Transformation of a non-enzymatic toxin into a toxoid by genetic engineering

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Curaremimetic toxins are typical non-enzymatic toxins that bind to their target [the nicotinic acetylcholine receptor (AChR)] through multiple residues. Nevertheless, we show that the concomitant substitutions of only three of the ten functionally important residues of such a toxin sufficed to cause an affinity decrease of the toxin for AChR that is higher than four orders of magnitude. Despite these triple mutations, the overall conformation of the mutated protein remains similar to that of a related recombinant toxin, as judged from both circular dichroism analysis and investigation of antigenicity, using monoclonal and polyclonal antibodies. Furthermore, we show that the detoxified toxin is capable of eliciting antibodies that neutralize the binding of a wild-type toxin to AChR. Therefore, transformation of a non-enzymatic toxin into a toxoid can be achieved, like in the case of enzymatic toxins, by introducing a small number of mutations at positions identified to be critical for expression of toxicity.

Keywords: snake toxin/site directed mutagenesis/toxoid

Introduction

The use of living vectors expressing selected recombinant antigens appears to be a promising approach for immunizing humans or animals against various pathogens, including toxic proteins (Pozzi et al., 1992; Walker et al., 1992; N’Guyen et al., 1993; Stover et al., 1993; Gomez-Duarte et al., 1995). For evident safety reasons, it is of primary importance that the immunogen produced by a living organism is devoid of toxic activity. In the case of toxic proteins, two situations can be encountered. First, the toxin possesses enzymatic activity and its pathogenicity is directly associated with expression of this activity. In principle, therefore, abolition of the latter should suffice for detoxification and indeed, this strategy has been successfully applied to bacterial enzymes such as pertussis toxin (Pizza et al., 1989; Loosmore et al., 1990; Rappuoli et al., 1992), cholera toxin (Hase et al., 1994; Fontana et al., 1995) or Shiga-like toxin (Gordon et al., 1992), in which mutations of a few (one or two) crucial catalytic positions were sufficient to fully inactivate the toxins. Second, the toxins exert their function without expression of any enzymatic activity but simply by binding to a biological target. This is the case, for example, for some bacterial toxins, such as Staphylococcal toxins (Bonventre et al., 1995; Ulrich et al., 1995), cytolyisins (Bhakdi et al., 1996) and of a large proportion of animal toxins, including most snake and scorpion toxins (Ménez et al., 1991). Then, a related question is to what extent the primary structure of such a toxin has to be modified in order to abolish its toxicity but not its ability to elicit neutralizing antibodies. The goal of the present paper is to address this question, with the future objective of testing the immunizing properties of the resulting recombinant toxoid expressed at the surface of a non-pathogenic Gram positive bacteria.

The antigen that was selected in the present study is erabutoxin a (Ea), a curaremimetic protein isolated from the venom of the sea snake Laticauda semifasciata. Several similar toxins have been isolated from venom of elapid and hydrophid snakes. Ea has no enzymatic activity and exerts its lethality by binding tightly and specifically to peripheral nicotinic acetylcholine receptors (AChR) with an equilibrium dissociation constant of 70 pM (Weber and Changeux, 1974; Chang, 1979). Upon binding, therefore, the toxin prevents acetylcholine recognizing its target and hence induces flaccid paralysis of the skeletal muscles. Ea possesses 62 residues and four disulphide bonds (Endo and Tamiya, 1991). The three-dimensional structure of Ea and of closely related homologs has been elucidated by both NMR spectroscopy (Zinn-Justin et al., 1992; Hatanaka et al., 1994) and X-ray diffraction studies (Low et al., 1976; Tsernoglou and Petsko, 1976; Low and Corfield, 1986; Arnoux et al., 1994). It is composed of three adjacent loops rich in β-pleated sheet, connected to a small globular core containing the four invariant disulphide bridges (Figure 1). The region by which Ea binds to AChR and hence exerts its lethal action has been previously localized on the basis of extensive mutational analysis (Hervé et al., 1992; Pillet et al., 1993; Tréméau et al., 1995; Ducancel et al., 1996). This region includes 10 functional residues spread on the three loops, forming a homogeneous surface of at least 680 Å² (Tréméau et al., 1995), which covers a large proportion of the concave face of the leaf-like shaped toxin.

The immunological properties of a large number of curaremimetic proteins highly homologous to Ea have been previously described. Thus, it has been reported that the toxin–AChR interaction is inhibited by polyclonal (Abe and Tamiya, 1979; Boulain et al., 1982) and monoclonal antibodies (Boulain and Ménez, 1982; Boulain et al., 1982; Tréméau et al., 1986). Also, epitopes recognized by monoclonal antibodies which neutralize curaremimetic proteins have been identified (Boulain et al., 1982; Tréméau et al., 1986; Ducancel et al., 1996). These epitopes are all topographical, being composed of several contiguous residues. These immunological studies indicate that elicitation of neutralizing antibodies requires the toxin structure to be in a native-like conformation, ruling out the possibility of preparing toxoids by introducing mutations that would cause toxin unfolding, such as deletion of one or more disulphides. However, since protein–protein interactions may be energetically driven by a small subset of most critical functional residues (Novotny et al., 1989; Clackson and Wells, 1995), we reasoned that concomitant mutations of a small number of such critical residues should considerably affect the binding affinity of a
toxic protein for its target. In agreement with this view, we show that introduction of concomitant mutations at three critical positions of Ea sufficed to destroy most of the capacity of the toxin to bind AChR. Moreover, we show that despite the presence of these three substitutions, the modified protein retains both the overall structure of the wild-type toxin and an ability to induce antiserum that neutralizes the toxicity of the wild-type toxin.

Material and methods

Site directed mutagenesis, plasmids and bacterial strains
DNA manipulations were carried out according to Sambrook et al. (1989). Site-directed mutagenesis experiments were performed using the Mutagen Kit in vitro mutagenesis kit from BioRad. For additive mutations on Ea encoding cDNA (Tamiya et al., 1985; Ducancel et al., 1989), we used successively the following single-strand oligonucleotides probes, synthetized by Bioprobe Systems. Mutated codons are indicated in bold excepted for EaΔSer18.

EaΔSer18 5′-ACTAAAGTTCACGCTGACGAGC-3′
Ea Lys27 Glu 5′-CTCCAGCTATAACGAGCAATGGAGCGAT-3′
Ea Arg33 Glu 5′-GGGAAGGAGTTTCGAGGAGACTATAATTT-3′
Ea Lys47 Ala 5′-TGCCCCACATGTCGCGCCCGGTATTAAA-3′

After digestion with KpnI and BamHII, the 0.2 kb fragments encoding Ea61 (EaΔSer18) and Ea61.3 (EaΔSer18, K27E, R33E, K47A) cDNAs were purified and inserted into a pET3a derived plasmid (Drevet et al., 1997), used as described by Studier et al. (1990). This construction allowed the production, in E.coli BL21(DE3) transformed cells, of a fusion protein successively composed of the ZZ domain from Staphylococcal protein A (Nilsson et al., 1987), a linker peptide and the toxin.

Production and purification of fusion proteins
Cells were cultured at 37°C in TSB medium (Difco) supplemented with 200 mg/l ampicillin. When OD₆₀₀ nm reached 0.5, induction was triggered with IPTG at a 0.5 mM final concentration. After 3 h of culture, cells were harvested, centrifuged and resuspended in lysis buffer composed of 30 mM Tris, 5 mM EDTA, 20% sucrose, pH 8, supplemented with 0.5 mM PMSF. Cell walls were disrupted by three cycles of freezing and thawing. DNA was then precipitated by protamine sulfate at 2 mg/ml final concentration. After centrifugation for 30 min at 10 000 r.p.m., the supernatant was applied to an IgG-Sepharose column (Pharmacia) equilibrated with 50 mM phosphate buffer, pH 7.6, 150 mM NaCl, containing 0.05% Tween 20. The column was washed with the same buffer until the OD₂₇₈ nm reached the baseline. Two volumes of renaturation buffer (0.1 M phosphate buffer, pH 8, containing 2 mM GSH, 4 mM GSSG and 5 mM EDTA) were then passed through the column. After 24 h incubation at room temperature, the gel was washed with 3 volumes of 5 mM ammonium acetate buffer, pH 5, and the hybrid was then eluted with 0.5 M acetic acid, pH 3.4. After lyophilization, the eluted
protein was dissolved in 0.1 N HCl and submitted to CNBr cleavage, as previously described (Trémeau et al., 1995).

**Purification and characterization of recombinant materials**

The recombinant toxin Ea61 was purified on a reverse phase HPLC column (Vydac C4) equilibrated in 0.1% trifluoroacetic acid (TFA). Elution was performed using a 0.1% TFA/CH3CN/H2O gradient, as previously described for the mutants of Ea (Trémeau et al., 1995). Slight modifications were introduced for the purification of recombinant Ea61.3. After treatment with CNBr, the mixture was chromatographed on an IgG-Sepharose column, using the same conditions as described above, to retain the ZZ moiety. The eluted material was then applied on a toxin-specific Mab (M1x1)-affinity column, prepared by coupling 10 mg M1x1 to 1 g CNBr-activated Sepharose (Pharmacia). Buffers and successive steps were identical to those used with the IgG-Sepharose column. Eluted Ea61.3 was finally separated by chromatography on a Vydac HPLC column, as described for Ea61. Each mutant was investigated by amino acid analysis, SDS–PAGE and CD spectra analysis. The dichroic spectra were recorded at 20°C using a CD VI Jobin-Yvon diograph, at a protein concentration of 5 × 10^−5 M.

**Binding to monoclonal antibody M1x1 and to AChR**

The affinity of the recombinant proteins for M1x1 monoclonal antibody (Boulain et al., 1982) was determined by radioimmunoassay, on the basis of competition experiments with 3H-labelled α-toxin, a curaremimetic toxin from Naja nigricollis venom, as a radioactive tracer (Ménez et al., 1971). Specific binding to AChR-rich membranes was also determined from competition experiments (Faure et al., 1983), using the same 3H-labelled curaremimetic protein. AChR-rich membranes were prepared according to Sobel et al. (1980). Equilibrium dissociation constants were determined according to Ishikawa et al. (1977).

**Immunization procedure**

Two month old male rabbits (Blanc de Bouscat) were injected with 100 µg (15 nmol) recombinant Ea61 emulsified in complete Freund’s adjuvant (CFA). They were reimmunised twice at 3-week intervals with 200 µg (30 nmol) Ea61 in incomplete Freund’s Adjuvant (IFA). Rabbits were bled 2 weeks after the last injection. Balb/c mice (five animals per group) were injected subcutaneously with 5 µg (0.75 nmol) Ea61 or Ea61.3 in CFA, then re-immunized twice with the same quantity of Ea61 or Ea61.3 in IFA, at 3 week intervals. Mice were bled 3 weeks after the last injection.

**Enzyme linked immunoassay**

Microwell ELISA plates were coated overnight at 4°C with either Ea61 or Ea61.3, at concentrations of 0.3 µg/ml in 50 mM phosphate buffer, pH 7.4. Plates were saturated with 100 mM phosphate buffer, pH 7.4, containing 0.3% BSA. Serum was serially diluted in 100 mM phosphate buffer containing 0.1% BSA and incubated overnight at 4°C. Wells were washed five times with 10 mM Tris buffer, pH 7.4 containing 0.05% Tween 20. Binding of rabbit antiserum IgG was detected using goat anti-rabbit peroxidase conjugate (Jackson) at a dilution of 1/10 000. Murine antibodies were detected using a biotinylated goat anti-mouse IgG antibody, at a dilution of 1/2000, and a streptavidin–peroxidase conjugate diluted 1/2000 (Amersham, UK), each added for 30 min at room temperature. After washing, 10^−3 M 2,2’-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid) (ABTS) was added and coloration was monitored at 414 nm after 30 min incubation. The titre of the sample was defined as the highest serum dilution giving an absorbance value of 0.6 above the negative control. This control was made with antisera from rabbit immunized with CFA only.

**Competition experiments**

Competition experiments were performed on plates coated with Ea61, by incubating anti-Ea61 polyclonal serum diluted at the titre with serial dilutions of soluble Ea61 or Ea61.3.

**In vitro neutralization assay**

Neutralization experiments were performed in vitro by determining the capacity of varying dilutions of antiserum to inhibit the binding of radiolabelled Eb to AChR. The recombinant Eb was produced and purified as described for Ea. Its radiolabelling was performed in an Eppendorf tube on the walls of which were adsorbed 40 µg 1,3,4,6-tetracholoro-3 quintet acid (Iodogen, Pierce Chemical Company). Eb (4 nmol) was incubated for 20 min at room temperature with 2 nmol Na1 and 0.15 nmol Na125I (0.3 mCi, Dupont-NEN) in 40 µl of 0.2 M phosphate buffer, pH 7.4. The labelled toxin was purified by exclusion chromatography on a Sephadex G10 column, equilibrated with the same phosphate buffer. In neutralization assays, 125I erabutoxin was incubated with serial dilutions of antisera overnight at 4°C. Then, AChR-rich membranes were added and incubated for 2 h at room temperature. Filtration of the incubation mixtures was performed as described for competition experiments with AChR. Radioactivity was measured using a LKB-Wallac γ counter.

**Results**

**Choice of the mutations**

On the basis of site-directed mutagenesis experiments (Hervé et al., 1992; Pillet et al., 1993; Trémeau et al., 1995; Ducancel et al., 1996), we previously delineated the region by which erabutoxin a (Ea) from Laticauda semifasciata binds to AChR. The toxin–AChR interaction is mediated by a multipoint attachment which involves Gln7, Ser8, Gln10, Lys27, Trp29, Asp31, Arg33, Ile36, Glu38 and Lys47. As shown in Figure 1, all these residues except Ser8 have their side-chain oriented toward the same side of the plane, defined by the five β-sheet strands present on the three loops. The side-chain of Ser8 is nearly within the plane of the sheet. In view of the involvement of so many functional residues, it was unlikely that a single mutation in the toxin would cause a satisfactory detoxification. In fact, previous studies have shown that a single mutation generates affinity decreases ranging between 1.5- and 680-fold (Ducancel et al., 1996). Therefore, we decided to mutate simultaneously several functionally critical residues of Ea. Four positions looked most attractive. These are Ser8 and the three basic residues, Lys27, Arg33 and Lys47. Although substitution of Ser8 into threonine causes the largest affinity decrease (680-fold), we decided not to retain this mutation because it was unclear as to whether or not the conformation of the toxin was affected by this mutation (Pillet et al., 1993).

The choice for the mutations to be introduced was rather straightforward. We first decided to reverse the positive charges at positions 27 and 33, by replacing the lysine and arginine by glutamic acids, each individual mutation causing affinity decreases by factors of 320 and 170, respectively (Pillet et al., 1993). Though charge inversion of Lys47 leads to an affinity decrease as high as 120-fold (Ducancel et al., 1996), we decided to mutate this residue into the neutral alanine, which
caused a 30-fold affinity decrease, only. We reasoned that in the wild-type erabutoxin, the positively charged residues 27 and 47 are spatially close to each other, being separated by only 7.4 Å (Low et al., 1976). The proximity of these two positively charged residues was possible as a result of a particular local environment, which made the pK of Lys-27 relatively low (pK = 9.7) (Ui et al., 1982). However, it was uncertain that the presence of two negative charges at these positions could be equally well accommodated by the toxin structure.

One additional mutation was introduced in Ea without the intention of decreasing the toxic activity but instead of generating an epitope that was topographically unrelated with the functional site of Ea. This epitope is recognized by Mx1, a monoclonal antibody that was initially generated against toxin α from Naja nigricollis (Boulain et al., 1982). The epitope is composed of residues that are located in the highly constrained core region (Figure 1). For the epitope to be recognized by Mx1, the toxin must be folded into a native-like structure. The epitope therefore appears to be a nice conformational probe of the three-dimensional structure of the toxin (Boulain et al., 1982). Ea did not possess this epitope (Boulain et al., 1982). However, by simply deleting Ser18, Ea became as potent as toxin α for binding to Mx1 and its affinity for AChR remained unaffected (Zinn-Justin et al., 1994). In other words, deletion of a single residue sufficed to generate the desired epitope without affecting the capacity of Ea to recognize AChR. Ea with Ser18 deleted was referred to as Ea61, whereas Ea61, with triple additional mutations at positions 27, 33 and 47, was referred to as Ea61.3.

Production and characterization of recombinant Ea61 and Ea61.3

Soluble Ea61 and Ea61.3 were produced as fusion proteins in the cytoplasm of Escherichia coli. Their N-terminal part was fused to a synthetic IgG-binding domain of Staphylococcal protein A, named ZZ (Nilsson et al., 1987). This fusion system offers several advantages, including the possibility to both increase the solubility of the toxin moiety in the bacteria and to purify, in a single step, the hybrid from cytoplasmic extracts, using an IgG-bound affinity column (Trémeau et al., 1995). Disulfide bridges of the immobilized toxin were formed directly on the column by passing an appropriate oxidizing medium through it (Trémeau et al., 1995; Drevet et al., 1997). The production yield of the hybrid proteins were 60 and 90 mg/l of culture for ZZ–Ea61 and ZZ–Ea61.3, respectively. Upon treatment with CNBr the hybrids were then cleaved and the resulting recombinant Ea61 and Ea61.3 were purified as described in the Material and Methods section. The two purified mutants displayed all the expected chemical properties regarding their molecular weight and amino acid composition (not shown).

The far UV circular dichroic spectrum of Ea61.3 displayed a positive band around 197 nm and a negative one at 215 nm, like both recombinant Ea and Ea61 (Figure 2). Therefore, Ea61.3, Ea61 and Ea adopt a similar secondary structure. One may notice, however, slight differences between the spectra of the three proteins, the ellipticity at 197 nm being respectively weaker and greater for Ea61.3 and Ea61, as compared with Ea. However, previous NMR studies have shown that the overall conformation of Ea61 is similar to that of Ea (Zinn-Justin et al., 1994), suggesting that the observed differential ellipticities do not reflect a major conformational difference between the proteins. To further validate this conclusion, we investigated the effect of the presence of the three mutations on the overall conformation of the toxin, by comparing the antigenicity of Ea61 and Ea61.3, using a rabbit polyclonal antiserum raised against Ea61. The polyclonal antiserum is diluted 1/9.10^5. Binding of the diluted serum to coated Ea61 is inhibited by varying amounts of soluble recombinant Ea61 and Ea61.3. After washings, antibodies bound specifically to the coated antigen were revealed as described in Material and Methods.

Fig. 3. Binding of Ea61 and Ea61.3 to the rabbit polyclonal antiserum raised against Ea61. The polyclonal antiserum is diluted 1/9.10^5. Binding of the diluted serum to coated Ea61 is inhibited by varying amounts of soluble recombinant Ea61 and Ea61.3. After washings, antibodies bound specifically to the coated antigen were revealed as described in Material and Methods.
The activity of a curaremimetic protein can be determined by measuring its ability to inhibit, at equilibrium and in a dose-dependent manner, the binding of radioactive toxin α (Figure 4). Therefore, despite Ea and toxin α differing by 16 mutations, the core regions of the two toxins are structurally similar. Unexpectedly, however, upon introduction of only three additional mutations at positions 27, 33 and 47, the affinity of the toxin for Mx1 was slightly higher (2–3-fold), as compared with those of the wild-type toxin. This result indicates that some long distance effects may occur between residues of the concave face of the toxin and residues of its core, in agreement with previous data (Faure et al., 1983). Therefore, if the concomitant mutations at positions 27, 33 and 47 had no detectable effect on the overall conformation of the toxin, they may produce a slight conformational effect, yet uncharacterized, in its core region.

**Ea61.3 exhibits no detectable binding activity for AChR**

The activity of a curaremimetic protein can be determined by measuring its ability to inhibit, at equilibrium and in a dose-dependent manner, the binding of radioactive toxin α to AChR. Previous data revealed that this inhibition activity directly reflects the lethal property of the toxins, as determined under in vivo conditions (Ishikawa et al., 1977). As shown in Figure 5, Ea61 and toxin α are similarly potent inhibitors, the IC50 values associated with their inhibitory activities being close to 10⁻⁸ M and the lowest detectable inhibitory concentrations being close to 10⁻⁹ M. In sharp contrast, a concentration of Ea61.3 as high as 10⁻⁵ M had no effect on the binding of the toxin to the receptor, indicating that Ea61.3 is at least 10²-fold less potent than Ea61 at binding to AChR. As a result, Ea61.3 is anticipated to be extremely weak in terms of in vivo toxicity. Using the linear correlation that was previously observed between equilibrium dissociation constants of curaremimetic proteins on AChR and their LD₅₀ values in mice (Ishikawa et al., 1977), we predicted the LD₅₀ of Ea61.3 to be higher than 8000 µg per 20 g mouse, by contrast with a LD₅₀ of 2.5 µg per 20 g mouse for native Ea. Unfortunately, however, Ea61.3 was not available in sufficient quantity to determine experimentally its lethal potency in vivo.

**Ea61.3 retains immunological properties of Ea61**

We injected separately Ea61 and Ea61.3 into Balb/c mice and measured the resulting antibody titres by ELISA. As shown in Figure 6A, the sera from mice immunized with Ea61 cross-reacted with both Ea61 and Ea61.3. However, the antibody titres toward the two antigens were substantially different, with average titres being respectively equal to 1/161 000 and 1/26 000 (Student’s test P < 0.025) suggesting that introduction of one or more of the three mutations affected the capacity of the antigen to recognize some antibody populations raised by Ea61. One may notice that such a difference was not observed with rabbit polyclonal anti serum raised against Ea61 (see above), suggesting that the differential effect observed in Figure 6A cannot be generalized to all host species. In contrast...
to what was observed in Figure 6A, Figure 6B shows that sera from mice immunized with Ea61.3 reacted similarly with both Ea61 and Ea61.3, the average titres being 1/17 500 and 1/21 000, respectively. In both cases, however, the titres were substantially lower than estimated against Ea61 with anti-Ea61. Therefore, introduction of one or more of the triple mutations affected the capacity of Ea61 to stimulate the immune system of Balb/c mice.

Mice antisera raised against Ea61 and Ea61.3 were separately pooled to determine their capacity to inhibit the interaction of radioactive recombinant erabutoxin b (Eb) with AChR. Ea and Eb have similar secondary and tertiary structures (Corfield et al., 1989) and virtually identical affinities for AChR (Ishikawa et al., 1997). The only difference between the two toxins is that position 26 is occupied by an histidine in Eb and an asparagine in Ea. The presence of the imidazole ring at position 26 allows iodination of Eb without affecting its binding capacity to AChR (Sato et al., 1970). Neutralization by immune sera is given as a function of antibody units which compensate the differences between anti-Ea61 titres in the two groups of sera. As shown in Figure 7, both antisera raised against Ea61 and Ea61.3 inhibit the binding of 125I-labelled Eb to AChR. However, the two antisera did not display similar neutralizing potencies, the antisera raised against Ea61 being approximately 10-fold more potent than the ansera raised against Ea61.3. As control experiments, we found that (i) preincubation of the antisera with an excess of soluble Ea61.3 abolished their inhibitory capacity and (ii) low dilutions (1/10) of antisera raised after injection of CFA alone did not inhibit the binding of radioactive Eb to AChR. Together, these data demonstrate the specificity of the neutralizing activities of the antisera raised against Ea61 and Ea61.3.

Discussion

In principle, the lethal property of a toxin is expected to be abolished by mutating residues that are involved in expression of toxicity. If, concomitantly, it is desirable that the inactivated toxin remains capable of eliciting neutralizing antibodies, the mutations have to be introduced with particular care. Such a delicate mutational strategy has been successfully applied to a number of enzymatically active toxins, including pertussis toxin (Loosmore et al., 1990), cholera toxin (Fontana et al., 1995), heat-labile toxin from E.coli (Pizza et al., 1994) and a bee venom phospholipase A2 (Dudler et al., 1995). In all these cases, a few mutations have been directed to residues involved in catalysis, an activity which constitutes an absolute requirement for the lethal character of the toxin to be expressed. Therefore, enzymatically active toxins have been transformed into toxoids, by minimal engineering of their catalytic site.

Not all toxic proteins are enzymes. A number of them exert their function by simply binding to a molecular target whose biological activity is thus blocked. For such toxins to be inactivated, a sufficient modification of their target-specific recognition domain needs to be achieved. According to crystallographic studies of molecular complexes, including protein–protein complexes (Janin and Chothia, 1990), the domain by which a protein recognizes its target usually involves multiple residues, raising the question as to the choice and number of residues that need to be mutated for the protein to be unable to recognize its target. Using a snake toxin as a prototype of non-enzymatic toxins, we showed that three mutations only, introduced concomitantly at residues identified to be functionally critical, sufficed to dramatically reduce the binding activity of the toxin.

Erabutoxin a (Ea), a sea snake toxin, appeared most appropriate for this investigation for at least two reasons. First, it has no enzymatic activity, exerting its toxic function by blocking specifically peripheral nicotinic acetylcholine receptor (AChR) with a high affinity, characterized by a $K_d$ value towards *Torpedo marmorata* AChR close to 70 pM (Pillet et al., 1993). Second, its AChR recognition domain has been well delineated by extensive mutational analyses (Pillet et al., 1993; Tréméau et al., 1995; Ducancel et al., 1996). Ten functional residues have been thus identified among which Lys27, Arg33 and Lys47 play a major binding role (Tréméau et al., 1995; Ducancel et al., 1996). This observation agrees with a number of recent observations concerning other protein–protein complexes, whose stabilization seems to be energetically driven by a small subset of interacting residues (Novotny et al., 1989; Clackson and Wells, 1995). Evidently, such critical residues constituted attractive elements to cause the largest possible decrease in binding affinity. As previously mentioned (see Results section), these critical residues, Lys27, Arg33 and Lys47 were concomitantly mutated, not in Ea itself, but in a mutant called Ea61 in which Ser18 was deleted, thus generating an epitope in the Ea core, apart from the AChR recognition domain.

Concomitant introduction of a glutamic acid at positions 27 and 33, together with introduction of an alanine at position 47, caused an affinity decrease of Ea for AChR by at least four orders of magnitude. The same individual mutations at positions 27, 33 and 47 respectively caused affinity decreases of 170-, 320- and 30-fold. It is unclear, however, as to whether or not the large affinity decrease associated with the three mutations results from an addition of the individual mutation effects. Using the linear correlation previously established between toxicity and binding affinity for AChR (Ishikawa et al., 1977), we estimated the LD$_{50}$ of Ea61.3 to be higher than 400 mg/kg mouse. The large amount of material which
was therefore required to achieve in vivo experiments was not available. However, considering that short-chain toxins, including Ea, have low affinities for human AChR (Ishikawa et al., 1985), we considered that the dramatic affinity decrease that is caused upon introduction of the three mutations makes Ea61.3 a safe protein to ultimately express it in a living vector. This project will, of course, be submitted to the approval of the committee of evaluation of Biohazards.

The three mutations had little effect on the toxin secondary structure as judged from circular dichroic analyses. Also, they did not affect the overall antigenicity of the toxin, as deduced from binding experiments made with polyclonal antibodies raised in rabbits against Ea61. These findings agree with the observations that both Ea61 and Ea61.3 cross-reacted with anti-Ea61 and anti-Ea61.3 antisera raised in mice. Therefore, we conclude that the overall conformation of Ea61.3 is similar to that of Ea61, implying that the low affinity of Ea61.3 for AChR is essentially, if not only, a direct consequence of the modifications of the three positively charged side chains that have been previously identified as functionally critical (Trémeau et al., 1995; Ducancel et al., 1996).

We noted, however, a number of small deviations regarding the antigenic and immunogenic properties of Ea61.3, as compared with Ea61. First, it was less potent at recognizing the murine antisera raised against Ea61, suggesting that one or more of the three mutations affected the capacity of the toxin to recognize murine antibodies raised against the parental toxin. Second, we found that a core-specific murine monoclonal antibody had slightly higher (2–3-fold) affinity for Ea61.3 as compared with Ea61, indicating that mutations occurring in the AchR binding domain could alter locally and at distance the toxin structure. Third, although Ea61.3 was still capable of eliciting antibodies, its immunogenicity in Balb/c mice was lower than that of Ea61, as judged from differential antibody titres raised against these two proteins. Presumably, therefore, some mutations affected some stages of the humoral response like, for instance, during presentation to T cells. In this respect, one should recall that the immunogenic properties in Balb/c mice of the highly homologous toxin α from Naja nigricollis have been previously studied in detail (Léonetti et al., 1990; Maillère et al., 1995). Two major T cell epitopes have been delineated between residues 24 and 49 of toxin α. Since the region 24–49 is highly homologous in both Ea61 and toxin α, it is likely that it plays a comparable role for both toxins, in terms of toxin-specific T-cell stimulation in Balb/c mice. Interestingly, it was previously shown that substitutions of a single residue, at various positions of a peptide encompassing this region, including for example the replacement of Arg33 into alanine, abolished the capacity of the peptide to stimulate toxin α-specific T-cells (Maillère et al., 1995). Therefore, mutation of Arg33 into Glu, as it occurs in Ea61.3 is likely to also cause a great change in the capacity of the corresponding processed peptide to stimulate toxin-specific T-cells. It is possible, therefore, that variations in antigenicity and immunogenicity associated with introduction of the three mutations may be associated with the use of inbred Balb/c mice as immunizing hosts whose MHC II molecules specificity exhibit a preference for basic residues (Sette et al., 1989; Bogen and Lambris, 1989; Schild et al., 1995). This is in agreement with the observation that Ea61 and Ea61.3 display similar crossreactivity toward anti-Ea61 antisera raised in rabbits, in contrast to what was observed with anti-Ea61 antisera raised mice.

Murine antisera raised against Ea61.3 were less potent at neutralizing the native toxin as compared with those elicited against Ea61. Different explanations may account for this phenomenon. First, antibodies that recognize the region of Ea61.3 where the three mutations were introduced fail to recognize the corresponding region in Ea61. However, this situation may not constitute an inconvenience. When generated against the native toxin, such antibodies are complementary to the site by which the toxin interacts with AChR (Trémeau et al., 1986; Charpentier et al., 1990; Ducancel et al., 1996). As a result, they partially mimic AChR and may elicit anti-idiotypic antibodies which, by acting like the toxins may block AChR (Ducancel et al., 1996). The antibodies elicited by Ea61.3 against the mutated residues, Glu27, Glu33 and Ala47, are unlikely to recognize the parent toxin which possesses Lys27, Arg33 and Lys47. As a result, the effective neutralizing antibodies elicited by Ea61.3 should mostly bind to areas that are topographically unrelated with the AChR binding site, being thus devoid of the above undesirable effects. A second explanation is that some mutations have locally affected the structure of other toxin regions. Our experiments carried out with Mrz1 support this possibility. This antibody recognizes the toxin core which was previously proposed to be important for the recognition of neutralizing antibodies (Boullain et al., 1982; Boullain and Ménez, 1982; Gatineau et al., 1988). Therefore, introduction of a small number of mutations within the functional domain of a non-enzymatic toxin might have a number of consequences that could alter not only its immunogenicity but possibly also, the quality of elicited antibodies.

In conclusion, we showed for the first time that a non-enzymatic toxin can be transformed into a toxoid by mutating a few positions that have been previously identified as being critical for the toxin to bind to its target. Therefore, like in the case of enzymatic toxins, generation of a toxoid by recombinant technology can be achieved using a similar approach, which consists of identifying a subset of functionally important residues and in mutating some of them. Having developed the tools described in this paper, we are now currently investigating the possibility of producing Ea61.3 at the surface of a living vector and comparing its immunogenic potency with that of the free toxoid.

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References
