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Characterization and neutralization of *Nemopilema nomurai* (Scyphozoa: Rhizostomeae) jellyfish venom using polyclonal antibody

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ABSTRACT

Jellyfish stings have often caused serious health concerns for sea bathers especially in tropical waters. In the coastal areas of Korea, China and Japan, the blooming and stinging accidents of poisonous jellyfish species have recently increased, including *Nemopilema nomurai*. We have generated a polyclonal antibody against *N. nomurai* jellyfish venom (NnV) by the immunization of white rabbits with NnV antigen. In the present study, the antibody has been characterized for its neutralizing effect against NnV. At first, the presence of NnV polyclonal antibody has been confirmed from the immunized rabbit serum by Enzyme linked immunosorbent assay (ELISA). Then, the neutralizing activities of the polyclonal antibody have been investigated using cell-based toxicity test, hemolysis assay, and mice lethality test. When the polyclonal antibody was preincubated with NnV, it shows a high effectiveness in neutralizing the NnV toxicities in a concentration-dependent manner. Moreover, we explored proteomic analyses using 2-D SDS-PAGE and MALDI-TOF mass spectrometry to illustrate the molecular identities of the jellyfish venom. From this, 18 different protein families have been identified as jellyfish venom-derived proteins; the main findings of which are matrix metalloproteinase-14, astacin-like metalloprotease toxin 3 precursor. It is expected that the present results would have contributed to our understandings of the envenomation by *N. nomurai*, their treatment and some valuable knowledge on the pathological processes of the jellyfish stinging.

1. Introduction

It is approximately estimated that in excess of 10,000 jellyfish stingings occur in each year and up to now more than 63 victims had met sudden and painful deaths in Australian waters (Tibballs, 2006). In contrast with the fatal stingings of chirodropid jellyfish species, such as *Chironex fleckeri* and *Carukia barnesi*, there have been relatively fewer reported human casualties caused by non-chirodropid jellyfish (Fenner and Williamson, 1996). Up to now, however, at least 14 deaths have been reported in Asian Pacific waters even by non-chirodropid jellyfish species, including *Nemopilema nomurai* (Fenner and Williamson, 1996).

Nemopilema nomurai is one of the largest jellyfish species, with a maximum bell size up to 2 m and weights up to 200 kg (Kawahara et al., 2006a). Recently, unusual large blooms of *N. nomurai* have occurred for the recent several years in East China Sea, Yellow sea, and East Sea (Uye, 2008)

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and the number of patients stung by the jellyfish has increased correspondingly. In the summer of 2012, an 8-yr old girl has died from the jellyfish stinging in a west coast beach of Korean peninsula, which is presumed to be caused by *N. nomurai* (unpublished). The symptoms caused by this jellyfish stings are usually very painful with a strong burning sensation, followed by an erythematous eruption with small vesicles (Kawahara et al., 2006b). Furthermore, it has been previously reported that NnV contains a variety of bioactive proteins which are cytotoxic, hemolytic and/or cardiotoxic (Kang et al., 2009) (Kim et al., 2006). Currently, there is no specific antidote therapy which is available for jellyfish envenomed patients except antivenom treatment. Therefore, the recognition of life-threatening effects of jellyfish venom demonstrates the need for broadening the specificity of the existing product and/or developing additional specific jellyfish antivenom.

Up to now, CSL box jellyfish antivenom is the only accessible therapeutic agent which is manufactured worldwide for the treatment of box jellyfish envenomations. This ovine IgG antivenom has been used since 1970 with questionable success. Several studies reported CSL box jellyfish antivenom neutralized the toxic effects of the venom both *in vitro* and *in vivo* (Baxter and Marr, 1975) (Tibballs et al., 1998). While Endean and Sizemore (Endean and Sizemore, 1988) observed the antivenom was less effective in *in vivo* studies. Thus, the role of antivenom in the treatment of jellyfish stings remains controversial and their treatment effectiveness largely depends on the antivenom potency (Currie, 2003). However, antivenom is still widely used because there is no other vaccine or other effective agent which is generally available against animal venoms (WHO, 1969). Therefore, the production and characterization of polyclonal antibodies and their ability to neutralize the toxicological effects and to map the structural aspects of NnV are the first step in furthering our understanding of jellyfish toxin's mode of action which in turn should permit the progress of more effective remedies against *N. nomurai* jellyfish stingings.

In the present study, the rabbit antiserum raised, in our laboratory, against NnV was investigated for its ability to neutralize the cytotoxicity, hemolytic activity and mice lethality of this venom. Moreover, we also employed proteomics, and established a preliminary protein profile for NnV using 2-D electrophoresis and immunoblot combined with MALDI-TOF analysis.

2. Materials and methods

2.1. Chemicals and reagents

Alsever's solution, phosphate buffered saline (PBS), and Freund's adjuvant were purchased from Sigma–Aldrich Inc. (St. Louis, MO, USA). All other reagents used were of the purest grade available.

2.2. Jellyfish collection and preparation

Mature specimens of *N. nomurai* jellyfish were captured from Korea Strait along the coasts of Tongyoung in September, 2007. The tentacles dissected from the jellyfish were stored in ice and transferred immediately to our laboratory for further preparation. Nematocysts were isolated from the dissected tentacles as described by (Bloom et al., 1998) with a slight modification. In brief, tentacles were gently swirled with the addition of distilled water, then stood still for 1–2 h to remove debris and sea water. After decanting the supernatant, tentacles settled down at the bottom were mixed with 2 × (v/v) distilled water and shaken vigorously for 3 min. The detached nematocysts were separated by filtering tentacle preparation through 4 layers of medical gauze. This was repeated for two more times with additional distilled water to harvest nematocysts from the tentacles. The filtrates were centrifuged (700 × *g*) at 4 °C for 20 min and the pellets (nematocysts) were lyophilized and stored −20 °C until use.

2.3. Venom extraction and preparation

Venom was extracted from the freeze-dried nematocysts using the technique described by (Carrette and Seymour, 2004) with a minor modification. In brief, venom was extracted from 50 mg of nematocyst using glass beads (approximately 8000 beads; 0.5 mm in diameter) and 1 ml of ice-cold (4 °C) phosphate buffered saline (PBS, pH 7.4). These samples were shaken in a mini bead mill at 3000 rpm for 30-s intervals for five times with intermittent cooling on ice. The venom extracts were then transferred to a new Eppendorf tube and centrifuged (22,000 × *g*) at 4 °C for 30 min. This supernatant was used as *N. nomurai* jellyfish venom (NnV) for the present study. Protein concentration of the venom was determined by Bradford method (Bradford, 1976) (Bio-Rad, C.A. USA) and the venom was used based on its protein concentration.

2.4. Production of polyclonal antibodies to NnV

Polyclonal antibody against NnV (antigen) was obtained by immunizing female New Zealand white rabbits (2–2.5 kg of body weight) with NnV as previously described elsewhere. Briefly, the rabbits were initially immunized with two intramuscular injections of venom (200 μg) dissolved in ice-cold (4 °C) PBS and complete Freund's adjuvant in a ratio of 1:1. Immunization was repeated with three fortnightly using the same amounts of antigen in incomplete Freund's adjuvant. The blood samples were taken from marginal ear veins of the rabbits at the time points of both before immunization (Before) and after final immunization (After), then stored at 4 °C overnight. Following centrifugation (10,000 × *g*) at 4 °C for 10 min, the serum was obtained by decanting the supernatant and the polyclonal antibody level in the serum was determined by ELISA method. The obtained antibody-containing serum was stored in aliquots at −20 °C for later use.

2.5. Enzyme-linked immunosorbent assay (ELISA)

The immunoreactivity of in-house generated polyclonal antibody to NnV antigen was assessed by using indirect ELISA method. Briefly, various concentrations of NnV (3 mg/ml ~ 1 ng/ml) were dissolved and diluted in 0.05 M carbonate bicarbonate buffer, and then the wells of the