

Epitope mapping of major insect allergens (chironomid hemoglobins) with monoclonal antibodies

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Monoclonal antibodies (mABs) were raised against the physically and chemically well-defined insect allergen, component CTT III from Chironomus thummi thummi (CTT) hemoglobin. With these mABs, we observed epitopes present in as many different hemoglobin (Hb) molecules from the widespread insect family Chironomidae as possible. These Hbs were identified as important allergens in previous studies. Twenty mABs from seven different clones were tested for specificity against the CTT Hbs III, IIIa, IV, and I to X, as well as against Hbs from 14 other chironomid species; mABs 1 to 3 were found to be specific for an epitope that was expressed on the CTT III component as well as in Hbs from three other species. mABs 5 to 7 and 15 recognized an epitope expressed in CTT III, CTT IIIa, CTT IV, and in Hbs from 13 other species. mABs 8 to 14 and 16 to 20 demonstrated heteroclitic behavior, that is, they bound CTT IIIa better than the immunogen CTT III. Reactivity of mAB 6 with a clinically relevant epitope was demonstrated by up to 15% inhibition of antibody binding by human IgE from sensitized patients. Our results demonstrate that clinically relevant allergenic epitopes can be localized on different molecules with mABs. This could be important in the development of immunotherapy protocols with highly purified or fragmented allergens. (J ALLERGY CLIN IMMUNOL 1987;80:876-83.)

The noncell-bound Hbs of Chironomidae, an insect family distributed worldwide, behave as potent inhalant allergens and are associated with several environmental and occupational diseases.¹⁻⁴ Larvae from one European species, CTT, possess at least 12 monomeric or dimeric Hb components (CTT I to X). The amino acid sequences demonstrate each component to have a unique primary structure.⁵ The three-dimensional structure of one monomeric component has been resolved at high resolution (1.4 nm).⁶

Recently, we elucidated the antigenic structures of various clinically important antibody-binding sites within well-defined regions of the Hbs CTT IV, CTT VI, and CTT VIII from CTT by studying fragments that bind human IgE and IgG antibodies.⁷ One

Abbreviations used

CTT:	<i>Chironomus thummi thummi</i>
FCS:	Fetal calf serum
Hb:	Hemoglobin
IB:	Immunoblot technique
mAb:	Monoclonal antibody
PBST:	50 mmol/L Na ₂ HPO ₄ /NaH ₂ PO ₄ , pH 7.5, 150 mmol/L NaCl, 1% Tween 20, 0.1% NaN ₃ , and 0.1% bovine serum albumin
PEG:	Polyethylene glycol
PBS:	Phosphate-buffered saline

antigenic determinant from CTT IV, comprising 11 continuous amino acids (position 91 to 101), was also found within the homologous three-dimensional structure of Hb CTT III. This fragment demonstrates characteristics that are typical for antigenic peptides, for example, a predominance of polar or hydroxy amino acids, a highly accessible surface location, the possibility of linkage to other molecules, and high thermal mobility factors that allow flexibility and site adaptation of the molecule.

Further elucidation of the structure and physico-

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TABLE I

Clone	Subclone	mAB No.	% Binding to solid-phase antigens					
			CTT Hbs				Sperm whale	Mouse
			III	IIIa	IV	I-X	myoglobin	Hb
H2D6	H2D6B1	1	100	—	20	8.5	0	0
	H2D6G3	2	100	—	24	8.5	0	0
	H2D6G10	3	100	0	18	9.0	0	0
H3E11	H3E11E6	4	0	0	0	0	0	0
H4B2	H4B2C7	5	100	—	64	47	13	0
	H4B2H2	6	100	35	70	60	10	0
	H4B2F9	7	100	—	70	57	12	0
H7D9	H7D9F7	8	100	325	0	92	0	0
H7E10	H7E10B8	9	100	310	0	90	0	0
	H7E10D8	10	100	240	0	98	0	0
	H7E10E9	11	100	—	0	96	0	0
	H7E10H2	12	100	250	10	100	0	0
	H7E10H7	13	100	—	10	100	0	0
	H7E10H12	14	100	270	0	100	0	0
H8G9	H8G9D3	15	100	35	65	65	0	0
H8H1	H8H1A10	16	100	—	0	100	0	0
	H8H1D7	17	100	—	0	90	0	0
	H8H1F9	18	100	250	0	80	0	0
	H8H1F11	19	100	—	0	100	0	0
	H8H1F12	20	100	230	0	100	0	0

Reactivity of 20 mAB with different solid-phase antigens by the RAST method. With the exception of clone H3E11 (negative control), the binding to immunogen CTT III is taken as 100%. (Counts per minute obtained with the individual mAB against CTT III ranged from 11,000 to 25,000.) Amino acid analysis of hydrolyzed RAST disks demonstrated the amount of Hb CTT III, IIIa, or IV covalently bound to the individual paper disk to be 0.04 nmol plus/minus 10% of each chain.

chemical characteristics of the hemoglobin allergens, especially of the antibody-binding sites, should help to provide greater insight into the immunopathogenesis of IgE-mediated diseases. In this article we describe the results of an investigation into the antigenic sites of the CTT III molecule and other chironomid Hbs using monoclonal antibodies as precise probes for checking sequences and structures at the molecular level.⁸⁻¹² Using the discriminatory capacity of the mAB, we searched for specific determinants that are expressed on different chironomid Hbs. We were able to identify antigenic determinants specific for CTT III as well as antigenic determinants found on different Hbs from CTT and other chironomid species. In addition, competitive inhibition was demonstrated between human IgE antibodies and mABs.

MATERIAL AND METHODS

Patients

A mixed serum pool obtained from eight fish enthusiasts was used for studying cross-reactivity between human IgE and mAB. All these patients had suffered from bronchial

asthma with or without rhinitis, conjunctivitis, and wheal-and-flare reactions after contact with freeze-dried chironomid larvae, a common fish food. In addition, all exhibited positive results in prick testing and RAST investigations with the crude extract of CTT larvae.

Preparation and purification of Hb extracts

Preparation of crude extracts of Chironomidae larvae from different species and further fractionation in monomeric and dimeric Hbs was carried out as described previously.^{1,7}

Isolation and purification of different Hbs, especially of CTT III, IIIa, and IV, were performed on DEAE cellulose exchanger (DEAE cellulose, preswollen, Pharmacia, Uppsala, Sweden) with Tris buffer and salt gradients.¹³ The homogeneity of the individual Hb components was always checked by polyacrylamide gel electrophoresis and with the IB. In addition, highly purified components were checked by N-terminal degradation of the globin.

Animals and cell lines

BALB/c mice were obtained from the Tierversuchsanstalt, Hannover, Federal Republic of Germany. The my-

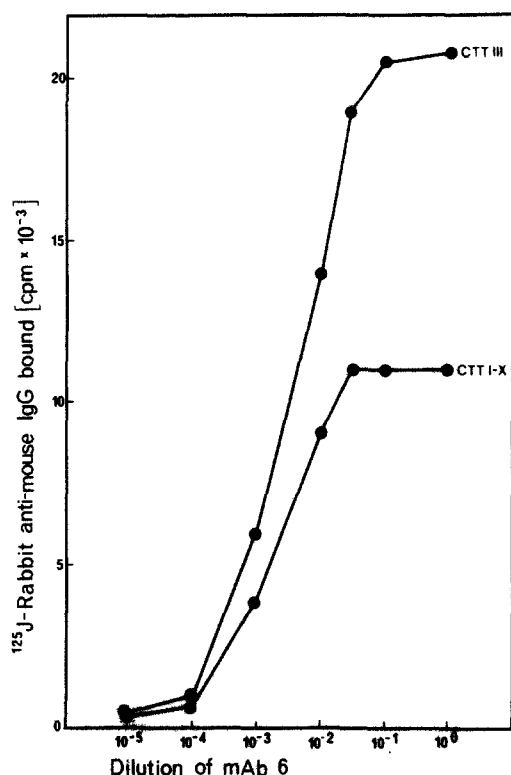


FIG. 1. Binding of mAb 6 to solid-phase antigens CTT III and CTT I to X.

eloma cell line P3x63Ag8U1 was a kind gift from Prof. Dr. A. Porschmann, Universitätsklinik, Hamburg-Eppendorf, Federal Republic of Germany.

Cell-culture conditions

Twenty percent FCS-RPMI 1640 medium, used in the production of mAb, contained 100 ml of RPMI 1640 with 20% (vol/vol) FCS, 10,000 units each of penicillin and streptomycin, 2 mmol/L of glutamine, and 1 mmol/L of Na-pyruvate (all reagents from Gibco, Eggenstein, Federal Republic of Germany). HT medium was 20% FCS-RPMI 1640 with 15.5 mg/L of thymidine (Sigma Chemical Co., Deisenhofen, Federal Republic of Germany) and 54.4 mg/L of hypoxanthine (Sigma Chemical Co.).

HAT medium was HT medium with 176 µg/L of aminopterin.

Production of mAb

BALB/c mice were injected intraperitoneally with 1 mg of CTT III Hb in PBS and complete Freund's adjuvant (1:1). Booster injections were administered on days 8 and 29 with the same amount of antigen and incomplete Freund's adjuvant. On day 32, 10⁶ splenic lymphocytes were fused with 10⁷ P3x63Ag8U1 cells by mixing with 500 µl of 50% PEG in RPMI 1640 and incubating for 3 minutes at 37° C. The cells were centrifuged for 5 minutes at 400 g, and the

PEG was diluted dropwise with 10 ml of RPMI 1640. The mixture was washed once, suspended in 62 ml of HAT medium, and plated out in 100 µl samples in a microtiter plate. Cells were cultivated at 37° C in a CO₂ incubator. Seven positive clones were subcloned with the method of limiting dilutions, and 20 subclones fulfilling monoclonal criteria were selected (Table I).¹⁴ After 14 days, the hybridoma supernatants were screened in an ELISA dot test with CTT I to X and CTT III as antigens.¹⁵

Isotype determination

The subclones were subclassed by agar-agar double diffusion.¹⁶

RAST

The RAST method in which extracts of chironomid larvae or highly purified Hbs are bound to CNBr-activated paper disks¹ was used for antibody characterization. The binding of mAbs to solid-phase allergens was measured by incubating the solid-phase allergens overnight with supernatant antibodies diluted in 100 µl of RPMI medium. A dilution of 1:20 was used unless it is otherwise indicated. Each disk was then washed three times with PBST, incubated in 100 µl of ¹²⁵I-labeled rabbit antimouse IgG (New England Nuclear, Boston, Mass., 1.6 × 10⁵ cpm in 100 µl of PBST) for 3 hours, and washed, and the bound radioactivity was measured in a gamma counter. Culture media and FCS were always tested in parallel. Background remained in the range of 200 to 500 cpm; all values <1000 cpm were considered to be negative. In order to estimate the amount of Hb covalently bound to the paper disks, 15 disks coupled with chains of Hb CTT III, Hb IIIa, or Hb IV were hydrolyzed in constant boiling hydrochloric acid at 110° C for 20 hours. The hydrolysates were centrifuged, and aliquots were investigated by means of automatic amino acid analysis.

RAST inhibition

Competitive inhibition by human IgE of supernatant antibodies binding to solid-phase-bound CTT III was performed as follows: 25 µl of pooled or individual IgE serum from patients with type I allergy against CTT Hbs was incubated with serially diluted mAb 6 (final volume 100 µl; Fig. 5, B) and solid-phase allergen CTT III during a period of 16 hours. Alternatively, 0.5 µl of supernatant mAb 6 was incubated with different volumes of IgE (see Fig. 5, A). After three washes with PBST, the bound mAbs were detected with ¹²⁵I-labeled rabbit antimouse IgG (1.6 × 10⁵ cpm in 100 µl) titrated with 0.1 mg of human immunoglobulin (Intraglobin, Biotest Pharmaceuticals, Dreieich, Federal Republic of Germany) to reduce background binding of human immunoglobulin. The amount of human IgE was detected with ¹²⁵I-labeled rabbit antihuman IgE (Phadebas RAST Kit, Pharmacia Diagnostics, Uppsala, Sweden). A series of controls was performed for each IgE-binding assay. For these controls, we used individual and pooled serum samples from atopic individuals not sensitized to CTT Hb and from nonallergic individuals, replacement

of mAB 6 by mAB B01, specific for an epitope on timothy-grass pollen (see Fig. 5, C), and replacement of each of the assay components individually by PBS.

Polyacrylamide gel electrophoresis and immunoblotting

Hbs were separated by polyacrylamide gel electrophoresis under native conditions with a discontinuous buffer system according to the method of Ornstein,¹⁷ Davies,¹⁸ and Baur et al.¹⁹

For blotting and immunologic detection, pure components or electrophoretically separated Hbs were transferred to nitrocellulose sheets¹⁹; 0.4 cm wide strips were incubated with 10 μ l of supernatant mAB in 2 ml of PBS overnight, washed with PBST, incubated 3 hours with 6 to 8 $\times$ 10⁴ cpm of ¹²⁵I-labeled rabbit antimouse IgG (New England Nuclear), washed, and then were autoradiographed on Kodak (Atlanta, Heidelberg, Federal Republic of Germany) XAR films.

RESULTS

Characterization and specificity of mAB

Mouse mABs were produced with Hb component CTT III as immunogen. The hybridoma supernatant fluids from growing cultures were screened with ELISA to test each well separately for antibodies against CTT Hbs. Seven positive clones were obtained, selected out, and subcloned with the method of limiting dilution. Twenty subclones were cultivated, and monoclonal antibodies in their supernatants were used in the investigations (Table I). All antibodies were in the IgG1 subclass.

Reactivity of mAB with CTT Hbs

The specificity of the mAB was tested with RAST against CTT III, IIIa, IV, and I to X as solid-phase antigens and the IB. Amino acid analysis of the solid-phase antigens after hydrolysis of prepared disks demonstrated that the amount of the different Hbs bound to the disks was very similar (0.04 nmol plus/minus 10%). Thus, the comparative binding intensity to the different Hbs could be assessed directly from the amounts of mAB bound to the different disks. With the exception of No. 4 (negative control), all the mABs reacted with CTT III (Table I). Fig. 1 illustrates a typical binding curve for the clone 6 mAB at different dilutions.

The binding to the total Hb fraction of CTT I to X was 60% of the binding to CTT III. In contrast, binding of mABs 1 to 3 to CTT I to X was only 10% of that to CTT III (Table I). These antibodies also demonstrated a very weak reaction to CTT IV (Table I). The mABs 1 to 3 recognized a band in the IB of electrophoretically separated total Hb whose electro-

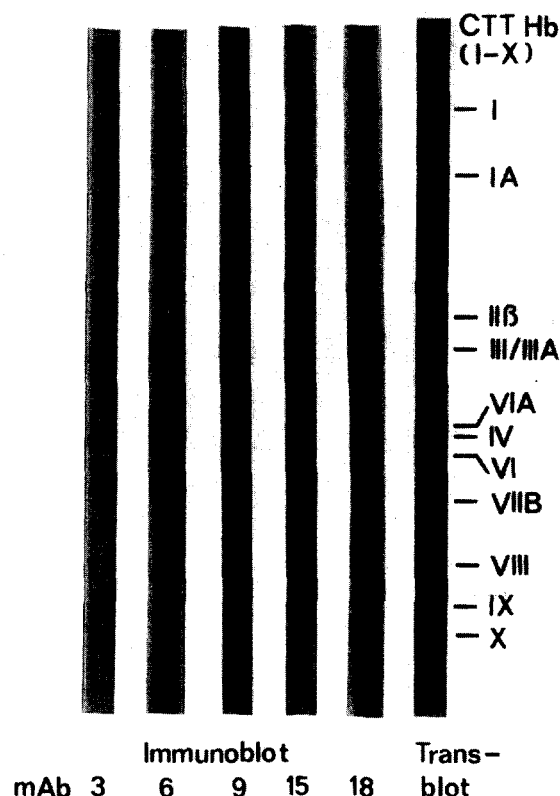


FIG. 2. Immunoblot illustrating the binding of mAB 3, 6, 9, 15, and 18 with CTT Hb I to X (lane 1 to 5) and proteinblot of CTT Hb I to X (lane 6).

phoretic mobility was the same as that of CTT III (Fig. 2). The mABs 5 to 7 demonstrated an intermediate binding to the whole Hb (I to X), a nearly equivalent binding to CTT IV (70%), a weaker binding to CTT IIIa (35%; Table I), and recognized bands in the IB equivalent to components III and IV as well as a band localized between CTT VI and VIIb (Fig. 2). mAB 8 demonstrated an equivalent binding to CTT I to X, but heteroclitic binding to CTT IIIa. A similar binding pattern was observed for mABs 9 to 14 (Table I). They all demonstrated no, or only a weak (10%) binding, to CTT IV and recognized a major band in the IB whose electrophoretic mobility was the same as that of CTT III (Fig. 2). The mAB from clone 15 had a binding pattern similar to that of mABs 5 to 7, and those from mABs 16 to 20, a pattern similar to that of clones 9 to 14 (Table I; Fig. 2). Thus, the mABs 1 to 20 can be divided into five groups (Table I) according to their specificity. The supernatants from clones 3, 6, 9, 15, and 18 were selected for further investigations.

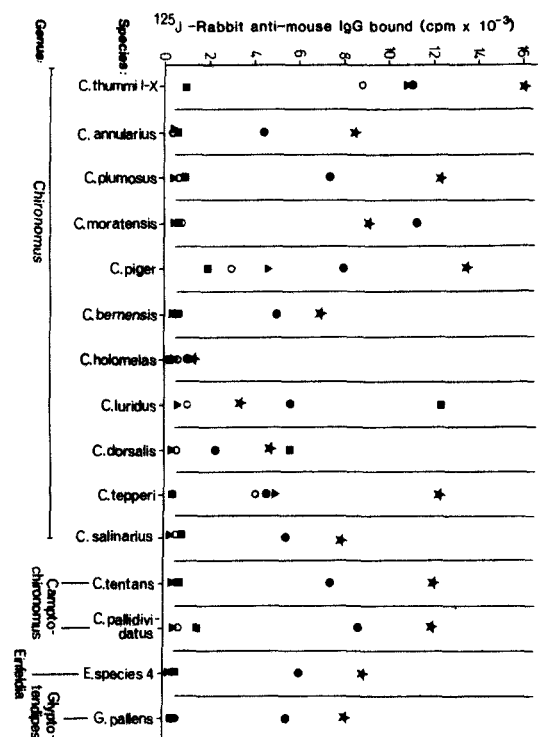


FIG. 3. Reactivity of five groups of mAB raised against CTT III with crude extracts of different chironomid species, listed according to their evolutionary relationship: mAB 3 (■), mAB 6 (●), mAB 9 (▲), mAB 15 (★), and mAB 18 (○).

Cross-reactivity of CTT mABs with Hbs from other chironomid species

The reactivity of mABs 3, 6, 9, 15, and 18 with larvae extracts from 14 different chironomid species was tested. The binding of mABs 3 and 6 was investigated further with the IB.

With RAST, mAB 3 bound high to Hbs from *C. luridus*, intermediate to Hbs from *C. dorsalis*, and weaker to Hbs from *C. piger* (Fig. 3). In each case, one Hb band was recognized in the IB (Fig. 4, lanes 1 to 4); mAB 6 reacted with components from 13 species but not with Hbs from *C. holomelas* (Fig. 3). In general, more than one Hb component was recognized in the IB (Fig. 4, lanes 5 to 16), mAB 9 recognized Hbs from *C. piger* and *C. tepperi*, and mABs 15 and 18 demonstrated nearly the same specificity as mABs 6 and 9, respectively.

Binding to nonchironomid hemoproteins

All mABs were investigated for reaction with mouse Hb and were found negative; mABs 5 to 7 reacted weakly with sperm whale myoglobin (Table I).

Inhibition of binding of human IgE by mAB

In order to test whether the determinant recognized by mAB 6 is also recognized by anti-CTT antibodies from allergic patients, human antibodies and mAB 6 were tested in vice versa inhibition studies. First (Fig. 5, A), 50 μ l of pooled or individual human sera containing specific IgE was demonstrated to inhibit the binding of mAB to CTT III disks by about 15%. In contrast, individual serum samples from atopic persons not sensitized to CTT Hb and pooled sera from nonallergic individuals demonstrated no inhibition. Second, the binding of 25 μ l of IgE sera from sensitized patients was tested in the presence of different concentrations of mAB 6 (Fig. 5, B); mAB 6 inhibited binding of the specific IgE sera in a dose-dependent manner. In a further test, mAB 6 was replaced by mAB B01 (specific for an epitope on timothy-grass pollen). This mAB had no effect on the binding of IgE antibodies to CTT III disks (Fig. 5, C).

DISCUSSION

Monoclonal supernatant antibodies were prepared from 20 different clones that were raised with the structurally well-defined allergen CTT III as immunogen. The clones comprised five groups with different specificities. mAB binding to a characteristic epitope of component III of the CTT Hb was identified (mABs 1 to 3) as well as mABs that recognized a determinant present on CTT III, CTT IV, and CTT IIIa (mABs 5 to 7 and 15).

The primary structures of the CTT Hbs III and IV are very similar.⁵ They differ in only 16 of 136 amino acids and have long sequences (up to 25 amino acids) that are identical. In contrast, CTT III and IIIa differ widely.¹³ If the CTT IIIa homologue is matched to CTT III,⁵ then CTT IIIa is found to be extended at the N-terminal end by eight amino acids and at a C-terminal end by five amino acids. Only 42 of the 136 amino acids in CTT III have a position identical to that in IIIa. These similarities and differences in the primary structures of CTT IV, III, and IIIa presumably explain why the mABs 5 to 7 and 15 demonstrate equivalent binding to CTT IV and only moderate binding to CTT IIIa.

mABs 8 to 14 and 16 to 20 demonstrated heteroclicity. On average they bound 250% stronger to CTT IIIa than to the immunogen CTT III. Direct differentiation in the immunoblot was not possible because the two components have virtually identical electrophoretic mobilities under the conditions used (Fig. 2).¹³

There are two possible explanations for this ab-

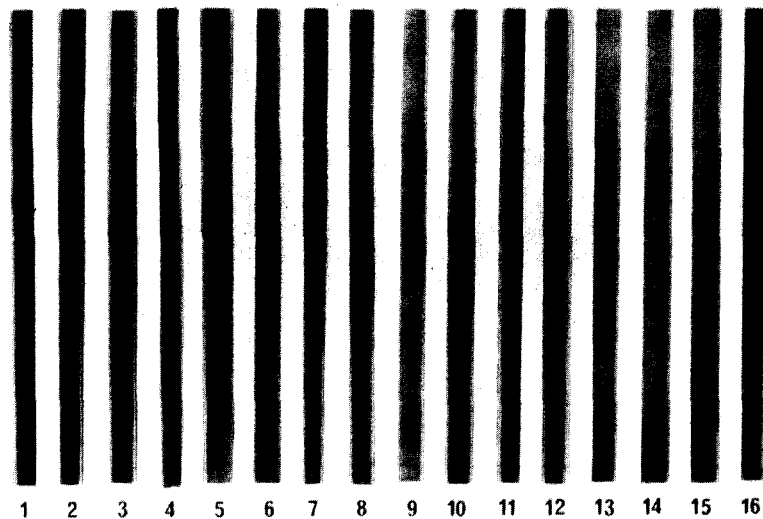


FIG. 4. Immunoblot illustrating the binding of mAB 3 (lanes 1 to 4) and mAB 6 (lanes 5 to 16) with Hbs of different chironomid species. (Only positive nitrocellulose strips are illustrated.) Lanes 1, CTT I to X; 2, *C. piger*; 3, *C. luridus*; 4, *C. dorsalis*; 5, *C. annularius*; 6, *C. plumosus*; 7, *C. moratensis*; 8, *C. piger*; 9, *C. bernensis*; 10, *C. luridus*; 11, *C. dorsalis*; 12, *C. tepperi*, 13, *C. tentans*; 14, *Einfeldia* species 4; 15, *G. pallens*; and 16, CTT I to X.

normal binding behavior. The first is that the immunogen CTT III was contaminated with CTT IIIa and that antibodies were raised against the latter component. N-terminal degradation of the immunogen, however, demonstrated that there was no detectable contamination with CTT IIIa. Furthermore, the total extent of binding to CTT I to X makes it unlikely that the mABs were specifically directed against the IIIa component, since this comprises <1% of total Hb.¹³ The second possibility is that the mABs really demonstrated heteroclitic binding. mABs with heteroclitic binding properties have been described by other investigators.^{20, 21} For example, mAB raised against hen egg white lysozyme bind >125% stronger to closely related lysozymes,⁹ and a mAB against sperm whale myoglobin only recognizes the immunogen when it is adsorbed by polystyrene.²⁰ In the latter case it is believed that the mAB recognizes a sequential epitope that is only readily accessible when the molecule is adsorbed onto a surface. This could be true for our heteroclitic-binding mAB. The possibility that the CTT III/IIIa conformation is altered by the binding to CNBr-activated disks, thus changing the three-dimensional configuration, will be investigated in further experiments. In addition, further competitive inhibition studies should help clarify the specificity of the effect.

We have described the presence of cross-reactivity

between Hbs from different chironomid species in previous articles.^{19, 22} Human IgE from patients exposed to *Cladotanytarsus lewisi* in the Nile region, for example, reacts with extracts from CTT. Thus, it is evident that the same determinants are expressed on Hbs from different chironomid species and recognized by immunoglobulins. Our investigation of the reactivity of mAB 3, 6, 9, 15, and 18 with Hbs from 15 different chironomid species demonstrated clearly the presence of cross-reactivity and common expression of particular epitopes.

mAB 3 recognized a band in Hb from *C. luridus*, *C. dorsalis*, and *C. piger*, indicating that one epitope characteristic for component III from CTT is also expressed in Hbs from these species.

The determinant recognized by both mABs 6 and 15 with minor differences in binding intensity is apparently present in many Hbs from other chironomid species. As many as six bands were recognized by these mABs in immunoblot studies (Fig. 4, lanes 5 and 6). It appears unlikely that this resulted from nonspecific binding in view of the different reaction behavior and the lack of a reaction with Hb from *C. holomelas* and the mouse. The only cross-reaction observed with nonchironomid Hbs was a weak reaction between mABs 5 to 7 and sperm whale myoglobin that is a monomer (Table I). Thus, mABs 5 to 7 and 15 appear to bind to a segmental or topographic

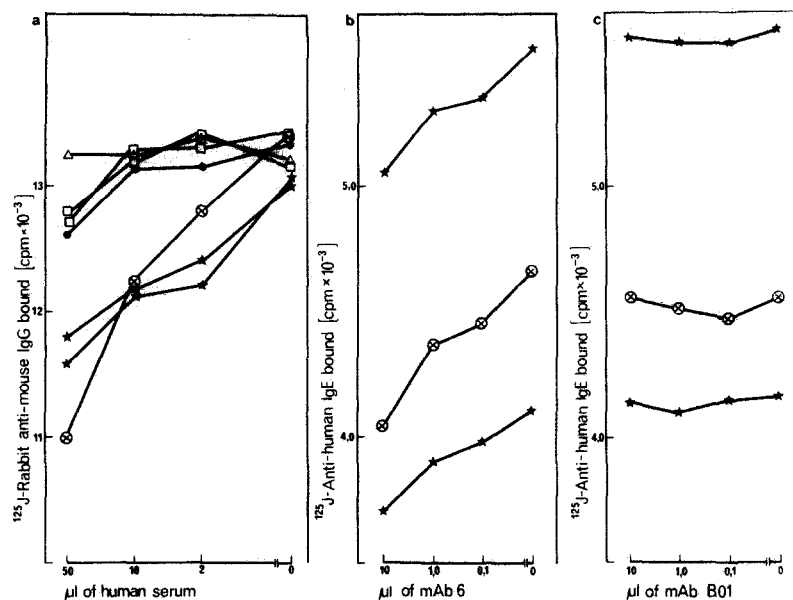


FIG. 5. A-C. RAST inhibition studies on the cross-reactivity between human IgE sera and mAB 6. Solid-phase antigen CTT III was incubated with **A**, 0.5 μl of mAB 6 and dilutions of different human sera. **B**, With 25 μl of human IgE sera and dilutions of mAB 6. **C**, With 25 μl of IgE sera and dilutions of mAB B01, specific for an epitope on timothy-grass pollen. Symbols indicate pooled IgE sera from sensitized patients ($n = 8$), ($\otimes$), individual IgE sera from sensitized patients ($\star$), individual sera from atopic persons not sensitized to CTT Hb ($\square$), pooled control sera from nonallergic persons ($\bullet$), and PBS instead of serum (Δ).

epitope that is present with a similar superficial location and homologous primary structure in many different chironomid Hbs.

A further interesting question was whether both mAB 6 and antibodies in IgE sera of patients bind to the same epitope, in other words, whether a single antigenic and allergenic determinant occupies the same site on the immunogen/allergen CTT III. The competitive inhibitor studies demonstrated that binding of mAB 6 was inhibited by up to 15% by IgE antibodies from sensitized patients (Fig. 5, A). Similar results were obtained in the vice versa experiments, that is, binding of pooled as well as of individual IgE sera was inhibited by mAB 6 (Fig. 5, A and B). Although the inhibition rates obtained were relatively low, it must be taken into account that patient serum contains antibodies against many different CTT Hb epitopes and that the human and monoclonal antibodies probably have different affinities for the same epitope. The specificity of the binding and the inhibiting capacity of mAB 6 in these experiments was further confirmed by the finding that the nonrelevant mAB B01, which is specific for an epitope on timothy-grass pollen, did not have a similar effect.

The aim of these studies was to produce mAB that could be used to identify one or more clinically relevant determinants distributed as widely as possible

in different chironomid Hbs, thus identifying suitable protein structures for therapeutic approaches. The reactivity of mAB 5 to 7 and 15 provides evidence for the likely existence of such epitopes. Further experiments will be aimed at the use of these mABs to identify and localize the common epitope(s) in the components III, IV, and probably IIIa.

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