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Guam Seaweed Poisoning: Hemolysis Neutralization Assay for Marine Toxins

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Abstract—The hemolytic action of palytoxin was exploited to develop a simple and sensitive assay with specificity based on a palytoxin neutralizing monoclonal antibody. A dilute suspension (0.2% v/v) of washed mouse erythrocytes in phosphate buffered saline was incubated with or without purified palytoxin at 37 °C in round bottom microtiter trays. Control wells produced a visible, discrete red cell pellet by 2 h; in the presence of palytoxin, the visible cell pellet was lysed. At 10 pg/mL palytoxin, the pellet is lysed by 4 h, and at the detection limit of activity, 1 pg/mL, lysis required 24 h. Hemolysis was inhibited by including 25 μg/mL of a neutralizing anti-palytoxin monoclonal antibody in the assay medium, thereby providing specificity for palytoxin. The assay selectively detected palytoxin in crude aqueous ethanolic extracts of *Palythoa tuberculosa*. Hemolytic activity associated with a crude extract of the seaweed *Gracilaria tsudai* was not inhibited by anti-palytoxin monoclonal antibody. The assay provides a simple, rapid, inexpensive and sensitive screening method for samples containing primarily palytoxin as the hemolytic agent.

It is an honor to have been invited to present evidence to this conference which suggests that the Guam *Gracilaria*-associated seaweed toxin is not palytoxin. Palytoxin is one of the toxins occasionally associated with ciguatera-like seafood poisoning incidents (Fukui & Murata et al. 1987, Fukui & Yasumura et al. 1987, Kodama et al. 1989, Noguchi et al. 1987, Yasumoto et al. 1986). When it was suspected that palytoxin might be the cause of the seaweed poisoning incident here on Guam, Hawaii Biotech was requested to test Guam seaweed extract samples with an enzyme immunoassay which we developed (Bignami et al., 1992). The results of these tests were inconclusive but suggested that a palytoxin-like molecule might be present. In an effort to further test these samples with a more sensitive test, we developed a palytoxin-specific hemolysis neutralization assay.

Palytoxin is a very unusual molecule. It is a potent non-protein ordinarily isolated from tropical *Palythoa* soft corals. Palytoxin has also been identified in certain fish and crab species in the human diet. Although human intoxication by palytoxin appears to be rare, the symptoms are severe and have occasionally resulted in death.

Detection of palytoxin in food samples is a laborious process requiring extraction and high performance liquid chromatography (HPLC) analysis. More rapid alternative methods for detecting palytoxin have been reported including a radioimmunoassay (Levine et al. 1981) and the enzyme immunoassays to which I referred.

A German group described the *in vitro* hemolytic activity of palytoxin against the erythrocytes of various mammalian species over ten years ago (Habermann, et al. 1981; Ahnert-Hilger et al. 1982). This effect was species, time and temperature dependant and was enhanced by the presence of sodium, calcium and borate ions in the test medium. Under optimal conditions hemolysis was detected at two hours and nearly complete by eight hours. We have developed a palytoxin-specific neutralizing monoclonal antibody which will competitively inhibit hemolysis *in vitro* (Hewetson et al. 1989). This monoclonal antibody, which we have named 73D3, is thought to react only with palytoxin and closely related compounds. Palytoxin induced hemolysis and the inhibition of this hemolysis is readily detectable with the unaided eye using a microtiter tray assay format which I will describe here. This method provides an inexpensive non-radioactive rapid screen for detecting palytoxin in picomolar concentrations. It can also detect other hemolytic toxins, including the one found in the toxic seaweed from Guam, though in this case not as a specific assay.

I would now like to describe this assay method and illustrate how truly simple the test is. Mouse blood is diluted to 0.2% (v/v) in phosphate buffered saline containing calcium chloride and sodium metaborate, and 50 μL aliquots are added to the wells of sterile round-bottom 96-well microtiter trays. The test samples, also diluted in PBS/calcium/borate, are added in 50 μL aliquots to the wells containing red cell suspensions. The plates are then incubated at 37 °C for up to 24 h. To illustrate this procedure, purified palytoxin and a *Palythoa tuberculosa* extract were assayed. Fig. 1 is a photo of the microtiter plate used in this assay after incubation for 4 h. On the right hand side of this plate (columns 9–12) there are increasing concentrations of palytoxin with a uniform number of red blood cells into each well. In wells that did not receive hemolytic amounts of palytoxin a discrete red cell pellet forms in about 2 hours when the cells settle out by gravity. As the dose of palytoxin is increased, one sees a destruction of this red cell pellet through hemolysis. In the presence of a palytoxin specific antibody, however, there is approximately a 500-fold concentration range in which the hemolysis does not occur (columns 9 & 10). This is the index that shows that the material being tested contains palytoxin.

To determine whether this procedure could also be performed with a natural extract rather than purified toxin, we treated a sample of *P. tuberculosa* with 70% (v/v) aqueous ethanol and tested the extract (Fig. 1, columns 1–4) as described. What is seen is a pattern similar to that achieved with the purified toxin, in which there is a zone of hemolysis which is protected in the presence of the neutralizing antibody. We also tested an aqueous fraction of the *Gracilaria tsudai* seaweed extract (columns 5–8) and, in this particular example, we see a marginal level of hemolysis at a 1 in 1000 dilution of the extract. Although it may not show clearly

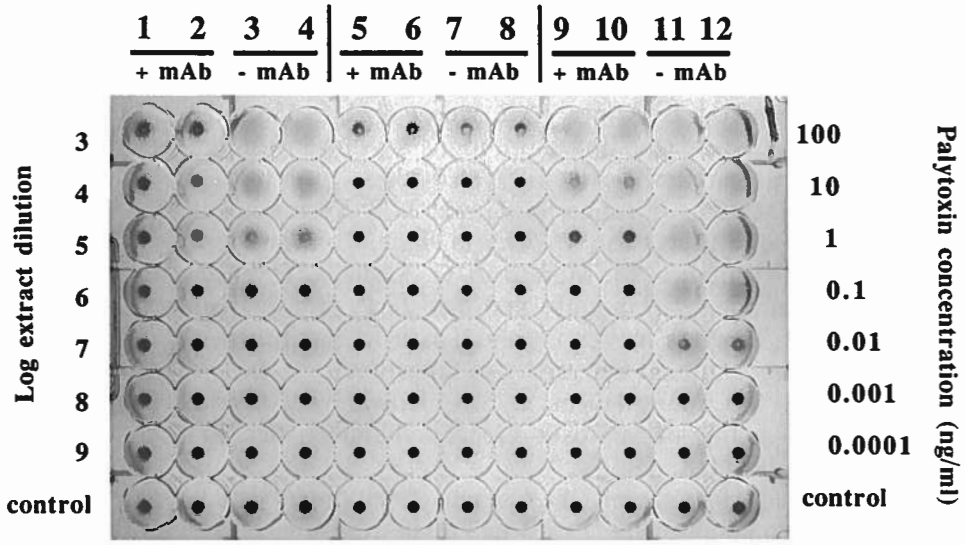

Figure 1. Microtiter plate hemolysis neutralization assay. The hemolytic effect of increasing dilutions (rows 3–9) of *Palythoa tuberculosa* extract (columns 1–4) or *Gracilaria tsudai* extract (columns 5–8) was compared to purified palytoxin (columns 9–12). Each extract and purified palytoxin dilution was tested in duplicate with (columns 1, 2, 5, 6, 9, 10) or without (columns 3, 4, 7, 8, 11, 12) 25 μg/mL of 73D3 monoclonal antibody. Control cultures contained PBS/calcium/borate with or without monoclonal antibody. Hemolytic activity is apparent in wells lacking a distinct cell pellet.

in this figure, no protection was provided by the anti-palytoxin antibody. When the aqueous extract of *G. tsudai* was tested in a more concentrated range, there was clear evidence of hemolysis without any palytoxin-type neutralization occurring when treated with neutralizing antibody (data not shown). We also tested an organic fraction of the authentic *G. tsudai* extract which apparently did not possess hemolytic activity. This is in contrast to an assay demonstration set up at the University of Guam Marine Laboratory yesterday, which exhibited hemolytic activity in certain organic fractions of samples retained from the poisoning incident. It is possible that the organic phase samples which were shipped to Hawaii had degraded before they could be tested. Even though this assay is not specific for *G. tsudai* toxins, it is a very sensitive, rapid and straightforward method which can be used to screen seaweed samples for the presence of hemolytic material. A positive assay result therefore suggests the presence of a hemolytic toxin, even though its precise identity is not known.

Note: A complete description of this assay methodology is included in a short communication accepted for publication in *Toxicon.*

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