

REVERSAL OF PACIFIC CIGUATOXIN-1B EFFECTS ON MYELINATED AXONS BY AGENTS USED IN CIGUATERA TREATMENT

by

Évelyne BENOIT (1), Dominique LAURENT (2), César MATTEI (1),
Anne-Marie LEGRAND (3) & Jordi MOLGÓ (1)

ABSTRACT. Ciguatera fish poisoning is a distinctive form of ichthyosarcotoxism characterised mainly by gastrointestinal and neurological disturbances. The ciguatoxins, responsible for this poisoning, are complex polyethers produced by toxic strains of the dinoflagellate *Gambierdiscus toxicus*. These toxins are increased to dangerous levels for man during their transmission through herbivorous and carnivorous fish, various species being contaminated. The known molecular target of ciguatoxins is the voltage-gated Na⁺ channel. During the action of these toxins, the permanent opening of channels, at the resting membrane potential, produces a continuous entry of Na⁺ ions in excitable cells causing a marked increase in membrane excitability and in cellular volume. To precise the neurocellular basis of the efficacy of some agents used in clinical and traditional treatments of ciguatera, their effects were studied on frog myelinated axons exposed to Pacific ciguatoxin-1B (CTX-1B). During the action of this toxin, the increase in axonal volume and membrane excitability was reversed by lidocaine (a local anaesthetic), by CaCl₂ and by hyperosmotic external solutions (containing D-mannitol, sucrose or tetramethylammonium chloride). The CTX-1B-induced hyperexcitability of the membrane was also reversed by extracts of *Argusia argentea* leaves or *Davallia solida* rhizomes, used traditionally in New-Caledonia. It is concluded that the various agents studied are able to counteract the neurocellular effects of CTX-1B in myelinated axons. These results are of particular interest since they provide a scientific basis to understand the beneficial action of therapeutic agents used in the treatment of ciguatera fish poisoning.

RÉSUMÉ. Réversibilité des effets de la ciguatoxine-1B du Pacifique sur les axones myélinisés par des agents utilisés dans le traitement de la ciguatera.

La ciguatera est une forme particulière d'ichtyosarcotoxisme principalement caractérisée par des troubles gastro-intestinaux et neurologiques. Ce sont les ciguatoxines, polyéthers complexes produits par des variétés toxiques du dinoflagellé *Gambierdiscus toxicus*, qui en sont responsables en se concentrant pour atteindre des doses dangereuses pour l'homme lors de leur transfert dans de nombreuses espèces de poissons herbivores et carnivores. La cible moléculaire connue des ciguatoxines est le canal Na⁺ sensible au potentiel de membrane. Durant l'action de ces toxines, l'ouverture permanente des canaux au potentiel de repos de la membrane, produit une entrée continue d'ions Na⁺ dans les cellules excitables ce qui augmente notablement l'excitabilité membranaire et le volume cellulaire. Dans le but de préciser les bases neurocellulaires de l'efficacité de certains agents utilisés dans le traitement clinique et traditionnel de la ciguatera, leurs effets ont été étudiés sur des axones myélinisés de grenouille préalablement soumis à l'action de la ciguatoxine-1B du Pacifique (CTX-1B). L'augmentation du volume axonal et de l'excitabilité de la membrane, produite par cette toxine, a été neutralisée par la lidocaïne (anesthésique local), le CaCl₂, et les milieux extracellulaires hyperosmotiques contenant du D-

(1) Laboratoire de Neurobiologie Cellulaire et Moléculaire, UPR 9040, CNRS, Bât. 32, 91198 Gif-sur-Yvette, FRANCE. [benoit@nbcn.cnrs-gif.fr]

(2) Laboratoire des Substances Naturelles, Institut de Recherche pour le Développement (ex ORSTOM), BP A5, 98848 Nouméa, NOUVELLE-CALÉDONIE.

(3) Unité d'Océanographie Médicale, Institut de Recherches Médicales Louis Malardé, BP 30, Papeete, Tahiti, POLYNÉSIE FRANÇAISE.

mannitol, du saccharose ou du chlorure de tétraméthylammonium. L'hyperexcitabilité membranaire, produite par la CTX-1B, a également été supprimée par les extraits de feuilles d'*Argusia argentea* ou de rhizomes de *Davallia solida*, utilisés dans la médecine traditionnelle en Nouvelle-Calédonie. En conclusion, les divers agents étudiés sont capables de neutraliser les effets neurocellulaires de la CTX-1B au niveau des axones myélinisés. Ces résultats sont particulièrement intéressants puisqu'ils apportent une base scientifique nécessaire à la compréhension de l'action bénéfique des agents thérapeutiques utilisés de manière encore empirique dans le traitement de l'ichtyosarcotoxisme de type ciguatera.

Key words. Ciguatera fish poisoning - Ciguatoxins - Na⁺ channels - Myelinated axons - Therapeutic agents - Cellular electrophysiology - Axonal volume.

Ciguatera fish poisoning is a distinctive and common form of a widespread human seafood intoxication associated with a polymorphic syndrome, which includes severe gastro-intestinal and neurological disturbances and develops after consumption of various contaminated species of tropical and subtropical coral reef fish. The research on ciguatera has mainly been carried out in the Pacific area where the intoxication has been directly linked to the benthic dinoflagellate *Gambierdiscus toxicus*, known to produce the ciguatoxins which, through the marine food chain, are transmitted to herbivorous and carnivorous fish and ultimately to man (reviewed by Russell and Egen, 1991; Swift and Swift, 1993; Glaziou and Legrand, 1994).

Ciguatoxins, purified from wild toxic *G. toxicus* dinoflagellates or from Pacific and, more recently, from Caribbean poisonous fish, are complex lipid-soluble highly oxygenated cyclic polyether compounds (see Murata *et al.*, 1990; Lewis *et al.*, 1998). Previous studies identified ciguatoxins as potent voltage-dependent Na⁺ channel-activating toxins. Indeed, ciguatoxins have been reported to bind with high affinity to the receptor-site 5 of the neuronal voltage-gated Na⁺ channel-protein (Lombet *et al.*, 1987; Poli *et al.*, 1997; Dechraoui *et al.*, 1999). Moreover, these toxins cause membrane depolarisation which in turn triggers spontaneous and/or repetitive action potential discharges in excitable cells (Bidard *et al.*, 1984; Molgó *et al.*, 1990; Benoit and Legrand, 1994; Hamblin *et al.*, 1995; Benoit *et al.*, 1996; Hogg *et al.*, 1998; Mattei *et al.*, 1999). In addition, swelling of motor nerve terminals and myelinated axons occurred during the action of ciguatoxins (Molgó *et al.*, 1994; Benoit *et al.*, 1996; Mattei *et al.*, 1997, 1999). These toxin effects were directly related to an increased membrane Na⁺ permeability due to persistent activation of Na⁺ channels at the resting potential where these channels are normally closed. These neurocellular actions of ciguatoxins are consistent with the neurological alterations reported in patients suffering from ciguatera fish poisoning (Allsop *et al.*, 1986; Cameron *et al.*, 1991).

Treatment of ciguatera fish poisoning is primarily supportive. Traditionally, herbal remedies are used and about 100 plants, including *Argusia argentea* and *Davallia solida*, are reputed to be active in the South Pacific (Laurent *et al.*, 1993). Various other therapeutic agents (such as local anaesthetics, calcium gluconate, vitamins, amitriptyline, glucose and D-mannitol) have been found to be effective to treat the neurological symptoms of ciguatera (Palafox *et al.*, 1988; Pearn *et al.*, 1989; Russel and Egen, 1991; Swift and Swift, 1993; Blythe *et al.*, 1994; Poli *et al.*, 1997).

The experiments to be described here were done to provide a rational basis for understanding the beneficial action of the above agents when used clinically or traditionally. For this purpose, we investigated whether some of them could reverse the effects of Pacific ciguatoxin-1B (CTX-1B), considered as the major ciguateric ichthyotoxin in the Pacific

area, on membrane excitability and axonal volume of myelinated nerve fibres.

MATERIALS AND METHODS

The experiments were performed on single myelinated axons isolated from the sciatic nerve of adult frogs (*Rana esculenta*) of 20-30g body weight.

Solutions, agents and ciguatoxin

The external standard physiological solution had the following composition (in mM): NaCl 111.5, KCl 2.5, CaCl₂ 1.8, HEPES 10 (buffered at pH 7.4 with NaOH). In some experiments, the osmolality of the external solution was increased by about 50% with 100mM D-mannitol, 100mM sucrose or 50mM tetramethylammonium chloride. In other experiments, the external CaCl₂ concentration was raised to 5.4mM.

Extracts of *Argusia argentea* leaves and *Davallia solida* rhizomes were prepared according to folk remedies: 20g of turned yellow leaves of *Argusia argentea* were cut in small pieces and put to boil in 250ml of water for 30 min, and 25g of *Davallia solida* rhizomes were beaten to a pulp and put to macerate in 250ml of water for 30 min. The two solutions were then filtrated and kept at -20°C until used for experiments.

Pacific ciguatoxin-1B (CTX-1B) was extracted and highly purified from poisonous moray-eels (*Gymnothorax javanicus*), as previously described (Murata *et al.*, 1990). The dry toxic extracts were dissolved in ethanol and the solution was divided into several samples. The ethanol was then evaporated. Samples were kept dry at -20°C and diluted immediately before experiments with external solutions.

Electrophysiology

Single myelinated axons of about 0.5mm length were isolated and mounted in a five-compartment chamber. The nodal membrane resting and action potentials were recorded using a conventional current-clamp technique (see Mattei *et al.*, 1999). Briefly, the normal resting potential of axons was assumed to be -70mV and, if not otherwise stated, the node of Ranvier under investigation was stimulated at a frequency of 0.5 Hz. The fibre ends were cut in a solution containing 120mM KCl, which was used in the end pools throughout the experiments performed near 18°C.

Confocal laser scanning microscopy

For imaging myelinated axons, we used confocal laser scanning microscopy and the fluorescent dye FM1-43 to stain their plasma membrane, as previously described (Benoit *et al.*, 1996). Briefly, a desheathed piece of the sciatic nerve of about 2mm long was pinned to the bottom of a rhodorsil-lined Plexiglas chamber and axons were gently teased apart from the main trunk. Before imaging, preparations were exposed for 15 min to 2μM FM1-43, dissolved in standard physiological solution, and thereafter rinsed with dye-free solution. A Sarastro-2000 laser confocal (Molecular Dynamics), mounted on a NIKON optiphot-2 upright microscope equipped with a x 40 water-immersion objective (0.75 numerical aperture), was used to collect series of optical sections of living axons and subsequent three-dimensional digital reconstruction of their structure by a look-through projection. The experiments were carried out near 22°C.

Results were quantified on the same axon, before and during the various treatments, by measuring the nodal length (L) and the nodal diameter (D). The nodal volume (V) was

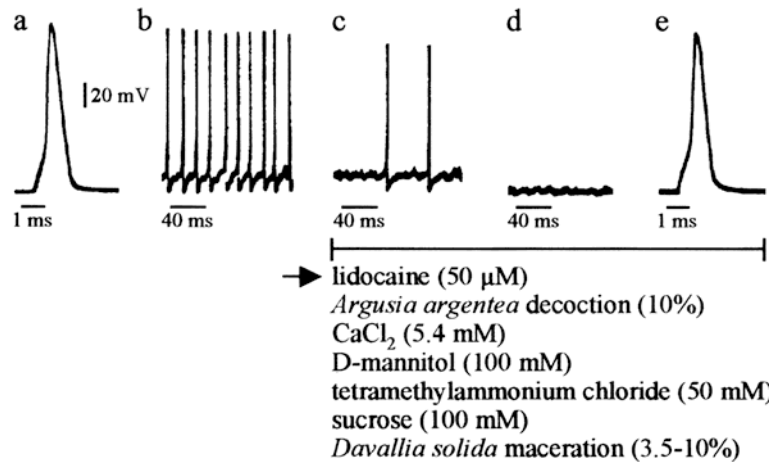


Fig. 1. Reversal of the CTX-1B-induced hyperexcitability of the nodal membrane by lidocaine. **a**: Control action potential evoked by a 0.5 ms depolarizing stimulus. **b**: Spontaneous and repetitive action potentials recorded in the presence of CTX-1B (10 μM). **c-d**: Decrease of frequency (C) and suppression (D) of spontaneous action potentials after addition of lidocaine (50 μM) to the external solution containing CTX-1B. **e**: Action potential evoked by a 0.5 ms depolarizing stimulus in the presence of lidocaine and CTX-1B.

then calculated as: $V_{max} = L(D/2)^2$. Statistical analysis of data was done using the Student's *t*-test (two-tailed). Data were considered significant at $p < 0.05$.

RESULTS

CTX-1B (2.5–10 μM), when added to the solution bathing current-clamped single myelinated axons, produced a small depolarisation of the nodal membrane (~3 mV) which triggered spontaneous and repetitive action potentials at a frequency of about 100 Hz (Fig. 1b). These repetitive action potentials occurred for periods up to 90 min in the absence of any electrical stimulation. It is worth noting that, under control conditions, action potentials were elicited only after brief depolarising stimuli (Fig. 1a). The nodal membrane hyperexcitability induced by CTX-1B could be markedly suppressed by various agents. Thus, addition of the local anaesthetic lidocaine (50 μM) or *Argusia argentea* leaves (10% of the decoction) to the external solution, as well as raising the external $CaCl_2$ concentration (from 1.8 to 5.4 mM) and increasing the osmolality of the external solution by about 50% with D-mannitol (100 mM), tetramethylammonium chloride (50 mM) or sucrose (100 mM), decreased in frequency and finally completely suppressed (in less than 5 min) the spontaneous action potentials elicited by CTX-1B (Fig. 1c, 1d). A similar effect, but with a longer latency (15–20 min), was observed after the addition of *Davallia solida* rhizomes (3.5–10% of the maceration) to the external solution. Under these various conditions, action potentials could still be evoked by electrically stimulating the axons (Fig. 1e). This was consistently observed in at least three different experiments. The inhibitory action of the above mentioned agents on spontaneous repetitive action potentials, except that of *Davallia solida* rhizomes, was reversible upon washing them out from axons.

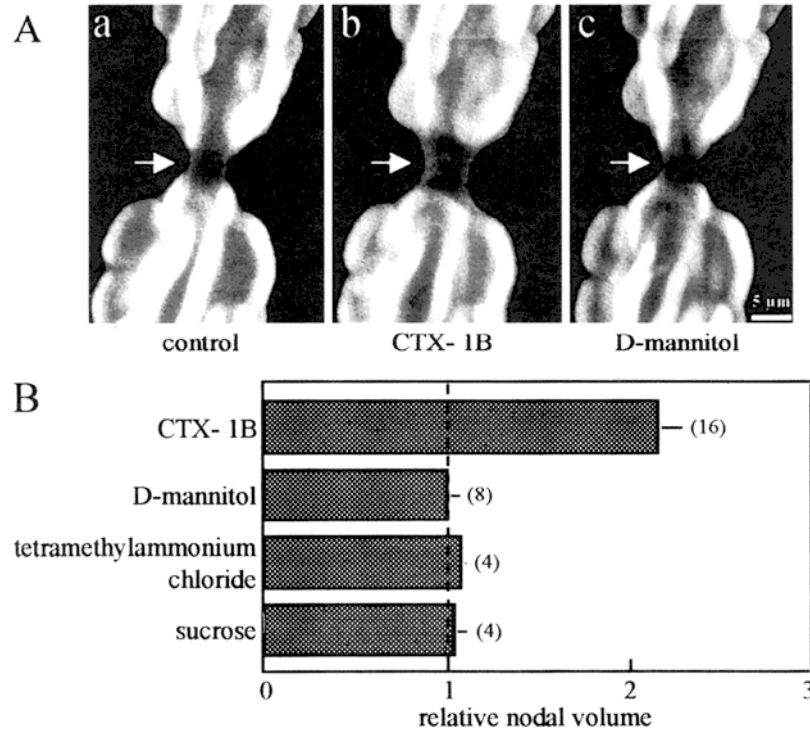


Fig. 2. Axonal swelling of nodes of Ranvier induced by CTX-1B (10 μ M) and its reversal by hyperosmotic external solutions. **A:** Look-through projections of a myelinated axon under control conditions (a), after 90 min exposure to CTX-1B (b) and after 60 min addition of 100 μ M D-mannitol to the external solution containing the toxin (c). The node of Ranvier (arrows) corresponds to the interruption of the myelin sheath layers which surround the axon. **B:** The relative nodal volume, normalized with respect to its control value, was determined before and after addition of 100 μ M D-mannitol, 50 μ M tetramethylammonium chloride or 100 μ M sucrose to the external solution containing CTX-1B. Mean \pm SEM of data obtained from n different axons (numbers in parentheses).

Addition of CTX-1B (10 μ M), to the medium of FM1-43-stained myelinated axons, produced a marked swelling of the nodes of Ranvier as revealed using confocal laser scanning microscopy. No alteration was detected in the morphology of internodal parts of axons covered by myelin sheath layers (Fig. 2A, 2B). The quantification of this effect, by calculating the nodal volume before and at various times after the addition of CTX-1B to the external solution (see Materials and Methods), revealed that the toxin caused, within 60-90 min, about two-fold increase in this parameter (Fig. 2B). Such increased nodal volume resulted from an enhancement of both length, and although to a lesser extent, diameter of nodes of Ranvier. The nodal swelling of myelinated axons produced by CTX-1B could be completely reversed with external solutions in which the osmolality was increased by about 50% with 100 μ M D-mannitol, 50 μ M tetramethylammonium chloride or 100 μ M sucrose (Fig. 2A, 2C). The reversal occurred within 30-60 min exposure with each of these agents. At that time, the nodal volume recovered values statistically similar to those obtained under control conditions, i.e. before CTX-1B application (Fig. 2B).

DISCUSSION

The present results indicate that CTX-1B, by activating voltage-dependent Na⁺ channels, increases the nodal membrane excitability of myelinated axons and produces a continuous Na⁺ entry into axons, through Na⁺ channels opened not only at the resting potential, but also during spontaneous action potentials. This leads to an increased intra-axonal Na⁺ concentration that disturbs the osmotic equilibrium between intra- and extra-axonal media, causing an influx of water. This influx of water, which is responsible for the long-lasting axonal swelling of nodes of Ranvier, is probably necessary to restore both the osmotic equilibrium and the internal Na⁺ concentration to its initial level.

The beneficial action of the therapeutic agents studied (i.e. lidocaine, CaCl₂, D-mannitol, sucrose, tetramethylammonium chloride, *Argusia argentea* leaves and *Davallia solida* rhizomes), in the treatment of ciguatera fish poisoning, may be explained in part by their ability to reverse the effects of CTX-1B in myelinated axons. They decrease the toxin-induced increased excitability of the nodal membrane to a level similar to that under control conditions (i.e. by suppressing only the spontaneous action potentials produced by CTX-1B but not those elicited by depolarising stimuli). In addition, some of the agents shrink nodes of Ranvier previously swollen by the toxin. This is of particular interest since it provides a rational basis for the use of the above mentioned therapeutic agents to treat the neurological symptoms of ciguatera fish poisoning.

Previous *in vitro* studies have reported the potency of hyperosmolar D-mannitol to reverse electrophysiological and morphological effects of ciguatoxins not only in myelinated axons but also in motor nerve terminals *in situ* and other excitable cells (see references in Mattei *et al.*, 1999) although this agent was shown to be ineffective, *in vivo*, to treat ciguatoxin-intoxicated rats (Purcell *et al.*, 1999). In contrast, the efficacy of traditional remedies has not been so widely studied despite the beneficial action of *Argusia argentea* extracts observed in mice intoxicated with ciguateric toxins (Amade and Laurent, 1992).

In conclusion, these studies not only may help to better understand the cellular and molecular factors involved in the toxic effects of ciguatoxins, but also provide an insight into the therapeutic basis of agents used for treating ciguatera fish poisoning symptoms.

Acknowledgements. □ This work was funded in part by a grant (981051 to J.M.) from the Direction des Systèmes des Forces et de la Prospective. C.M. was supported by a fellowship from the Direction des Recherches Etudes et Techniques.

REFERENCES

- ALLSOP J.L., MARTINI L., LEBRIS H., POLLARD J., WALSH J. & S. HODGKINSON, 1986. □ Neurologic manifestations of ciguatera. 3 cases with a neurophysiologic study and examination of one nerve biopsy. *Rev. Neurol. (Paris)*, 142: 590-597.
- AMADE P. & D. LAURENT, 1992. □ Screening of traditional remedies used in ciguatera fish poisoning treatment. *In: Recent Advances in Toxinology Research* (Gopalakrishnakone P. & C.K. Tan, eds), 2: 503-508.
- BENOIT E. & A.M. LEGRAND, 1994. □ Gambiertoxin-induced modifications of the membrane potential of myelinated nerve fibres. *Mem. Queensl. Mus.*, 34: 461-464.

- BENOIT E., JUZANS P., LEGRAND A.M. & J. MOLGÓ, 1996. □□ Nodal swelling produced by ciguatoxin-induced selective activation of sodium channels in myelinated nerve fibers. *Neuroscience*, 71: 1121-1131.
- BIDARD J.N., VIJVERBERG H.P.M., FRELIN C., CHUNGUE E., LEGRAND A.M., BAGNIS R. & M. LAZDUNSKI, 1984. □□ Ciguatoxin is a novel type of Na⁺ channel toxin. *J. Biol. Chem.*, 259: 8353-8357.
- BLYTHE D.G., FLEMING L.E., AYYAR D.R., DE SYLVA D., BADEN D. & K. SHRANK, 1994. □ □ Mannitol therapy for acute and chronic ciguatera fish poisoning. *Mem. Queensl. Mus.*, 34: 465-470.
- CAMERON J., FLOWERS A.E. & M.F. CAPRA, 1991. □□ Electrophysiological studies on ciguatera poisoning in man (Part II). *J. Neurol. Sci.*, 101: 93-97.
- DECHRAOUI M.Y., NAAR J., PAUILLAC S. & A.M. LEGRAND, 1999. □□ Ciguatoxins and brevetoxins, neurotoxic polyether compounds active on sodium channels. *Toxicon*, 37: 125-143.
- GLAZIOU P. & A.M. LEGRAND, 1994. □□ The epidemiology of ciguatera fish poisoning. *Toxicon*, 32: 863-873.
- HAMBLIN P.A., MCLACHLAN E.M. & R.J. LEWIS, 1995. □□ Sub-nanomolar concentrations of ciguatoxin-1 excite preganglionic terminals in guinea pig sympathetic ganglia. *Naunyn Schmiedeberg's Arch. Pharmacol.*, 352: 236-246.
- HOGG R.C., LEWIS R.J. & D.J. ADAMS, 1998. □□ Ciguatoxin (CTX-1) modulates single tetrodotoxin-sensitive sodium channels in rat parasympathetic neurones. *Neurosci. Lett.*, 252: 103-106.
- LAURENT D., BOURDY G., AMADE P., CABALION P. & D. BOURRET, 1993. □□ La gratte ou ciguatera. Ses remèdes traditionnels dans le Pacifique Sud. 150 □□, Paris: ORSTOM.
- LEWIS R.J., VERNOUX J.P. & M. BRERETON, 1998. □□ Structure of Caribbean ciguatoxin isolated from *Caranx latus*. *J. Am. Chem. Soc.*, 120: 5914-5920.
- LOMBET A., BIDARD J.N. & M. LAZDUNSKI, 1987. □□ Ciguatoxin and brevetoxins share a common receptor site on the neuronal voltage-dependent Na⁺ channel. *F.E.B.S. Lett.*, 219: 355-359.
- MATTEI C., BENOIT E., JUZANS P., LEGRAND A.M. & J. MOLGÓ, 1997. □□ Gambiertoxin (CTX-4B), purified from wild *Gambierdiscus toxicus* dinoflagellates, induces Na⁺-dependent swelling of single frog myelinated axons and motor nerve terminals *in situ*. *Neurosci. Lett.*, 234: 75-78.
- MATTEI C., MOLGÓ J., MARQUAIS M., VERNOUX J. & E. BENOIT, 1999. □□ Hyperosmolar D-mannitol reverses the increased membrane excitability and the nodal swelling caused by Caribbean ciguatoxin-1 in single frog myelinated axons. *Brain Res.*, 847: 50-58.
- MOLGÓ J., COMELLA J.X. & A.M. LEGRAND, 1990. □□ Ciguatoxin enhances quantal transmitter release from frog motor nerve terminals. *Br. J. Pharmacol.*, 99: 695-700.
- MOLGÓ J., JUZANS P. & A.M. LEGRAND, 1994. □□ Confocal laser scanning microscopy: a new tool for studying the effects of ciguatoxin (CTX-1b) and mannitol at motor nerve terminals of the neuromuscular junction *in situ*. *Mem. Queensl. Mus.*, 34: 577-585.
- MURATA M., LEGRAND A.M., ISHIBASHI Y., FUKUI M. & T. YASUMOTO, 1990. □□ Structures and configurations of ciguatoxin from the Moray eel *Gymnothorax javanicus* and its likely precursor from the dinoflagellate *Gambierdiscus toxicus*. *J. Am. Chem. Soc.*, 112: 4380-4386.
- PALAFIX N.A., JAIN L.G., PINANO A.Z., GULICK T.M., WILLIAMS R.K. & I.J. SCHATZ, 1988. □ □ Successful treatment of ciguatera fish poisoning with intravenous mannitol. *J.A.M.A.*, 259: 2740-2742.
- PEARN J.H., LEWIS R.J., RUFF T., TAIT M., QUINN J., MURTHA W., KING G., MALLETT A. & N.C. GILLESPIE, 1989. □□ Ciguatera and mannitol: Experience with a new treatment regimen. *Med. J. Aust.*, 151: 77-80.
- POLI M.A., LEWIS R.J., DICKEY R.W., MUSSER S.M., BUCKNER C.A. & L.G. CARPENTER, 1997. □□ Identification of Caribbean ciguatoxins as the cause of an outbreak of fish poisoning among U.S. soldiers in Haiti. *Toxicon*, 25: 733-741.
- PURCELL C.E., CAPRA M.F. & J. CAMERON, 1999. □□ Action of mannitol in ciguatoxin-intoxicated rats. *Toxicon*, 37: 67-76.
- RUSSELL F.E. & N.B. EGEN, 1991. □□ Ciguateric fishes, ciguatoxin (CTX) and ciguatera poisoning. *J. Toxicol. □□ Toxin. Rev.*, 10: 37-62.

SWIFT A.E.B. & T.R. SWIFT, 1993. *Ciguatera. J. Toxicol. Clin. Toxicol.*, 31: 1-29.

Reçu le 01.04.2000.

Accepté pour publication le 20.06.2000.