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Neutralization of Saxitoxin by Anti-Saxitoxin
Rabbit Serum: The Effect of Antiserum on Toxin Binding to
the Sodium Channel and on Toxicity in Mice ^{1,2}

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ABSTRACT

S.R. DAVIO. Neutralization of saxitoxin by anti-saxitoxin rabbit serum: The effect of antiserum on toxin binding to the sodium channel and on toxicity in mice. Toxicon _____, _____, 19____. This study examined the ability of anti-saxitoxin rabbit serum to neutralize saxitoxin, both in vitro and in vivo. In vitro, two rabbit antisera decreased (³H)-saxitoxin binding to the toxin's site of action--the sodium channel of rat brain membranes. The more potent of these sera, antiserum A, when combined with saxitoxin in vitro, decreased saxitoxin's toxicity based on mouse bioassay. Antiserum A also neutralized saxitoxin in vivo as illustrated by the fact that mice injected i.p. with antiserum A (1:4) survived an injection 1 hr later of 16.7 µg saxitoxin/kg (an LD₅₀, s.c.). Finally, antiserum A prevented death when injected i.v. immediately after s.c. injection of 16.7 µg saxitoxin/kg; antiserum injected by the i.p. and i.m. routes caused no significant increase in survival. This study indicates that antiserum can neutralize saxitoxin in vitro and in vivo and, therefore, warrants further study as a therapeutic agent for saxitoxin poisoning.

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FOOTNOTES

¹ In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals", as promulgated by the Committee on the Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council. The facilities are fully accredited by the American Association for Accreditation of Laboratory Animal Care.

² The views of the author do not purport to reflect the positions of the Department of the Army or the Department of Defense.

INTRODUCTION

Saxitoxin (Fig. 1) is one of the most lethal non-protein toxins known ($LD_{50} \sim 11.6 \mu\text{g/kg}$, i.p., mouse, personal observations). This toxin blocks the sodium channel of nerve and muscle membranes and thereby prevents action potential propagation necessary for nerve conduction and muscle contraction (KAO, 1966). Saxitoxin is produced by dinoflagellates of the genus Gonyaulax (SOMMER et al., 1937; SCHANTZ et al., 1966). These dinoflagellates can contaminate shellfish which, when eaten by humans, can cause numbness, paralysis, and even death due to respiratory arrest (EBRIGHT, 1930; FOPKISS et al., 1979; BOWER, et al., 1981). The primary therapy for saxitoxin poisoning is artificial respiration; saxitoxin has no known antidote (HALSTEAD, 1967).

A previous study (JOHNSON et al., 1964) demonstrated that immunizing rabbits with a saxitoxin-formaldehyde-bovine serum albumin conjugate produced antiserum capable of protecting mice against lethal doses of saxitoxin. In the present study, I examined this observation in greater detail. Specifically, two anti-saxitoxin rabbit sera were tested for their ability to decrease saxitoxin binding to the nerve membrane sodium channel in vitro. Based on these binding studies, the more potent of these sera was tested for its ability to neutralize saxitoxin in vitro as determined by bioassay. Finally, this antiserum was tested for its ability to neutralize saxitoxin in vivo and prevent lethality. These latter studies examined the effects of antiserum injected either before or after injection of toxin. Based on these studies, I have drawn conclusions concerning passive immunotherapy as a method of treatment for saxitoxin poisoning.

MATERIALS AND METHODS

Saxitoxin.

Saxitoxin for [^3H]-labeling was obtained from the Edgewood Arsenal, Aberdeen Proving Ground, MD 21010. Saxitoxin used to determine the specific activity of [^3H]-saxitoxin was obtained from Dr. James E. Gilchrist, Food and Drug Administration (FDA), Cincinnati, Ohio 45226.

Antisera.

Antiserum A (Bio-Metric Systems, Inc., Eden Prairie, MN, USA) was obtained from rabbits which had been immunized with a saxitoxin-bovine serum albumin conjugate (saxitoxin-BSA) prepared as described by CARLSON et al. (1984). Antiserum B (Dr. Fun Sun Chu, University of Wisconsin, Madison, WI, USA) was obtained from rabbits which had been immunized with another saxitoxin-BSA conjugate prepared using the method of JOHNSON et al. (1964).

[^3H]-Saxitoxin and Membranes.

[^3H]-saxitoxin was prepared by Amersham Corporation using the exchange labeling procedure of RITCHIE, et al. (1976). The [^3H]-saxitoxin was subsequently purified by high-performance liquid chromatography (HPLC) on a 250 mm x 4.6 mm (I.D.) Partisil 10 SCX column (Whatman Chemical Separations, Inc.). The [^3H]-saxitoxin was eluted from the column with a linear gradient of 0 - .4 M KH_2PO_4 , pH 4.0, at 1 ml/min. The final product had a radiochemical purity of 58% using the procedure of CATTERALL and MORROW, (1978). The specific activity was 14 Ci/mmol as determined by displacement from rat brain membranes by standard solutions of unlabeled saxitoxin. Rat brain membranes were prepared from 250-300 g male Sprague-Dawley rats using the procedure of KRUEGER, et al. (1979).

[³H]-Saxitoxin Binding Measurements.

One-half milliliter of rat brain membranes (0.5 mg protein/ml) was combined with 0.5 ml [³H]-saxitoxin (5.6 nM) and 0.5 ml diluted antiserum. All components were in 20 mM HEPES, 140 mM NaCl, 6 mM EDTA, pH 7.4 (isotonic HEPES buffer). After equilibration for 1 hr at 4°, 1 ml of each suspension was filtered through a BSA-pretreated (see below) GF/F filter. Each filter was washed rapidly with 15 ml ice-cold HEPES buffer. The radioactivity of each filter, due to membrane-bound [³H]-saxitoxin, was quantitated by scintillation counting. Membrane-bound [³H]-saxitoxin was expressed as a fraction of that bound in the absence of antiserum, i.e., B/B₀. The GF/F filters used in this assay were pre-soaked overnight at 4° in isotonic HEPES buffer containing 1% BSA. This pretreatment was done to prevent non-specific binding of [³H]-saxitoxin-antibody complexes to the filter. The toxin-binding capacity of each antiserum was calculated by dividing the antibody-associated [³H]-saxitoxin by the volume of antiserum which caused half-displacement of membrane-bound [³H]-saxitoxin. Antibody-associated [³H]-saxitoxin was calculated as the difference: [³H]-saxitoxin_{total} - ([³H]-saxitoxin_{membrane-bound} + [³H]-saxitoxin_{free}).

Animal Studies

All animal studies used fed 25-30 g Swiss-Webster mice (Charles River Laboratories, Inc., Wilmington, MA, USA). Injections of saxitoxin and antiserum consisted of 0.1 ml volume. Dilutions of antiserum and saxitoxin were made in sterile isotonic saline. Unless noted otherwise mice were challenged with saxitoxin doses ranging from 16.2 to 16.7 µg/kg. These doses were determined from preliminary studies to be approximate LD₉₉ doses for either i.p. - or s.c. - injected toxin. Lethality was determined 24 hr after

injection with saxitoxin.

The toxin-neutralizing capacity of antiserum A was calculated from two sets of lethality data by using equation(1):

$$\text{toxin neutralizing capacity} = (TD - LD_{50}) \times \frac{\text{average weight}}{\text{per mouse}} \times 1/EV_{50} \quad (1)$$

where TD, total dose of toxin, and LD_{50} were in $\mu\text{g/kg}$; average weight per mouse was in kg; and EV_{50} was the volume of antiserum in ml which reduced lethality to 50%. The EV_{50} was estimated by eye from data in Figs. 3 and 4. Equation (1) assumes that at 50% lethality the antiserum has neutralized enough saxitoxin to reduce the total dose to an LD_{50} dose. Preliminary studies determined that the LD_{50} for saxitoxin injected s.c. was $12.9 \mu\text{g/kg}$ (95% confidence interval $12.3 - 13.4 \mu\text{g/kg}$). The LD_{50} for saxitoxin injected i.p. was $11.6 \mu\text{g/kg}$ (95% confidence interval $11.2 - 12.1 \mu\text{g/kg}$).

RESULTS

In Vitro [^3H]-Saxitoxin Binding Experiments.

Saxitoxin-specific antibodies must be capable of decreasing saxitoxin binding to the sodium channel if they are to counteract the effects of the toxin in vivo. On this premise I measured [^3H]-saxitoxin binding to sodium channels in rat brain membranes in the presence of dilutions of two saxitoxin-specific antisera (Fig. 2). Both antisera decreased saxitoxin binding to the sodium channel. Antiserum A, 1:24000, and antiserum B, 1:2040, decreased saxitoxin binding to 50% of the control level. Based on these data, antiserum A was calculated to have a toxin-binding capacity of $\sim 11.4 \mu\text{g}$ saxitoxin/ml; antiserum B, $\sim 0.97 \mu\text{g}$ saxitoxin/ml. Due to its higher toxin-binding capacity, antiserum A was used in the remaining studies in this paper.

In Vitro Neutralization of Saxitoxin by Antiserum A.

To determine if antiserum A could decrease the toxicity of saxitoxin, 16.2 µg/kg doses of toxin were combined with various dilutions of antiserum, incubated, and bioassayed by i.p. injection into mice (Fig. 3). As expected, animals injected with saxitoxin + control rabbit serum (1:2) showed 0% survival. However, animals injected with saxitoxin + 1:10- and 1:8-, and 1:4- diluted rabbit antiserum showed 50, 80, and 100% survival, respectively. Based on these data, the in vitro toxin-neutralizing capacity of undiluted antiserum A was calculated (see Materials and Methods) to be 12.7 µg saxitoxin/ml antiserum. Note that this value is very close to the toxin-binding capacity, 11.4 µg saxitoxin/ml, determined from the above binding experiment.

In Vivo Neutralization of Saxitoxin by Antiserum A.

To determine whether antiserum can neutralize saxitoxin in vivo, mice were injected i.p. with dilutions of antiserum A and injected s.c. 1 hr later with 16.7 µg saxitoxin/kg (Fig. 4). Dilutions of antiserum A from 1:16 through 1:8 produced partial protection against this dose of toxin. Mice injected with a 1:4 dilution of antiserum A showed 100% survival (n = 10). Control serum, as expected, afforded no protection. Using these data the in vivo toxin-neutralizing capacity of antiserum A was calculated to be 12.8 µg saxitoxin/ml.

To determine the time course for the apparent passive immunity afforded by antiserum A, animals were injected i.p. with antiserum A (1:4) and, at designated times later, injected s.c. with 16.5 µg saxitoxin/kg (n = 10 for each time point). Animals injected with antiserum 1 hr or 1 week before saxitoxin challenge showed 90 and 80% survival, respectively. Animals pre-injected with antiserum at 6, 24, 48 and 72 hr all showed 100% survival. This

result indicates that the passive immunity afforded by antiserum injection takes effect rapidly and is persistent over a period of days.

To approximate the maximum level of protection afforded by passively-administered antiserum A, ten animals were injected with 100 μ l undiluted antiserum and challenged 6 hrs later, s.c., with 2.1, 4.2, or 6.3 times the normal LD₅₀ dose of 12.9 μ g/kg (s.c.) (low numbers of animals were used due to the limited supply of antiserum). Two animals out of two injected with 2.1 times the normal LD₅₀ survived; four out of six injected with 4.2 times the normal LD₅₀ survived; zero out of two injected with 6.3 times the normal LD₅₀ survived. Thus, the LD₅₀ was increased approximately four-fold in animals pre-injected with undiluted antiserum A. This is roughly consistent with the level of protection expected on the basis of the in vivo toxin-neutralizing capacity of antiserum A. One-tenth milliliter of serum in a 27 g mouse (average weight) should be capable of neutralizing 47.4 μ g saxitoxin/kg ($0.1 \text{ ml antiserum/animal} \times 12.8 \text{ } \mu\text{g saxitoxin/ml} \times 1 \text{ animal}/.027 \text{ kg} = 47.4 \text{ } \mu\text{g saxitoxin/kg}$). This would result in a 4.7-fold shift in the LD₅₀.

Finally, an experiment was conducted to determine the most effective route of antiserum administration when antiserum is given after exposure to toxin. Mice were injected s.c. with 16.7 μ g saxitoxin/kg, followed within 30 sec by injection of antiserum A (1:8) by the i.m., i.p., or i.v. routes. Animals injected with antiserum by the i.m. and i.p. routes showed 0-10% survival (n = 10 for each route). Moreover, times-to-death were not significantly increased in these groups of animals. However, animals injected with antiserum A (1:8) through the tail vein (i.v.) showed 100% survival (n = 9), clearly indicating that this is the most effective route of administration for post-toxin injection of antiserum.

DISCUSSION

The purpose of this study was to examine the ability of anti-saxitoxin rabbit serum to neutralize saxitoxin both in vitro and in vivo. The investigation began at the molecular level and showed that two rabbit antisera decreased binding of [3 H]-saxitoxin to specific sites in rat brain membranes--sites known to be associated with sodium channels (WEIGELE and BARCHI, 1978; TALVENHEIMO et al., 1982; GOLDIN et al., 1980; HENDERSON et al., 1974). The [3 H]-saxitoxin-rat brain membrane binding assay was used to calculate and compare the toxin-binding capacities of the two antisera. The toxin-binding capacity of antiserum A calculated from these binding data was close to the toxin-neutralizing capacity found using the more conventional method of test tube neutralization followed by bioassay (11.4 vs 12.7 μ g saxitoxin/ml, respectively). This suggests that the binding method is a valid approach for quantitating the amount of toxin-neutralizing antibody in a given serum. This procedure could be used in any number of situations requiring the screening for and quantitation of antibodies to saxitoxin; e.g., in the screening of hybridoma clones for monoclonal antibodies to saxitoxin.

While the [3 H]-saxitoxin binding assay demonstrated antiserum A to be more potent than antiserum B, these results cannot be used to draw conclusions regarding the antigenicity of the respective saxitoxin-BSA conjugates. This is because the antigens were synthesized by separate laboratories which used different immunization protocols.

Current therapy for saxitoxin poisoning includes gastric lavage, to remove contaminated shellfish, and artificial respiration. There is no definitive antidote for saxitoxin poisoning (HALSTEAD, 1967). The results in this paper indicate that antiserum may be an effective therapeutic agent. Most relevant to this is the observation that antiserum injected i.v. after a

lethal injection of saxitoxin resulted in 100% survival. The effect of antiserum injected i.v. must be essentially immediate since saxitoxin injected s.c. normally kills mice within 5-10 minutes. In cases where humans eat saxitoxin-contaminated shellfish, symptoms appear within minutes of ingestion while death can occur anywhere from 1-12 hours later (KAO, 1966; HALSTEAD, 1967). This should be sufficient time to intervene with injection of antiserum. This mode of therapy would be consistent with that used against another neurotoxin, botulinum toxin, which is combated using immune horse serum (ELLIS, 1962).

While the data demonstrate that antiserum A can counteract saxitoxin in vivo, this particular antiserum may not be effective against the many other "saxitoxin-like" paralytic shellfish poisons (PSPs) produced by Gonyaulax dinoflagellates and associated with toxic shellfish (KOEHN et al., 1982; SHIMIZU, 1979). CARLSON et al. (1984) have shown that antiserum A does not bind neosaxitoxin (N-1 hydroxysaxitoxin). Neosaxitoxin and related toxins are major components in some toxic shellfish (SHIMIZU, 1979). Thus, a true antidote for the PSPs must have a broader reactivity.

In conclusion, the data in this paper demonstrate that rabbit antiserum to a saxitoxin-BSA antigen can neutralize saxitoxin in vitro and in vivo. The data indicate that passive immunity through antiserum injection is a therapeutic concept for saxitoxin poisoning which warrants further study.

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FIGURE LEGENDS

FIG. 1. THE CHEMICAL STRUCTURE OF SAXITOXIN (SCHANTZ et al., 1975).

FIG. 2. THE EFFECT OF ANTISERA ON [^3H]-SAXITOXIN BINDING TO RAT BRAIN MEMBRANES.

Results are expressed as the ratio B/B_0 where B and B_0 represent binding of [^3H]-saxitoxin to membranes in the presence and absence of antiserum, respectively.

FIG. 3. % SURVIVAL OF MICE INJECTED WITH PRE-MIXED SAXITOXIN + ANTISERUM A.

Saxitoxin and diluted antiserum were combined and incubated at 37° for 1 hr, then 0.1 ml antiserum/toxin mixture was injected per animal, i.p. The average dose of toxin was $16.2 \mu\text{g/kg}$. Five animals were used to determine each point. The average weight per animal was 27.6 g.

FIG. 4. % SURVIVAL OF MICE PRE-INJECTED WITH ANTISERUM A FOLLOWED BY INJECTION OF SAXITOXIN

Dilutions of antiserum A were injected i.p. 1 hr before s.c. challenge with saxitoxin ($16.7 \mu\text{g/kg}$). Antiserum and toxin injections each consisted of 100 μl volume. Ten animals were used to determine each point. The average weight per animal was 27.0 g.







