

Epitope mapping with peptides of *Chi t I* component III and immunomodulation of the *Chi t* immune response

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Background: Hemoglobins of the Diptera family are known as aggressive inhalant allergens.

Methods: The whole sequence of the insect allergen *Chi t I* component III was studied for T-cell epitope activity in cell cultures of 15 sensitized patients with a panel of 17 overlapping synthetic peptides and 12 peptides obtained by tryptic cleavage.

Results: All but one of the peptides tested were found to be immunogenic. The nonstimulatory sequence 102-109 and two other tryptic peptides (69-90 and 110-135) were used in inhibition experiments. The *Chi t I*-induced proliferation was downregulated by all tested peptides when compared with stimulation with *Chi t I* alone.

Conclusions: Because stimulation with phytohemagglutinin or tetanus toxoid was not influenced by the above-mentioned peptides, this mechanism seems to be specific for the antigen. Specific immunomodulation with nonstimulating peptides may offer new therapy approaches. (*J ALLERGY CLIN IMMUNOL* 1993;92:334-9.)

Key words: Allergen, T-cell epitope, *Chi t I* component III, immunomodulation

The Diptera family *Chironomidae* induces allergic reactions in approximately 20% of exposed persons.¹ Relevant exposure can be occupational, environmental, or hobby-related.² Hemoglobins of these insects have been identified as the causative allergenic substances.³ The hemoglobin *Chi t I* comprises 12 homologous components of the species *Chironomus thummi*.⁴ *Chi t I* component III, which has already been analyzed by x-ray studies,⁵ is now studied in detail. Component III and a mixture of its peptides obtained by tryptic cleavage were found to stimulate peripheral blood lymphocytes (PBLs) of sensitized patients.⁶

Looking for T-cell epitopes, we tested individual tryptic peptides of *Chi t I* component III in the lymphocyte transformation test. Twelve overlapping synthetic peptides of 21 amino acid lengths (20 amino-acid length for peptides 21-40 and 41-60) were synthesized and tested as well. Fur-

Abbreviations used

Chi t I: Hemoglobin of the species *Chironomus thummi thummi*
MHC: Major histocompatibility complex
PBLs: Peripheral blood lymphocytes
SI: Stimulation index
TT: Tetanus toxoid

thermore, four additional peptides were used for detailed immunogenic studies of C-terminal epitopes. One peptide (98-111) was synthesized corresponding to a Rothbard motif.⁷ The experimentally determined epitopes were compared with T-cell epitope predictions of Rothbard and Taylor⁷ and of Margalit et al.⁸

Inhibition studies with three tryptic peptides provided evidence for specific downregulation of the *Chi t I* immune response with peptides. Regarding the epitope 90-110, it could be shown that a part of this sequence with little or no stimulation capacity still functions well as an inhibitor.

METHODS

Human PBLs were obtained from 15 patients who had bronchial asthma, rhinitis, and/or conjunctivitis as a result of contact with fish food. Each subject had a positive RAST and skin test results for *Chi t I* and showed a stimulation index (SI) with *Chi t I* component

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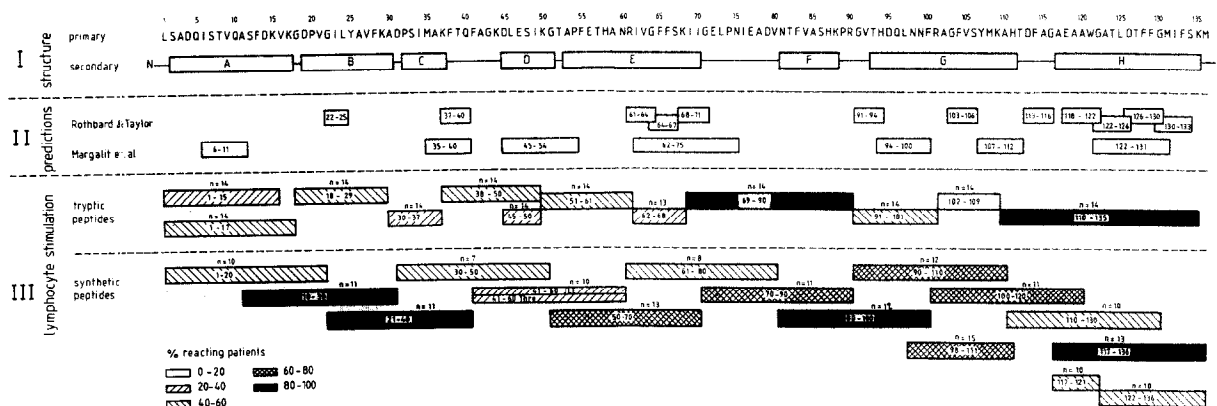


FIG. 1. The 136 amino acids of *Chi t I* component III contain eight α -helical structures (A to H). T-cell epitope predictions according to Rothbard and Taylor⁷ and Margalit et al.⁸ are compared with results of lymphocyte transformation test. An SI of >2 with peptides obtained by tryptic cleavage and an SI of >1.5 with synthetic peptides were regarded as positive. Because *Chi t I* component III consists of a neutral exchange isoleucine/threonine in position 57,²⁶ peptide 41-60 was synthesized with isoleucine, as well as with threonine. With the exception of sequence 102-109, all peptides stimulated lymphocytes in at least 20% to 40% of patients.

III of at least 3.0 in the lymphocyte transformation test.⁶ Lymphocytes were separated by Ficoll density centrifugation, washed twice with Hanks' balanced salt solution, and suspended in RPMI 1640 (Gibco, Eggenstein, Germany) supplemented with glutamine (Gibco), human AB serum (Baynisches Rotes Kreuz, Munich, Germany), and streptomycin/penicillin (Gibco).

In 96-well, round bottom plates, 5×10^4 cells per well were added to 100 μ l allergen. The allergen concentration was 0.32 μ mol/L for *Chi t I* and *Chi t I* component III and 3.2 μ mol/L for the peptides. Inhibition studies were performed: (1) by adding the respective peptide (3.2 μ mol/L) to *Chi t I*-stimulated cells and (2) by adding the respective peptide (3.2 μ mol/L) to phytohemagglutinin (5 μ g/ml) or tetanus toxoid (TT) (5 μ g/ml) stimulated cells.

The control samples contained RPMI instead of the allergen. Cultures were incubated in triplicate at 37°C in a humidified atmosphere of 5.8% CO₂ for five days. Thirty-seven kilobecquerels of tritiated thymidine (NEN, DuPont, Dreieich, Germany) per well were added, and cell proliferation was determined by tritiated thymidine uptake during the last 6 hours of the culture period. After the plates were frozen each well was harvested on a cellulose filter, treated with scintillation fluid (Quickszint 1, Zinser Analytic Frankfurt, Germany), and counted with a β -counter (LKB, Turbo, Finland).

Cleavage of *Chi t I* components was performed enzymatically with trypsin (5 hours at 37°C; Boehringer-Mannheim GMBH, Mannheim, Germany) and arginine protease (1 hour at 20°C; 4 hours at 37°C, Boehringer-Mannheim GMBH). The cleavage procedure was stopped by lyophilization, and fragments were isolated by high-performance liquid chromatography.⁶

Synthetic peptides were prepared by Fmoc chemical

strategy and purified by high-performance liquid chromatography.⁹ All tryptic and all but three synthetic peptides (41-60 Thr, 41-60 Ile, and 110-130) underwent amino acid analysis. Reaction of PBLs is given as SI (SI = counts per minute with allergen related to counts per minute without allergen). Background activity is between 500 and 1000 cpm, indicating an SI of 2.0 for 1000 to 2000 cpm. SIs of responding patients showed a significant difference between the mean maximal counts of wells that contained the peptides and the wells that contained RPMI at the $p < 0.05$ level (Student's t test).

RESULTS

Fig. 1 summarizes the results of the epitope mapping compared with the predictions of Rothbard and Taylor⁷ and Margalit et al.⁸ The predictions of Rothbard and Taylor⁷ comprise a certain motif (glycine followed by two hydrophobic amino acid residues), whereas Margalit et al.⁸ define epitopes in regions with amphipathic helices. The percentage of patients reacting to each peptide is given. Because six nonexposed control subjects showed SIs between 0.8 and 1.8 with the tryptic peptides, values above 2.0 were regarded as positive. With the exception of sequence 102-109, each tested peptide functions well as a stimulator. Regions of high immunogenicity, which stimulated PBLs in the majority of patients, were detected in the C-terminal sequence and in region 69-90.

In control subjects PBLs were found not to be stimulated by synthetic peptides (SI ≤ 1), indicating a positive reaction above 1.5. Synthetic peptides stimulated PBLs in at least 20% to 40% of

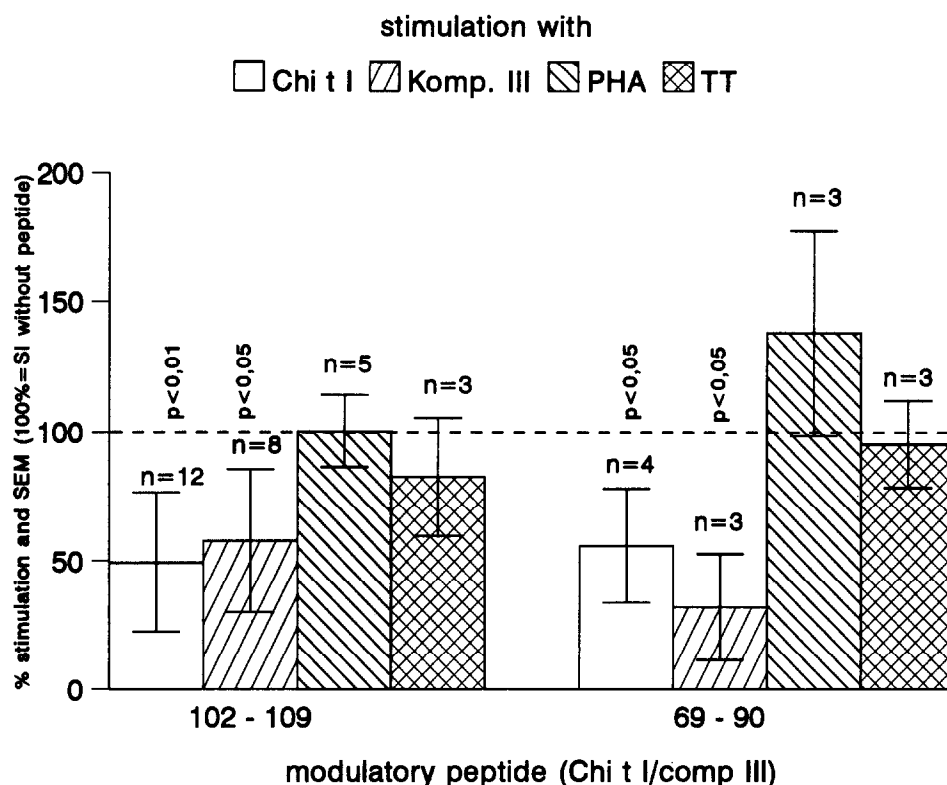


FIG. 2. Significant downregulation of the *Chi t I* or *Chi t I* component III induced tritiated thymidine uptake by two tryptic peptides. The modulatory functions of one stimulatory peptide (69-90) and one nonstimulatory (102-109) peptide were tested with *Chi t I* component III, phytohemagglutinin, and TT. Phytohemagglutinin-induced and TT-induced proliferation were not influenced by these peptides, proving the specificity of the reaction. The number of independent tests with PBLs from different patients is indicated as *n* over each bar. Regarding *Chi t I* downregulation, similar results were obtained with the stimulatory peptide 110-135. A value of 100% indicates the SI of stimulation in the absence of peptides.

tested patients. Comparison of the predicted with identified epitopes reveals that computer algorithms are not sufficient in this case. However, identified immunodominant regions such as the C-terminal site, which stimulated PBLs of all tested patients, are indicated by multiple epitope predictions (Fig. 1).

The stimulation capacity of sequence 102-109, compared with sequence 90-110 or 98-111, is dramatically reduced (Fig. 1). Nevertheless, this short region functions well in downregulation of the *Chi t I* or *Chi t I* component III induced lymphocyte proliferation (Fig. 2).

Fig. 3 shows the degree of reaction to the tryptic peptides in 14 patients tested. These data make clear that immunodominant regions are characterized by many reacting patients, as well as by high individual responses. Nevertheless, some peptides (1-15, 45-50) also induce strong cell proliferation in only a few patients.

Fig. 2 demonstrates the inhibition of the *Chi t I*

induced tritiated thymidine uptake with one stimulatory (69-90) and one nonstimulatory (102-109) peptide at a concentration of 3.2 $\mu\text{mol/L}$. A significant inhibition of between 50% and 70% was obtained with each peptide ($p < 0.05$). Similar results were found for the stimulatory peptide 110-135 in *Chi t I*-stimulated cultures (percent stimulation with *Chi t I* and peptide 32.5 ± 10.3 [$x \pm \text{SEM}$; compared with *Chi t I* alone = 100%, $n = 8$]). Peptides 69-90 and 110-135 were used for inhibition studies as examples of well-stimulating peptides. We chose these peptides because of their immunodominance (recognized by 85.7% and 100% of the patients). The phytohemagglutinin-induced and TT-induced proliferation were not influenced by the nonstimulatory peptide 102-109 and the stimulatory sequence 69-90. These results show that only the specific immune response can be blocked. As shown by peptide 102-109 (Fig. 4), this downregulation is dose-dependent. An individual, donor-specific pattern

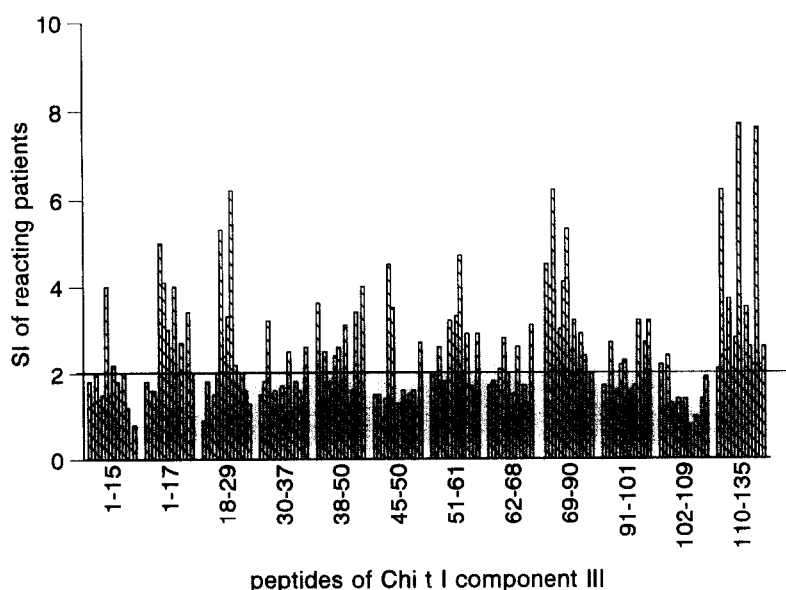


FIG. 3. Degree of reaction to 12 tryptic peptides in 14 patients. Because control subjects showed no reactions above 1.8, SIs above 2.0 were regarded as positive.

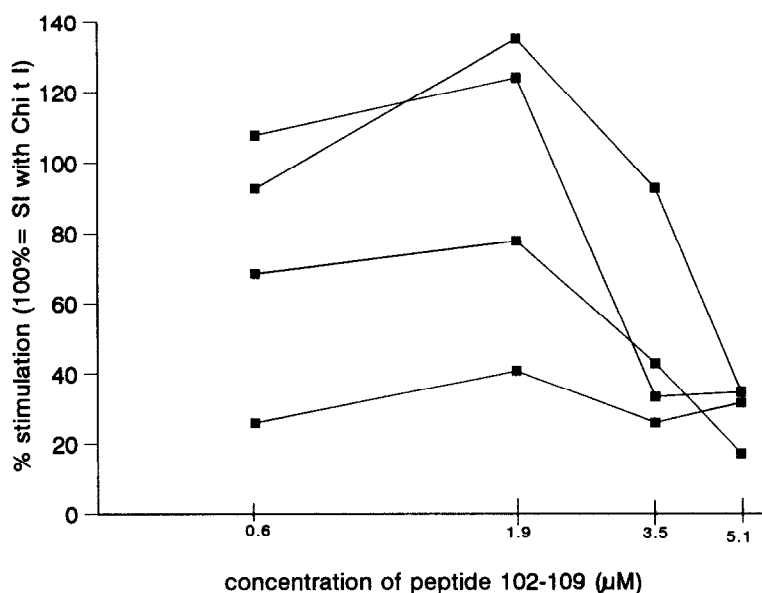


FIG. 4. Dose-dependent downregulation of *Chi t I* induced tritiated thymidine uptake with peptide 102-109 in four patients.

of downregulation was measured. The concentration of 3.2 $\mu\text{mol/L}$ was found to produce optimal results in three of four tested patients.

DISCUSSION

With the use of a panel of synthetic and naturally derived peptides the whole *Chi t I* component III was mapped for T-cell epitopes.

In contrast to studies on single, defined epitopes,¹⁰ *Chi t I* component III contains a lot of

T-cell epitopes. Regarding computer algorithms, a low agreement of predicted sites and experimentally defined epitopes was found. These predictions proved to be insufficient for human T-cell lymphotropic virus proteins and *Mycobacterium tuberculosis* antigen.^{11, 12} Many more epitopes, as predicted, were defined in the lymphocyte assay. As already mentioned for other antigens,^{10, 12} within *Chi t I* component III, we can only differentiate between immunodominant regions that

stimulate PBLs in the majority of patients and less immunogenic regions. However, when predicted sites according to Rothbard and Taylor⁷ were combined with those of Margalit et al.,⁸ the agreement with our findings is much higher and gives an idea of the high immunogenicity of *Chi t I* component III. Whether immunodominant parts of the molecule include more often predicted sites (such as the C-terminal end of *Chi t I* component III) remains to be determined. This high amount of immunogenic regions in the chironomid hemoglobin yields strong reactions in most patients even in the case of different HLA types. Our previous studies revealed a significant association between the immune response to *Chi t I* and HLA-DR and HLA-DQ alleles¹³ in monosensitized subjects. In addition, antibodies to DR and in part also to DQ and DP were able to reduce the lymphocyte response to *Chi t I*.¹⁴

Regarding our results, the length of T-cell epitopes does not seem to be restricted, because even the short peptides 45-50 and 117-121 are capable of stimulating PBLs in approximately 40% of the tested patients. But an optimal length of 8 to 12 amino acids, as suggested by Rothbard and Taylor,⁷ seems to validate major histocompatibility complex (MHC) binding. Indeed, immunodominance was only measurable in peptides of 19 to 25 amino acid lengths (10-30, 21-40, 80-100, 110-135, and 117-136). In addition, T-cell epitopes may contain "core regions," which are specifically involved in peptide binding and "tail regions," which are important with regard to protein stabilization.¹²

These results may explain the high percentage of patients reacting to any synthetic peptide. Each peptide with a length of 20 amino acids may contain some residues involved in binding, and all of these peptides may be structurally stable. T-cell epitopes are mostly expected to be linear. However, three-dimensional structures are also important, as demonstrated by clones that recognize myoglobin but not apomyoglobin, which has an identical primary but different tertiary structure.¹⁵ However, as consequence of structural features or direct involvement in binding as shown in our experiments for the peptides 102-109/90-110 and 1-15/1-17, the lack of only a few amino acids may dramatically reduce stimulation capacity.

Regarding therapy, it will be of great interest to create peptides with low or even no immunogenicity that are still capable of blocking the immune response. That peptide-based therapies should be possible has already been mentioned by

Wraith et al.¹⁶ They succeeded in competitively inhibiting T-cell reactivity in vitro and in vivo with peptides not stimulating myelin basic protein-specific T-helper cells but binding to the MHC. Using this type of peptide-based strategy, they were able to block the induction of experimental autoimmune encephalomyelitis in mice. Adorini et al.¹⁷ and Adorini and Naggi¹⁸ also observed that immunogenic and nonimmunogenic peptides of lysozyme can compete with each other for MHC binding in vivo.

That inhibition may be due to competition at the MHC is shown for *Dermatophagoides* peptides by O'Hehir et al.¹⁹ as well. Huang and Marsh²⁰ could also prove with *Amb a V* clones that inhibition occurs at the MHC binding site. As described by Wraith et al.¹⁶ inhibition may also involve the T-cell receptor, however. De Magistris et al.²¹ described antagonists of the T-cell receptor that act as antigen analogs. According to our studies, the site of competition may be the MHC molecule, as well as the T-cell receptor. However, as shown with one stimulatory peptide (69-90) and the nonstimulatory peptide (102-109), downregulation is highly specific because phytohemagglutinin-induced and TT-induced proliferation are not influenced.

In any case, specific downregulation of the *Chi t I* immune response is possible with each of the three tested peptides. Because T-cell epitopes are defined by cross reaction with the whole molecule, it has to be assumed that *Chi t I*-induced proliferation is inhibited by stimulating peptides. But nonstimulating peptides may not interact with any cell. This does not seem to be true for the sequence 102-109, because this peptide is able to block the immune response despite its nonstimulatory feature. In addition, no data exist on the question of whether the peptides tested in vitro also occur in vivo. Perhaps only a few of these sequences will be immunogenically relevant. As shown for the MHC molecule HLA-A 2.1, each bound peptide was nine residues long and shared a structural motif.²² However, naturally processed peptides bound to MHC class II molecules were found to display a high degree of heterogeneity in length (13 to 25 residues) and in the site of terminal truncation.^{23, 24} As outlined by Chiczy et al.,²⁵ it is possible that class II peptides are derived from a complex random mixture.

In conclusion, there is evidence that evaluation of specific downregulation of the lymphocyte response will be a first step toward a new therapeutic strategy. Epitope prediction may be helpful in

identification and characterization of immunogenic sites. Moreover, further studies should be focused on the question of whether special structural features are responsible for allergenicity.

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