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A COMMON EPITOPE ON MAJOR ALLERGENS FROM NON-BITING MIDGES (CHIRONOMIDAE)

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Abstract—A synthetic peptide corresponding to sequence 91–101 of the *Chironomus thummi thummi* haemoglobins (*Chi t I*) components III and IV was used to investigate binding and cross-reactivity with polyclonal human IgE and rabbit IgG antibodies and murine IgG₁ subclass monoclonal antibodies (MABs). The synthetic peptide reacted with antibodies from all three mammals. The specificity of the reaction, especially that with IgE antibodies was shown by dose dependent inhibition with native *Chi t I* component III. Epitope(s) reacting with these antibodies were also found in haemoglobins from 14 of the 15 chironomid species analyzed. The synthetic peptide III/IV 91–101 enabled the identification of an important antigenic/allergenic determinant of the broadly distributed insect family Chironomidae. The antigenic potency of this synthetic peptide as shown by testing with human IgE, rabbit IgG and mouse MABs, and the widespread occurrence of the epitope in an identical or homologous sequence and/or superficial location, qualifies the peptide for therapeutic applications in medicine.

INTRODUCTION

The non-cell bound haemoglobins (Hbs) of larval extracts from the insect family Chironomidae are known to be aggressive inhalant allergens (Freeman, 1950; Kay *et al.*, 1978; Baur *et al.*, 1982; Kagen *et al.*, 1984; Ito *et al.*, 1986). The Hbs from the species *Chironomus thummi thummi* (*Chi t I*, Marsh *et al.*, 1987) have been investigated thoroughly and their primary structures elucidated by Braunitzer and co-workers (Goodman *et al.*, 1983). We have described the enzymatic and chemical cleavage of the components IV, VI, and VIII from *Chi t* extracts and the immunological recognition of fragments with human IgE (Baur *et al.*, 1982). We also characterized for *Chi t I* the antigenic region of component IV, amino acid positions 91–101, described its physical and chemical properties and localized the surface position in the three-dimensional structure (Baur *et al.*, 1986). The same sequence is also found in the corresponding region of the component III. This allergenic fragment, III IV 91–101, possesses the typical properties of an antigenic site (Berzofsky, 1985) including a predominance of hydroxy or polar amino acids, a surface location, accessibility, high segmental mobil-

ity and the possibility of linkage to other molecules by hydrogen bonds or solvent clusters (Baur *et al.*, 1986). We have now synthesized this region.

It is well known that the polymorphic Hbs of many chironomid species are cross-reactive (Kay *et al.*, 1983; Baur *et al.*, 1983; Tee *et al.*, 1985). For example, human IgE from Sudanese patients exposed to *Cladotanytarsus lewisi* reacts well with extracts from *Chi t* larvae and IgE antibodies from our Munich patients bind to larvae extracts from different foreign species for example from U.S.A., Africa or Japan (Baur *et al.*, 1983; Tee *et al.*, 1985). The cross-reactivity of chironomid allergens was also confirmed by an investigation with MABs (Mazur *et al.*, 1987).

In this report we used the synthetically produced allergenic site III/IV 91–101 and analyzed its antibody reactivity.

Our results show that this synthetic peptide bound well to human IgE, rabbit IgG and two MABs. Remarkably these binding MABs react also with Hb epitopes of 14 chironomid species.

MATERIALS AND METHODS

Preparation of antigens

Extracts from larvae were fractionated into monomeric and dimeric Hbs on Sephadex G75 at pH 6.0. Further separations of monomeric Hbs and purification of components III and IV were performed on DEAE-cellulose exchanger (DEAE-cellulose, pre-swollen, obtained from Pharmacia Up-

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Abbreviations: CNBr, cyanogen bromide; *Chi t*, *Chironomus thummi thummi*; Hb, haemoglobin; MAB(s), monoclonal antibody(ies); PBS, phosphate-buffered saline; PBST, phosphate-buffered saline including 0.1% Tween 20.

psala, Sweden) with Tris buffer and salt gradients (Baur *et al.*, 1982).

The homogeneity of the individual Hb components was routinely checked by polyacrylamide gel electrophoresis under native conditions with a discontinuous buffer system (Mazur *et al.*, 1987). The purity of individual components was further investigated by N-terminal degradation after Edman (Edman and Henschen, 1970). The amino acids were identified as their phenylthiohydantoin derivatives by HPLC (Zimmermann and Pisano, 1977).

Preparation of the synthetic peptide III/IV 91-101

Peptide synthesis was performed in a 430 A peptide synthesizer (Applied Biosystems, Weiterstadt, F.R.G.) using Merrifield solid phase synthesis (Steward and Young, 1984) with the following modifications: to complete the coupling reactions double-couple cycles were used for each amino acid; in order to avoid sterical problems the first coupling was done in dimethylformamide, the second in methylene chloride.

N-t-butoxy-carbonyl (N-t-Boc)-groups were removed by treatment with 25% trifluoroacetic acid in methylene chloride for 20 min. A four-fold excess of N-t-Boc amino acids according to the first residue was used; these were converted to symmetrical amino acid anhydrides by treatment with dicyclohexylcarbodiimide (Aldrich). Completeness of coupling and deprotection reactions were monitored by ninhydrin colour tests (Kaiser *et al.*, 1970). All amino acids were purchased from Bachem AG, Bubendorf, Switzerland; N-Aoc-tosyl-arginine-resin was purchased from Peninsula Laboratories. St. Helens, U.K.; solvents were obtained from Merck AG, Darmstadt, F.R.G. Permanent side chain protection groups and the resin were cleaved from the peptide in an anisole suspension at 0°C with anhydrous hydrogen fluoride (Matheson). After precipitation and extensive washing in ethylacetate the peptide was lyophilized, extracted with 1.5% NH_4HCO_3 and purified.

Preparation of solid phase antigens

Purified Hb and the synthetic peptide III/IV 91-101 were coupled to CNBr activated paper discs by incubating 200 μg of antigen with 25 mg discs in 0.1 M NaHCO_3 (Ceska and Lundkvist, 1972; Baur *et al.*, 1982).

Antisera

(a) *IgE patients' sera.* A mixed serum pool obtained from five allergic fish enthusiasts, was used as well as individual sera from other allergic patients. All the patients had suffered from bronchial asthma with or without rhinitis, conjunctivitis, and wheal and flare reactions following contact with freeze-dried chironomid larvae. In addition, all showed positive results in prick testing and RAST investigation with a Hb fraction from *Chi t* larvae.

(b) *Rabbit anti-*Chi t* I immunoglobulins.* Antisera to *Chi t* I allergens were raised in rabbits by injection of 1 mg *Chi t* I components I-X, emulsified 1:1 in complete Freund's adjuvant. Boosting injections were prepared with incomplete Freund's adjuvant and given at two-weekly intervals. Anti-*Chi t* I serum was obtained from the marginal vein of the ear 1 week after each booster.

(c) *Monoclonal antibodies.* Monoclonal antibodies against *Chi t* I component III were prepared as described by Mazur *et al.* (1987). Briefly, BALB/c mice were given 0.1 mg component III in PBS and Freund's adjuvant (1:1) on days 1, 8 and 29. On day 32, 10^8 splenic lymphocytes were fused with 10^7 P3 $\times$ 63Ag8U1 cells by mixing with 500 μl 50% polyethylene glycol in RPMI 1640 and incubating for 3 min at 37°C. The cells were centrifuged and the polyethylene glycol diluted dropwise with 10 ml RPMI 1640. After one wash the mixture was suspended in 62 ml HAT-medium (20% FCS-RPMI 1640 with 15.5 mg/l thymidine, 54.4 mg/l hypoxanthine and 176 $\mu\text{g/l}$ aminopterin), and plated out in 100 μl samples in microtiter plates. Cells were cultivated at 37°C in a CO_2 incubator. Positive clones were subcloned using the method of limiting dilutions, after Köhler and Milstein (1975). Twenty subclones fulfilling monoclonal criteria were selected and characterized according to their reaction to the components III, III a and IV and divided into five groups: MABs 3, 6, 9, 15 and 18. All antibodies belonged to the G_1 subclass (Mazur *et al.*, 1987).

Antibody binding assay

(a) *Determination of human IgE to *Chi t* I allergens.* Human IgE antibodies were measured by IgE RAST (Phadebas RAST Kit, obtained from Pharmacia Diagnostics, Uppsala, Sweden; Ceska and Lundkvist, 1972; Baur *et al.*, 1982) with the commercially available reference standards used for evaluation of antigenic reactivity (U/ml). Sera which gave IgE units >4 were diluted and retested. The results were expressed as units/ml undiluted serum. A series of controls was performed for each IgE binding assay. For these controls we used individual and pooled serum samples from atopic patients not sensitized to non-biting midge allergens and from non-allergic individuals.

(b) *Determination of rabbit IgG.* Solid phase antigens were incubated for 16 hr in 100 μl of rabbit *Chi t* I antiserum diluted 1:1000 in PBST-BSA. Each disc was then washed three times with PBST, incubated in 100 μl ^{125}I labelled protein A (1×10^5 cpm, New England Nuclear) for 2.5 hr washed and the bound radioactivity measured in a gamma counter.

(c) *Determination of MABs.* The amount of MABs bound to solid phase antigens was detected with ^{125}I labelled rabbit anti-mouse IgG (Mazur *et al.*, 1987). The supernatant antibodies were diluted 1:20 in RPMI medium and incubated overnight with solid phase antigens. Each disc was then washed three

Synthetic peptide III/IV 91-101: Gly-Val-Thr-His-Asp-Gln-Leu-Asn-Asn-Phe-Arg

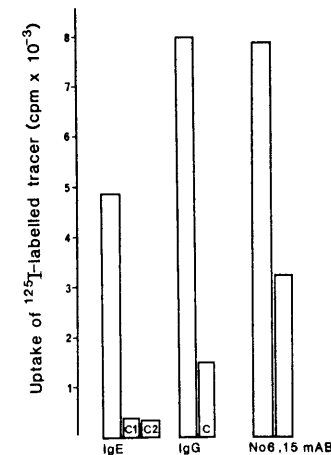


Fig. 1. Binding of human specific IgE, rabbit anti-*Chi t* I IgG, MAB 6 and 15 to solid phase bound synthetic peptide III/IV 91-101 from *Chi t* I components III and IV. Control sera from atopic patients sensitized to common allergens but not to Chironomidae (C1), sera from healthy controls (C2) and from preimmune rabbits (C). For MABs see Fig. 2.

times with PBST, incubated in 100 μl ^{125}I labelled rabbit anti-mouse IgG (NEN) for 3 hr, then washed and the bound radioactivity was measured. For controls, culture media and a MAB (clone H3E11, MAB No. 4) without specific binding to *Chi t* I were used. Background remained in the range of 200-500 cpm.

Competitive inhibition assay

Various amounts of *Chi t* I component III in 50 μl PBST (0.001-1 mg/ml) were incubated with 50 μl serum from chironomid allergic patients for 2 hr and

then added to solid phase bound HPLC purified peptide III/IV 91-101 or component III. The remaining protocol was similar to RAST.

RESULTS

Allergic and antigenic reactivity of the synthetic peptide III/IV 91-101 with antibodies from sensitized patients, immunized rabbits, and with MABs

(a) *Human IgE antibodies.* The capacity of the synthetic peptide III/IV 91-101 to bind to human IgE antibodies was evaluated by RAST. A value of 1.5 U/ml (corresponding to 4800 cpm bound ^{125}I labelled anti-IgE) was determined using a pool of five sera from *Chi t* I sensitive patients (Fig. 1). No positive reactions were obtained with sera from healthy controls (Fig. 1, C2) or atopic subjects who had never been in contact with chironomids (Fig. 1, C1). The values from these sera were around 0.02 IgE U/ml, i.e. in the range of background.

The reactivity of individual sera from sensitized patients with the synthetic peptide III/IV 91-101 was also investigated. Fifteen sera with specific RAST uptakes of ^{125}I labelled anti-IgE, all ≥ 2 on the Pharmacia 0-4 + Phadebas RAST scale, were analyzed for binding to the synthetic peptide. The sera showed marked differences in binding capacity, with values from 0.06 to 5.5 IgE U/ml (Table 1). The activity of the synthetic peptide was expressed as a percentage of that bound by intact component IV (Table 1, lane 6).

(b) *Rabbit IgG.* To examine the binding ability of polyclonal immunoglobulins from another mammal we used a rabbit anti-*Chi t* I serum pool ($N = 3$). Using this serum we detected a five times higher amount of bound ^{125}I labelled protein A than in the preimmune serum pool (Fig. 1).

(c) *MABs.* MABs from 20 subclones raised against the component III were analyzed for their binding to solid phase bound antigen III/IV 91-101. We found the MABs 5, 6, 7 and 15 to have a significant binding

Table 1. Reactivity of IgE antibodies from 15 patients sensitized to *Chi t* I with solid phase components I-X, III, IV and the synthetic peptide III/IV 91-101. Activity of the synthetic peptide is expressed as the percentage of the component IV response

Patients	I-X	Measurement of specific IgE to solid phase allergens (RAST U/ml)			% Activity of synthetic peptide III/IV 91-101
		Components III	IV	Synthetic peptide III/IV 91-101	
1	24.0	16.5	15.0	0.42	2.8
2	87.0	70.0	64.0	3.65	5.7
3	7.0	2.9	2.3	0.24	10.4
4	21.0	12.3	11.5	1.52	13.2
5	22.0	3.7	7.2	0.14	1.9
6	36.0	16.6	17.1	1.40	8.2
7	49.0	30.0	24.5	3.70	15.1
8	66.0	25.0	27.0	0.70	2.6
9	205.0	168.0	90.0	5.50	6.1
10	57.0	21.5	29.0	1.28	4.4
11	6.7	2.9	6.7	0.20	3.0
12	3.3	1.2	1.4	0.06	—
13	140.0	65.0	73.0	1.26	1.7
14	58.0	34.0	18.0	1.30	7.2
15	2.0	0.72	1.3	0.06	—

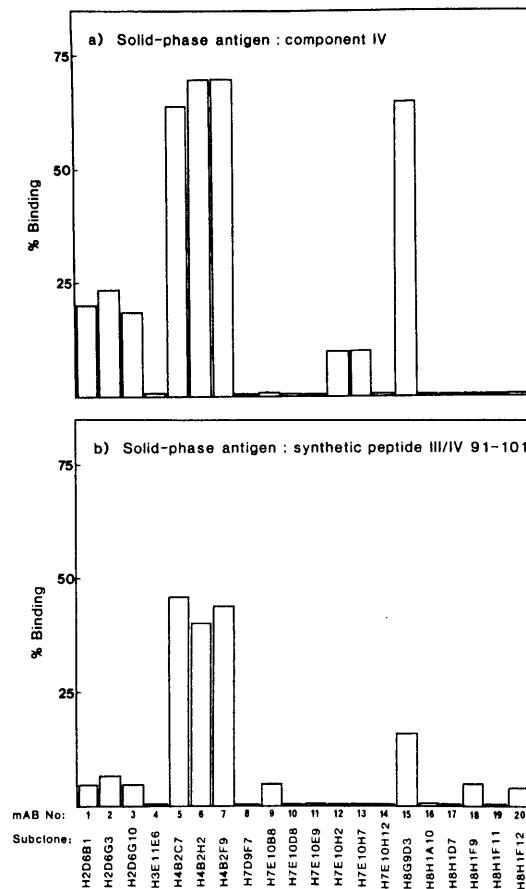


Fig. 2. Binding of 20 MAbs to the component IV (a) and the synthetic peptide III/IV 91-101 (b). The results are expressed as percent radioactivity bound in relation to the immunogen component III, which is taken as 100%.

capacity (Figs 1 and 2b). This is expressed in Fig. 2b as a percentage of the ^{125}I labelled anti-mouse IgG bound by the immunogen component III.

Reactivity of human IgE antibodies and MAbs with individual *Chi t 1* components

In order to estimate the binding of IgE antibodies and MAbs to the synthetic peptide III/IV 91-101, we investigated their behaviour with individual *Chi t 1* components. The binding of human IgE from individual patients to native Hb molecules III and IV (solid phase bound) was in the range of 0.72-168 IgE U/ml (Table 1). Of the 20 MAbs analyzed, only seven showed clear binding to component IV (Fig. 2a) at levels of 17-70% of binding to the immunogen component III.

Detection of autoantibodies and unspecific cross-reactivity

Human IgE, rabbit IgG and MAbs were investigated for binding to solid phase human Hb, rabbit Hb, mouse Hb, and sperm whale myoglobin. All reactions were negative.

Competitive inhibition assay

The results of RAST inhibition experiments are shown in Fig. 3. Preincubation of sera from two chironomid allergic patients with native Hb component III resulted in a dose dependent inhibition of IgE binding to synthetic peptide III/IV 91-101. Inhibition to *Chi t 1* component III was stronger.

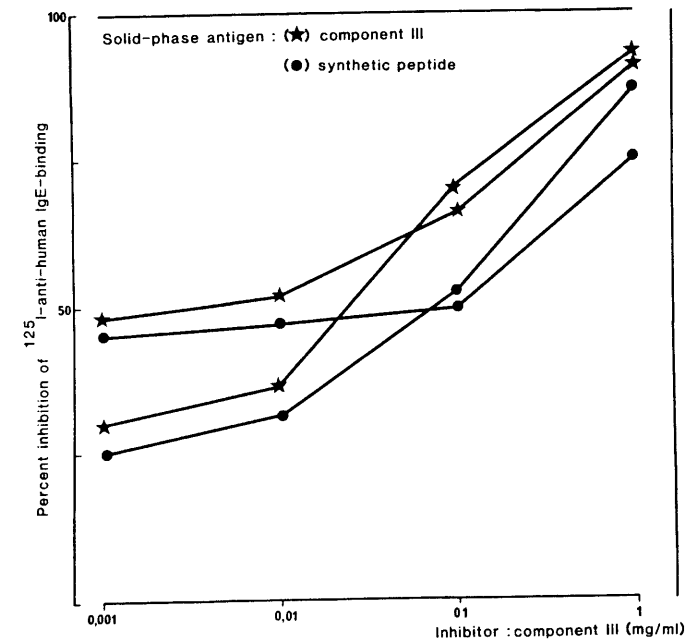


Fig. 3. Inhibition of binding of human IgE antibodies (two patients sensitized to chironomid allergens) to solid phase bound (●) synthetic peptide III/IV 91-101 and (★) component III.

DISCUSSION

In this study we investigated the immunoreactivity of the synthetically produced peptide III/IV 91-101 with different classes of antibodies, namely polyclonal IgE and IgG and monoclonal IgG, raised against native Hbs. The results show that anti-*Chi t 1* antibodies bind to both the natural (Baur *et al.*, 1986) and the synthetic peptide.

The antibodies tested were derived from quite unrelated mammals (humans, rabbits, mice) but all recognized the synthetic peptide. These results may be interpreted as evidence for an immunodominant antigenic determinant of chironomid allergens. The response also appears to be independent of the method of antigen uptake; our atopic patients inhaled the environmental allergens and absorption took place along the airways, whereas the animals received Hb directly in the blood stream.

As expected there was no cross-reactivity between anti-*Chi t 1* antibodies and mammalian Hb or myoglobin. Chironomidae and mammals are widely separated on the evolutionary scale. Investigations using a variety of proteins and their homologues, such as myoglobin (Cooper *et al.*, 1984; Rodda *et al.*, 1986) or cytochrome *c* (Jemmerson and Margoliash, 1979), have shown that the selection of immunoreactive sites depends on the evolutionary relationship between

protein antigen and the homologous host protein (Benjamin *et al.*, 1984; White *et al.*, 1978). In other words the host preferentially produces antibodies against determinants which are not homologous with its own proteins.

The reactions of MAbs with the synthetic peptide III/IV 91-101 was very specific. Only those MAbs (No. 5, 6, 7 and 15; Fig. 2b) which clearly bound to the component IV also bound to the peptide (Fig. 2a,b). These MAbs also recognized determinants in larval extracts from 14 out of the 15 chironomid species investigated (Mazur *et al.*, 1987). This immunological cross-reactivity between phylogenetically widely separated chironomid species confirms the existence of common epitopes on chironomid allergens and the fact that they have been at least partially conserved during evolution.

The synthetic peptide also bound human anti-chironomid IgE. The results of the investigations with a serum pool were further confirmed by analyzing 15 individual sera from chironomid sensitized patients. Furthermore, the specificity of the IgE binding was verified by dose dependent inhibition with native component III.

Thus the results clearly identify the fragment III/IV 91-101 as an IgE combining site, probably present in many chironomid Hbs. It is possible that the un-

decapeptide does not necessarily mimic the complete structure of the corresponding epitope(s) in the native Hb molecules and that only a few of the peptide residues are involved in antibody binding. The linear peptide could either be a continuous or discontinuous determinant, or part of a longer determinant.

The use of synthetic peptides offers considerable advantages in immunological research, especially for the exact identification of antibody combining sites, since it is easy to synthesize the large quantities of distinct peptides using a systematic substitution approach (Geysen, 1985; Geysen *et al.*, 1987; Getzoff *et al.*, 1987; Van Regenmortel, 1987).

The ability to produce individually relevant antigenic determinants of environmental allergens in a pure state offers incomparable possibilities for research into the pathogenetic mechanism of allergic diseases and for application in immunotherapy.

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