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A Goldfish Model for Evaluation of the Neurotoxicity of ω-Conotoxin GVI A and Screening of Monoclonal Antibodies

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A Goldfish Model for Evaluation of the Neurotoxicity of ω-Conotoxin GVI A and Screening of Monoclonal Antibodies. ADEYEMO, O. M., SHAPIRA, S., TOMBACCINI, D., POLLARD, H. B., FEUERSTEIN, G., AND SIRÉN, A-L. (1991). *Toxicol. Appl. Pharmacol.* **108**, 489–496. The neurotoxicity of ω-conotoxin (ω-CgTx), a potent neuronal voltage-sensitive calcium channel blocker, was measured using a new bioassay. ω-CgTx was administered intraperitoneally (ip) to goldfish weighing approximately 1.6 g, and dose-related changes were observed over a 2-hr period. ω-CgTx induced time- and dose-dependent abnormal swimming behavior (ASB) and mortality. The antitoxin activity of the antibodies was investigated *in vivo* by either (1) preincubation of the antibody with ω-CgTx at 4°C overnight, or (2) pretreatment with antibody, 30 min before ω-CgTx injection in a 10:1 antibody/ω-CgTx molar ratio. The LD50 dose of ω-CgTx in goldfish was 5 nmol/kg ip, and preincubation of monoclonal antibody (50 nmol/kg ip) with ω-CgTx (5 nmol/kg ip) significantly ($p < 0.05$) reduced mortality, ASB, and toxicity time. The antitoxin activity of the monoclonal antibodies evidenced in the goldfish bioassay was further tested in the conscious rat. In the rat, the increases in mean arterial pressure and heart rate induced by ω-CgTx (0.03 nmol/rat icv) were significantly ($p < 0.02$ and $p < 0.01$, respectively) attenuated by preincubation of the toxin with the antibody (0.3 nmol/rat). We conclude that the goldfish bioassay provides a simple, accurate, and inexpensive *in vivo* model for the study of the toxicity of ω-CgTx. © 1991 Academic Press, Inc.

ω-Conotoxin GVI A (ω-CgTx) is a naturally occurring peptide of 27 amino acids produced by the piscivorous marine mollusc, *Conus geographus* (Olivera *et al.*, 1984, 1985). Several studies have shown that ω-CgTx is an irreversible blocker of the N- and L-type voltage sensitive calcium channels in neural tissue (McCleskey *et al.*, 1987; Cruz *et al.*, 1987; Rosario *et al.*, 1989). This neurotoxin has a wide distribution of binding sites in the central nervous system especially in the hippocampus (Dooley *et al.*, 1988a; Kerr *et al.*, 1988), where it has been shown to cause specific neuronal death especially in the CA3 region (Shapira *et al.*, 1990b). ω-CgTx has also been shown to inhibit voltage-sensitive calcium channel-mediated release of neurotransmitters in some brain areas (Miller, 1987; Dooley *et al.*, 1987,1988b; Woodward *et al.*, 1988; Feuerstein *et al.*, 1990) by interfering with the depolarization-induced calcium influx necessary for neurotransmitter release (Reynolds *et al.*, 1986).

Although extensive *in vitro* studies have been conducted to explore the nature of the neurotoxicity of ω-CgTx (Dooley *et al.*, 1987; McClesky *et al.*, 1987; Feuerstein *et al.*, 1990), very few data are available on its *in vivo* effects (Olivera *et al.*, 1985; Rosario *et al.*, 1989; Shapira *et al.*, 1990a). This may be due in part

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to the fact that systemic administration of ω-CgTx has been shown to be ineffective in rodents (Olivera *et al.*, 1985; Shapira *et al.*, 1990a). The present experiments were therefore first aimed at establishing a simple *in vivo* bioassay to detect ω-CgTx toxicity. We achieved this aim by intraperitoneal administration of the toxin into the goldfish; a species which we believed would be suitable for studying ω-CgTx neurotoxicity by systemic administration due to its relatively permeable blood–brain barrier compared to the mammalian nervous system (Bradbury, 1979). We then proceeded to use the bioassay to select monoclonal antibodies against ω-CgTx by screening for their antitoxin activity. The goldfish as an *in vivo* model has been successfully used for neural regeneration studies in optic nerve and optic tectum (Arora and Sperry, 1962; Springer and Agranoff, 1977; Springer *et al.*, 1977; Schmidt, 1979; summarized in Powers, 1989). We were also aware that the piscine systems had provided the original preparations for studies on voltage-controlled Na^+ and K^+ channels (Rosenberg *et al.*, 1984), and acetylcholine receptors (Whittaker, 1987).

In the present paper, we describe the development of the goldfish as a subject for the sought after *in vivo* bioassay of ω-CgTx toxicity. We report parallels between the goldfish model and the rat; also, the accuracy, precision, and reproducibility of the goldfish model contribute to its analytic value.

MATERIALS AND METHODS

Animals. Goldfish, *Carassius auratus,* were obtained from a local pet store and maintained in dechlorinated tap water at room temperature. Regular gold fish food was provided once a day. All of the fish were housed for at least 24 hr in the laboratory before experimentation. The average weight of the goldfish was approximately 1.6 g (n = 300).

Male Sprague–Dawley rats (Taconic Farms, Germantown, NY) weighing 350 ± 5 g (n = 20) were housed in a temperature-controlled room with a 12/12-hr light/dark cycle and access to food and water *ad libitum.*

Dose-response studies in goldfish. A dose-dependent toxicity profile of ω-CgTx in goldfish was established by the administration of graded doses of the toxin intraperitoneally (ip) in a total volume of 10 μl to 20 goldfish per dose level. A water tank measuring 18 × 9 × 7.5 inches housed each group for each dose level. The toxin administration in the goldfish, which was held upside-down in wet gauze for a brief injection time, was such that the head of the fish was oriented toward the investigator while a 27-gauge needle attached to a 50-μl Hamilton syringe, away from the gills, delivered the predetermined toxin concentration intraperitoneally. Incidence of mortality was recorded over a 2-hr period of observation.

Another group of goldfish with 10 fish per dose level was individually (one fish per experiment, therefore, 10 experiments per dose) injected and toxic signs were evaluated as the time for occurrence of abnormal swimming behavior (ASB) over a 2-hr period of observation. A normal swimming behavior in the goldfish is typified by gentle rocking motions of the body to either side; however, this approximately 5° tilting of the body to either side from the vertical is quickly and easily reversed and stabilized. Also, another observable characteristic of normal swimming in the goldfish is the ability to stay at one spot and display "stationary swimming" (absence of forward, backward, or sideways motion) while completely afloat using only the fast moving fins for balancing.

The abnormal swimming behavior in goldfish showing signs of toxicity to ω-CgTx is characterized by a significant loss of the righting reflex to the rocking motion (see above). Equally, the ability to engage in stationary swimming is completely absent in the goldfish showing toxicity to ω-CgTx, thereby resulting into persistent sinking. When the inability to balance the tilting of the body to either side, coupled with persistent sinking, occurred, the fish usually lost its escape mechanism from such external stimuli as touch, bright light, or gentle taps on the containing water tank. The time span between the toxin injection and the observation of ASB was recorded as the toxicity time.

To further quantify the effect of ω-CgTx in the goldfish, the toxicity was calculated as the inverse of the toxicity time (e.g., Pollard, 1988). Thus, a fish not showing toxic signs within the observation period of 2 hr would exhibit a toxicity of nearly zero, whereas a goldfish whose toxicity time was 25 min would exhibit a toxicity of 0.04 min^{-1}.

Evaluation of monoclonal antibody effects in goldfish. The raising of monoclonal antibodies against ω-CgTx was accomplished by using an established procedure, details of which have been described in Tombaccini *et al.* (1990). Monoclonal antibodies produced by this method were then tested for antitoxin activity as follows. Two groups of goldfish of 40 per group were used for this study and each group was then divided into four subgroups of 10 each (see Table 1). Group I: The anti-ω-CgTx monoclonal antibodies were premixed with the LD50 dose of the ω-CgTx in goldfish in a molar ratio of 10:1. This mixture was incubated at 4°C for 12 hr or overnight before ip administration into subgroup one. A second subgroup received ω-CgTx only, a third subgroup received monoclonal antibody

TABLE I

TREATMENT PROTOCOLS FOR MONOCLONAL ANTIBODIES AND ω-CgTx IN GOLDFISH

	Antibody + toxin	Toxin only	Antibody only	Saline	N
I. Premix[a]					
1	X	—	—	—	10
2	—	X	—	—	10
3	—	—	X	—	10
4	—	—	—	X	10
II. Pre-Rx[b]					
1	X	—	—	—	10
2	—	X	—	—	10
3	—	—	X	—	10
4	—	—	—	X	10

[a] Preincubation of antibodies with the ω-CgTx at 4°C for 12 hr.
[b] Pretreatment with antibodies 30 min prior to ω-CgTx injection.

only, while the fourth subgroup received saline. Group II: Subgroup one was pretreated with monoclonal antibody (molarity was 10× higher than that of the toxin) followed by ω-CgTx ip injection 30 min later. The other three subgroups received ω-CgTx, monoclonal antibody, and saline, respectively. These two groups were monitored for abnormal swimming behavior, toxicity time, and mortality data over the 2-hr observation period as described earlier.

Further evaluation of the monoclonal antibody for antitoxin activity in conscious rats by intracerebroventricular injections. Rats divided into four groups (four to six in each) were anesthetized with an intramuscular injection of ketamine (130 mg/kg) and acepromazine (1.3 mg/kg). A stainless-steel guide cannula for intracerebroventricular (icv) administration of drugs into the right lateral ventricle was then implanted on the skull (Sirén and Feuerstein, 1988). The following coordinates in reference to the bregma were used: −1.2 mm lateral and −0.8 mm anterior–posterior (Paxinos and Watson, 1986). The position of the icv cannula was verified in all rats used in the study by icv injections of methylene blue in a volume of 10 μl.

On the experimental day, 5–6 days after implantation of the icv cannula, the right femoral artery was cannulated with a PE-50 catheter under halothane (2% in oxygen) anesthesia. The catheter was tunneled under the skin and allowed to exit at the back of the neck. All rats were allowed 3–5 hr to fully recover and stabilize from surgery. Subsequently, the toxin, antibody, or vehicle was administered icv (10 μl/rat over 30–35 sec). MAP and HR were monitored with a Narcotrace 80 computerized physiograph (Narco Scientific, Houston, TX), and sampled automatically at 30–60 sec intervals by a Northstar–Hazeltine computer. All rats were observed for 2 hr, after which all surviving rats were further monitored for 48 hr and finally euthanized with 1 ml of T-61 euthanasia solution given ip (Hoechst, Somerville, NJ).

Drugs used. ω-Conotoxin GVI A (Peninsula Laboratories, Inc., Belmont, CA) was reconstituted in 0.9% (w/v) saline and kept at 4°C for use. Anti-ω-conotoxin monoclonal antibody was raised and characterized in our laboratory (Tombaccini *et al.*, 1990).

Statistical analysis of the data. Data in the text and figures are mean ± SE for the indicated number of goldfish or rats. Mortality data comparisons were done with the Fisher's Exact Probability Test (2 × 2 table), while analysis of variance (ANOVA) followed by the Student–Newman–Keul test for multiple comparisons and ANOVA with repeated measures was used where appropriate. A significant change was accepted at $p < 0.05$.

RESULTS

Dose–response studies for ω-conotoxin in goldfish. Goldfish were injected with different doses of ω-CgTx, ip, and the time measured thereafter until onset of abnormal swimming behavior. The lethal effects of these doses were also assessed. The toxicity time, the time interval until onset of abnormal swimming behavior, proved to be dose dependent, as shown in Fig. 1. The shape of this curve was typical in character of curve shapes found for other time-dependent intoxicants (e.g., Pollard, 1988). The approximate TTD50 (median toxicity-time dose) was calculated to be 3.3 nmol/kg ip. Similarly, the approximate LD50 of ω-CgTx in goldfish was 5 (3.1–6.6) nmol/kg ip

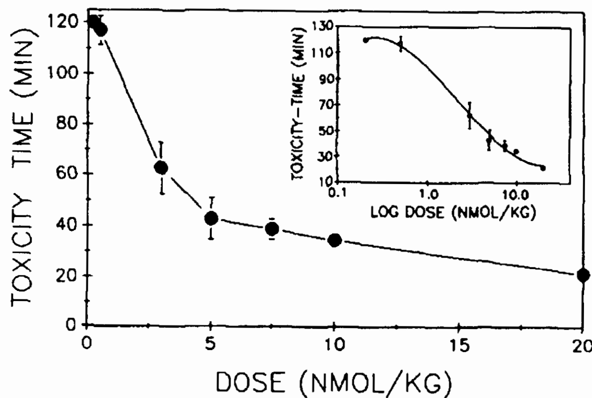

FIG. 1. Toxicity time dependence on the dose of ω-conotoxin GVI A. Toxicity time is the time between the toxin injection and the observance of abnormal swimming behavior in the goldfish. Number of goldfish (n) is 10 per dose level, mean ± SE. Insert shows same data represented on a logarithmic scale.

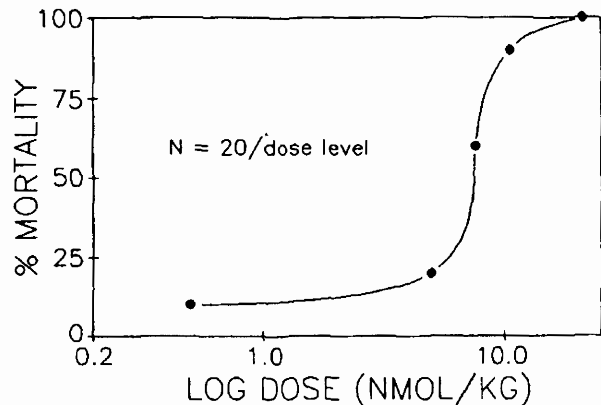

FIG. 2. Dose–response curve of ω-conotoxin GVI A in goldfish. The approximate LD50 value within a 2-hr period of observation is calculated using the Spearman–Karber method. Number of goldfish (n) is 20 per dose level.

(Spearman–Kärber method; Finney, 1978) within a 2-hr period of observation (Fig. 2).

A more quantitative relationship between dose and toxicity time was achieved by expressing the response as the toxicity, the inverse of the toxicity time, at different doses. As shown in Fig. 3, the data could be fitted to a straight line, with a correlation coefficient of 0.91 and a y-intercept of 0.0112, min^{-1}, corresponding to 90.91 min. Thus, the method could be used to measure toxicity times of less than 91.0 min with the observed accuracy. The precision of this method calculated from the distribution of toxicity values about the mean (standard error) for all dose levels was less than 10%.

Antitoxin activity of monoclonal antibodies in goldfish. Following preliminary studies with a set of monoclonal antibody clones raised against ω-CgTx, one clone (ω1-20-18) was chosen for detailed analysis. The antibody ω1-20-18, alone at a dose of 50 nmol/kg, ip, had no intrinsic toxic effect on the goldfish (Fig. 4). The LD50 dose of ω-CgTx (5 nmol/kg, ip) was chosen for these experiments since it was close to the TTD50. The toxicity of 5 nmol/kg dose of ω-CgTx was 0.0233, min^{-1} (i.e., 43 min to observe ASB), and all fish were affected. Incubation *ex vivo* of this dose of the toxin together with 50 nmol/kg of the antibody ω1-20-18 for 12 hr at 4°C before injection resulted into a substantial reduction ($p < 0.01$) of toxicity (Fig. 4, far right bar). Thus, 70% of the treated fish showed no signs of toxin treatment, while the remaining 30% exhibited a decline in toxic response by nearly 50% to 0.0123, min^{-1} (81.3 min to observe ASB) (Fig. 4, far right bar).

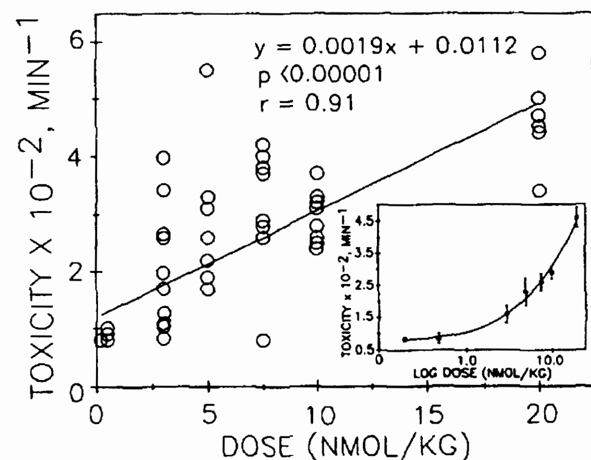

FIG. 3. Relationship between toxicity (1/toxicity time) and the dose of ω-conotoxin GVI A (from data in Fig. 1). The solid line shows the fit of data to the straight line equation $y = mx + B$ by regression analysis. r = regression coefficient, and p = level of significance. Insert shows same data represented on a logarithmic scale. Circles represent individual data points. The number of subjects (n) per each dose level is 10 but appears to be less due to instances of superimposition of one or more equal data points.

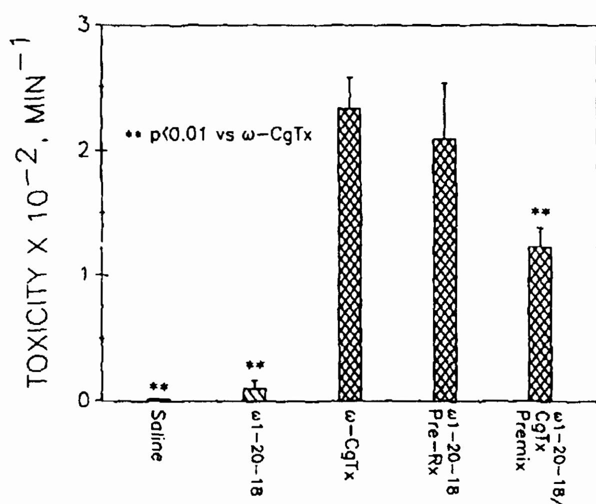

FIG. 4. Effect of the monoclonal antibody (ω1-20-18) against ω-conotoxin GVI A (50 nmol/kg) on the toxicity of ω-conotoxin GVI A (ω-CgTx) (5 nmol/kg, ip) in goldfish. Each bar represents the mean ± SE of 10 goldfish (10 experiments). Pre-Rx, pretreatment with antibody ω1-20-18; Premix, preincubation of ω-conotoxin GVI A with antibody ω1-20-18 at 4°C for 12 hr. Significance versus ω-conotoxin GVI A only is indicated by asterisks (Dunnett's test).

We then proceeded to investigate the possible protective effect of pretreatment of the goldfish with monoclonal antibody ω1-20-18. The fish were treated with the antibody (50 nmol/kg, ip) 30 min before ω-CgTx administration at the 5 nmol/kg dose. In this experiment, 90% of the antibody pretreated goldfish exhibited toxic effects of the ω-CgTx, and with a toxicity indistinguishable from that of the toxin alone (Fig. 4). Although 10% of the goldfish were protected from toxicity as measured by abnormal swimming behavior, ω-CgTx-induced mortality significantly increased from 50% with toxin alone to 90% in antibody pretreated fish suggesting that the toxin and the antibody have an additive effect on goldfish mortality (Fig. 5).

Action of monoclonal antibody (ω1-20-18) and ω-CgTx on the rat. As reported previously from our laboratory (Rosario *et al.*, 1989; Shapira *et al.*, 1990a), intracerebroventricular injection of ω-CgTx in the rat produces characteristic behavioral and autonomic responses, including a persistent shaking of the head, neck, and limbs as well as dose-related increases in MAP and HR. In the present study, we observed 100% mortality at doses higher than 0.3 nmol/rat and 0% mortality at doses less than 0.03 nmol/rat as previously reported (Shapira *et al.*, 1990a). Injection of ω-CgTx (0.03 nmol/rat, icv) resulted in a significant pressor response, peaking about 30 min post-treatment and subsiding after 60 min. Over the period of observation, a sustained increase in heart rate was also noted. However, preincubation of ω-CgTx (0.03 nmol/rat) with ω1-20-18 (0.3 nmol/rat) significantly attenuated the pressor response (Fig. 6, upper pannel) and the increases in heart rate (Fig. 6, lower panel). Consistently, icv injections of saline, or ω1-20-18 alone, did not have any effects on MAP and HR of the rat. Thus, the antitoxin effects of monoclonal antibody (ω1-20-18), initially observed in the goldfish model, seemed to have predictive value when tested in the rat.

DISCUSSION

The present study supports the concept that the goldfish bioassay model system can be used

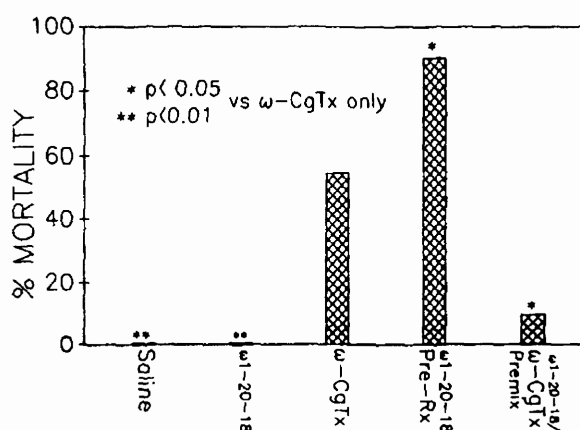

FIG. 5. The influence of antibody ω1-20-18 (50 nmol/kg, ip) on the lethal effect of ω-conotoxin GVI A (ω-CgTx) (5 nmol/kg, ip) in goldfish. n = 10/experiment. Pre-Rx, pretreatment with antibody ω1-20-18; Premix, preincubation of ω-conotoxin GVI A and antibody ω1-20-18 at 4°C for 12 hr. Significance versus ω-conotoxin GVI A only is indicated by asterisks (Fisher Exact probability test, 2 × 2 table).

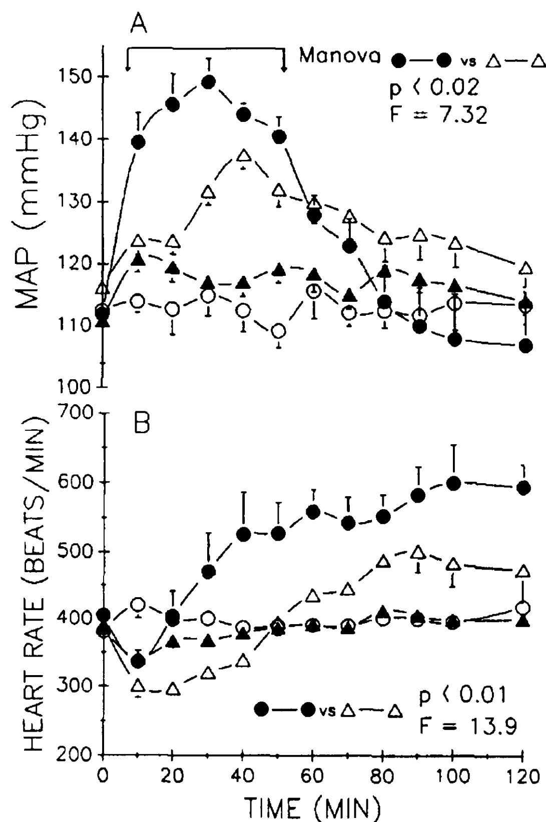

FIG. 6. Effect of preincubation of ω-conotoxin GVI A with antibody ω1-20-18 on mean arterial pressure (A) and heart rate (B) in the conscious rat. Solid circles denote the effect of ω-conotoxin GVI A (0.03 nmol/rat icv) alone, open triangles denote the effect of preincubation of ω1-20-18 (0.3 nmol/rat icv) with ω-conotoxin GVI A (0.03 nmol/rat icv), solid triangles denote the effect of ω1-20-18 (0.3 nmol/rat icv) alone, and open circles denote the effect of saline alone. n = 4–6; statistical analysis by ANOVA with repeated measures.

to predict toxic effects of ω-CgTx in the rat. Some of the advantages of the goldfish model are that it possesses a relatively limited blood–brain barrier (Bradbury, 1979) and expresses some useful behavioral and pharmacologic parallels to the higher vertebrates. For example, in the work shown here we have found that toxicity of ω-CgTx injected ip into the goldfish occurs in about the same concentration range as that previously noted for this toxin when injected icv into the rat (Rosario *et al.*, 1989; Shapira *et al.*, 1990a). Therefore, similar sites of action may exist in both fish and rat, the difference only being the limited access to the brain in the rat. Thus, the neuropharmacology and neuroimmunology of charged toxins such as ω-CgTx can be studied by systemic injection into goldfish.

A second important parallel between goldfish and rat is the observation that the monoclonal antibody against ω-CgTx (ω1-20-18) interferes with toxin activity if prereacted prior to injection, either ip into the goldfish, or icv into the rat. This result further substantiates the goldfish model as an analytical system with remarkable powers of prediction for the higher vertebrates, at least insofar as ω-CgTx and monoclonal antibody against the toxin are concerned.

The present results also demonstrate that while preformation of the antibody/ω-CgTx complex attenuates toxic effects, both in goldfish and in rat, pretreatment with the antibody seemed to lack any protective effect. In goldfish, pretreatment with the antibody was even more harmful, increasing significantly the mortality to ω-CgTx (at the LD50 dose). On the other hand, while all goldfish were affected by the treatment with toxin alone, pretreatment with the antibody tended to protect 10% of the fish from any observed effect.

The inability of the antibody pretreatments to protect the goldfish against the toxic effects of ω-CgTx might be due to an interaction at the ω-CgTx binding site. The antibody might be binding at an allosteric site on this receptor. The antibody binding might therefore induce a conformational change in the receptor which enhances the binding of ω-CgTx and thereby the toxicity. Second, the antibody might bind an endogenous ω-CgTx-like substance in the fish therefore making more of the injected toxin available at the receptor site. On the other hand, attenuation of the toxic response to ω-CgTx in the goldfish following *ex vivo* incubation of the antibody–toxin mixture could be due to the formation of an antibody–ω-CgTx complex that may not fit properly into the binding site on the receptor.

In this present bioassay, the accuracy (r = 0.91) and precision (<10% coefficient of variation about the mean) for analysis of ω-CgTx effects in the goldfish after ip injections

also seem to emphasize the analytic values of this system. It may be that the parallels found here between the goldfish model and the rat's CNS may extend to other neurotoxins. It remains for future studies to reveal whether parallels to such action may be found in higher vertebrates, either by injection into the brain of living animals or by studies using the antibody on cultured cells from nervous tissue. We are currently following such a course of study.

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