



ANALYSIS OF B-CELL EPITOPES IN THE N-TERMINAL REGION OF *Chi t I* COMPONENT III USING MONOCLONAL ANTIBODIES

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Abstract—The hemoglobins of the midge *Chironomus thummi thummi* (*Chi t I*) are known to cause immediate-type hypersensitivity reactions in humans. Further knowledge of the antigenic sites of such allergens will provide new therapeutic approaches. The aim of our study was to identify and characterize linear B-cell epitopes of the hemoglobin component III of *Chi t I* (136 amino acid residues). Using the antigenic index algorithm of Jameson and Wolf (Jameson and Wolf (1988) *Comput. Appl. Biosci.* 4, 181–186), three linear binding sequences of this allergen molecule were predicted. Two mouse monoclonal antibodies (mAbs 3 and 6) raised against purified *Chi t I* component III were investigated by ELISA for their binding to nine synthetic peptides 19–21 residues in length, covering nearly the whole sequence of component III. MAb 6 recognized only one peptide (11–30) while mAb 3 bound to both N-terminal peptides (1–19 and 11–30), suggesting that the antibody binding site is located in the overlapping region. This assumption could be confirmed in ELISA with solid phase-bound recombinant peptides (RP) as well as in inhibition studies with free tryptic peptides indicating that identification of these linear B-cell epitopes is neither influenced by the method of peptide production nor by the kind of used immunoassay. To define the essential amino acid residues we investigated mAbs with solid phase-bound overlapping octamers. In the case of mAb 3, amino acids experimentally identified as essential for antibody binding (aa 13–17) are identical with those residues predicted as a B-cell epitope with the antigenic index of Jameson and Wolf.

Key words: *Chironomus thummi thummi* hemoglobins (*Chi t I*), monoclonal antibodies (mAbs), B-cell epitopes, synthetic peptides.

INTRODUCTION

Detailed knowledge of the molecular structures of allergens, in particular the antibody binding sites or B-cell epitopes, is useful for the development of new therapeutic strategies for allergic diseases (Zhang *et al.*, 1992). Epitopes can be subdivided into sequential and conformational epitopes (Laver *et al.*, 1990; Mazur *et al.*, 1990). In recent years, analyses primarily based on the crystal structures of antibody–antigen complexes have shown epitopes to be predominantly conformational and containing more or less dominating elements of sequential nature (Davies and Padlan, 1990).

The aim of our study was to identify and characterize linear B-cell epitopes of *Chironomidae* hemoglobin. *Chironomidae*, a family of non-biting midges, are distributed worldwide in areas abundant in water (Baur *et al.*, 1983). The non-cell bound hemoglobin of the species *Chironomus thummi thummi* (*Chi t I*) which comprises three monomeric and nine homodimeric components is known to be an aggressive inhalant allergen (Liebers and Baur, 1994; Mazur *et al.*, 1988; Tee *et al.*, 1985; Brasch *et al.*, 1992). *Chi t I* component III, a monomer of 136 amino acid residues which had been analysed by X-ray crystallography (Steigemann and Weber, 1979), was studied in detail. First, linear B-cell epitopes of the hemoglobin component III were predicted by the antigenic index algorithm of Jameson and Wolf (Jameson and Wolf, 1988; Isringhausen-Bley *et al.*, 1993). All known predictive algorithms were derived from the observed correlation between the location of sequential epitopes and structural parameters such as hydrophilicity, accessibility and mobility. Therefore, the method of Jameson and Wolf generates values for hydrophilicity and surface accessibility and combines these values with those for regional backbone flexibility and predicted secondary structure (Lal and Griffiths, 1991). Using this algorithm, three epitopes containing residues 14–17, 45–50 and 89–91 were calculated. Synthetic peptides

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 Abbreviations: *Chi t I*, *Chironomus thummi thummi* hemoglobins; mAb(s), monoclonal antibody(ies); RP, recombinant peptides; BrCN, cyanogen bromide; CLIA, chemiluminescence immunoassay.

with appropriate specific antibodies are often used for the experimental identification of linear B-cell epitopes. In the case of the allergens *Fel d 1* (van't Hof *et al.*, 1993), *Der p 1* (van't Hof *et al.*, 1991) *Cor a 1* and *Bet v 1* (Elsayed *et al.*, 1989) epitopes were found by this technique. In our study, we investigated the antigenic potency of the N-terminus of *Chi t 1* component III by using mouse monoclonal antibodies and synthetic peptides. Furthermore, results were confirmed by using recombinant and tryptic peptides.

MATERIALS AND METHODS

Antigens

Chi t 1 component III. Using frozen midge larvae (*Chironomus thummi thummi*), hemoglobins were isolated by ammonium sulfate precipitation of the hemolymph followed by fractionation into monomeric and dimeric components by gel filtration on Sephadex G-75. Purification of *Chi t 1* component III was performed on a DEAE exchanger (DEAE Sephacel). The purity of the hemoglobin component was checked by polyacrylamide gel electrophoresis under native conditions with a discontinuous buffer system (Braun *et al.*, 1968).

Synthetic peptides. The peptides corresponding to amino acid sequences 11–30, 31–50, 41–60, 51–70, 71–90, 80–100, 91–110 and 110–130 of *Chi t 1* component III (Table 1) were assembled by solid phase synthesis using an automated peptide synthesizer (peptide synthesizer 432A, ABI, Weiterstadt, Germany) and N- α -fluorenyl-methoxycarbonyl (Fmoc)-protected amino acids. For activation 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) was used. The deprotection of Fmoc-group was carried out in piperidine/dimethylformamide (2:8, v/v). Cleavage of the peptides from the resins and the side chain deprotection were performed in a mixture of trifluoroacetic/thioanisole/Ethandithio (90:5:5, v/v/v) within 2 hr at room temperature. The peptides were purified by reversed-phase high performance liquid chromatography (HPLC) on a silica bead C18 column (20 $\times$ 250 mm) and identified by ion-spray mass spectroscopy on an API III

triple-quadrupole mass spectrometer with an IonSpray interface (Sciex, Toronto, Canada). The purity of peptides was over 75% as determined by analytical reversed-phase HPLC (C18 column, 4 $\times$ 250 mm). The peptide 1–19 (Table 1) was a gift from Dr P. Rücknagel, Max-Planck Institute for Biochemistry, Martinsried. For exact epitope mapping we used overlapping octamers received from CHIRON Mimotopes, Australia.

Tryptic peptides. After removing the heme group of purified *Chi t 1* component III (Braun *et al.*, 1968), the globin chain was digested by trypsin (2% w/w, Cooper Biomedical) in sodium carbonate-bicarbonate buffer (50 mM, pH 9.0) at 37°C for 5 hr. The cleavage was checked by HPLC. Peptide separation was carried out by reversed-phase HPLC on a LiChrosolv 60 RP select B column (4 $\times$ 12.5 mm) using LKB-Pharmacia equipment. Peptides were eluted with a 0–66% ethanol gradient in 0.1% HCl at a flow rate of 0.8 ml/min. The peptide fractions were identified by amino acid analysis.

Recombinant peptides. Plasmid constructions of the recombinant peptides 1–17 (RP 1–17) and 61–80 (RP 61–80) of *Chi t 1* component III were constructed as LacZ-hybrids. Synthetic oligonucleotides corresponding to these amino acid residues were set blunt ended into the unique SmaI site of the β -galactosidase expression vector pPR 2 (Rihs and Peters, 1989; Rihs *et al.*, 1991a). Purification of *Chi t 1*- β -galactosidase fusion proteins was carried out on *p*-aminophenyl (or benzyl)-1-thio- β -D-galactoside sepharose (pAPT or pABTG, Sigma, Deisenhofen, Germany) using the crude extract of a 500 ml-culture induced by 0.5 mM isopropyl- β -D-thiogalactopyranoside (IPTG) (Rihs and Peters, 1989; Rihs *et al.*, 1991b). The correct sequence of the insert aa 1–17 in plasmid pPR 2 was confirmed by restriction analysis and Sanger sequencing. The insert aa 61–80 was checked by restriction analysis.

Monoclonal antibodies (mAbs)

To prepare monoclonal antibodies against *Chi t 1* component III, BALB/c mice were injected intraperitoneally 0.1 mg component III in PBS and Freund's adjuvant (1:1, v/v) on days 1, 8 and 29. Three days later, spleen cells from immunized mice were fused with P3 $\times$ 63Ag8U1 myeloma cells using polyethylene glycol in RPMI 1640. Hybridomas were selected in HAT-medium. Cells were cultivated at 37°C and 5% CO₂ atmosphere (Mazur *et al.*, 1988). MABs 3 and 6 reacted to *Chi t 1* component III while mAb 4, raised against a single allergen of *Phleum pratense* (*Phl p V*) was used as control antibody.

Preparation of solid phase antigens for ELISA

Purified hemoglobin component III as well as synthetic and recombinant peptides were coupled to BrCN activated paper disks (Schleicher & Schuell, diameter: 6 mm) by incubating antigen (component III and synthetic peptides, 0.5 mg; recombinant peptides, 1 mg) with 25 disks in 1 ml 0.1 N NaHCO₃ (Baur *et al.*, 1982).

ELISA

The binding of monoclonal antibodies to solid phase antigens was measured by incubating the allergen-coated paper disks overnight with 50 μ l hybridoma culture supernatant. Each disk was washed three times with 2 ml PBST [0.05 M sodium phosphate, 0.15 M NaCl (PBS); 0.1% BSA; 0.1% Tween 20; pH 7.5] and then incubated with 50 μ l alkaline phosphatase-conjugated goat anti-mouse IgG (Sigma, 1:1000 with 0.05 M Tris, 0.01 M MgCl₂, pH 8.0) for 3 hr at room temperature. After three washes with PBST, 200 μ l alkaline phosphatase substrate *p*-nitrophenyl phosphate (PNPP, 1 mg/ml 10% diethanolamine buffer) was added for 90 min at 37°C. The reaction was stopped by adding 1 ml 1 N NaOH per tube. The optical density (OD) was measured at 405 nm. Results were expressed as the mean of duplicate tubes.

CLIA (chemiluminescence immunoassay) inhibition test with tryptic peptides

Polystyrol tubes were coated overnight with 5 μ g *Chi t 1* component III in 200 μ l sodium carbonate-bicarbonate buffer (25 mM, pH 9.6). Tubes were saturated with 500 μ l incubation buffer (IB: PBS containing 0.1% Tween 20 and 0.5% BSA). Purified tryptic peptides (2 and 8 nM) in 100 μ l IB were mixed with 100 μ l mAb-supernatant (1:1000 with IB) and incubated for 16 hr at 4°C. After two washes with 500 μ l PBST, the preincubated peptide/mAb-solution was added to the coated tubes. After 1.5 hr the tubes were washed three times and then incubated with 1.25 ng acridinium-labelled anti-mouse IgG (Molecular Light Research Products, Bioanalysis, Cardiff, U.K.) in 200 μ l IB for 1 hr at room temperature. After three washes, 100 μ l

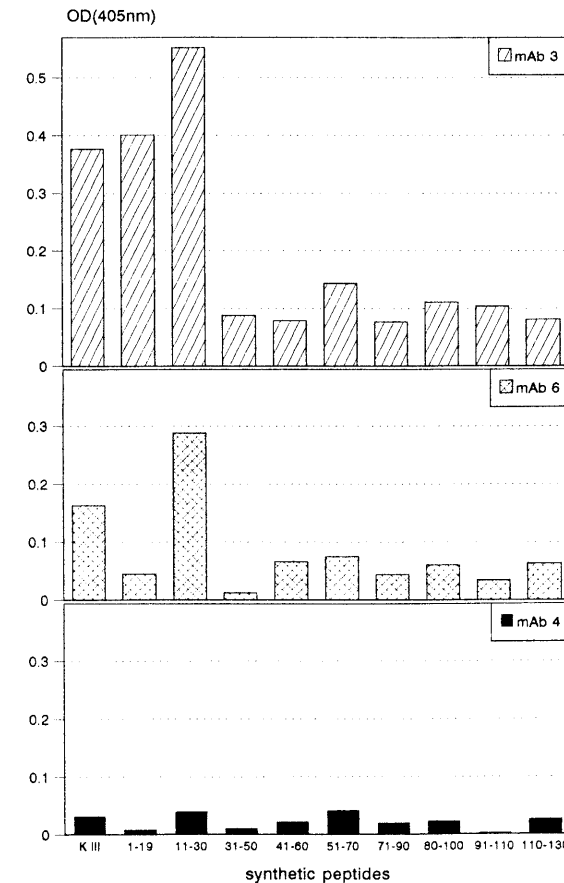


Fig. 1. Binding of mouse monoclonal antibodies (mAbs) 3, 6 and 4 to solid phase-bound component III and nine synthetic peptides (19–21 residues) covering the sequence of *Chi t 1* component III. Mabs 3 and 6 are raised against component III while mAb 4 acts as control antibody (raised against *Phl p V*).

Table 1. Amino acid sequences of synthetic peptides of *Chi t 1* component III

N-terminal residue	C-terminal residue	Sequence
1	19	LSADQISTVQASFDKVKGD
11	30	ASFDKVKGDPVGILYAVFKA
31	50	DPSIMAKFTQFAGKDLESIK
41	60	FAGKDLESIKGTAPFETHAN
51	70	GTAPFETHANRIVGFFSKII
71	90	GELPNIEADVNTFVASHKPR
80	100	VNTFVASHKPRGVTHDQLNMF
91	110	GVTHDQLNMFVAGFVSYMKA
110	130	AHTDFAGAEAAWGATLDTFFG

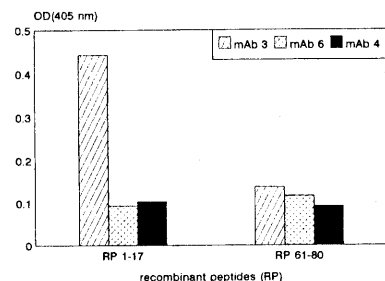


Fig. 2. Binding of mAbs 3 and 6 to solid phase-bound recombinant peptide (RP) 1-17 measured by ELISA. Reaction specificity was confirmed by using control mAb 4 as well as another recombinant peptide (RP 61-80).

of distilled water were added, and bound acridinium-labeled antibodies were measured by a Magic Lite Analyzer II (Ciba Corning, Fernwald, Germany). The released photons of light were counted and expressed as relative light units (RLU).

RESULTS

MAbs 3, 4 and 6 were analysed by ELISA for their binding to nine solid phase-bound peptides with 19 to 21 amino acid lengths covering nearly the whole sequence of *Chi t I* component III (Fig. 1). Mab 3 reacted to peptide 1-19 as well as to peptide 11-30 while mAb 6 only recognized peptide 11-30. No reactivity with *Chi t I* derived peptides was obtained with the control antibody mAb 4. Furthermore, mAbs 3, 6 and 4 were tested with recombinant peptides (RP) by ELISA. In this experiment, monoclonal antibody 3 bound to RP 1-17 (Fig. 2). In the test with RP 61-80 serving as control and with solid phase β -galactosidase alone (data not shown) no antibody binding was detected. The control antibody mAb 4 as well as mAb 6 reacted neither to RP 1-17 nor to RP 61-80.

In order to confirm the results of ELISA with solid phase-bound antigens we performed a CLIA inhibition

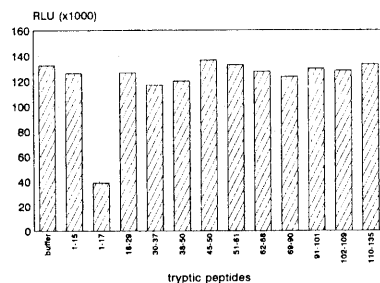


Fig. 3. Inhibition of binding of mAb 3 to solid phase-bound *Chi t I* component III by tryptic peptides measured by CLIA. Two nanomolar peptides were used.

Table 2. Binding of mAbs 3 and 6 to solid phase-bound overlapping synthetic octamers as well as to component III. Amino acids essential for antibody binding are written in bold face

Number	Octamer	Absorbance with		Prediction ^a antigen index
		mAb 3	mAb 6	
1	LSADQIST	0.088	n.d. ^b	0.000
2	SADQISTV	0.112	n.d.	0.600
3	ADQ/STVQ	0.100	n.d.	0.000
4	DQISTVQA	0.154	n.d.	0.600
5	QISTVQAS	0.104	n.d.	-0.450
6	ISTVQASF	0.154	n.d.	-0.450
7	STVQASFD	0.152	n.d.	-0.600
8	TVQ4SFDK	0.126	n.d.	-0.150
9	VQ4SFDKV	0.114	n.d.	0.450
10	QASFDKVK	0.335	0.064	0.850
11	ASFDKVKG	0.342	0.042	1.300
12	SSDKVKGD	0.469	0.076	1.300
13	FDKVKGDP	0.350	0.098	1.300
14	DKVKGKPV	0.115	0.042	1.300
15	KVKGDPVG	0.159	0.066	0.900
16	VKGD/PVGI	0.130	0.066	0.600
17	KGDP/VGIL	0.105	0.064	0.850
18	GDP/VGILY	0.105	0.036	0.100
19	DPVG/ILYA	0.128	0.034	-0.600
20	PVG/LYAV	n.d.	0.059	-0.600
21	VGILYAVF	n.d.	0.100	-0.600
22	GILYAVFK	n.d.	0.468	-0.600
23	ILYAVFKA	n.d.	0.324	-0.450
24	LYAVFKAD	n.d.	0.080	-0.450
25	YAVFKADP	n.d.	0.072	-0.150
<i>Chi t I</i> component III (136 aa)		0.435	0.442	

^aThe predicted antigenic index value for each residue is aligned with the fourth residue (italicised) in each of the synthetic octamers.

^bn.d. = Not determined.

test with tryptic peptides (Fig. 3). The binding of mAb 3 to solid phase *Chi t I* component III was inhibited up to 38% by 2 nM peptide 1-17 but not by peptide 1-15. A concentration of 8 nM peptide 1-17 reduced the binding of mAb 3 up to 93% indicating a dose-dependent inhibition. In CLIA-autoinhibition experiments with the native *Chi t I* component III, binding of mAb 3 was inhibited from 14% (inhibitor concentration: 0.05 nM) to 94% (inhibitor concentration: 500 nM) (data not shown).

To define the minimal epitopes we investigated those amino acid regions which were recognized by mAbs 3 and 6 with solid phase overlapping octamers. Table 2 shows that mAb 3 bound to octamers 10-17, 11-18, 12-19 and 13-20 indicating that amino acid residues 13-17 are essential for antibody binding. In octamer-ELISA, mAb 6 reacted to synthetic peptides 22-29 and 23-30 suggesting that the minimal epitope of mAb 6 contains the amino acid residues 23-29. Using the X-ray data we created a computer display of component III and localized B-cell epitopes in the three-dimensional structure which is shown in Fig. 4.

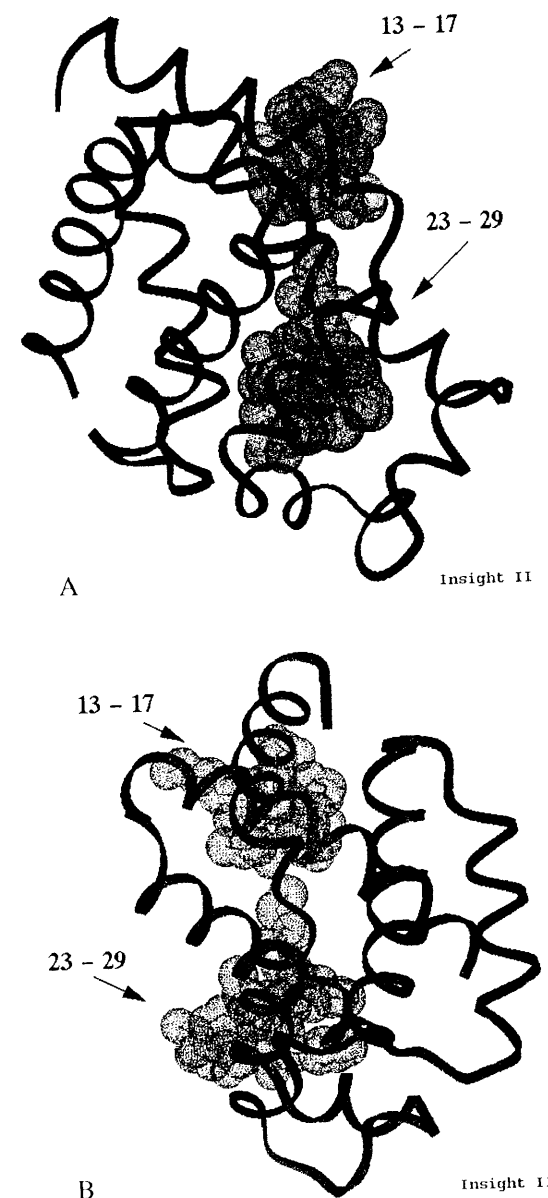


Fig. 4. Three-dimensional structure of *Chi t I* component III at 1.4 Å resolution (Steigemann and Weber, 1979) using the computer program 'Insight II' (BIOSYM). The molecule is shown in ribbon structure with van der Waals dot representation in the regions of amino acids 13-17 and 23-29 (mAb binding sites). It can be seen that both epitopes are located at the surface of the molecule. Figs A and B show the same molecule rotated by an angle of 180 degrees.

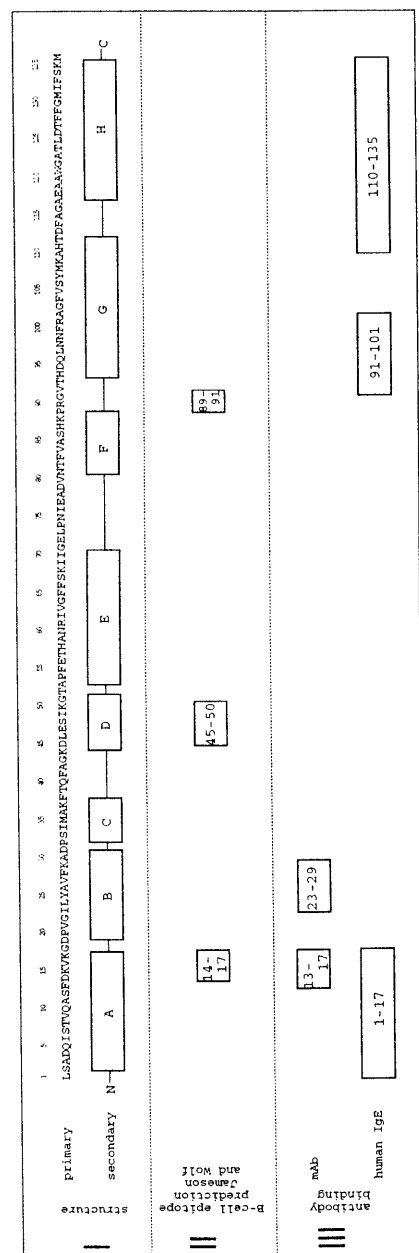


Fig. 5. (I) Sequence of 136 amino acid residues of *Chi t I* component III containing eight α -helices determined by X-ray crystallography; (II) Linear B-cell epitopes predicted by the antigenic index algorithm of Jameson and Wolf; and (III) Experimentally found B-cell epitopes. The epitopes including the amino acids 13-17 or 23-29, respectively were determined in this work using mAbs 3 and 6. Binding sites for human IgE antibodies were identified using the RAST method with solid phase tryptic peptides according to Mazur *et al.* (1990).

Our results and those of previous studies are summarized in Fig. 5. This illustration shows the sequence of 136 amino acids of *Chi t I* component III (Goodman *et al.*, 1983) and positions of eight α -helical structures detected by X-ray crystallography (Steigemann and Weber, 1979). The four predicted epitopes and the experimentally detected B-cell epitopes are demonstrated. In addition to the epitopes 13-17 and 23-29 determined by mAbs, the amino acid residues 1-17, 91-101 and 110-135 of component III are identified as B-cell epitopes for human IgE antibodies (Mazur *et al.*, 1990).

DISCUSSION

This paper describes B-cell epitope analysis of the allergen molecule *Chi t I* component III by using monoclonal antibodies and synthetic, recombinant as well as tryptic peptides. Independent of the method of peptide production and of antigen-antibody binding measurement we obtained corresponding results.

The binding of mAb 3 to the synthetic peptides 1-19 and 11-30 implied that the antibody binding site is located in the overlapping peptide region. This assumption was confirmed by an inhibition test with tryptic peptides showing that the binding of mAb 3 to component III was not inhibited by the peptide 18-29. In ELISA with solid phase-bound recombinant peptides (RP), mAb 3 reacted to RP 1-17. This finding in combination with the results shown in Fig. 1 suggested that a B-cell epitope between the amino acid residues 11 and 17 exists. Negative results with RP 61-80 as well as with β -galactosidase alone excluded a reaction between mAb 3 and the β -galactosidase molecule and confirmed the specificity of the antibody binding. A CLIA inhibition test with tryptic peptides was also performed. In this investigation, mAb 3 was inhibited by peptide 1-17 but not by peptide 1-15 indicating that the amino acid residues 16 (V) and 17 (K) belong to the epitope recognized by mAb 3 or that these amino acids stabilize the adjoining epitope structure.

The majority of mAbs raised against intact proteins is thought to be specific for conformational epitopes and not to react to linear peptide fragments derived from the antigen (van Regenmortel, 1992). However, mAb 3 recognized both the intact protein and linear peptides. This finding implies that these two molecules possess more or less identical epitopes. In this connection it is important to note that a comparison of the secondary structure between NMR analysis of synthetic peptides with 20 amino acid lengths of *Chi t I* component III and the known X-ray data of the corresponding segments in the native protein shows a good correlation (Czisch *et al.*, 1993). As previously reported (Spangler, 1991), the ability of many antipeptide mAbs to bind the cognate protein in ELISA may depend on denaturation of the antigen as a consequence of adsorption to solid phase surfaces. Therefore, it is very interesting that mAb 3 reacted with solid phase-bound peptides as well as soluble peptides suggesting that the procedure of solid phase binding does in this case not destroy the B-cell epitopes.

In order to identify those amino acid residues which were essential for antibody binding, we examined monoclonal antibodies with overlapping synthetic octamers by ELISA. The findings presented in Table 2 demonstrate that the five amino acid residues F D K K V K corresponding to positions 13-17 are involved in the binding of mAb 3. The monoclonal antibody mAb 6 which in ELISA with peptides of a length of 20 residues only reacted to peptide 11-30, showed positive results with the synthetic octamers 22-29 and 23-30. Therefore, the amino acids 23-29 (I L Y A V F K) were identified to be essential for mAb 6 binding.

The comparison between the position of identified B-cell epitopes and the secondary structure of *Chi t I* component III determined by X-ray crystallography (see Fig. 5) shows that the binding sites of mAbs as well as of human IgE antibodies are localized in helical regions. In NMR analysis of synthetic peptides of component III, the fragment 1-19 also showed preference for an α -helix. For peptide 11-30, indications for a helix were also detected (Czisch *et al.*, 1993).

It should be emphasized that the N-terminal amino acids, recognized by mAbs and human IgE, are also known as T-cell epitopes (Liebers *et al.*, 1993). It would be interesting to investigate in an experimental model whether peptide 1-17 can be used to influence the immune response, i.e. to downregulate the production of IgE antibodies (Nakagawa and Gershwin, 1993).

Using the antigenic index of Jameson and Wolf, we predicted only one B-cell epitope in the N-terminus of *Chi t I* component III, in the region of amino acids 14-17. This calculation showed a high degree of concordance with the experimentally identified mAb 3 binding site containing residues 13-17. Other authors also found a good correlation between predicted and determined immunodominant amino acids (Briggs *et al.*, 1993). In the case of the mAb 6 epitope (residues 23-29) no antigenic potency was calculated. In other instances, however, nonpredictive epitopes have demonstrated immunodominance (Lal and Griffiths, 1991). In addition to the calculation of Jameson and Wolf, nearly 20 other algorithms exist obtaining a rate of correct prediction between 50 and 60% (Pellequer *et al.*, 1991). Thus, B-cell epitope prediction provides a useful indication of immunodominant protein regions but cannot replace the experimental epitope mapping. No calculation has yet considered experimentally determined secondary structure data. Therefore, more successful B-cell epitope prediction methods may be found when scales and information of known secondary protein structures are combined.

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SYNTHETIC PEPTIDE ANTIGENS OF TETANUS TOXIN

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Abstract—In this study the immunochemical structure of the heavy chain polypeptide from tetanus toxin was studied. Numerous antigenic determinants were identified by probing a set of overlapping peptides derived from the amino acid sequence of tetanus toxin with polyclonal anti-toxin antibody preparations. Synthetic antigens representing continuous epitopes were prepared and used to immunize mice. The capacity of the resulting anti-peptide antibodies to react with tetanus toxin *in vitro* and *in vivo* was determined. The majority of antibodies bound to tetanus toxin and three epitopes capable of eliciting neutralizing antibodies were identified.

Key words: tetanus toxin, peptide, vaccine, epitope mapping.

INTRODUCTION

Intracellular tetanus toxin, whose primary structure is known at the gene level (Eisel *et al.*, 1986; Fairweather and Lyness, 1986), consists of a single polypeptide chain with an M_r of ca. 151,000. Upon bacterial cell lysis the extracellular toxin molecule, consisting of disulphide-linked light (M_r ca. 52,000) and heavy chains (M_r ca. 98,000), is produced. The arrangement of cysteine residues within the toxin molecule has been elucidated (Kriegstein *et al.*, 1990; Fischer and Howden, 1993).

Tetanus toxin has played an important role in the development of totally synthetic vaccines. Because of tetanus toxoid's utility as a carrier for other peptide vaccines (see e.g. Jolivet *et al.*, 1987), some effort has been made to identify promiscuous T_H epitope peptides from the amino acid sequence of tetanus toxin (Demotz *et al.*, 1989; Ho *et al.*, 1990; Valmori *et al.*, 1992). On the other hand, we are aware of only one report where an attempt was made to identify potential tetanus synthetic peptide vaccine candidates (Habig *et al.*, 1987). Many laboratories have produced monoclonal antibodies against tetanus toxin and the incidence of neutralizing and protective antibodies amongst them appears to be quite high (Ahnert-Hilger *et al.*, 1983; Kenimer *et al.*, 1983; Sheppard *et al.*, 1984; Volk *et al.*, 1984). These

results, as well as some successes with peptide vaccines from other viral and bacterial toxins (Arnon, 1991) indicate that it should be possible to design a tetanus synthetic vaccine consisting of peptides corresponding to important epitopes of this protein. In the present study we describe the results of our work towards this goal.

Tetanus vaccines in current use consist of chemically inactivated toxin obtained from cultures of *Clostridium tetani*. Apart from the hazards inherent in this vaccine production process, adverse clinical reactions to tetanus vaccination may be due to the fact that tetanus toxin becomes conjugated to bacterial cell debris during the formaldehyde toxoiding process. Well-defined homogeneous recombinant vaccines would be more satisfactory and it has been shown that such an approach is possible (Fairweather *et al.*, 1987). An oral tetanus vaccine consisting of a live attenuated *Salmonella typhimurium* carrier has also been proposed (Fairweather *et al.*, 1990).

MATERIALS AND METHODS

Amino acid analysis was carried out according to the Pico-Tag™ method (Bidlingmeyer *et al.*, 1986). Peptide sequence analyses were conducted according to a method which we have described earlier (Fischer and Howden, 1989).

Epitope mapping

Overlapping hexapeptides (four-residue overlap) covering the entire sequence of tetanus toxin heavy chain were synthesized on blocks of derivatized pins. For this purpose a commercial kit including amino acid derivatives, control pins and antibody, as well as synthesis software was obtained from Cambridge Research Biochemicals Ltd, Harston, Cambridge, England. Peptide assemblies, N-terminal acetylation, final deprotection and immunoassay procedures were carried out following

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Abbreviations: BSA, bovine serum albumin; DMF, *N,N*-dimethylformamide; ELISA, enzyme-linked immunosorbent assay; Fmoc, 9-fluorenylmethoxycarbonyl; HPLC, high-performance liquid chromatography; KLH, keyhole limpet haemocyanin; MBS, *m*-maleimidobenzoyl *N*-hydroxysuccinimide ester; MLD, minimal lethal dose; PBS, phosphate-buffered saline; TFA, trifluoroacetic acid; Trt, trityl.