Molecular decoys: Ligand-binding recombinant proteins protect mice from curarimimetic neurotoxins

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ABSTRACT Mimic ligand-binding sites of the nicotinic acetylcholine receptor bind d-tubocurarine and α-bungarotoxin in vitro. Injection of such binding sites into mice could act as molecular decoys in vivo, providing protection against toxic ligands. This hypothesis of molecular "decoyance" has been tested in >250 mice. Bacterially produced cholinergic binding sites provided a 2-fold increase in the survival rate of animals challenged with curarimimetic neurotoxins. Possible considerations for decoy designs and their applications are discussed.

The nicotinic acetylcholine receptor (AcChoR) is by far the most extensively studied receptor today (1-4). Since the appearance of the complete amino acid sequence of this receptor, much work has been done with the intent to correlate structures with function. Over the past years we have systematically studied the AcChoR from the electric organ of the fish Torpedo californica so as to identify and characterize the ligand-binding site. By applying protein blot analyses (5), an essential component of the cholinergic binding site has been mapped to the area of residues 180-200 of the receptor's α-subunit (6-10). This region has been found to bind curarimimetic neurotoxins such as d-tubocurarine and the snake-derived α-toxins, α-bungarotoxin (α-BTX) and α-cobratoxin (α-CTX).

Whereas the main thrust of our research has been to elucidate the fundamental principles that govern protein-ligand recognition, it was thought that the toxin-binding proteins we have isolated may also have therapeutic potential, the concept being that binding sites that mimic the native receptor could serve as molecular decoys. Administering such decoys to animals might provide protection against neurotoxins. This hypothesis, termed molecular "decoyance," has been tested and evidence is presented here describing its application in vivo. A preliminary report of this study has been presented (11, *, 1).

MATERIALS AND METHODS

Production of R4137. Oligonucleotides corresponding to the α-amino acid sequence 184-200 were synthesized on an Applied Biosystems 380B DNA automated synthesizer by Ora Goldberg (Weizmann Institute of Science). The synthetic double-stranded DNA was then cloned into the Smal I site of the bacterial expression vector pATH2 (10, 12, 13). Escherichia coli HB101 competent cells were then transformed with these plasmids and α-BTX-binding transformants were selected by toxin overlay of colony blots as described (10). A preparation enriched for the fusion protein (designated R4137) was prepared as follows. Starter cultures of R4137 clones were grown overnight on Luria broth medium containing ampicillin (30 μg/ml) and then used to inoculate (1:100) M9 medium (50-500 ml) supplemented with 0.5% (wt/vol) Casamino acids, thiamine (5 μg/ml), and 3-β-indoleacrylic acid (5 μg/ml) as described (10). The cultures were grown at 33C for 12 hr and reached a density of 1-2 OD600. The cells were then pelleted by centrifugation and washed in phosphate-buffered saline. They were finally suspended to 1/10th their original volume in a high salt (500 mM NaCl) phosphate buffer (pH 7.4) containing 1 mM EDTA, 1 mM 1,4-dithiothreitol, 1 mM phenylmethylsulfonyl fluoride. The cells were disrupted by sonication in a Branson sonicator operated at 1/3rd its maximal output (0.5-1 min on ice). The pelletable material was separated from the soluble fraction by centrifugation (10 min at 3000 rpm in a Sorvall SS-34 rotor). The pellet was then resuspended by sonication in water. The suspension was centrifuged as described above and the supernatant was collected. This fraction was found to be highly enriched in R4137. It was either used directly or stored at 4°C until use.

Polyacrylamide Gel Electrophoresis and Protein Blotting. Toxin overlays of protein blots were done essentially as described (6, 10). Total bacterial homogenates (equivalent to 0.5 OD600) or the enriched preparation of R4137 were separated on 10% polyacrylamide slab gels as described (6, 14). The gels were blotted to nitrocellulose membrane filters (Schleicher & Schuell; 0.45-μm BA85) with a blot apparatus equipped with an electrode array designed to produce a gradient electric field (50-8 V) (15). The blots were quenched with 1% hemoglobin in phosphate-buffered saline and probed with 125I-labeled α-BTX (1-5 x 10^6 cpm; 1-5 x 10^-9 M) overnight. The filters were washed in ice-cold buffer and autoradiographed.

Binding Assays. In principle, toxin binding to the R4137-enriched fraction was measured by the Schmidt and Raftery method (16). The band ligand was separated from the free ligand via vacuum filtration through positively charged modified nylon membranes (Zetabind, AMF-CUNO, Meriden, CT). In this manner, kinetic and equilibrium constants could be assayed even for complexes with relatively low affinities.

Injection of Mice. Male and female mice (5 wk old; 20-25 g) were used. Both inbred (BALB/c) and outbred (CD1) strains were tested. Mice were first injected intraperitoneally with either R4137 or a placebo—a similar fraction derived from bacteria transformed with the unmodified vector pATH2 [these cells have no toxin-binding capacity (10)]. Five minutes later, all the mice were challenged with various amounts of d-tubocurarine or α-CTX. The dose was gauged

Abbreviations: AcChoR, acetylcholine receptor; α-BTX, α-bungarotoxin; α-CTX, α-cobratoxin.


RESULTS

The region of residues 180–200 of the α-subunit of the AcChor derived from \textit{T. californica} has been found to bind curarinimetic neurotoxins (9, 10, 17). Indeed, recombinant fusion proteins expressing this peptide sequence bind α-BTX (ref. 10; see also ref. 18). R4137 is such a fusion protein. It demonstrates that the 17-amino acid sequence Trp-Lys-His-Trp-Val-Tyr-Tyr-Thr-Cys-Cys-Pro-Asp-Thr-Pro-Tyr-Leu-Asp (positions 184–200) is sufficient for α-BTX binding. This binding is highly specific, with \( K_d = 1.5 \times 10^{-7} \text{ M} \) (see Fig. 1).

Although this binding is appreciably less than that of the intact receptor, it does resemble the binding characteristics of the complete α-subunit (437 amino acids) of AcChor (compare with ref. 6).

The pharmacology of R4137 toxin binding reflects that of the competitive antagonist binding site of the native receptor (unpublished data). The kinetic binding constants of R4137 for α-BTX and the IC\(_{50}\) values for d-tubocurarine and α-CTX competitions are given in Table 1.

As shown in Fig. 2, a highly enriched fraction of R4137 can be prepared from \textit{E. coli} transformants. Cells grown under inductive conditions produce a 36-kDa fusion protein as a major band. This polypeptide is unique for toxin binding. In high salt buffer (500 mM NaCl), this protein is less soluble than it is in low salt. This characteristic has been exploited and, by differential centrifugation and sonication, a soluble fraction containing predominantly R4137 is easily obtained (lanes A and C).

In view of the fact that R4137 binds curarinimetic neurotoxins, it was conceivable that it may serve as a decoy, having a protecting effect in vivo, should it be injected into animals. Therefore, 5-wk-old BALB/c males were injected with R4137 or a similar fraction derived from pATH2-transformed bacteria. Five minutes after “priming” of the mice, they were challenged with d-tubocurarine and the rate of death was recorded for each group. As shown in Fig. 3, R4137 had a profound effect (>3-fold increase) on the survival rate of the mice. To ascertain that this effect was not peculiar to males or unique to inbred mice, males and females of CD1 outbred mice were also tested. The results for >200 mice injected with d-tubocurarine are given in Table 2. A 2- to 4-fold increase (average, 3.52) in survival rate was seen for all cases in which mice were treated with R4137.

The binding of R4137 to d-tubocurarine has an affinity of \( \approx 10^{-4} \text{ M} \). α-CTX binds R4137 with \( K_d = 10^{-6} \text{ M} \), but the native receptor binds this antagonist substantially more tenaciously (\( K_d = 10^{-9} \text{ to } 10^{-10} \text{ M} \)). It was therefore of

\[ R_{2} = 2.6 \times 10^{-7} \text{ M} \]
\[ K_d = 1.5 \times 10^{-7} \text{ M} \]

Table 1. Characteristics of α-BTX binding to R4137

<table>
<thead>
<tr>
<th>Kinetic constants</th>
<th>( K_{on} = 8538 \text{ M}^{-1} \text{sec}^{-1} )</th>
<th>( K_{off} = 1.128 \times 10^{-4} \text{sec}^{-1} )</th>
</tr>
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<tbody>
<tr>
<td>Equilibrium constant</td>
<td>( K_d = 1.5 \times 10^{-7} \text{ M} )</td>
<td>( K_l = 1.5 \times 10^{-7} \text{ M} )</td>
</tr>
<tr>
<td>Competition</td>
<td>IC(_{50}) (d-tubocurarine) = 5 \times 10^{-4} \text{ M}</td>
<td>IC(_{50}) (α-CTX) = 2.6 \times 10^{-6} \text{ M}</td>
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Fig. 1. Scatchard analysis of α-BTX binding to R4137. The enriched fraction of R4137 (\( \approx 500 \mu\text{g} \); see Fig. 2) was incubated with different concentrations (10\(^{-10}\)–10\(^{-3}\) M) of \(^{125}\)I-labeled α-BTX until equilibrium was achieved (30 min). Net bound α-BTX was determined by adding a 1000-fold excess of nonradioactive α-BTX and the bound vs. free toxin for each point was calculated.

to cause 80% death of untreated mice. The toxins were administered to the animals by subcutaneous injection at the nape of the neck. The animals were observed and the number of deaths during the course of 2 hr after the toxin injection was recorded. All the survivors were kept at least 24 hr and in some cases for as long as 1 week.

Fig. 2. Preparation of an enriched fraction for R4137. \textit{E. coli} HB101 were transformed with a pATH2 plasmid containing DNA corresponding to the α-amino acid sequence 184–200 (designated R4137) of the \textit{T. californica} AcChoR. The bacteria were cultured on M9 medium to induce expression. The cultures were harvested, and the bacteria were then sonicated and centrifuged. The pellet was extracted with water to give a soluble fraction enriched in the fusion protein. The enriched fraction (lane A) was used in the experiments described. The extent of enrichment is appreciated by comparison with the original total cell homogenate (lane B). A 10% polyacrylamide gel stained with Coomassie brilliant blue (lanes A and B) or blotted and overlaid with \(^{125}\)I-labeled α-BTX is shown (lanes C and D correspond to blots of lanes A and B, respectively. Arrowhead indicates the position of the fusion protein R4137. Numbers on left are kDa.

Fig. 3. Effect of R4137 on the survival rate of d-tubocurarine-injected mice. Two groups of BALB/c mice (35 in each group) were injected with either pATH2 or R4137 (\( \approx 3 \text{ nmol of α-BTX binding sites per mouse} \)) interperitoneally. Five minutes later, the mice were given \( d \)-tubocurarine (\( \approx 15 \text{ nmol; 9 µg per mouse, subcutaneously} \)). The number of survivors as a function of time after the injection of toxin is shown (data are derived from experiments 2 and 3 of Table 2).
Table 2. Mimic binding sites as protectants against neurotoxins

<table>
<thead>
<tr>
<th>Bacterial protein (i.p.)</th>
<th>Strain</th>
<th>Sex</th>
<th>Toxin (s.c.)</th>
<th>Dose, mg/kg</th>
<th>Survivors, n</th>
<th>Mice, n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exp.</td>
<td></td>
<td></td>
<td>R4137</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>pATH2</td>
<td>BALb/c</td>
<td>M</td>
<td>Tubo</td>
<td>0.40</td>
<td>10</td>
</tr>
<tr>
<td>2</td>
<td>pATH2</td>
<td>BALb/c</td>
<td>M</td>
<td>Tubo</td>
<td>0.36</td>
<td>10</td>
</tr>
<tr>
<td>3</td>
<td>pATH2</td>
<td>BALb/c</td>
<td>M</td>
<td>Tubo</td>
<td>0.36</td>
<td>25</td>
</tr>
<tr>
<td>4</td>
<td>pATH2</td>
<td>CD1</td>
<td>M</td>
<td>Tubo</td>
<td>0.36</td>
<td>37</td>
</tr>
<tr>
<td>5</td>
<td>pATH2</td>
<td>CD1</td>
<td>F</td>
<td>Tubo</td>
<td>0.29</td>
<td>20</td>
</tr>
<tr>
<td>6</td>
<td>pATH2</td>
<td>CD1</td>
<td>M</td>
<td>α-CTX</td>
<td>0.15</td>
<td>10</td>
</tr>
<tr>
<td>7</td>
<td>pATH2</td>
<td>CD1</td>
<td>M</td>
<td>α-CTX</td>
<td>0.15</td>
<td>21</td>
</tr>
</tbody>
</table>

Mice were injected with R4137 or pATH2 preparations as indicated and challenged 5 min later with d-tubocurarine (Tubo) or α-CTX. The number of survivors 2 hr after the toxin injection is given.

interest to test whether R4137 would have any protecting effect on mice injected with this toxin. It is shown in Table 2, 61 CD1 male mice were tested. For the course of the experiment (2 hr after toxin injection), R4137 had a dramatic effect. However, by 24 hr after the toxin was injected, all 61 mice were found dead. This transient effect of R4137 most probably reflects dissociation of the toxin from the decoy and gradual lethal accumulation of the α-CTX at the neuromuscular junction. No such effect was found in the d-tubocurarine-tested mice, and survivors appeared to be healthy even 1 wk after the experiment.

**DISCUSSION**

*In vitro* binding of ligands with isolated receptors is a necessary step toward understanding recognition processes. However, isolated functional fragments of receptors can only be effective as clinical reagents if they combine and inactivate toxins or pathogens in *vivo*. The experiments presented here clearly demonstrate that isolated cholinergic-binding sites fulfill this requirement as they protect mice against curimimetic neurotoxins. Such mimic binding sites act as molecular decoys, which intercept the toxin in the blood, preventing it from reaching its target—the neuromuscular junction. This decoy action therefore has therapeutic potential. The production of highly effective decoys, however, must take into account a number of parameters. Moreover, it should be understood that R4137 is but an intermediate tool that, by its genetic manipulation, will allow us to design more efficient cholinergic decoys.

The first issue to be addressed should be the binding affinity. Peptide sequences are currently being tested for preferred toxin-binding characteristics so as to more effectively compete against the native receptor. Furthermore, as is indicated by the experiments with α-CTX, longer-lived decoys are needed. This could be achieved by making synthetic peptides containing D amino acids. Alternatively, based on the fusion protein blueprint, organic molecules—i.e., not proteinaceous—could be designed to satisfy the physicochemical requirements of a decoy that must form a functional interface with the toxin. In addition, a decoy might compete against the native receptor for the endogenous ligand. This objection may not be too serious, as agonists often bind substantially less efficiently than do antagonists. Indeed, R4137 appears to bind acetylcholine with very low affinity (10⁻⁷ M; unpublished data). Finally, fusion proteins such as R4137 might have immunogenic properties, making them less than suitable for clinical use (as would be intact AcChOR, which could elicit myasthenia gravis). Organic decoys or short peptide decoys should be less problematic in this respect. Modified decoys containing asialo-glycoconjugates might also assist in clearance of the decoy–toxin complex.

The applications of decoys could be numerous. The most obvious is the fact that d-tubocurarine is routinely used in surgery as a neuromuscular blocking agent, and a decoy based on R4137 could be extremely useful as its antidote. Moreover, decoys should be effective in combating infectious disease. Many pathogens elicit their effects by exploiting natural membrane receptors. Rabies virus, for example, has been found to bind to AcChOR, and curimimetic neurotoxins can compete for this binding (19). Thus R4137-based decoys should intervene in rabies infections. Most intriguing is the fact that CD4, the T4 antigen of lymphocytes, has been found to be the human immunodeficiency virus receptor (20, 21). CD4-based decoys should therefore be anticipated to be helpful in treating acquired immunodeficiency syndrome (AIDS).

The concept of molecular “decoy” has been promoted and demonstrated to be feasible in *vivo*. Continuous study of the basic principles of ligand binding and the striving to understand the fundamentals ruling protein interaction with their surroundings will provide the know-how necessary for the design of efficient decoys, allowing their potential to be realized.

**Note Added in Proof.** Recently, conditions were established in which mice were injected with a lethal dose of α-CTX. One hour later, they were given either the placebo or the cholinergic decoy. These treatments resulted in 100% death or 90% survivors, respectively, showing that the decoy can serve as a genuine antidote.

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