

## REVIEW

# Chironomidae haemoglobin *Chi t I*—characterization of an important inhalant allergen

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Within the millions of insect species, *Chironomidae* represent a family of non-biting midges (order: *Diptera*) found worldwide. In the sub-family *Chironominae*, the trachea system is reduced suggesting a physiological role of its highly polymorphic haemoglobins for oxygen storage and transport. Their larvae living in swampy, often polluted water, require the binding of oxygen with high affinity. The thoroughly investigated species *Chironomus thummi* (Fig. 1) express 12 homologous haemoglobins (*Chi t I*, with its components: I, IA, II $\beta$ , III, IIIA, IV, VI, VIIA, VIIB, VIII, IX, X). These non-cellbound chromoproteins are synthesized in the fat body and represent approximately 80% of all proteins in the larvae haemolymph [1]. They consist of 136–151 amino acid residues and have molecular weights of 16 000 or 32 000 kDa for the monomeric and homodimeric forms respectively. In comparison, tetrameric human haemoglobin has a molecular weight of 67 000 kDa [1]. Haemoglobins are rather conservative proteins. The functional characteristics of oxygen binding are closely related to their structure. The large quaternary structural change between the low-affinity deoxy and high-affinity oxy state is induced by far smaller tertiary structural changes [2]. All globin genes are thought to be derived from one ancestral globin gene by duplication and mutation. It is assumed that they spread into different branches (plant, insects, molluscs, polychaets and later mammals) about one billion years ago.

Due to intensive genetic, structural, immunological and clinical investigations, haemoglobins of *Chironomidae* have become one of the best characterized allergens inducing IgE-mediated diseases in humans [3–27]. Our studies with *Chi t I*-component III give evidence for genetic restriction and a variety of B-cell and T-cell epitopes within this molecule. A detailed analysis of its immunogenic structures should contribute to develop new aspects of therapeutic strategies.

### Clinical data and antibody results

Since 1978 when the first two cases of *Chironomus thummi* allergy had been identified clinically and by allergy tests

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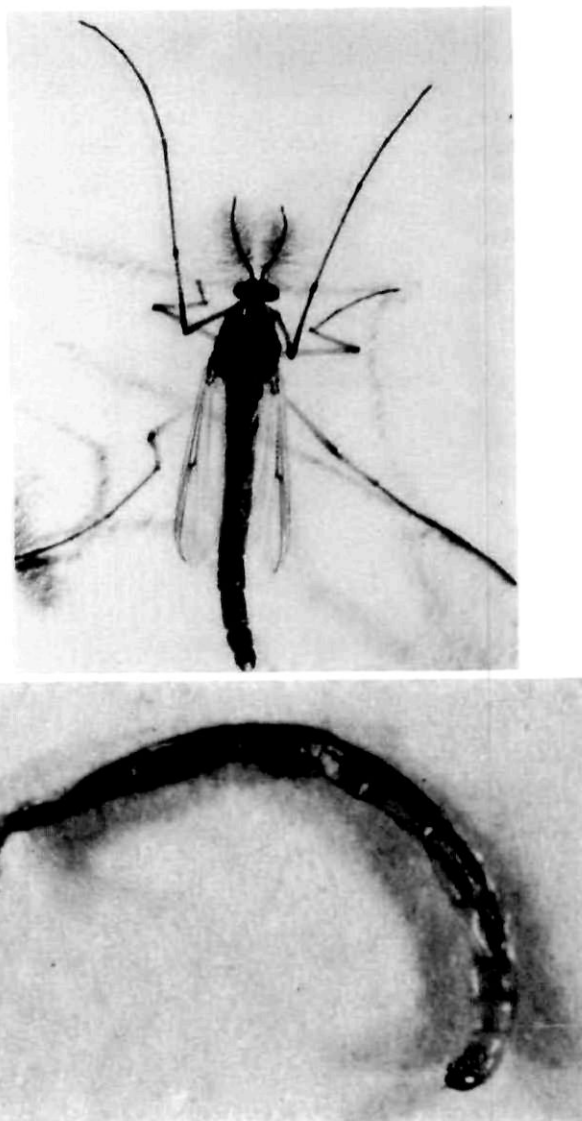


Fig. 1. Larvae and adult midge (male) of *Chironomus thummi*. The length of each is approximately 12 mm.

[3], sera of 642 *Chi t I*-exposed subjects have been examined in our department. It could be shown that approximately 20% of exposed subjects develop immediate type hypersensitivity with significant levels of specific IgE antibodies. Contact takes place in areas

abundant in water where seasonally an extraordinary number of *Chironomidae* midges occur, e.g. in Japanese rice fields [28,29,30], the Nile region of Sudan [31,32] or in lake areas in Wisconsin [33] (Table 1). In Germany, *Chi t I* allergy is widespread among fish breeders since larvae of *Chironomidae* are frequently used as fish food. Lyophilized products of these larvae produce airborne dust containing a high content of haemoglobin. People engaged in fish food production and animal trade are frequently affected due to the high exposure levels [11].

Patients with *Chi t I*-induced immediate type-hypersensitivity predominantly suffer from rhinitis and conjunctivitis followed by urticaria and bronchial asthma (Fig. 2). IgE antibodies determined by RAST or EAST methods were shown to correspond closely to skin-prick test results and correlate with exposure-related symptoms [21]. On the other hand, *Chi t I*-specific IgG antibodies were measurable in a sub-group of exposed people irrespective of the presence of symptoms.

Several cases were studied by bronchial challenges which revealed *Chi t I* to be an extremely potent allergen. Immediate asthmatic reactions or rhinitis were induced by less than 10 ng of the *Chi t I* allergen (Fig. 3). We observed that immediate type symptoms, as well as the level of specific IgE antibodies are related to the exposure degree, whereas a time-dependent risk of sensitization could not be verified [21]. Correspondingly, the number of subjects with complaints increased with rising exposure degree. Specific IgE-antibody levels above 3.5 U/ml preponderated in high exposure groups. Furthermore, these groups comprise a significantly higher proportion of asthmatic patients [21].

Crossreactivity between the individual 12 homologous *Chironomus thummi* haemoglobins (components of *Chi t I*) indicated in direct IgE measurements was confirmed by RAST inhibition [8]. It should be noted that haemoglobins are degraded during *Chironomidae* metamorphosis, the ensuing products in adult midges still reveal the capacity of binding human IgE antibodies [34]. IgE binding patterns of patients exposed and sensitized to individual *Chironominae* species (e.g. to *Chironomus thummi* or *Cladotanytarsus lewisi*) give evidence for crossreactivity even between distantly related species [8,14].

Further studies including larval and/or midge extracts of 33 *Chironomidae* species, of European, Japanese and North American origin showed that human IgE antibodies of German fish fanciers and US patients recognize antigenic sites (epitopes) present in nearly each of these insects belonging to different sub-families. This crossreactivity correlates to some extent with the evolutionary relationship of the investigated species which in turn is associated with the genome and structural diversity of the

Table 1. Investigations on hypersensitivity to *Chironomidae* larvae or midges. On average, approximately 20% of exposed people are sensitized

| Author                         | Study group (n)              | Country   | <i>Chironomidae</i> species tested | Exposure to insect stage | Contact due to   | Subjects studied by skin-prick test (n) | IgE antibody    | Sensitized subjects (n) |
|--------------------------------|------------------------------|-----------|------------------------------------|--------------------------|------------------|-----------------------------------------|-----------------|-------------------------|
| Knüsel J. et al. (1983) [69]   | Adults                       | Germany   | Crude extract/ <i>C. thummi</i>    | Larvae                   | Hobby            | 8                                       | 4 (RAST)        | 8                       |
| Schou C. et al. (1991) [71]    | Adults                       | Sweden    | <i>C. thummi</i>                   | Midges                   | Environment      | 71                                      | 71 (Magic lite) | 55                      |
| Tee R.D. et al. (1985) [32]    | Adults                       | Sweden    | <i>C. lewisi</i> <i>C. thummi</i>  | Midges                   | Environment      | 26                                      | 5 (RAST)        | 12                      |
| Adachi Y. et al. (1990) [66]   | Children                     | Japan     | <i>C. plumosus</i>                 | Midges                   | Environment      | 718                                     | 0 (RAST)        | 200                     |
| Ferro G. et al. (1993) [68]    | Adults                       | Italy     | <i>C. salinarius</i>               | Larvae                   | Environment      | 680                                     | 0               | 36                      |
| Baur X. et al. (1992) [11]     | Adults                       | Germany   | <i>C. thummi</i>                   | Larvae                   | Hobby/occupation | 95                                      | 290 (RAST)      | 95                      |
| Baur X. et al. (1992) [11]     | Adult atopies                | Italy     | <i>C. thummi</i>                   | Midges                   | Environment      | 0                                       | 94 (RAST)       | 9                       |
| Baur X. et al. (1992) [11]     | Adult atopies                | Japan     | <i>C. thummi</i>                   | Midges                   | Environment      | 0                                       | 229 (EAST)      | 10                      |
| (in cooperation with D. Marsh) |                              |           |                                    |                          |                  |                                         |                 |                         |
| Baur X. et al. (1992) [11]     | Clinically sensitized adults | Wisconsin | <i>C. thummi</i>                   | Midges                   | Environment      | 3                                       | 3 (RAST)        | 3                       |
| (in cooperation with S. Kagen) |                              |           |                                    |                          |                  |                                         |                 |                         |
| Total                          | 2119                         |           |                                    |                          |                  | 1601 (75.5%)                            | 696 (32.8%)     | 428 (20.2%)             |

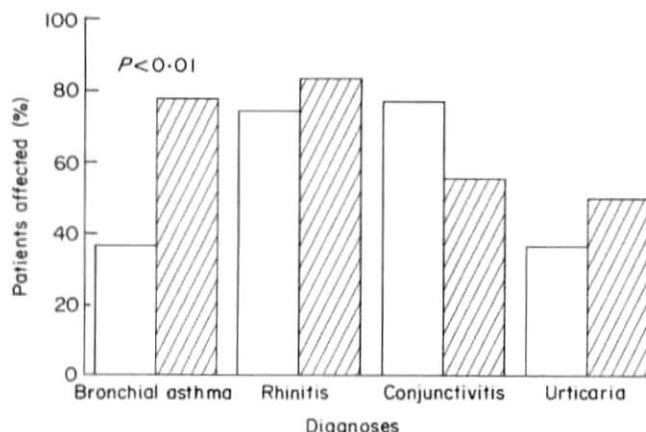


**Fig. 2.** Diagnoses of 95 *Chi t I*-sensitized German fish breeders or workers in fish food factories. The 21 occupationally exposed subjects showed a significantly higher prevalence of bronchial asthma ( $P < 0.05$ ). *Chi t*-contact due to □, Hobby. ■, Occupation.

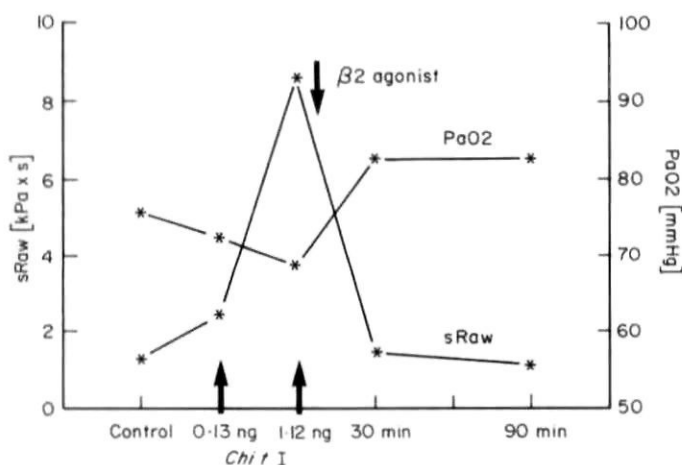


**Fig. 3.** Inhalative challenge with a total of 1.25 ng allergen (*Chi t I*) in an allergic fish breeder. A strong immediate asthmatic reaction was obtained. sRaw = specific airway resistance measured by a body plethysmograph (Jaeger, Wuerzburg, Germany; normal range of sRaw  $< 1.2 \text{ kPa} \times \text{s}$ ).

haemoglobin molecule [8]. It was also reported that direct measurement of human IgE antibodies as well as RAST-inhibition experiments and moAb assays show crossreactivity between components in crude extracts of the *Chironomidae* species *Chironomus thummi* on the one hand and in shrimps on the other hand [2,35]. This crossreactivity which has not been further substantiated with purified allergens and more sophisticated immunological methods, is obviously not related to haemoglobins. Crossreactivity between *Chironomus*, the silk moth *Antheraea pernyi* [8], sperm whale myoglobin and bovine haemoglobin [36] was excluded.

There was also evidence for a common T-cell recogni-

tion determinant in *Chironomus yoshimatsui* and *Dermatophagoides pteronyssinus*, the most important allergen sources in Japan [37]. According to the authors, the sensitization to *Dermatophagoides pteronyssinus* could promote a secondary IgE response to *Chironomus yoshimatsui* and vice versa, although there is no crossreactive IgE. The role of crossreactive T-cells in the immunopathogenesis of allergic diseases remains to be clarified, however.

#### Structure of *Chi t I* component III and its peptides

There is evidence that structural properties of proteins are important for allergenicity. It could be shown, e.g. for Melittin, a crucial bee venom allergen, that T- and B-cell epitopes as well as secondary structure elements such as  $\alpha$ -helices are necessary for the IgE response in mice [38].

The primary structures of all *Chi t I* components were first described by Braunitzer's group [39]. Homology of the primary structure between individual *Chi t I* components is between 28 and 99%. Furthermore, their amino acid compositions correspond roughly to the evolutionary interspecies relationship with other globins [27]. Compared with the human haemoglobin  $\beta$ -chain, a similarity of 16% (23 residues) to *Chi t I* component III exists [39]. More recently, polyacrylamide gel electrophoresis (PAGE) studies of haemoglobin extracts and especially modern gene sequencing have indicated a high degree of homology between haemoglobin DNA sequences of related species in *Chironomidae* [40,41].

The 3D-structure of *Chi t I* component III was evaluated by X-ray crystallography analyses [2] (Fig. 4). It is a globular protein consisting of eight helical and six non-helical segments. The theme group is located within a hydrophobic pocket and covalently bound to a histidine residue.

In our approaches to determine T- and B-cell epitopes, peptides either free or absorbed to a solid phase were used. These binding measurements raise the question whether allergen fragments are similar in structure to the respective sequence in the haemoglobin. Therefore, we performed studies of secondary structure elements with circular dichroism as well as NMR (nuclear magnetic resonance) in seven peptides comprising almost the whole sequence of *Chi t* component III [42,43]. Lengths of the peptides were 19 or 20 residues. NMR showed the peptides to exist in water predominantly in random coil conformations. However, each fragment also exhibits more or less structured conformation. The latter are similar to those of corresponding fragments in the intact protein.

The best defined structure, an  $\alpha$ -helix, was found for the N-terminal fragment 1–19 which contains T-cell and B-cell epitopes [43].

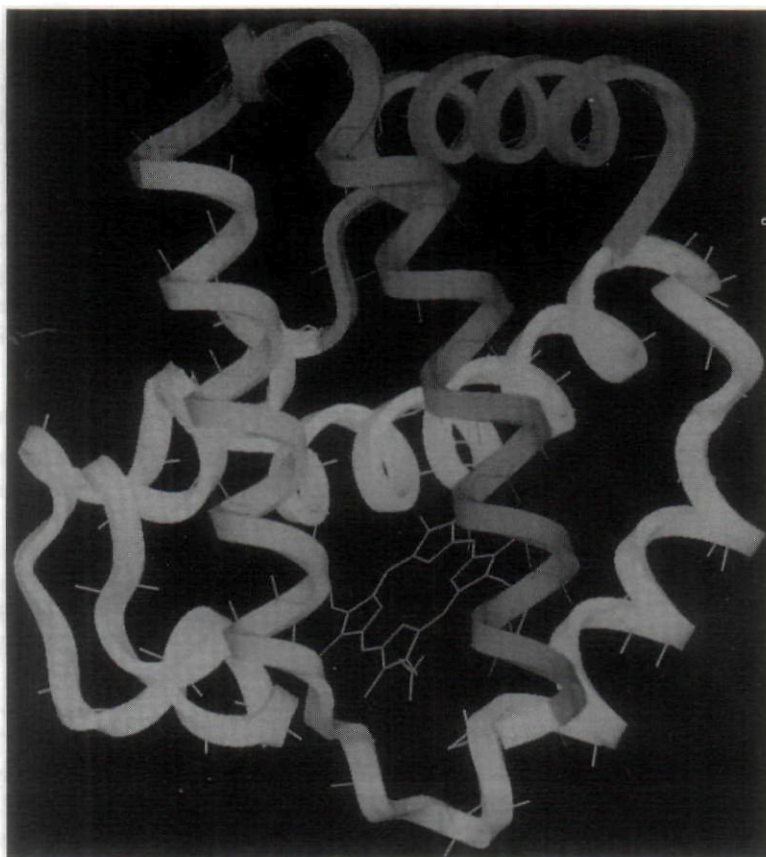


**Fig. 4.** Three-dimensional structure of *Chi t I* component III as evaluated by Steigemann and Weber [2]. The carbon backbone is schematically represented. The theme group is surrounded by eight  $\alpha$ -helices. Three T-cell epitopes are indicated in grey and black.

#### Epitope mapping

The specific recognition of allergen molecules by T- and B-cells refers to restricted sites within the molecule, the antigenic determinants or T- and B-cell epitopes. B-cell epitopes are mainly conformational (=discontinuous), i.e. they consist predominantly of residues brought together by the folded peptide chain. T-cell sites are thought to be continuous (linear peptide sequences). While B-cell epitopes are predominantly on surface-exposed regions of the intact allergen molecule, T-cell epitopes are derived by allergen degradation (processing). To induce an allergenic immune response which results in immediate type sensitization, T-cell as well as B-cell epitopes of the causative allergen have to be present. The T-cell receptor manifests specificity for a given antigenic peptide as well as for the allele of MHC molecule to which it is bound. The process of antigen fragmentation and formation of peptide-MHC complexes takes place in antigen presenting cells (APC), e.g. macrophages, B-cells and dendritic cells. Exogenous antigens such as allergens, are internalized by the APC from the extracellular space resulting predominantly in peptide-MHC class II complexes which are recognized by CD4+ cells since CD4 acts as a co-receptor for MHC-class II molecules.

#### Predictions

A general relationship between antigenicity and various structural parameters such as hydrophilicity or amphipathicity of protein structures was assumed [44]. Based on a limited amount of experimentally defined epitopes, different algorithms have been developed to predict sites specifically recognized by the immune system.

Apart from the occurrence of continuous or discontinuous formation, B-cell epitopes are mainly found in surface-exposed, highly mobile, hydrophilic regions. Epitope predictions are therefore based on the prognosis of these physical properties. Summing up respective data, the algorithm of Jameson and Wolf [45], the so-called antigenic index which takes a window of six neighbouring amino acids into consideration, can be calculated along the allergenic polypeptide. Regions with high antigenic indices ( $> 1.0$ ) are supposed to be bound by antibodies. For example, a B-cell epitope is expected at the *N*-terminus of *Chi t I* component III involving amino acid residues 14 to 17 [46]. This predicted epitope could be confirmed experimentally. However, we identified also B-cell epitopes within areas of low antigenic indices, e.g. in the *N*-terminus (residues 25–31). This shows that the algorithms are hitherto of limited value (Fig. 5). None of

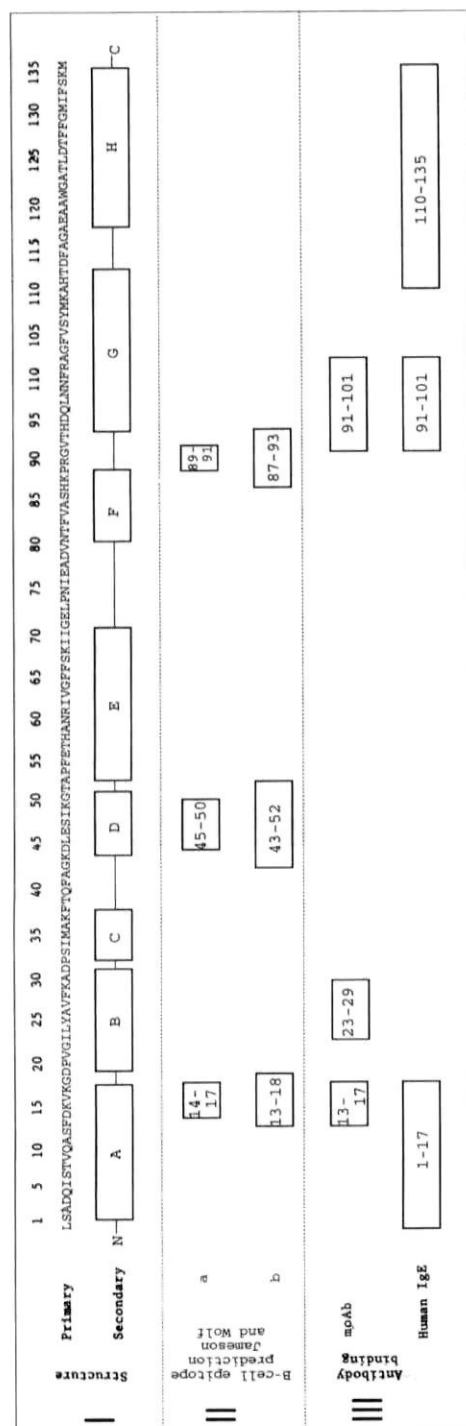


**Fig. 5.** B-cell epitope mapping of *Chi t I* component III: The 136 amino acids of *Chi t I* component III contain eight helical regions (A-H). According to Jameson and Wolf [45], three B-cell epitopes are expected. Hydrophilicity was predicted either to (a) Kyte Doolittle or (b) Hopp and Woods. Linear B-cell epitopes identified so far are located within the N- and the C-terminal region of the molecule [15].

those currently available gives a high level of correct prediction of B-cell epitopes.

The same is true for T-cell epitopes. Although amphipathic helices [47] as well as a certain pattern of amino acid sequences (glycine followed by two hydrophobic residues [48]) were frequently found to behave as T-cell epitopes, many identified T-cell sites of *Chi t I* component III cannot be allotted to any of these concepts. Even if both algorithms are combined, there is only partial accordance between predicted and evaluated T-cell epitopes within *Chi t I* component III (Fig. 6).

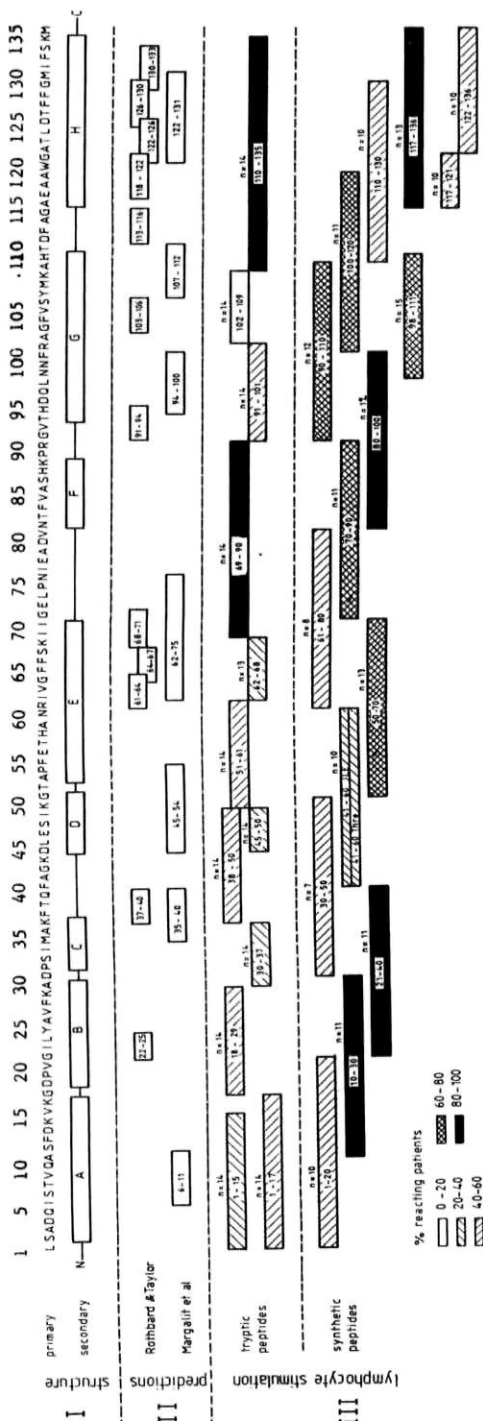
#### Experimental data

In recent years, the primary amino acid sequences of many different allergens have been made available offering the possibility, to perform studies with peptides regarding T- and B-cell epitope mapping. B-cell epitopes were studied with monoclonal antibodies as well as human IgE sera, for example in the pollen allergens *Cor a I*, *Bet v I* [49] and *Cyn d I* [50], the cat allergen *Fel d I* [51] and the major allergens of house dust mites *Der f I* [52] and *Der p II* [53].

The T-cell response was either investigated in the mouse system or by stimulating peripheral blood lymphocytes of allergic patients. For example studies of the *Amb a I* allergen of ragweed [54], of the house dust mite allergen *Der p II* [55] or the major allergen of bee venom, PLA2 [56], supported the common finding that T-cells are predominantly primed to a relatively small number of immunodominant peptides of an antigen.

Using tryptic digestion and overlapping synthetic 20mer-peptides of *Chi t I* component III, the stimulation capacity in peripheral blood lymphocytes of 14 patients was determined. In this way, nearly the whole sequence of component III could be shown to be antigenic. However, differences exist in the percentage of responding patients and the intensity of reaction. The N- and the C-terminal sequences of *Chi t I* component III as well as the central part of the molecule, namely residues 69–100 containing the heme binding region, were immunodominant [23] (Fig. 6).

Out of several B-cell epitopes, three could be verified with monoclonal antibodies, three also bound human IgE. Screening was performed using solid-phase bound tryptically derived as well as overlapping synthetic peptides of 20 and eight amino acid lengths respectively. Linear B-cell epitopes were localized within sequences 14–17, 23–29, 91–101 and 110–135 of *Chi t I* component III (Fig. 5) [15,24–27]. Each of these studies showed that recognition sites for T-cells and B-cells are frequently closely related or even identical in the allergen *Chi t I* component III.



### Expression of cell surface molecules after allergen stimulation

Lymphocyte surface markers such as the IL-2 receptor CD25, the low-affinity receptor for IgE CD23, or the activation marker HLA-DR likewise play a crucial role in initial steps of the immune response. The appearance of these molecules at a distinct developmental stage of lymphocytes suggests that they function as receptors for external signals directing cells to the next phase of response.

In the cell surface marker expression between allergic and non-sensitized donors, significant differences could be evaluated in response to *Chi t I*. In cultures of peripheral blood lymphocytes, *Chi t I* induced a significant increase of CD23, HLA-DR and CD25. On the other hand, the  $\alpha/\beta$  T-cell receptor decreased, probably due to internalization during signal transduction [22]. It can be assumed that such antigen-specific changes in the profiles of expressed lymphocyte surface markers may develop into valuable diagnostic indicators of sensitization.

### Genetic aspects of IgE-mediated hypersensitivity

Human leucocyte antigen genes are extremely diverse; distinct different forms of HLA molecules occur in different individuals. This genetic polymorphism results in individual immune response variations and might be an adaptive evolutionary mechanism to ensure immune recognition for a large variety of immunologic challenges and pathogens. Disease susceptibility that is associated with specific HLA genes can perhaps be viewed as an unintended consequence or bystander effect of maintaining a polymorphic array of HLA molecules in the population. More than 70 diseases having to some degree genetic associations with the HLA complex were described, for example autoimmune diseases like rheumatoid arthritis or type I diabetes [57].

**Fig. 6.** T-cell epitope mapping of *Chi t I* component III: when measuring the *Chi t I*-specific proliferation of peripheral blood lymphocytes (PBL), significant differences between patients, exposed and non-exposed controls were found [19,23]. T-cell epitope predictions are given along the structure of *Chi t I* component III. Using overlapping peptides for the stimulation of human PBL, nearly the whole sequence was found to be antigenic. A stimulation index of  $> 2$  with peptides obtained by tryptic cleavage and  $> 1.5$  with synthetic peptides was regarded as positive since controls were always below these borderlines. Since *Chi t I* component III consists of a neutral exchange isoleucin/threonine in position 57, peptide 41–60 was synthesized with isoleucin as well as with threonine. With the exception of sequence 102–109, every peptide stimulates lymphocytes at least in 20–40% of the patients.

Furthermore, molecular genetic studies of human immune responsiveness revealed significant associations between particular class II HLA phenotypes and the presence of specific serum IgE antibodies to several highly purified allergens. This refers for example to the pollen allergens from ragweed (*Amb a* V; DR 2) or *Lolium perenne* (*Lol p* I, *Lol p* II; DR 3) [58,59,60]. Those associations indicate a very small number of major epitopes of the antigen to be recognized by just a few class II molecules.

Our genetic examination of 188 *Chi t* I exposed individuals by means of polymerase chain reaction, and hybridization with sequence-specific oligonucleotides revealed a significant predominance of the HLA-types DRB1\*0101, DQB1\*0501 and DQA1\*0101 within subjects exclusively sensitized to *Chi t* I [61,62]. Since these DR- and DQ-genes are in strong linkage disequilibrium, which denotes the tendency for certain alleles to occur more frequently together on the same chromosome than expected by chance, these data do not permit to postulate whether the DRB1, DQB1 or DQA1 represent the class II molecule presenting the respective *Chi t* I epitopes. However, *Chi t* I-specific T-cell proliferation was shown to be significantly higher in DRB1\*0101 positive subjects than in individuals of other haplotypes ( $n=38$ ). In addition, antibodies to HLA-DR gene products were able to inhibit T-cell proliferation significantly ( $n=9$ ), whereas antibodies to HLA-DQ showed interindividual different results [20]. Therefore, the HLA-DRB1\*1\*0101 gene seems to be at least the predominant gene for the immune response to *Chi t* I.

#### Immunomodulation and future aspects

A curative therapy for immediate type allergic diseases is not yet available. Exact diagnoses including identification of the causative allergen(s), remain most important. They are prerequisites for effective prevention measures with regard to the allergy-inducing agent(s). Hyposensitization is of limited value or even ineffective as shown in many controlled studies [10,63,64]. At present, different strategies are pursued by immunologists. Recent progress in understanding allergy has identified several possible targets for selective immune intervention: at the major histocompatibility complex on antigen presenting cells, at the T-cell receptors, at CD4 and CD8 and other accessory molecules.

We could previously show that downmodulation of *Chi t* I-specific T-cell proliferation with allergen-derived peptides is possible [23]. This means that competition of low or non-stimulating peptides with the allergen at the respective HLA-molecules or T-cell receptor sites may prevent the onset of the immunological cascade with subsequent reduction of hypersensitivity. Since Tetanus-

or Phytohaemagglutinin-induced T-cell proliferation was not influenced in our experiments, unspecific immunosuppression is not probable [23]. In accordance with our results, immunomodulation of the *Dermatophagoides*-specific T-cell response with MHC-binding peptides was observed [65]. Further investigations are necessary to determine whether peptides can also be used *in vivo* as immunomodulators or whether other strategies inducing anergy of the respective IgE-mediating TH-2 cells will be required.

Last but not least, it should be kept in mind that according to our clinical data from simple measures, such as the reduction of the allergen dose in the environment an impressive decrease in the prevalence of sensitization and the intensity of symptoms can be expected.

In conclusion, the well characterized allergen *Chi t* I may serve as a good model for future allergy research. Small larvae and midges which appear to be harmless, but elicit strong immune responses in susceptible people might offer new perspectives of understanding and curing the pathomechanisms of allergic diseases.

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