	W	Pr	W	Pr	W	Pr
Medium control	0	0	0	0	0	0	0	0
Medium + Ag.	1	0	0	0	0	0	0	0
Birch (1+4 days Stim.)	0	1	1	0	0	0	0	0
Mite (1+4 days Stim.)	13	9	2	0	0	0	1	0
Sum	14	10	3	0	0	0	1	0

Ag. = Antigen; Stim. = stimulated.

controls. Whereas only 1 control subject had one minor wheal to mite antigen at 60 min reading, 6 of 10 patients had whealing or itching reactions, and these occurred mostly for mite antigen, less so for birch antigen, and not at all for unstimulated supernatants, for medium alone, or for medium with antigen added. Whealing and itching was mostly detectable at 20 min and rarely at 60 min. There was no significant difference in the number of reacting patients comparing the applied supernatants from cells incubated for 1 or 4 days. The intensity of whealing and itching never exceeded

a score of 2 in individual patients and never a score of 1 in controls (table 3). Patients pretreated with antihistamines showed no reduction and even slightly more whealing and itching reactions (table 2).

Analysis of the supernatants for cytokines showed variable low levels of IL-6 (median 0.3 ng/ml) in patient and control supernatants with or without antigen stimulation. IL-8 secretion was in contrast markedly increased after allergen stimulation in both patients and controls with slightly higher levels in atopic patients (table 4). Levels were fur-

Table 4. IL-8 production (ng/ml) from unstimulated (–) or antigen (birch or mite)-stimulated (+) leukocytes of healthy controls (Co) and atopic dermatitis (AD) patients after 1 (D1) and 4 (D4) days of culture

	Mean	Range
Co D1 –	0.20	0.11–0.28
AD D1 –	0.33	0.19–0.32
Co D1 +	1.30	0.07–3.40
AD D1 +	3.18	1.05–11.0
Co D4 –	0.33	0.08–0.60
AD D4 –	0.28	0.13–0.44
Co D4 +	1.03	0.20–2.00
AD D4 +	2.81	0.17–12.00

thermore higher after 1 day than after 4 days of stimulation. Values varied, however, widely between individuals on a quantitative level and a correlation between IL-8 secretion and positive prick tests was only seen in patients with high IL-8 levels. Quantities of TNF α , GM-CSF and IL-2 were always below the levels of detection of commercial ELISAs or in vitro assays.

Discussion

The present study confirms our previous finding [6] that the majority of atopic eczema patients with positive immediate skin tests also respond to supernatants of their own stimulated leukocytes, while nonatopic controls fail to do so. In contrast, prick test results with histamine and substance P were lower in patients compared to controls, as previously observed by us and by others [6, 11, 12]. The reasons why not all patients reacted to their leukocyte supernatants remain elusive. Possibly, in vitro stimulation was not optimal regarding the dose of the antigen or its nature. Thus, extensive dose response curves could not be done in each patient because this would have exceeded the testing capacity of this study. Furthermore, since subjects had been screened with prick tests for mixed pollen of hazel, alder and birch, their specific responsiveness to birch might have been low or absent.

The ELISA analysis shows nevertheless that relatively large quantities of IL-8 were released into leukocyte supernatants, as observed previously with mitogen stimulation where high levels of IL-6 and IL-8 and no TNF α or IL-4 release had been induced [6]. A correlation between skin

test reactivities and IL-8 contents of the test samples was, however, not evident. Furthermore, allergens also induced the production of substantial quantities of this cytokine in nonatopic controls, suggesting a high allergenic potency, nonspecific stimulation or subclinical sensitization of these individuals. Similar observations have so far also been reported with IL-6 [13]. Since the timing as well as the quantity of secreted cytokines tested here fail to correlate with the skin test reactivity in the patients, one must postulate that cytokines other than those examined so far are responsible for the whealing and itching. Of the cytokines known until now, itching has been reported for IL-2 and GM-CSF only after local and systemic application [3–5]. These molecules can be excluded as possible candidates to explain the skin test reactions, on the basis of the present data. Alternatively, factors inducing histamine release from mast cells might be implicated, but in this case, cetirizine should have suppressed the skin test reactivity in the pretreated patients.

To sum up, we have shown here that peripheral blood mononuclear cells of atopic eczema patients can be stimulated to secrete IL-8 after antigen stimulation, that they produce only variable amounts of IL-6, and no measurable quantities of IL-2, TNF α and GM-CSF in this setting. Furthermore, we have demonstrated that these stimulated leukocyte supernatants contain agents that produce whealing and itching only in atopic eczema patients and have excluded that secondary release of histamine is involved. Possibly, because of the nonresponsiveness of some patients, specific reactivity of the tissue in the responsive patients or inhibitory factors in the nonresponsive patients might also explain the data. These unresolved aspects will have to be explored in future studies.

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