

Stimulation of Human Peripheral Blood Lymphocytes with Chironomid Hemoglobin Allergen (Chi t I)¹

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Abstract. Hemoglobins (Chi t I) of the dipteran species *Chironomus thummi thummi* are known to cause severe allergic diseases in humans. We tested the allergen-specific stimulation of human peripheral blood lymphocytes (PBL) by Chi t I and its nine main components. Further, we applied fragments of the well-analyzed component III, obtained by cleavage with trypsin as well as arginine protease. In this way, we screened the molecule in order to identify T-cell epitopes. The whole component was found to be immunogenic and to have regions demonstrating varying PBL stimulation. In addition, interindividual patterns of reactivity, probably due to genetic restriction, were found. A T-cell epitope could be shown to be within the site 98-111, as predicted by application of Rothbard's algorithms.

Introduction

Chironomidae are one of the largest insect families. They are distributed worldwide and are found in nearly all types of inland waters and some species in marine habitats. Larvae and adult midges of this Dipteran family are known to be aggressive inhalant allergens which cause severe bronchial asthma, rhinitis, conjunctivitis and urticaria [1-8]. In the larvae from the species *Chironomus thummi thummi*, which are often used in freeze-dried form as domestic fish food, most or all relevant allergens are hemoglobins (Chi t I) [9]. The primary structures of twelve individual hemoglobin components have been analyzed [10, 11], and the tertiary structure of component III was elucidated by X-ray analysis [12]. The following investigations describe the stimulation of human peripheral blood lymphocytes (PBL) with Chi t I, the nine main components of Chi t I, fragments of Chi t I components and the synthetic peptide 98-111. Allergen-specific stimulation was performed to determine T-cell epitopes within the hemoglobin molecule.

Materials and Methods

Human PBL were obtained from 40 patients suffering from bronchial asthma, rhinitis and/or conjunctivitis due to contact with fish food; all of them were RAST and skin test positive with Chi t I. In addition, we investigated PBL from 22 exposed but healthy and 18 non-exposed controls. Lymphocytes were separated by Ficoll centrifugation, washed twice with Hanks' solution and suspended in RPMI 1640 (supplemented with glutamine, human AB serum and penicillin/streptomycin) at a final concentration of 1×10^7 cells/ml for freezing and 5×10^7 cells/ml for the lymphocyte transformation test.

Lymphocyte transformation test: 100 μ l of the cell suspension were added to 100 μ l allergen (containing 1 μ g of Chi t I or its individual components or 0.032-1.3 nmol of the synthetic peptide). The control probes contained RPMI instead of the allergen. The cultures were incubated in triplicate or fourfold at 37°C in a humidified atmosphere of 5.8% CO₂ for 5 days. 0.074 MBq ³H-thymidine (NEN) per well were added, and cell proliferation determined by ³H-thymidine uptake during the last 6 h of the culture period. After freezing the plates, each well was harvested on a cellulose filter, treated with 2 ml scintillation fluid (Quickszint 1, Zinsser Analytic) and counted for 2 min with a β -counter (Beta-zint BF 5000).

The stimulation index (SI) relates results after stimulation with and without allergen. The coefficient of variation ($V = S/X \times 100\%$) of the triplicate and fourfold values was between 10 and 40% in all tests.

Chi t I was prepared as described [1], dissolved in a low molar ammonium bicarbonate buffer (< 0.05 molar; pH 9) to a final concentration of 500 μ g/ml and frozen in 20 μ l portions. They were thawed when required and further diluted with RPMI. A final

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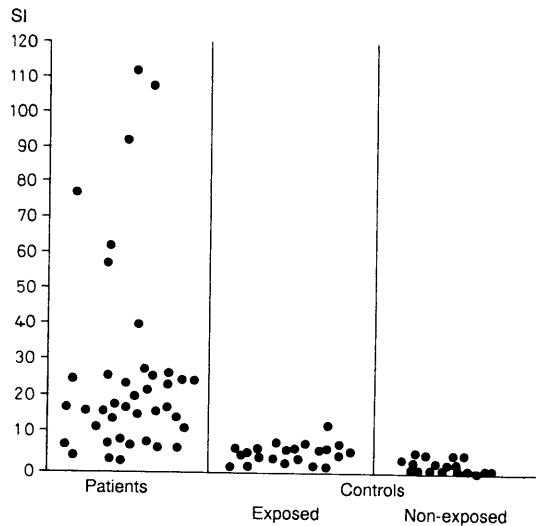


Fig. 1. Stimulation of human lymphocytes with chironomid hemoglobin (Chi t I) in three study groups. SI = relation of results after stimulation with and without the allergen Chi t I (5 µg/ml). An SI of 10 corresponds to 3,000–5,000 cpm.

concentration of 5 µg/ml (0.33 nmol/ml) was used, because 10 tested patients showed the optimal reaction at 0.33 nmol/ml.

Cleavage of Chi t I components was performed enzymatically with trypsin (5 h/37°C, Boehringer-Mannheim) and arginine protease (1 h/20°C, 4 h/37°C, Boehringer-Mannheim). The cleavage procedure was stopped by lyophilization. Nine fragments of component III with a minimum length of 7 amino acids were isolated by HPLC, and each fragment used for the stimulation assay. Seven healthy controls were used to determine the unspecific stimulation occurring with every peptide.

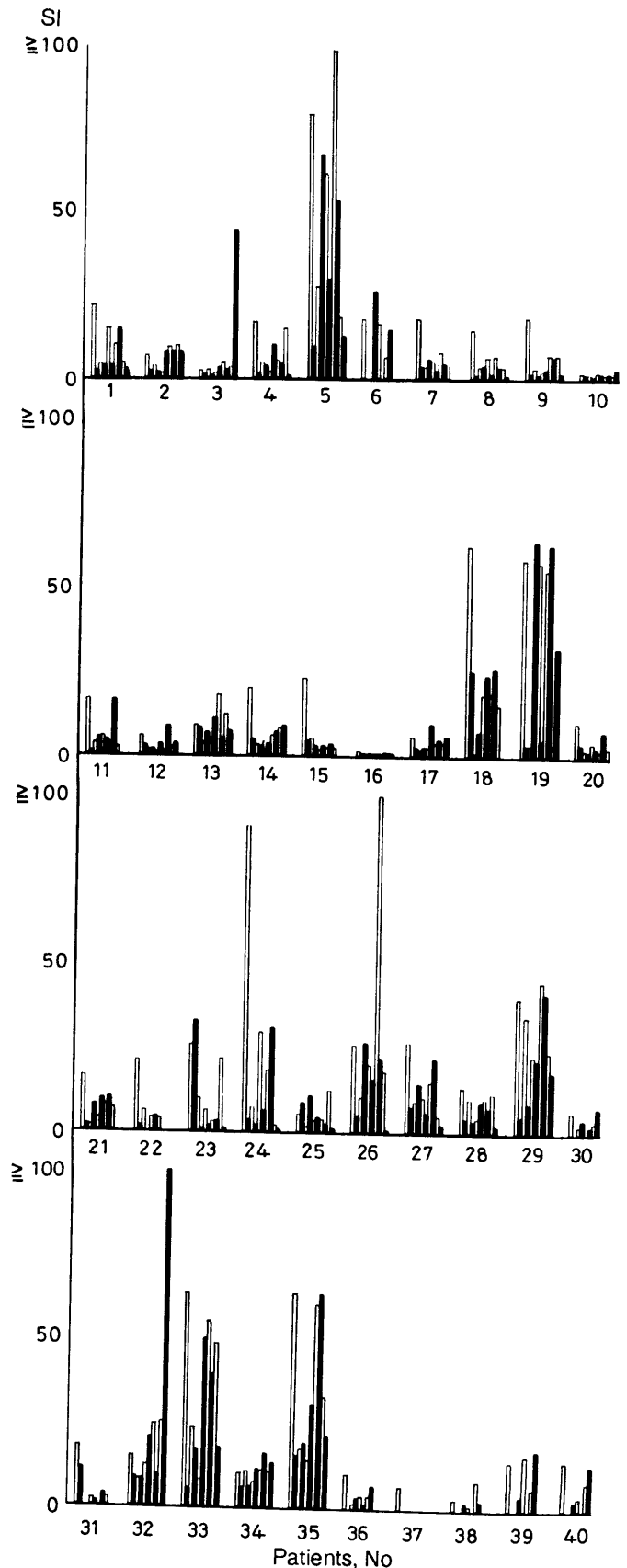
The Chi t I peptides were dissolved in ammonium bicarbonate buffer and lyophilized twice to remove the acid from the HPLC separation. Aliquots of 65 nmol were dissolved in 1 ml ammonium hydrogen carbonate buffer and frozen in portions of 100 µl. After thawing, the portions were diluted with RPMI (see lymphocyte transformation test).

The synthetic peptide 98–111 was a kind gift of Dr. S. Modrow, Max v. Pettenkofer-Institut, Munich, FRG.

Three groups of patients and controls were examined:

- (1) 40 symptomatic, exposed patients which are RAST and skin test positive for Chi t I (specific IgE ranges from 0.8 to 635 U/ml; specific IgG ranges from 0.06 to 10,300 U/ml);
- (2) 22 allergen-exposed, but healthy controls (specific IgE in RAST <0.35 U/ml; specific IgG ranges from 0.03 to 0.54 U/ml), and
- (3) 18 non-exposed persons.

Fig. 2. PBL stimulation of 40 patients with Chi t I and nine Chi t I components. SI values are attenuated to 100. Each row shows the response from 10 patients to the ten stimuli. For every patient, the ten stimuli are shown in the same order: Chi t I (□) component I (■); II (□); III (■); IV (□); VI (■); VII (□); VIII (■); IX (□); X (■).



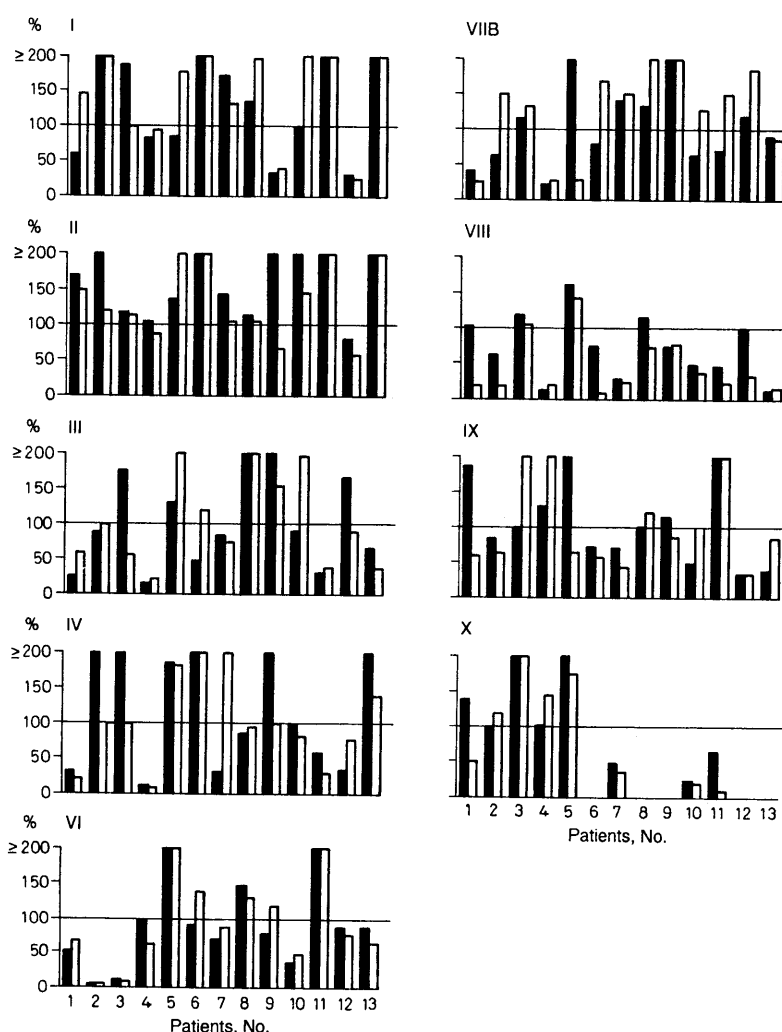


Fig. 3. PBL stimulation of 13 Chi t I-allergic patients with peptides of nine Chi t I components. The stimulation is expressed as a percentage of the stimulation with the native component. ■ = Arginyl peptides; □ = tryptic peptides. The results are attenuated to 200%.

20 patients (group 1) also showed sensitization to common allergens. 15 (38%) of them had specific IgG antibodies for Chi t I in addition to their specific IgE antibodies [13]. In the exposed control group, there were only 29% atopics, and 8% had Chi t I-specific IgG antibodies.

Results

SI with lymphocytes of 40 patients sensitized with Chi t I ranged from 5 to 100. The lymphocytes of only 3 patients barely responded ($SI < 5$, fig. 1). The SI of patients are significantly higher than those of exposed but healthy controls ($p < 0.01$, Duncan test). Mainly, non-exposed controls did not react. Using the nine main components of Chi t I separately, we found an individual stimulation pattern for each patient. Lym-

phocytes normally reacted to a greater extent with two or three components. VIIIB and VIII are most reactive; all patients responded to them, whereas for the other components the SI is sometimes below 1, especially with component X. In some cases, a higher SI was seen with the single component than with the whole Chi t I (fig. 2). Non-exposed controls did not react ($SI < 1.5$ /data not shown).

13 patients of whom enough PBL were available and who showed a $SI > 6$ with Chi t I were tested with the cleavage products of the nine components (fig. 3). The cleavages were at arginine and at arginine and lysine (smaller peptides). According to the results, three different groups of patients were obtained: group 1: SI remains the same; group 2: SI increases; and group 3: SI decreases. Three non-exposed control persons showed no reaction with the cleavage prod-

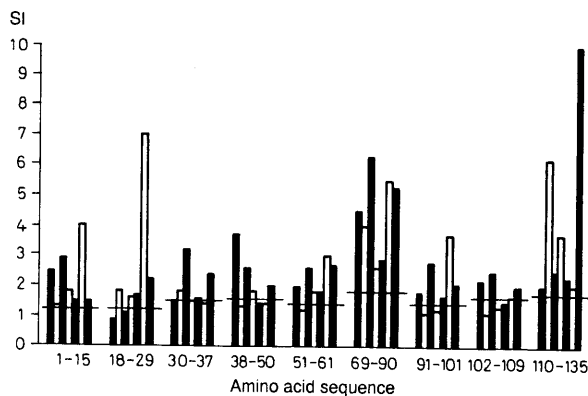


Fig. 4. PBL stimulation of 7 Chi t I-allergic patients with peptides of component III (mean values of two independent tests). Considering all 7 patients (shown consecutively for each sequence), the whole component is immunologically active. — = Mean of 7 non-exposed controls; ■ = patients 1, 3, 5, 7; □ = patients 2, 4, 6.

ucts of each component ($SI < 1.5$ /data not shown). Because the cleavage procedure with trypsin at component III does not destroy the lymphocyte response in most cases (8 of 13 patients) we started an epitope screening with the tryptic peptides of this well-known component.

The HPLC-isolated fragments of Chi t I component III gave rise to lymphocyte stimulations which again differed from patient to patient. The most reactive peptide was fragment 69–90 which shows a significant reaction in all tested patients (fig. 4).

Using Rothbard's algorithm for epitope prediction, there should be an epitope in component III at position 98–111 [14]. This peptide was synthesized and used for stimulation in the same patients who were tested with the isolated HPLC fragments and an additional panel of 5 patients. All tested patients showed a minimal SI of 3 with the whole component III or their cleavage products. Using different concentrations in 10 patients, it was seen that the optimum peptide concentration differs slightly between patients. In any case, lymphocytes from 9 patients showed a clear reaction at 3.2 nmol/ml (fig. 5), whereas the lymphocytes of non-exposed controls did not react ($SI < 1$, data not shown). Although the 7 tested patients did not react to peptide 102–109 (fig. 4), 5 of them responded to peptide 98–111. Thus Rothbard's prediction could be verified for this site.

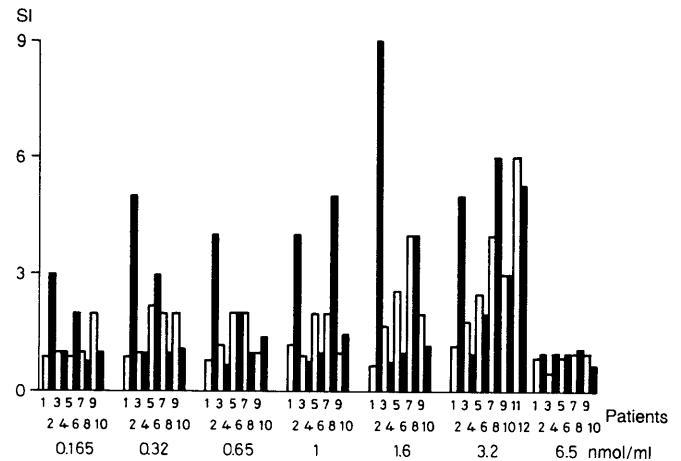


Fig. 5. PBL stimulation of 12 sensitized patients with the synthetic peptide 98–111 of component III. Different concentrations were used in 10 patients (shown consecutively), giving in most cases a maximum stimulation at 3.2 nmol/ml. This concentration was used in 2 additional patients. The first 7 patients are the same patients who were tested with the tryptic peptides (fig. 4).

Discussion

The allergen-specific proliferation of patients' lymphocytes after Chi t I exposure is mostly very high, indicating that chironomid hemoglobins are strong immunogens. Moreover, many people who have no sensitization to common allergens react to Chi t I; 49% of Chi t I-sensitized persons are non-atopic.

The proliferation of patients' lymphocytes is significantly higher than that of exposed and non-exposed controls. These findings agree with previous results [15, 16] using the house dust mites *Dermatophagoides pteronyssinus* and *D. farinae*. Only Cavaillon et al. [17] reported that PBL proliferation of *Dermatophagoides*-allergic persons was not significantly higher than that of healthy persons. However, he reported different mean values and a lack of significance possibly due to the small collective.

Comparing these examinations with our Chi t I study, it is also important to see that in case of the house dust mite only exposed controls can be tested because everybody is more or less intensively in contact with their immunogenic components. In any case, exposed controls show a low to moderate response to the allergen. However, our investigations included definitely non-exposed controls who did not respond.

Some patients showed higher SI values with single components than with Chi t I (fig. 2). Their immuno-

genic reaction is mainly triggered by the epitope(s) of one component which is present there in a higher relative concentration. In other patients, single hemoglobins reach only smaller values, and only the sum of all components with all epitopes approximates the relatively high proliferation due to Chi t I.

As mentioned, after enzymatic cleavage of the components, the SI changes in three ways. When the SI remains the same the amount of antigenic sites seems not to be influenced by the cleavage. When the SI with the fragments is lower than with the whole component the relevant topographic or linear T-cell epitopes are destroyed [18]. An increase in the SI after cleavage could possibly be explained by the fact that internal determinants become accessible to T cells; maybe the cleavage in vitro is more efficient than within the antigen-processing cell. It is also possible that antigenic sites, which are not important in the cell, will be accessible by stronger in vitro cleavage in the lymphocyte transformation test.

The SI value does not correlate with the specific IgE antibody level, as was reported in case of *D. pteronyssinus* and *D. farinae* [15, 16] and for *Amb a I* [19]. Besides, the correlations in these three studies are very weak or, excluding the healthy controls, really poor. However, such a relationship may be antigen dependent. Black and Marsh [19] could not correlate the SI with specific IgE-mediated sensitivities (skin test, histamine release) for either *Amb a V* or *Lol p I* allergen. In addition, it could not be confirmed that IgG antibodies are a prerequisite for proliferative responses. In our study, 38% of the patients and 8% of the exposed control group possess Chi t I-specific IgG [13]. 78% of the exposed controls show a low to moderate response to Chi t I, 22% did not react.

Each patient's lymphocytes exhibit an individual stimulation pattern. This may be due to genetic restriction. As already shown for other allergic diseases, there is often a restriction with respect to the HLA-D locus [20, 21]. In addition to the allergen, the HLA-D gene product (MHC class II molecule) which is expressed on B cells, macrophages and dendritic cells, is necessary to stimulate T-helper cells. It is likely that a certain epitope is recognized exclusively or predominantly by a certain MHC II molecule. Peptides may help to study the role of genetic restriction. However, it may not be possible to determine a preferred MHC II molecule for Chi t I because this allergen consists of many epitopes which probably show different restrictions. It seems that genetic restriction can only be

evaluated using small peptides: 6 patients showed the highest SI to the peptide 98–111; all express HLA-DQw 1 (fig. 5, patients 2, 7, 8, 10, 11 and 12). On the other hand, it is also possible that a single epitope is recognized by T cells in association with many different MHC class II molecules. Data supporting this hypothesis were reported for the malaria parasite *Plasmodium* [22]. Such epitopes would be good candidates for vaccines.

In this respect, our group has already started HLA typing with restriction fragment length polymorphism [23] and SSO hybridization [24] in these patients. We also intend to study the relevance of MHC II molecules in antigen recognition with monoclonal antibodies against different HLA-D gene products, as already done with Rye group I [25] and *D. farinae* allergen *Der f I* [26].

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