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Systemic toxic effects induced by the aqueous extract of the fire coral *Millepora complanata* and partial purification of thermostable neurotoxins with lethal effects in mice

Alejandro García-Arredondo [a], Alejandra Rojas-Molina [a,*], Moustapha Bah [a], César Ibarra-Alvarado [a], Marco Antonio Gallegos-Corona [b], Martín García-Servín [c]

[a] Laboratorio de Investigación Química y Farmacológica de Productos Naturales, Facultad de Química, Universidad Autónoma de Querétaro, Centro Universitario, Querétaro 76010, México
[b] Laboratorio de Patología Experimental, Facultad de Medicina, Universidad Autónoma de Querétaro, Querétaro 76176, México
[c] Instituto de Neurobiología, Universidad Nacional Autónoma de México, Campus Juriquilla, Querétaro 76201, México

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ABSTRACT

Millepora complanata is a cnidarian widely distributed in the coral reefs of the Mexican Caribbean. This species is popularly known as "fire coral", since contact with it causes severe pain, skin eruptions and blisters. Intravenous administration of of *M. complanata* aqueous extract induces violent convulsions and death in mice within 1 min (LD_{50} = 4.62 μg protein/g of body weight). Doses less than the LD_{50} produced histopathological damage in kidneys and lungs. Such histopathological damage was completely eliminated after incubation of the extract in heat denaturing conditions. Unexpectedly, the denatured extract conserved its lethal effect. These findings demonstrated that the extract contained hemolytic and phospholipase activities that might be responsible for the histopathological damage, and additionally it contained other unidentified thermostable toxins with lethal effects in mice. Chromatographic analysis of the extract led to the isolation of a 61 kDa vasoconstrictor protein. Furthermore, several non-peptidic vasoconstrictor fractions were separated. Particularly interesting was the fraction MC1-IIA obtained as a result of three-step chromatography processes (ion exchange, gel filtration and reverse phase). Like the original crude extract, this fraction induced vasoconstriction and delayed hemolysis and lethal effects in mice. A subsequent chromatographic analysis of MC1-IIA showed that this fraction contained at least four non-peptidic compounds. MS and NMR spectroscopic data analyses indicated that these metabolites were poly-oxygenated alkylbenzenes. The present study constitutes the first report of the presence of non-peptidic lethal toxins in an organism of the class Hydrozoa, and evidences the great structural diversity of the toxins produced by the *Millepora* species.

1. Introduction

Hydrocorals of the genus *Millepora* are commonly known as "fire corals" since they possess the ability to produce an intense burning sensation in human skin after contact (Sagi et al., 1987; Bianchini et al., 1988). Systemic symptoms caused in humans by contact with *Millepora* species are characterized by generalized malaise, nausea, vomiting and fever (Sagi et al., 1987; Moats, 1992; Prasad et al., 2006). It has also been reported that contact with these hydrocorals can result in renal disease (Prasad et al., 2006) and nerve dysfunction (Moats, 1992). The capacity of these cnidarians to induce their damaging effects is due to the release of not fully identified toxins from their cnidae (Wittle et al., 1974; Radwan, 2002; García-Arredondo et al., 2011; Rojas-Molina et al., 2012). Several toxinologic studies suggest that aqueous tissue extracts (referred to here as venoms) of *Millepora* species are mainly constituted by proteins. These studies have also demonstrated that, regardless of their geographical location, hydrocorals provoke similar toxic effects, which indicates that some relationship exists between their toxins. For instance, aqueous extract venoms of the Atlantic species (*Millepora alcicornis* and *Millepora tenera*) exhibit hemolytic and dermonecrotic properties, and when intravenously administered to mice, they induce forced respiration, convulsions and death within 30 s at lethal doses (Middlebrook et al., 1971; Wittle

* Corresponding author at: Laboratorio de Investigación Química y Farmacológica de Productos Naturales, Facultad de Química, Universidad Autónoma de Querétaro, Centro Universitario, S/N, Querétaro 76010, México. Tel.: +52 442 1921200x5527; fax: +52 442 1921302.
E-mail address: rojasa@uaq.mx (A. Rojas-Molina).

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et al., 1971; Wittle et al., 1974). Moreover, it was found that antisera from immunized rabbits, which received *M. alcicornis* and *M. tenera* venoms subcutaneously, were cross reactive (Wittle and Wheeler, 1974). In agreement with these results, Radwan (2002) observed immunological cross-reactivity between *Millepora platyphylla* and *Millepora dichotoma* venoms. Both venoms were lethal to mice and displayed hemolytic, vasopermeable, dermonecrotic and phospholipase A_2 (PLA_2) activities.

Our previous studies concerning *Millepora complanata* showed that the venom of this hydrocoral contains proteins that induce calcium-dependent contractions on the guinea pig ileum (Rojas et al., 2002) and the rat aorta (Ibarra-Alvarado et al., 2007). Vasoconstriction is caused primarily by 15 and 20 kDa proteins. In addition, the venom displays PLA_2 activity, and it contains a 30 kDa hemolysin (Ibarra-Alvarado et al., 2007), whose molecular weight is similar to that of Milleporin-1, a PLA_2 previously detected in *M. platyphylla* venom (Radwan and Aboul-Dahab, 2004).

The results of these earlier studies indicate that venoms of *Millepora* species contain toxins that induce lethality in mice, hemolysins with properties that include phospholipase activities that appear to dually exhibit or alternatively co-purify with hemolytic activity, as well as other proteins that elicit contractile effects on intestinal and vascular smooth muscles. Although the hemolytic and dermonecrotic activities of *Millepora* spp. crude venoms have been described, at present, the key tissues, which cause the systemic toxicity, are unknown.

In this paper, we present the first report of lethality, systemic toxicity and histopathological effects of the venom of *M. complanata* in mice. We also present evidence indicating that the aqueous extract venom of this hydrocoral contains lethal non-peptidic neurotoxins.

2. Material and methods

2.1. Specimen collection and crude extract preparation

Fragments of *M. complanata* were collected by SCUBA diving from coral reefs along the coasts of Puerto Morelos, Quintana Roo, México, in November 2008 at depths of 4–10 m. The fragments were immediately frozen in dry ice and transported to the laboratory where extraction was carried out.

Nematocyst discharge and aqueous extraction of coral tissue were accomplished by stirring the hydrocoral fragments in deionized water (pH 7) at 4 °C for 18 h. The extract obtained with this procedure was centrifuged at 3000 rpm (2060 ×*g*) for 15 min at 4 °C. This process was repeated twice, and the supernatant was lyophilized and stored at −70 °C. To determine the biological effects, the lyophilized product was dissolved in deionized water at a concentration of 150 mg/ml and centrifuged at 3000 rpm (2060 ×*g*) for 15 min at 4 °C. Then, the supernatant was filtered through a 0.45 μm pore filter (Millipore). This filtered solution was stored at −20 °C and was used to perform the bioassays. Protein content was measured using the Bradford method (Bradford, 1976).

2.2. Electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out as previously described (Laemmli, 1970; Schägger and Von-Jagow, 1987). Samples were diluted 1:1 in a sample buffer (Bio-Rad, Cat # 161-0737) containing β-mercaptoethanol, and heated at 95 °C for 5 min. Thereafter, samples (10 μg protein) were run in 12% polyacrylamide gels at 120 V for 2 h at 4 °C, using Tris–glycine as buffer (25 mM Tris, 192 mM glycine, pH 8.3; Bio-Rad, Cat # 161-0734). Protein bands were stained using Coomasie Blue. Molecular masses were determined by comparison with broad-range polypeptide standards (Bio-Rad, Cat # 161-0318).

2.3. Bioassays

2.3.1. Animals

For isolated rat aortic ring and hemolysis assays male Wistar rats, weighing 275 to 325 g, were used. For the lethality and dermonecrosis assays, male CD1 mice, weighing 30 g, were used. All animals were supplied by "Bioterio del Instituto de Neurobiología, Universidad Nacional Autónoma de México". The animals were maintained with free access to standard mouse food pellets and water *ad libitum*. Three animals per group were used, as stated in each experimental design. All experiments were performed in accordance with The Mexican Official Standard NOM-062-ZOO-1999 for the production, care and use of laboratory animals (Norma Oficial Mexicana, 2014) and the ethical standards for animal experiments in toxinological research recommended by the International Society of Toxinology (Meier et al., 1993).

2.3.2. Isolated rat aortic ring assay

Rats were sacrificed by decapitation. The descending thoracic aorta was removed and placed in an ice-cold, oxygenated Krebs–Henseleit solution (126.8 mM NaCl, 5.9 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 30 mM $NaHCO_3$ and 5 mM D-glucose; pH 7.4) and immediately flushed with the Krebs–Henseleit solution to prevent intravascular clot formation. The endothelium intact aorta was dissected free of connective tissue and cut into rings at 4 to 5 mm intervals. The aortic rings were mounted between stainless steel hooks and suspended in water-jacked, 7 ml organ baths containing the oxygenated (95% O_2 and 5% CO_2) Krebs–Henseleit solution at 37 °C. The tissues were allowed to equilibrate for 60 min under a resting tension of 1.5 g. During this period, the bathing medium was renewed every 15 min. After a final adjustment of the passive resting tension to 1.5 g, aortic segments were contracted with 100 mM KCl. Once a stable contractile tone was reached, the bathing medium was renewed to restore a resting tension of 1.5 g. After that, the tissues were contracted with 1 μM L-phenylephrine, the force of contraction was recorded and this contraction was set as 100%. The bathing medium was changed again to restore the original resting tension, and then the test samples (extract and chromatographic fractions) were added to the organ bath. The endothelial integrity of each vascular preparation was routinely checked in one representative aortic segment by reaching a ≥60% relaxation in response to the addition of 1 μM acetylcholine. The isometric tension was measured by a Grass FT03 Force-displacement transducer connected to a Grass 7D polygraph. The responses were expressed as a percentage of the initial contraction achieved with phenylephrine.

2.3.3. Hemolysis assay in rat erythrocytes

The hemolytic activity was monitored as described for the jellyfish venom *Cassiopea xamachana* (Torres et al., 2001). Briefly, samples for the assay contained a mixture (1 ml) of Alsever solution (120 mM D-glucose, 30 mM sodium citrate, 7 mM NaCl and 2 mM citric acid; pH 7.4) with 50 μl of a 1% suspension of rat erythrocytes and the required volume of the extract. These samples were incubated at 37 °C for 30 min. After centrifugation at 2500 rpm (1430 ×*g*) for 4 min at 4 °C, the A_{415} of the supernatant fluid containing the hemoglobin released from lysed erythrocytes was measured in a spectrophotometer (Lambda Bio, Perkin Elmer Co.). Each experiment was normalized with respect to complete hemolysis which was measured by diluting the erythrocyte sample in deionized water instead of the Alsever buffer. One hemolytic unit (HU_{50}) was defined as the amount of protein sample required to cause 50% hemolysis in a 1% erythrocyte solution at 37 °C for 30 min.

In order to evaluate if the hemolytic activity was related to some substances similar to palytoxin (PTX), the hemolytic assay was performed using a long term incubation time of 4 h at 37 °C, instead of the 30 min standard incubation time. In another set of experiments, 0.1 mM ouabain, a specific inhibitor of PTX, was added to the red cell