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Combining phage display and molecular modeling to map the epitope of a neutralizing antitoxin antibody

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Crotoxin is a potent presynaptic neurotoxin from the venom of the rattlesnake *Crotalus durissus terrificus*. It is composed of the noncovalent and synergistic association of a weakly toxic phospholipase A_2, CB, and a nontoxic three-chain subunit, CA, which increases the lethal potency of CB. The A-56.36 mAb is able to dissociate the crotoxin complex by binding to the CA subunit, thereby neutralizing its toxicity. Because A-56.36 and CB show sequence homology and both compete for binding to CA, we postulated that A-56.36 and CB had overlapping binding sites on CA. By screening random phage-displayed libraries with the mAb, phagotopes bearing the (D/S)GY(A/G) or AAXI consensus motifs were selected. They all bound A-56.36 in ELISA and competed with CA for mAb binding, although with different reactivities. When mice were immunized with the selected clones, polyclonal sera reacting with CA were induced. Interestingly, the raised antibodies retained the crotoxin-dissociating effect of A-56.36, suggesting that the selected peptides may be used to produce neutralizing antibodies. By combining these data with the molecular modeling of CA, it appeared that the functional epitope of A-56.36 on CA was conformational, one subregion being discontinuous and corresponding to the first family of peptides, the other subregion being continuous and composed of amino acids of the second family. Phage-displayed peptides corresponding to fragments of the two identified regions on CA reacted with A-56.36 and with CB. Our data support the hypothesis that A-56.36 and CB interact with common regions of CA, and highlight residues which are likely to be critical for CA–CB complex formation.

Keywords: epitope; molecular modeling; neutralizing antibody; phage display; toxin.

Crotoxin is the major lethal component of the venom of the South American rattlesnake *Crotalus durissus terrificus* (*C. d. t.*) [1]. It is a presynaptically acting phospholipase A_2 (PLA_2), a β-neurotoxin, which blocks acetylcholine release from nerve endings [2]. Crotoxin is made of the non-covalent association of two different subunits, CA and CB. CA is an acidic, nontoxic and nonenzymatic subunit that results from the proteolytic cleavage of a PLA_2-like precursor, which has been cloned and sequenced [3]. The enzymes involved in the proteolysis are as yet unidentified, thus a recombinant form of CA can not be obtained. Mature CA is composed of three disulfide-bridged peptide chains (α, β, γ) [4,5]. CB is a single-chain, basic and weakly toxic PLA_2. The toxicity of crotoxin results from the synergistic action of the two subunits, CA increasing the specificity of binding of CB at the neuromuscular junction and therefore its lethal potency. CA is also temporarily involved in the binding of crotoxin on its receptor before being released free in solution [6,7]. As shown for various isoforms of crotoxin, its neuromuscular blocking effect is correlated with the stability of the association of CA and CB in the crotoxin complex [8–10].

Severe envenomation following *C. d. t.* snakebites is commonly treated by intravenous injections of large doses of horse $F(ab)'_2$ prepared from animals hyperimmunized against the crude venom. Nevertheless, only 10–20% of the immune response is directed against the venom proteins used for immunization [11]. These heterologous antibodies are, therefore, poorly effective, immunogenic and might be responsible for adverse effects. Consequently, several attempts have been made to generate murine mAbs characterized by a higher neutralizing capacity against the lethal potency of crotoxin. Such molecules can be further engineered to produce Fab or single-chain variable fragment (scFv), which are less immunogenic and exhibit faster distribution and clearance kinetics than $F(ab)'_2$. Most of the crotoxin-neutralizing mAbs were found to react with the PLA_2 subunit CB, blocking its enzymatic activity or preventing crotoxin binding to its acceptor [12,13]. However, a mAb directed against the nontoxic subunit CA, A-56.36, has recently been shown to efficiently neutralize *in vivo* crotoxin toxicity by inducing the dissociation of the crotoxin complex and preventing its interaction with the crotoxin receptor [14]. A-56.36 is an IgG1 antibody binding to CA with a high affinity ($K_d = 5 \times 10^{-9}$ M) [13] and competing with CB for the binding to CA [10]. Furthermore, sequence similarity was found between some complementarity-determing regions of A-56.36 and two regions of CB, suggesting that A-56.36 and CB interact with the same or overlapping binding sites on CA [15]. Considering the biological activities of A-56.36, determining its epitope on CA should lead to definition of the region of CA involved in the association with CB and to the design of peptides which could be used in a vaccine approach to *C. d. t.* envenomation.

Among the several approaches used for epitope mapping, phage display techniques have proven to be successful in the determination of continuous epitopes, and of discontinuous epitopes when the three-dimensional structure of the antigen is

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Abbreviations: IPTG, isopropyl thio-β-D-galactoside; PLA_2, phospholipase A_2; SAS, solvent accessible surface; scFv, single chain variable fragment; MD, molecular dynamics.

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known [16,17]. This has also been used in the mapping of critical residues involved at the interface of protein–protein complexes [18]. Given that the three-dimensional molecular structure of the subunit CA has not yet been determined experimentally, nor has the epitope been characterized in any way, we combined phage-display library technology and molecular modeling methods in an attempt to characterize the molecular surface of CA interacting with A-56.36. For that purpose, we screened two nonapeptide libraries displayed on filamentous phages with A-56.36. Two families of peptides mimicking the epitope of A-56.36 on CA were identified. Phagotopes of both families were able to bind CB. In addition, we obtained a three-dimensional model of CA in which several consensus amino-acid residues from the two families of peptides were found to be solvent exposed and located on the α chain and β chain of CA. These residues form a patch of two subregions that may constitute a part of the mAb A-56.36 epitope, as well as of the surface area of CA that interacts with CB.

EXPERIMENTAL PROCEDURES

Reagents

Crotoxin was isolated from *C. d. t.* venom, as described previously [8] and its subunits prepared by ion-exchange chromatography in 6 M urea, as described by Hendon & Fraenkel-Conrat [19]. The anti-CA (A-56.36) and anti-CB (B-71.1) mAbs were obtained and purified, as described by Choumet *et al*. [13]. The synthetic peptides were ordered from Neosystem (France) and were more than 95% pure.

Biopanning

The phage nonapeptide libraries were a gift of R. Cortese (IRBM, Pomezia, Italy) and phage affinity selection for A-56.36 binding was conducted essentially as described previously [20]. Briefly, magnetic beads (Dynabeads M450, tosyl-activated, Dynal A.S., Norway) were coated with A-56.36 and blocked with UV-killed M13KO7 helper phage (5×10^{11} particles·mL^{-1}). The libraries ($10^{11}-10^{12}$ phage particles) were then added and bound phages were eluted with 0.1 N HCl, pH 2.2 buffer containing 1 mg·mL^{-1} BSA. Eluates were neutralized and titrated as transducing units. XL1-Blue cells were infected with eluates, plated and grown overnight at 37 °C. The next day, cells were scraped and an aliquot was used to inoculate a liquid culture. The culture was super-infected with M13KO7 helper phage, isopropyl thio-β-D-galactoside (IPTG; 0.1 mM final concentration) added, and grown for 5 h at 37 °C with strong agitation. Phage particles were isolated by poly(ethyleneglycol) precipitation and resuspended in Tris/NaCl (50 mM Tris/HCl, pH 7.5 containing 150 mM NaCl).

Colony blotting

After super-infection with helper phage, a culture sample was centrifuged to eliminate excess helper phage and the pellet was resuspended in 1 mL of culture medium. Aliquots were spread on agar plates and grown overnight at 37 °C, colonies were then transferred onto nitrocellulose filters (C-super Hybond, Amersham). Membranes were blocked with NaCl/P_i containing 3% BSA and 0.1% Tween 20 and phages were screened by an immunological procedure adapted from Christian *et al*. [21]. The mAb A-56.36 (3 mg·mL^{-1}) and the peroxidase-conjugated goat anti-(mouse Ig) IgG (Biosys, 1 mg·mL^{-1}) were diluted 1 : 2000 in NaCl/P_i containing 1% BSA and 0.1% Tween. The development of positive clones was performed with the ECL detection kit (Amersham).

DNA sequencing

Selected clones were analyzed by single-strand dideoxy-sequencing with the chain termination method [22] using the T7 DNA polymerase kit from Pharmacia.

Immunization of mice

CsCl-purified phage samples were injected in 2-month-old Biozzi mice [23–25]. Immunizations were made by intraperitoneal injection of 150-μL samples (containing 2×10^{12} TU) at day 0, 21, 42 and 79 without adjuvant, in four animals for each immunogen. Blood samples were taken at days 31, 52 and 90. Control animals were injected with the phage pC89.

Carboxymethylation of crotoxin subunit

The CA subunit of crotoxin was reduced and carboxymethylated as described previously [26].

ELISA assays

Binding of phage-displayed peptides to A-56.36. Microtitration plates (Nunc, Denmark) were coated overnight at 4 °C with avidin (10 μg·mL^{-1} in NaCl/P_i) and washed with buffer A (NaCl/P_i containing 0.1% Tween 20). Serial dilutions of phage-displayed peptides were pre-incubated overnight at 4 °C with biotinylated A-56.36 (5 μg·mL^{-1} in buffer B: buffer A containing 0.5% w/v gelatin). The coated plates were blocked with buffer B and the mixtures were allowed to react for 15 min at room temperature with the coating. The plates were then washed with buffer A prior to the addition of peroxidase-conjugated sheep anti-M13 immunoglobulins (Pharmacia) in buffer B. After 1 h at 37 °C and washes with buffer A, 100 μL of 0.1 M sodium citrate buffer pH 5.2 containing 0.2% orthophenylenediamine and 0.03% H_2O_2 were added and the reaction was stopped by the addition of 50 μL 3 M HCl. The optical density was measured at 490 nm.

Binding of immune sera to CA. Microtitration plates were coated overnight at 4 °C with CA (1 μg·mL^{-1} in NaCl/P_i). The coated plates were washed thoroughly with buffer A and the sera diluted in buffer B were added for a 2-h incubation at 37 °C. The plates were washed with buffer A prior to the addition of peroxidase-conjugated goat anti-mouse immunoglobulins (1 μg·mL^{-1} in buffer B).

Binding of phage-displayed peptides to CB. Microtitration plates were coated with avidin and all the following reactions were performed with a Tween 20 concentration of 0.5%. Serial dilutions of phage-displayed peptides were pre-incubated overnight at 4 °C with CB (100 μg·mL^{-1}) and biotinylated mAb B-71.1 (5 μg·mL^{-1}) in buffer B. The coated plates were then blocked with buffer B and the mixtures were allowed to react with the coating for 15 min at room temperature. The plates were then washed with buffer A prior to the addition of peroxidase-conjugated sheep anti-M13 immunoglobulins (Pharmacia, 1/2500 in buffer B).

Binding of A-56.36 to native or reduced CA subunit. Microtitration plates were coated overnight at 4 °C with native or reduced CA (1 μg·mL^{-1}) in NaCl/P_i. After washing and

blocking with buffer B, the plates were incubated for 1 h at 37 °C with serial dilutions of A-56.36 in buffer B. After washing, plates were incubated with peroxidase-conjugated anti-mouse immunoglobulins (Biosys, France, 1/1000).

Inhibition assays. Competition assays between the phage-displayed peptides and CA for the binding to A-56.36 were carried out as follows. Microtitration plates were coated with 100 μL of a solution of 1 $\mu g \cdot mL^{-1}$ CA (10^{-7} M) in NaCl/P_i. A-56.36 (20 $ng \cdot mL^{-1}$ in buffer B) (1.3×10^{-10} M) was incubated overnight at 4 °C with serial dilutions of phage particles. The coated plates were then blocked with buffer B and the mixtures were allowed to react with the coating 20 min at 4 °C. The plates were then washed with buffer A prior to the addition of β-galactosidase-conjugated anti-mouse immunoglobulins (Biosys, France) (1 $\mu g \cdot mL^{-1}$ in buffer B) for 1 h at 37 °C. After washing, the plates were incubated for 30 min at room temperature in the dark with 4-methylumbelliferyl-β-D-galactoside (Sigma) (0.6 mM) in 0.1 M phosphate buffer pH 7.4. After blocking the reaction with 50 μL of 2 M sodium carbonate, the fluorescence was recorded using a Spectroskan II fluorimeter (Labsystem, France), emission and excitation wavelengths being 360 and 448 nm, respectively [27]. The inhibition percentage was calculated as follows: $I\% = 100 \times (I_o - I_c)/I_o$, where I_o and I_c are the fluorescence signals without or with inhibitor, respectively. The binding affinity of mAb to phage-displayed peptides was not calculated because of the unknown number of peptides at the phage surface.

The IC_{50} of immune sera was determined in the same way. A fixed dilution of immune sera (1/200) was incubated with various concentrations of CA overnight at 4 °C. Aliquots of 100 μL of the mixtures were then incubated on CA-coated plates for 20 min at 4 °C. The plates were then washed with buffer A prior to the addition of β-galactosidase-conjugated anti-mouse immunoglobulins, as described above. The IC_{50} was expressed as the concentration of CA that inhibited half of the maximal ELISA response.

Competition assays performed between synthetic peptides and CA for their binding to A-56.36 were performed as follows. Microtitration plates were coated with CA. Biotinylated A-56.36 (25 $ng \cdot mL^{-1}$) was incubated with variable quantities of C3 and C12 synthetic peptides in NaCl/P_i containing 3% BSA and 0.1% Tween 20 (buffer C) overnight at 4 °C. After saturation with buffer C, peptide–antibody mixtures were allowed to react for 5 min at 4 °C with CA-coated plates. The plates were then washed with buffer A prior to the addition of a 1/1000 dilution of streptavidin-labeled peroxidase in buffer C.

Phospholipase A_2 assay

PLA_2 activity was measured using a fluorescent-labeled phospholipid [28]. The effect of immune sera or A-56.36 on the enzymatic activity of crotoxin was measured by analyzing the activity after incubating serial dilutions of sera or antibody with crotoxin for 1 h at 37 °C.

Molecular modeling

All calculations were carried out and visualized on an SGI graphics workstation. The commercial software package INSIGHT II 95.0 (MSI, Cambridge, UK) was used for all calculations. The model for the three-chain molecule CA, isoform 2 [26], was derived from a model for the one-chain precursor proCA, a seven disulfide-bonded PLA_2, by removing the proteolyzed peptides and mutating the remaining amino-acid residues to conform to the sequence of CA. The N-terminal residue of chains β and γ used for the calculations was glycine. The Renetseder *et al*. [29] method of numbering of amino-acid residues was used. The resulting entity was energy-minimized using the CFF91 force field. Atomic charges were taken from the CFF91 package. Cross-terms were not used and energy convergence was considered to have been obtained when the maximum derivative reached 0.416 $kJ \cdot Å^{-1}$ by the conjugate gradients method. In order to mimic the effects of solvent, a distance-dependent dielectric $\varepsilon = 4r$ was used, and the formal charges on all ionizable side chains were set to zero in order to avoid fictitious coulombic interactions between charged side chains and backbone polar groups. This pseudo gas force field produces reasonable solution conformations [30]. Thereafter, and in order to overcome certain potential energy barriers, three independent 100 ps molecular dynamics (MD) trajectories at 300 K with a 1 fs time step using the DISCOVER program were performed. A thermal equilibration lasting 1 ps was performed before each MD trajectory. Files were written every 10 steps. The last point in each trajectory was submitted to a final energy minimization using the same conditions as before, the best last point being taken as a representative model. No constraints whatsoever were introduced at any step of the calculations.

The relative solvent accessible surfaces (SAS) of the individual residues were calculated with the Access-Surf option of the ProStat menu of the HOMOLOGY module. All residues with a relative SAS ≥ 25% were considered as solvent exposed. The probe radius was of 1.4 Å. The atomic radii used are C 1.55 Å, H 1.10 Å, O 1.35 Å, S 1.81 Å and N 1.40 Å.

Statistiscal analysis

Differences between values were examined using the Student's *t*-test. Differences yielding *P*-values < 0.05 were regarded as significant.

RESULTS

Reactivity of A-56.36 to denatured CA

A-56.36 is able to dissociate the crotoxin complex by binding to its CA subunit. To determine whether the A-56.36 binding site was a continuous fragment of CA, we compared the antibody reactivity with native or denatured (reduced and carboxymethylated) CA. A-56.36 bound denatured CA with lower reactivity than native CA, the EC_{50} being, respectively, 10^{-7} and 10^{-10} M (data not shown), demonstrating that the A-56.36 epitope on CA was discontinuous and conformation dependent.

Selection of phage-displayed peptides binding A-56.36 and competition with CA

In order to find peptides mimicking this binding site, two nonapeptide libraries, an unconstrained one (L1) and a disulfide bridge-constrained one (L2), were screened by affinity to A-56.36. In both libraries, an increase in the eluate concentration after the second round of selection was observed. Nevertheless, the selection rate increased poorly in L2 to reach 8.7×10^{-4}, and analysis of eluted phage particles by colony blotting led to the isolation of weakly positive clones from which no consensus sequences could be derived. In contrast, the selection rate increased markedly for L1, indicating a significant enrichment of the eluate in specific clones. Fifty L1-positive clones were isolated by colony blotting after

Table 1. Phagotopes displayed on phage and selected using mAb A-56.36. ND, not determined.

Clone	Peptide sequence[a]	Frequency[b]	Binding to A-56.36[c]	Competition with CA[d]
C12	NKPNWDGYA	1/23	0.648	10.1 ± 0.2
C19	IQWDGYARQ	1/23	0.432	ND
C6	DYDGYGWRE	3/23	0.360	9.3 ± 0.2
C7	WSSGYGTGR	1/23	0.234	8.2 ± 1.0
C4	AYESGYNLP	1/23	0.252	9.7 ± 1.5
C3	KAAQIYSEP	8/23	0.684	20.0 ± 6.0
C14	YAADITNGL	3/23	0.648	11.0 ± 2.3
C9	CSAGICFSE	1/23	0.414	7.2 ± 1.6
Control peptide				
X1	WSLFLNHAE	–	0.198	0
Probe peptides[e]				
CA-a	^{23}SSYGCYCGAGGQGWPQDASDR^{43}	–	1.044	17.3 ± 3.6
CA-b	^{100}DKAAAICFRNSMDT^{112}	–	0.954	17.8 ± 0.4

[a] Residues selected two or more times at the same position are underlined. [b] Number of occurrences among the 23 sequenced positive clones. [c] ELISA measurements of the binding to A-56.36 expressed as A_{490}, SD ± 10%. [d] ELISA measurements of the competition with CA, expressed as the inhibition percentage of the binding of A-56.36 to CA-coated plates in the presence of a fixed amount of phages (10^{12} p.f.u.·mL^{-1}). Average values and SD on two experiments are reported. [e] Peptides derived from regions of CA which share many of the residues found in the phage-peptide sequences.

the second biopanning. Their reactivity was checked by ELISA and the 23 clones giving the strongest signals were sequenced. Among these, 19 clones presented sequence homologies (Table 1), whereas the remaining four were unrelated. Table 1 also shows X1, a clone selected from L1 using an irrelevant mAb and used as a negative control in binding assays to CA.

A competition assay with CA was used to examine whether the positive clones bound specifically to the CA binding site of A-56.36. Table 1 (column 5) shows that each was able to decrease the binding of A-56.36 to CA, the best reactivities being obtained for C12 and C3. Although in the 10–20% range, the inhibitory effects of all selected clones were significant, because the irrelevant phage X1 did not interfere with CA binding to the mAb. The highly multivalent display system utilized in these experiments might play an important role in the partial competition results obtained.

Sequence analysis of the selected clones allowed us to group them into two families presenting consensus motifs. The first family was characterized by the sequence (D/S)GY(G/A), the N-terminus of this consensus frequently containing aromatic residues such as Trp or Tyr. The best reacting clone of this

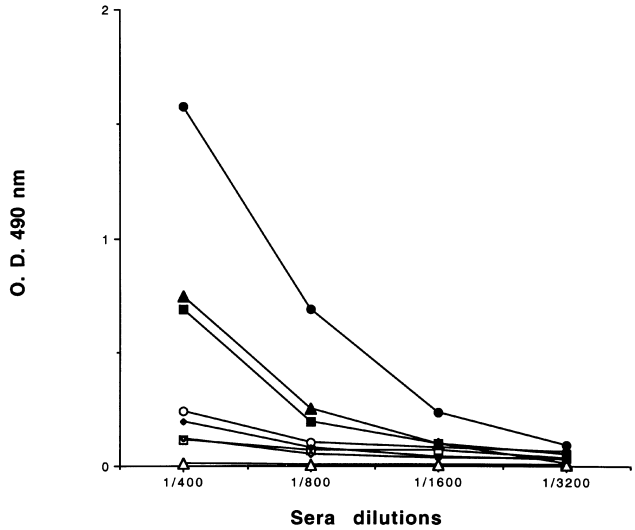

Fig. 1. Reactivity to CA of mice sera directed against phage-peptides C3, C12 and CAb. Microtitration plates were coated with CA and serial dilutions of pre-immune sera (open symbols) or immune sera (closed symbols) directed against phage pC89 (◇), phage-peptides C3 (○), C12 (□) or CA-b (△) were incubated for 1 h at 37 °C.

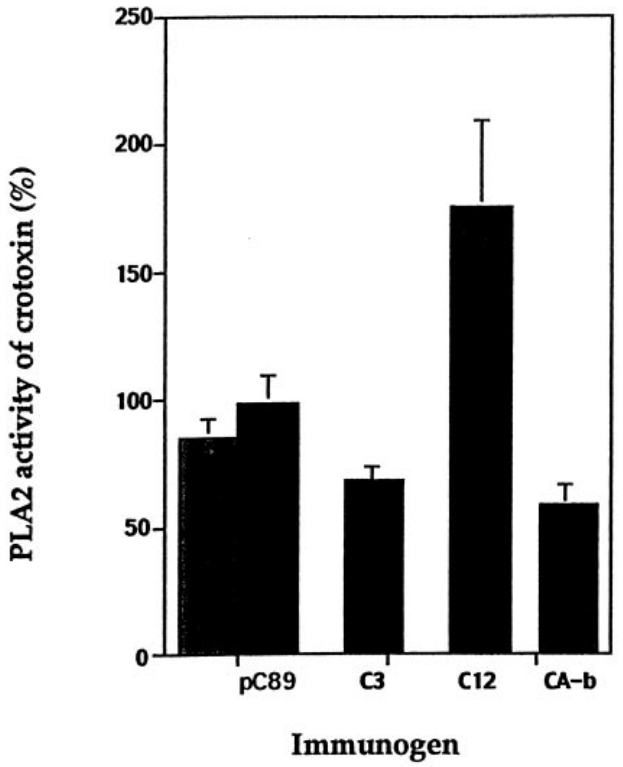

Fig. 2. Effect of the anti-mimotopes immune sera on crotoxin enzymatic activity. A pool of anti-pC89, anti-C12 and anti-C3 and pre-immune sera are compared, before the first injection (S0) and after one boost (S1). Results are expressed as the percentage of crotoxin-specific activity. The average and SD correspond to three independent experiments.

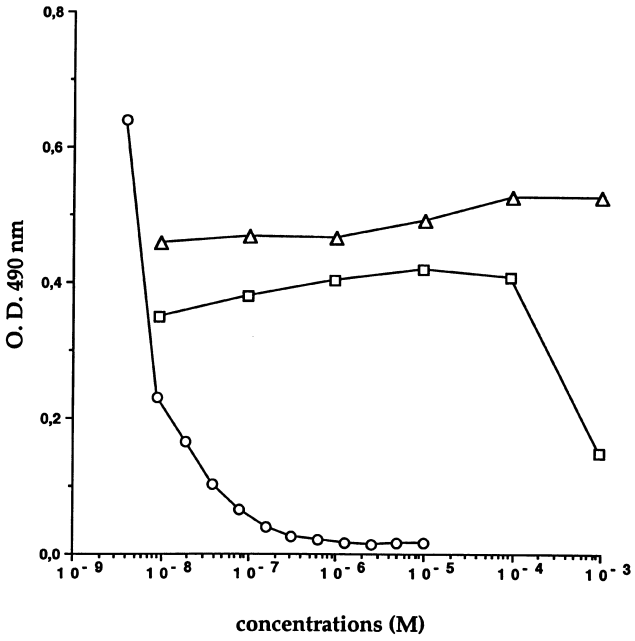

Fig. 3. Competition between CA and synthetic peptides C3 and C12 for the binding to the biotinylated A-56.36 antibody. CA was used to coat microtitration plates. Various molar concentrations of C12 (□) and C3 (△) synthetic peptides as well as CA (○) were incubated overnight at 4 °C with a fixed concentration of biotinylated A-56.36 and allowed to interact for 5 min on the CA-coated microtitration plates. The results of two independent experiments are presented.

family, C12, contained the sequence WDGYA. The second family was characterized by the consensus sequence AAXI and contained three clones, the first Ala missing in one of them. This group contained the peptide C3, which gave the highest ELISA signal for A-56.36 binding and for competition with CA (Table 1).

Immune sera elicited by the phage-displayed mimotopes of CA

From these results, it appeared that C12 and C3 may structurally mimic part of the antibody epitope on CA. To test whether they represent immunogenic equivalents of CA, the phage-displayed peptides were injected into Biozzi mice, in parallel with pC89 control phages, and the immune sera were tested by ELISA for binding to CA (Fig. 1). The immune sera elicited with C12 and C3 reacted significantly with CA, but with IC_{50} values of 2×10^{-7} M and 6×10^{-6} M, respectively.

A-56.36 has been shown to increase the PLA_2 activity of crotoxin, as measured using negatively charged phospholipids [14]. This is a consequence of the dissociation of the crotoxin complex, CB possessing a higher enzymatic activity than crotoxin. To examine whether the sera elicited by the phage-displayed peptides were able to increase the PLA_2 activity of crotoxin, a pool of the four anti-C12 and anti-C3 sera was tested and compared with a pool of pre-immune sera and of sera from mice immunized with the empty virion (pC89). Figure 2 shows that a significant increase in the crotoxin enzymatic activity was observed after pre-incubation of the toxin with anti-C12 sera, indicating a dissociation of the crotoxin complex. This effect was similar to that obtained when A-56.36 was incubated with crotoxin at a molar ratio of 5/1 [14]. Surprisingly, although C3 was found to bind A-56.36 with high reactivity, the anti-C3 sera failed to induce crotoxin dissociation.

Binding of synthetic peptides C3 and C12 to A-56.36

In order to establish whether the selected peptides retained their reactivity out of the phage context, C3 and C12 were synthesized chemically. The interaction of C3 and C12 with A-56.36 was tested in a competition ELISA (Fig. 3). When used at a concentration of 10^{-4} M, C12 could inhibit the binding of A-56.36 to CA. However, no competition was observed with C3, suggesting that the phage environment of C3 was involved in the peptide reactivity [31].

Sequence homology with CA of the phage-display selected clones

Because A-56.36 could bind reduced CA, although with lower affinity than native CA, we looked at whether the identified consensus sequences shared similarities with the CA primary sequence. A motif homolog to the consensus sequence AAXI of the second family (Table 1) was found at the C-terminus of the β chain of CA (Fig. 4). Interestingly, the preceding residue in

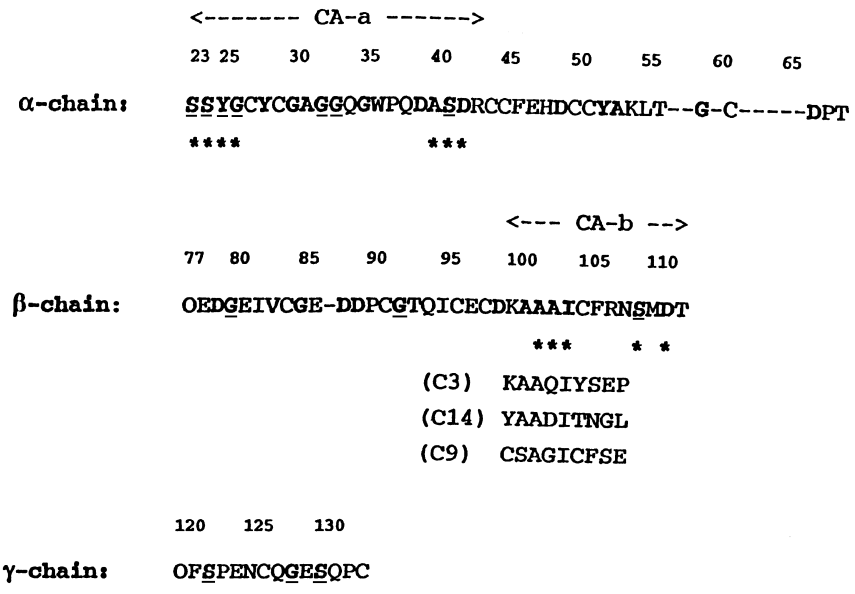

Fig. 4. Amino-acid sequence of CA. The α chain, β chain and γ chain composing the mature CA_2 isoform are shown. Sequences corresponding to CA-a and CA-b peptides are indicated by arrows and the consensus residue types from the two families of phagotopes of Table 1 are given in bold (W, D S, G, Y, A and I). An asterisk indicates the exposed residues contributing to the putative epitope. O indicates the cyclization of the N-terminal glutaminyl residue to pyrrolidone carboxylyl (pyroglutamyl) [26]. The numbering is taken from Renetseder *et al*. [29]; the left digit indicates the corresponding residue.

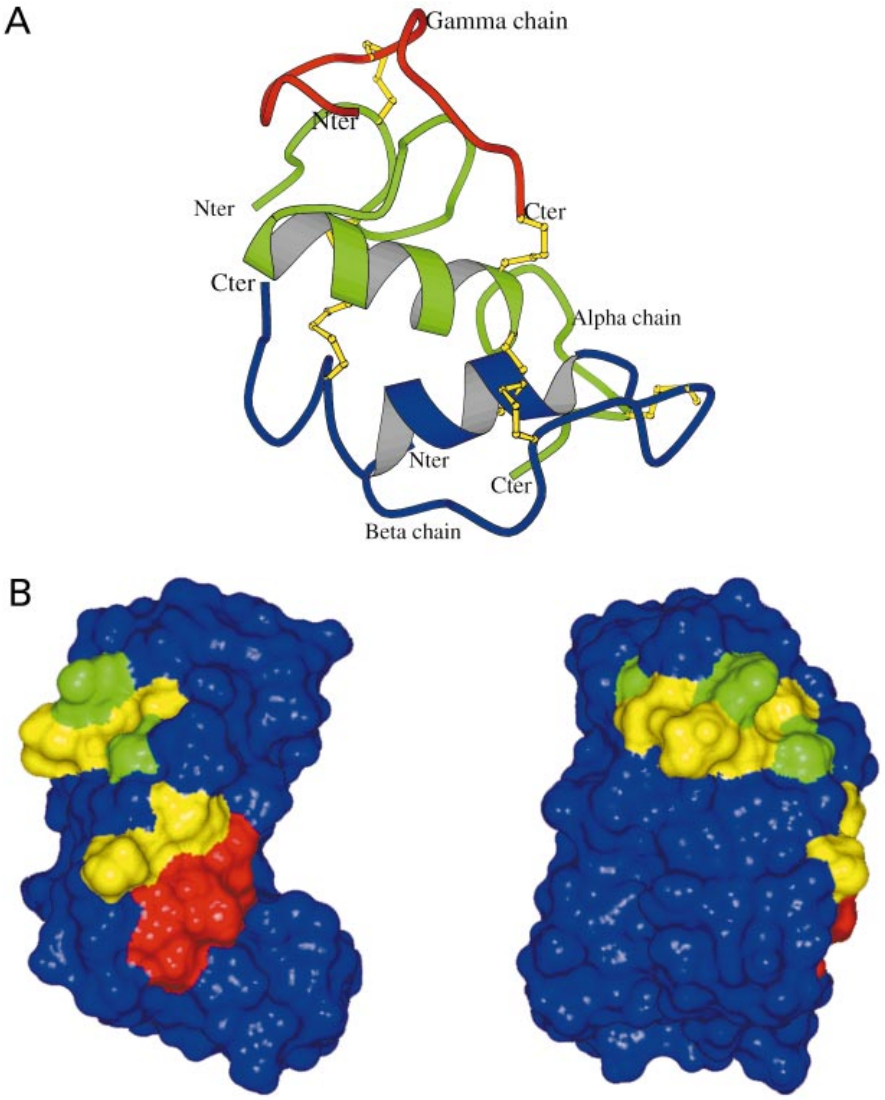

Fig. 5. Molecular modeling of CA. (A) The α chain, β chain and γ chain of CA are represented by green, blue and red ribbons, respectively. Cysteine bonds are in yellow. Exposed residues are for CA-α: S23–G26, G30–G32, Q34, W36, P37, Q38, A40, R43, A53, K54, L55, T56, G59, C61, D67 and T69; for CA-β: O77–G85, E87, D88, P90, C91, T93, K100, A103, I104, F106–T112; for CA-γ: O120, S122–G128, E130–P133. (B) Molecular surface representation of CA and rotated about 90° around the vertical axis. The putative mAb A-56.36 binding region II (residues K100, A103 and I104) is in red. Residues (Y25, G26, A40) and (S23, S24, D39, S41, Ser109, Asp111) from region I are in green and yellow, respectively.

CA-β was a lysine, so that the C3 sequence showed 44% identity to that region. C9 showed equivalent homology to CA, because it contained the alanine and isoleucine of the consensus sequence, extended with cysteine and phenylalanine residues. No significant homology between the peptides of the first family and CA could be established, the corresponding amino acids (in bold on Fig. 4) being found in distant regions in CA.

Epitope mapping using molecular modeling

Contrary to a previous model of the crotoxin complex based on a simple amino-acid replacement of the X-ray structure of *C. atrox* homodimeric PLA_2 [32], a three-dimensional model of CA taking into account the mobility of the N-terminal and C-terminal parts of each of the three chains composing CA was calculated. The resulting α chain is composed of a long unstructured segment (S23–D39) comprising two β turns, a 3.5-turn α helix (A40–K54) and an extended C-terminal part (L55–T69) (Fig. 5A). The N-terminal half of the β chain (Q77–Q94), which folds back into the molecule, is also a nonstructured segment, deriving from the β wing of PLA_2s. The C-terminal part of the β chain is composed of a kinked helix (I95–N108), and an extended segment (S109–T112). The γ chain (Q120–C134) comprises one turn of an α helix, from C126 to E130. All seven disulfide bridges present in PLA_2s are preserved in the CA model. Five of these bridges are interchain (two between chains α and γ and three between chains α and β) and two are intrachain (α–α, β–β).

Several types of consensus residue emerge from the two families of phagotopes in Table 1: Trp, Asp, Ser, Gly, Tyr and Ala from the first family, and Ala and Ile from the second family. Interestingly, the sequence Gly-Tyr always appears in the first family. In the deduced amino-acid sequences of the insert in several mAb-isolated phage-displayed peptides, the amino-acid residue pair Gly-Xaa is widely present [16,17]. Yet, in the actual sequence of the ferritin antigen, the dipeptide appears as Xaa-Gly [16]. This pair is indeed found in the α chain of the CA antigen as Tyr25-Gly26. In the three-dimensional 3D molecular model of CA, the residues of the first family of selected phagotopes are exposed to the solvent in several compact patches. In order to map the functional epitope in the model of CA, only that subset of the consensus residue types that formed a continuous surface region with the Tyr25-Gly26 pair was kept. Given the abundance of Ser and Asp residues in the region, we were unable to choose a unique set of residues. The set is thus constituted by either one or two of Ser23, Ser24, Asp39, Ser41, Ser109 and Asp111 residues for the (D/S) position, and Ala40 for the (G/A) position. The

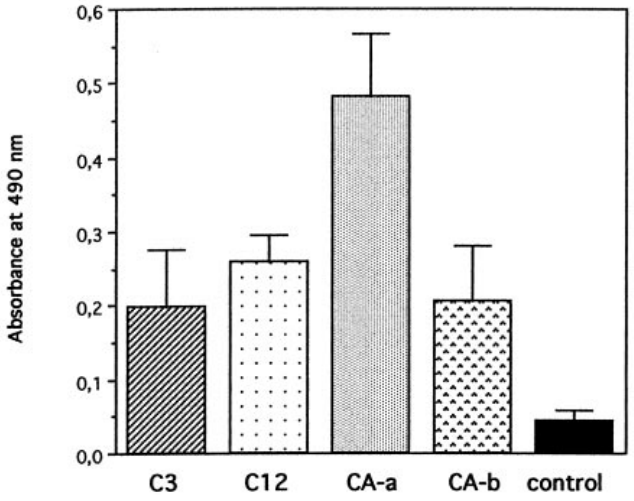

Fig. 6. ELISA measurements of the binding of C3, C12, CAa, CAb and control peptide X1 to CB. The binding to CB of phages expressing the peptides C3, C12, CA-a and CA-b was compared with the binding of the wild-type pC89 phage and of phagotope X1 (control). The results of three independent experiments are presented.

consensus amino acids AAXI of the second family of peptides are found in the C-terminal part of the β chain. Given the helicoidal structure of this segment in the CA model, only residues K100, A103 and I104 show good solvent exposition and form a continuous patch.

Figure 5B shows the SAS of all the above-mentioned residues on CA derived from the two families of phagotopes. They constitute two adjacent clusters at a relative orientation of $\approx 90°$ (regions I and II). The face of CA opposite to the mapped surface in the figure is almost completely devoid of any of the determined residue types.

Binding of CA-a and CA-b to A-56.36

CA-a and CA-b, two fragments of CA shown by molecular modeling to contain most of the amino acids selected by library screening, were subcloned in the pC89 vector. An ELISA using the recombinant phage particles showed that peptides CA-a and CA-b were able to bind A-56.36, with higher reactivity than C12 and C3, respectively. In addition, they competed significantly with CA for antibody binding (Table 1). When mice were injected with the CA-b clone, the resulting immune sera specifically bound CA, with a 10^{-6} M affinity (Fig. 1).

Binding of CA-a and CA-b to CB

If the antibody binds at or near the CA site interacting with CB, peptides mimicking the A-56.36 epitope on CA should bind to CB. To test this hypothesis, the binding of phage-displayed C3, C12, CA-a and CA-b to CB was examined by ELISA. Figure 6 shows that all these peptides bound CB significantly ($P < 0.05$), CA-a and C12 being the best reacting clones. Interestingly, C3 and CA-b showed equivalent ELISA signals, suggesting that C3 mimics this particular region of CA for binding to CB.

DISCUSSION

This study aimed to determine the residues composing the functional epitope of the crotoxin neutralizing mAb A-56.36 using phage display technology, and to locate these residues on a three-dimensional molecular model of CA. A-56.36, which is directed against the CA subunit of crotoxin, binds with a high affinity to the native form of CA. However, as shown here, A-56.36 binds weakly to CA when this subunit is reduced and carboxymethylated, suggesting that a continuous part of the epitope might still be recognized by the antibody in the denatured molecule.

Phage display technology has allowed the identification of two families of peptides reacting specifically with A-56.36. The alignment of the second family of phagotopes with CA was significant and led to a consensus sequence AAXI located in the C-terminal part of the β chain. This part of the β chain of CA seems to constitute the continuous portion of the epitope. Instead, no significant alignment between the consensus sequence of the selected phagotopes of the first family and CA was possible, suggesting that these phagotopes mimic discontinuous fragments of the natural epitope.

The three-dimensional structural model obtained for CA was used to select residues critical for A-56.36 binding. Our model shows that residues of the first consensus family are gathered in space in the folded molecule, are solvent exposed and constitute a compact region (region I, Fig. 5B). Four to five of these residues could be involved in the interaction with A-56.36. These include the Y25, G26 pair and A40 on the one hand, and a Ser and/or an Asp on the other hand. This zone is close enough to residues of the second family of peptides, located on the C-terminal part of the β chain, to form a molecular interaction zone. It is possible that the helicoidal structure of this part of the β chain (region II, Fig. 5B) may be in equilibrium with a nonperiodical conformation, leading to the complete presentation of the detected linear epitope KAAXI (100–104). Indeed spectroscopy experiments indicate modification of the secondary structure of CA upon complex formation [33]. In addition, several of the residues of the discontinuous epitope are located at the ends of the α and β chains, beyond the neighboring disulfide bridges. These ends, by nature very flexible, may allow the movement of anyone of the surrounding serine and aspartate residues towards the (Y25, G26) pair. As a consequence, at least eight residues selected from the random peptide libraries form a compact area on CA (four from each family), representing a surface of 370–400 $Å^2$. Moreover, in agreement with the overall amino-acid composition of protein determinants recognized by antibodies [34], polar/charged and hydrophobic residues are present in regions I and II, respectively. The reported number of residues that contact epitopes contain (15–22), as well as the dimensions of antigens in contact with the antibody (650–900 $Å^2$) [35], suggest that our mapped residues represent part of the whole contact epitope, composed of stretches of continuous amino acids (region II) and of amino acids that are distant in the primary sequence (region I). This is in agreement with previous studies that have shown that epitopes can be a conformational mixture of continuous and discontinuous subregions [16,36–38]. Because a functional binding epitope is smaller that the corresponding structural epitope [39], we might have already mapped a large part of the mAb A-56.36 functional epitope.

As A-56.36 and CB competed for their binding to CA, the putative epitope is expected to be located partially at the area of CA that binds CB. This is supported by two observations. First, phage-displayed peptides C12 and CA-a bound CB specifically. Secondly, anti-C12 immune sera induced crotoxin dissociation, as does A-56.36. In addition, given that CA-a bound CB more strongly than C12, amino acids other than those of the consensus are presumably involved in the binding of CA to CB. Otherwise, the weak binding of C3 and CA-b to CB is in

good correlation with the inability of anti-C3 and anti-CA-b sera obtained from mice immunized with the phagotopes to dissociate crotoxin. Thus, CB and A-56.36 are expected to show somewhat different binding patterns to CA, part of the α chain corresponding to CA-a gathering a larger number of residues critical for crotoxin complex formation than the part of the β chain corresponding to CA-b. Both chains seem to participate equally in the interaction of CA with A-56.36.

Lending credence to the proposition that several of the amino acids located on the identified surface are at the interface between CA and CB, our molecular dynamics calculations show that W36 remains exposed during the time range of the calculation, in agreement with fluorescence spectra indicating that the only Trp residue in CA is exposed to the solvent in the crotoxin complex and therefore outside the intersubunit binding site [33].

Immunization of mice with phage-displayed C3 and C12 led to the generation of antibodies cross-reacting with CA, suggesting that these sequences mimic parts of the discontinuous epitope of A-56.36 well enough to elicit a specific response to the original antigen, which is not always the case [40]. Thus, the phage displayed peptide C12 induces dissociating, and therefore potentially neutralizing, antibodies against crotoxin. Although the peptide-induced antibody response is of lower reactivity than the parental antibody's A-56.36, phage peptides having low affinities were shown to elicit a specific immune response in mice and to prevent symptoms of myasthenia gravis [41].

Finally, more direct evidence and further probing of the whole epitope will come from site-directed mutagenesis studies that await overcoming the difficulties inherent to expression of the three-chain mature CA molecule. Three-dimensional molecular modeling and mutant synthetic peptides derived from C12 may then help further in the design of peptides better mimicking the interface of CA that interacts with CB and capable of triggering an antibody response directed specifically towards the zone of interaction between CA and CB. These peptides could become appropriate candidates for the conception of a vaccine used in the treatment of toxin poisoning.

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REFERENCES

1. Slotta, K.H. & Fraenkel-Conrat, H. (1938) Schlangengifte III: Reinigung und Kristallization von Klapper Schlangengifte. *Ber. Dtsch. Chem. Ges.* **71**, 1076.
2. Vital-Brazil, O. (1966). Pharmacology of crystalline crotoxin. *Mem. Inst. Butantan São-Paulo* **33**, 981–992.
3. Aird, S.D., Kaiser, I.I., Lewis, R.V. & Krugger, W.G. (1985) Rattlesnake pre-synaptic neurotoxins: primary structure and evolutionary origin of the acidic subunit. *Biochemistry* **24**, 7054–7058.
4. Breithaupt, H., Rubsamen, K. & Habermann, E. (1974) Biochemistry and pharmacology of the crotoxin complex. *Eur. J. Biochem.* **49**, 333–345.
5. Bouchier, C., Boulain, J.-C., Bon, C. & Menez, A. (1991) Analysis of cDNAs encoding the two subunits of crotoxin, a phospholipase A_2 neurotoxin from rattlesnake venom: the acidic enzymatic subunit derives from a phospholipase A_2-like precursor. *Biochim. Biophys. Acta* **1088**, 401–408.
6. Délot, E. & Bon, C. (1993) Model for the interaction of crotoxin, a phospholipase A_2 neurotoxin, with presynaptic membranes. *Biochemistry* **30**, 10708–10713.
7. Krizaj, I., Faure, G., Gubensek, F. & Bon, C. (1997) Neurotoxic phospholipases A_2 ammodytoxin and crotoxin bind to distinct high-affinity protein acceptors in *Torpedo marmorata* electric organ. *Biochemistry* **36**, 2779–2787.
8. Faure, G. & Bon, C. (1988) Crotoxin, a phospholipase A_2 neurotoxin from the South American rattlesnake *Crotalus durissus terrificus*: purification of several isoforms and comparison of their molecular structure and of their biological activities. *Biochemistry* **27**, 730–738.
9. Faure, G., Harvey, A.L., Thomson, E., Saliou, B., Radvanyi, F. & Bon, C. (1993) Comparison of crotoxin isoforms reveals that stability of the complex plays a major role in its pharmacological action. *Eur. J. Biochem.* **214**, 491–496.
10. Choumet, V., Saliou, B., Fideler, L., Chen, Y.C., Gubensek, F., Bon, C. & Délot, E. (1993) Snake-venom phospholipase A_2 neurotoxins. Potentiation of a single-chain neurotoxin by the chaperon subunit of a two-component neurotoxin. *Eur. J. Biochem.* **211**, 57–62.
11. Rivière, G., Choumet, V., Audebert, F., Sabouraud, A., Debray, M., Scherrmann, J.-M. & Bon, C. (1997) Effect of antivenom on venom pharmacokinetics in experimentally envenomed rabbits: toward an optimization of antivenom therapy. *J. Pharmacol. Exp. Therap.* **281**, 1–8.
12. Choumet, V., Jiang, M.S., Radvanyi, F., Ownby, C. & Bon, C. (1989) Neutralization of lethal potency and inhibition of enzymatic activity of a phospholipase A_2 neurotoxin, crotoxin, by non-precipitating antibodies (Fab). *FEBS Lett.* **244**, 167–173.
13. Choumet, V., Faure, G., Robbe-Vincent, A., Saliou, B., Mazié, J.C. & Bon, C. (1992) Immunochemical analysis of a snake venom phospholipase A_2 neurotoxin, crotoxin, with monoclonal antibodies. *Mol. Immunol.* **29**, 871–882.
14. Choumet, V., Lafaye, P., Mazié, J.C. & Bon, C. (1998) A monoclonal antibody directed against the non-toxic subunit of a dimeric phospholipase A_2 neurotoxin, crotoxin, neutralizes its toxicity. *Biol. Chem.* **379**, 899–906.
15. Choumet, V., Lafaye, P., Demangel, C., Bon, C. & Mazié, J.C. (1999) Molecular mimicry between a monoclonal antibody and one subunit of crotoxin, a heterodimeric phospholipase A_2 neurotoxin. *Biol. Chem.* **380**, 561–568.
16. Cortese, R., Felici, F., Galfre, G., Luzzago, A., Monaci, P. & Nicosia, A. (1994) Epitope discovery using peptide libraries displayed on phage. *Trends Biotech.* **12**, 262–267.
17. Luzzago, A., Felici, F., Tramontano, A., Pessi, A. & Cortese, R. (1993) Mimicking of discontinuous epitopes by phage-displayed peptides I. Epitope mapping of human H ferritin using a phage library of constrained peptides. *Gene* **128**, 51–57.
18. DeLeo, F., Yu, L., Burritt, J.B., Loetterle, L.R., Bond, C.B. & Jesaitis, A.J. (1995) Mapping sites of interaction of p47-phox and flavocytochrome b with random-sequence peptide phage display library. *Proc. Natl Acad. Sci. USA* **92**, 7110–7114.
19. Hendon, R.A. & Fraenkel-Conrat, H. (1971) Biological role of the two components of crotoxin. *Proc. Natl Acad. Sci. USA* **68**, 1560–1563.
20. Felici, F., Luzzago, A., Monaci, P., Nicosia, A., Sollazzo, M. & Traboni, C. (1995) Peptide and protein display on the surface of filamentous bacteriophage. *Biotechnol. Annu. Review* **1**, 149–183.
21. Christian, R.B., Zuckermann, R.N., Kerr, J.M., Wang, L. & Malcolm, B.A. (1992) Simplified methods for construction, assessment and rapid screening of peptide libraries in bacteriophage. *J. Mol. Biol.* **227**, 711–718.
22. Sanger, F., Nicklen, S. & Coulson, A.R. (1977) DNA sequencing with chain-terminating inhibitors. *Proc. Natl Acad. Sci. USA* **74**, 5463–5467.
23. Biozzi, G., Mouton, D., Heumann, A.M., Bouthillier, Y., Stiffel, C. & Mevel, J.C. (1979) Genetic analysis of antibody responsiveness to sheep erythrocytes in crosses between lines of mice selected for high or low antibody synthesis. *Immunology* **36**, 427–438.

24. Demangel, C., Lafaye, P. & Mazié, J.C. (1996) Reproducing the immune response against the *Plasmodium vivax* merozoite surface protein 1 with mimotopes selected from a phage-displayed peptide library. *Mol. Immunol.* **33**, 909–916.
25. Demangel, C., Rouyre, S., Alzari, P.M., Nato, F., Longacre, S., Lafaye, P. & Mazié, J.C. (1998) Phage-displayed mimotopes elicit monoclonal antibodies specific for a malaria vaccine candidate. *Biol. Chem.* **379**, 65–70.
26. Faure, G., Guillaume, J.-L., Camoin, L., Saliou, B. & Bon, C. (1991) Multiplicity of acidic subunit isoforms of crotoxin, the phospholipase A_2 neurotoxin from *Crotalus durissus terrificus* venom, results from posttranslational modifications. *Biochemistry* **30**, 8074–8073.
27. Ternynck, T. & Avrameas, S. (1987) Dosages immunoenzymatiques quantitatifs. In *Techniques immunoenzymatiques*, pp. 41–58. Editions Inserm, Paris, France.
28. Radvanyi, F., Jordan, L., Russo-Marie, F. & Bon, C. (1989) A sensitive and continuous fluorometric assay for phospholipase A_2 using pyrene-labeled phospholipids in the presence of serum albumin. *Anal. Biochem.* **177**, 103–109.
29. Renetseder, R., Brunie, S., Dijkstra, B.W., Drenth, J. & Siegler, P.B. (1985) A comparison of the structures of phospholipase A_2 from bovine pancreas and *Crotalus atrox* venom. *J. Biol. Chem.* **260**, 11627–11634.
30. Smith, K.C. & Honig, B. (1994) Evaluation of the conformational free energies of loops in proteins. *Proteins* **18**, 119–132.
31. Jensen-Jarolin, E., Leitner, A., Kalchhauser, H., Zürcher, A., Ganglberger, E., Bohle, B., Scheiner, O., Boltz-Nitulescu, G. & Breiteneder, H. (1998) Peptide mimotopes displayed by phage inhibit antibody binding to Bet v1, the major birch pollen allergen, and induce specific IgG response in mice. *FASEB J.* **12**, 1635–1642.
32. Mascarenhas, Y.P., Stouten, P.F.N., Beltran, J.R., Laure, C.J. & Vriend, G. (1992) Structure function relationship for the highly toxic crotoxin from *Crotalus durissus terrificus*. *Eur. Biophys. J.* **21**, 199–205.
33. Hanley, M.R. (1979) Conformation of the neurotoxic crotoxin complex and its subunits. *Biochemistry* **18**, 1681–1688.
34. Padlan, E.A. (1992) Structure of protein epitopes deduced from X-ray crystallography. In *Structure of Antigens* (Van Regenmortel, M.H.V., ed.), pp. 29–42. CRC Press, Boca Raton, FL, USA.
35. Graeme Laver, W., Air, G.M., Webster, R.G. & Smith-Gill, S.J. (1990) Epitopes on protein antigens: misconceptions and realities. *Cell* **61**, 553–556.
36. Chothia, C. (1991) Antigen recognition. *Curr. Opin. Struct. Biol.* **1**, 53–59.
37. Raut, S. & Gratney, P.J. (1996) Evaluation of the fibrin binding profile of two anti-fibrin monoclonal antibodies. *Thromb. Haemostat.* **76**, 56–64.
38. Orlandi, R., Formantici, C., Ménard, S., Boyer, J.R. & Colnaghi, M. (1997) A linear region of a monoclonal antibody conformational epitope mapped on $p185^{HER2}$ oncoprotein. *Biol. Chem.* **378**, 1387–1392.
39. Jin, L., Fendly, B.M. & Wells, J. (1992) High resolution functional analysis of antibody–antigen interactions. *J. Mol. Biol.* **226**, 851–865.
40. Felici, F., Luzzago, A., Folgori, A. & Cortese, R. (1993) Mimicking of discontinuous epitopes by phage-displayed peptides. II. Selection of clones recognized by a protective monoclonal antibody against the *Bordetella pertussis* toxin from phage peptide libraries. *Gene* **128**, 21–27.
41. Balass, M., Heldman, Y., Cabilly, S., Givol, D., Katchalski-Katzir, E. & Fuchs, S. (1993) Identification of a hexapeptide that mimics a conformation-dependent binding site of acetylcholine receptor by use of a phage-epitope library. *Proc. Natl Acad. Sci. USA* **90**, 10638–10642.