Cell bioassay—a trial to develop new assay system for paralytic shellfish poisoning toxins (PSPs) and ciguatoxins, and comparison with other newly developed assays for PSPs.

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Monitoring programs for marine toxins in seafood were limited in large part to mouse bioassays in many countries. However, these assays were criticized from the viewpoints of animal welfare, cost, sensitivity, and efforts required for the assays. It is now clear that replacement of the assay would be desirable. Efforts are being made to develop new methods to replace or to complement mouse bioassays. Cell bioassay, one of those alternative assays, is reviewed here comparing with other methods.

The sodium ion channel

Paralytic shellfish poisons (PSP), as well as tetrodotoxin (TTX), are known to block voltage-sensitive sodium channels on the membrane of nerve and muscle cells and thus interferes with sodium ion influx to these cells. To the contrary, ciguatoxins and brevetoxins are sodium channel activators. The amino-acid sequence of sodium ion channels has been determined for human, rats, electric eels, and others. Mammalian sodium channels consist of large α (M. W. 260 K) and smaller β (M. W. 37-45 K) subunits. The α subunit has four repeats, each of which consists of six transmembrane segments. These four repeats are thought to form the pore of the channel protein. The site of block by TTX and saxitoxin (STX) of sodium channel was discovered to be located close to the mouth of pore of the sodium channel.

Cell bioassay

The principle of the cell bioassay for marine toxins is based on the effects of toxins on sodium ion channels. The original method was first developed by Kogure et al. for the detection of sodium channel blocking toxins such as PSPs. The antagonic activity of these toxins against the combined effects of veratridine, a sodium channel activator, and ouabain, a sodium-potassium pump blocker, was evaluated using a mouse neuroblastoma cell line, Neuro-2a. The existence of sodium channel blocking toxins prevents cell burst which was to be caused by an excess influx of sodium ion. In Kogure’s method, the results were scored by microscopically observed morphology of the cells. Then the method was further improved by introducing
colorimetric techniques by Jellet\textsuperscript{14} and a FDA group\textsuperscript{15}. Manger et al.\textsuperscript{15,16} extended the application of the cell assay to enhancers of sodium channel conductance such as ciguatoxins and brevetoxins, which in turn accelerate cell burst introduced by the treatment with ouabain and veratridine. They reported the detection limit for saxitoxin as 20 pg (equivalent to 0.4 µg/100 g tissue), ID\textsubscript{50} values as less than 0.25, 1 pg, and 4 ng for ciguatoxins CTX-1, CTX-2, and brevetoxin PbTx-1, respectively\textsuperscript{16}. The cell assay responds in a manner which indicates whether an unknown toxin belongs among either the “sodium channel blockers” or “sodium channel enhancers”.

The procedure of the cell assay is summarized. Mouse neuroblastoma cells, Neuro-2a (ATCC CCL-131), are grown in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 1 mM sodium pyruvate, 50 µg/ml streptomycin, and 50 units/ml penicillin. Cells are cultured at 37°C in CO\textsubscript{2} atmosphere (5%). For the assay, 96-well multiplates are seeded with cells at the concentration of 1×10\textsuperscript{5} cells per well in 200 µl of the same medium as that mentioned above except serum concentration (5%), and the cells are incubated overnight. The wells then receive 10 µl each of 10 mM ouabain and 1 mM veratridine, with or without 10 µl of sample. Cells are incubated at 37°C for 6 to 24 hours depending on the type of toxins to be assayed. Then the medium is removed by flicking the plates, and the wells receive 60 µl of 1 : 6

![](image)

**Fig. 1** An example of standard curve for brevetoxin, PbTx-1. Neuro-2a cells were seeded in the wells of microplates at the concentration of 1×10\textsuperscript{5} cells per well in 200 µl of the medium. The wells then received 10 µl each of 10 mM ouabain, 1 mM veratridine, and PbTx-1. The cells were further incubated for 18 hr at 37°C, and were assayed by MTT method. The results were converted to % control of ouabain/veratridine.
diluted 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT) stock solution (5 mg/ml PBS) in complete medium without serum. Plates are further incubated for about 20 min until sufficient MTT development occurs. The medium is removed again and replaced with 100 μl DMSO, and the plates are read at 570 nm with reference at 630 nm. The results of sample are calculated as % of ouabain/veratridine control.

Seafood Products Research Center of FDA has produced a draft standard operating protocol to facilitate standardization of the methodology and transfer of the procedure to other testing laboratories. To prepare the interlaboratory collaborative research, we have received a cell line from FDA and started preliminary assays for brevetoxin, PbTx-1. As is shown in Fig. 1, dose dependent acceleration of cell death against the toxin was observed. Since, as the cell passage number increases, the cells gradually become less sensitive to PbTx-1, ouabain and veratridine, each assay takes into account the toxin standard's performance. For this reason, standardization is crucial to ensure consistent assay results and will be an important factor in assuring acceptable inter-laboratory precision. This need to restandardize and the equipment and time requirements for cell culture are considered to be disadvantages of the cell bioassay. However, analysis times required per sample are comparable to mouse bioassay because far more samples can be treated in parallel by cell assay.

**Other assays for marine toxins**

There are also other attempts to develop new methods for detecting marine toxins. Advances in analytical methodologies have been reported. LC/MS was shown as a universal method for marine toxins. Capillary electrophoresis has been applied to PSP toxins, DSP toxins, and maitotoxin. Instrumental methods give accurate results and are quite useful to determine contaminating toxins. The primary disadvantage of HPLC and any separation-based methods is the need for multiple toxin standards. Most marine toxins are not available as standards, and those that can be purchased are only available at high cost. Legal restriction on the use of saxitoxin in Japan made the situation much worse. These effectively prevent use of any separation based methods in routine monitoring for marine toxins. In addition, the equipment are still expensive, complicated to maintain, and limited number of samples are able to be treated per day.

As immunological methods, enzyme immunoassay (EIA) against STX group on microtiter plates, membrane-based enzyme-linked immunofiltration assay, and ion sensitive field effect transistor (ISFET)-ELISA are reported. Detection limits for STX or neosaxitoxin (NEO) by these tests are reported in the low ng/ml or even pg/ml range, but detailed studies on the applicability to naturally contaminated materials and on the comparison with the mouse bioassay or HPLC have still been limited. Antibody specificity is the most decisive factor determining the suitability of immunochemical tests for PSP toxins. Quantitative agreement between EIA and mouse bioassay is influenced also by relative toxicities of PSP toxins in the bioassay.

Receptor assays for each of the major toxin classes have been developed over the past 5 years. A comparison study of the competitive binding assay with mouse bioassay and HPLC...
analysis for determination of PSP toxins in shellfish and algal samples was published in 1997. A similar receptor assay has been utilized with the mouse bioassay for red tide management in Chile since 1995. This type of assay has the same advantages as cell bioassay in the meaning that it detects binding to cell receptors on the living membrane. However, it does not transmit the effect of that binding on a functional membrane’s ion transport behavior.

Summary

Advantages and disadvantages of above mentioned methods are summarized in Table 1. Since the supply of purified standard toxins are still very limited, there is a strong need for seafood toxin detection methods that do not require all corresponding toxin standards and yet provide enough information that is useful for monitoring purposes. From this aspect, and also from the point that it uses an intact living membrane system which responds not just to the initial binding but to the toxin effect on membrane mediated ion transport, cell bioassay seems to be one of the most promising methods at present among various trials to replace mouse bioassay.

Table 1 Comparison of methods for PSP detection

<table>
<thead>
<tr>
<th>method</th>
<th>advantages</th>
<th>disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>mouse bioassay</td>
<td>react to all PSPs, easy extraction</td>
<td>ethical problem, less sensitive</td>
</tr>
<tr>
<td>analytical method</td>
<td>accurate determination</td>
<td>expensive instruments, limited standard supply,</td>
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<tr>
<td>HPLC, NMR, Mass spectral analysis,</td>
<td></td>
<td>complicated clean-up</td>
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<tr>
<td>cell bioassay</td>
<td>correlate to in vivo neurotoxicity</td>
<td>time consuming, cell maintenance</td>
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<tr>
<td>binding assay</td>
<td>sensitive, rapid</td>
<td>use of RI, additional clean-up required</td>
</tr>
<tr>
<td>immunoassay</td>
<td>sensitive, rapid, easy (kit)</td>
<td>limited standard supply, low correlation with in vivo toxicity</td>
</tr>
</tbody>
</table>

References

21) Quilliam, M. A.: JAOAC Int. 81, 142–151 (1998)