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U.S. DEPARTMENT OF DEFENSE

SMALL BUSINESS INNOVATION RESEARCH PROGRAM
PHASE 1 — FY 1989
PROJECT SUMMARY

Topic No. A89-082 Military Department/Agency Army

Name and Address of Proposing Small Business Firm

Standard Scientifics, Inc.

925 Webster St.
Needham, MA 02192

Name and Title of Principal Investigator

Chia-Ling Hu, President

Proposal Title

A Novel Immunoassay for Palytoxin

Technical Abstract (Limit your abstract to 200 words with no classified or proprietary information/data.)

Phase I was highly successful in meeting its goals. The purpose of this research effort is the development of a novel, non-isotopic immunoassay for the detection of palytoxin. This assay must be sufficiently sensitive, specific and robust to meet the unique demands of field use. Two solid-phase immunoassay approaches were planned for evaluation using: (1) an immobilized antigen surface and (2) an immobilized antibody surface. During Phase I, the immobilized antigen approach was very successful. It attained RIA sensitivity (0.05 pmol) with a one hour assay time. Preliminary results also indicated that the use of unextracted serum did not interfere with the analysis. For feasibility studies on the antibody-coated surface approach, the FLIA has produced comparable sensitivity, but longer assay time at present. Studies using purified antibody are underway and are expected to improve significantly on the preliminary results obtained during the first monthly reporting period. During Phase II, we plan to improve the assay for palytoxin with specific emphasis on developing it as a field test by: developing a qualitative dipstick assay, optimizing the quantitative assay, and applying the FLIA to the palytoxin to maximize the robustness of the method.

Anticipated Benefits/Potential Commercial Applications of the Research or Development

Once fully developed, this immunoassay for palytoxin can be used by the Department of Defense to detect exposure to palytoxin in the field. It may also have commercial value in countries where palytoxin can contaminate food sources.

List a maximum of 8 Key Words that describe the Project.

Palytoxin, Immunoassay, Non-radioactive, Non-enzymatic

Contents

PHASE I FINAL REPORT

Summary:

Phase I was highly successful in meeting its goals. The purpose of this research effort is the development of a novel, non-isotopic immunoassay for the detection of palytoxin. This assay must be sufficiently sensitive, specific and robust to meet the unique demands of field use: e.g., fast, accurate results are required with limited access to controlled laboratory facilities. Two solid-phase immunoassay approaches were planned for evaluation using: (1) an immobilized antigen surface and (2) an immobilized antibody surface. During Phase I, the immobilized antigen approach was very successful. It attained RIA sensitivity (0.05 pmol) with a one hour assay time. Preliminary results also indicated that the use of unextracted serum did not interfere with the analysis.

For feasibility studies on the antibody-coated surface EIA, the antibody was extensively affinity purified to remove non-IgG and anti-BSA antibodies. Studies using purified antibody are underway and are expected to improve significantly on the preliminary results obtained during the first monthly reporting period.

During Phase II, we plan to improve the assay for palytoxin with specific emphasis on developing it as a field test by: developing a qualitative dipstick assay, optimizing the quantitative assay, and applying the FLIA™, the novel method of Standard Scientifics, to the palytoxin to maximize the robustness of the method. A summary table showing the current status of the assay and plans for Phase II and Phase III is presented in Table I.

Identification and Significance of the Problem:

Palytoxin (PTX), the most toxic non-proteinaceous animal toxin known to man (Habermann, 1989), has been isolated from zoanthid corals of the genus Palythoa. It has not been determined if palytoxin is produced by the coral itself, or by a biological entity associated with the coral.

Palytoxin is toxic if administered intravenously (LD_{50}= 0.2 µg/kg in mouse; Moore and Scheuer, 1971) or by intraperitoneal injection (mouse; Habermann, 1989). It is less toxic orally (Wiles *et al*, 1974). The degree of inactivation is dependent on the length of time palytoxin is exposed to the acidic contents of the stomach (Wiles *et al*, 1974; Levine *et al*, 1988).

Table I. Palytoxin Detection, System Development--An Assessment of the Current (Phase I) Accomplishments and Future (Phase II & III) Objectives.

Key Features	Stages of Development Phase I	Phase II	Phase III
Sensitivity	0.05 pmol	0.01 pmol	0.01 pmol
Specificity*			
Palytoxin	0.05 pmol	0.01 pmol	0.01 pmol
Maitotoxin	2.9 nmol	>20.0 nmol	>20.0 nmol
Okadaic Acid	10.0 nmol	>20.0 nmol	>20.0 nmol
Operator Skill	B.S., trained	untrained	untrained
Reagent Stability	2 mo., 4 °C		
Unopened kit	--	6 mo., ambient	1 yr., ambient
Opened kit	--	3 wk., ambient	2 mo., ambient
Temperature for Assay	lab. room temp.	ambient (4-40 °C)	ambient (4-40 °C)
Instrument Requirement			
Qualitative	--	non-instrumented	non-instrumented
Quantitative	microtiter plate reader	microtiter plate reader	digital biosensor

* minimum detectable

Palytoxin causes depolarization and contraction of cardiac, smooth and striated muscles. It also depolarizes nervous tissues and causes changes in sodium and potassium ion fluxes (Habermann, 1989). Although palytoxin has direct effects on a variety of tissues, palytoxin toxicity has not clearly been attributed to any single effect. What is known is that palytoxin binds to the surface and produces depolarization in all cell types studied by binding to the Na^+, K^+-ATPase (Habermann, 1989). Its lethal effects are likely caused by acute heart failure produced by direct myocardial depression and severe myocardial ischemia caused by profound coronary vasoconstriction (Ito *et al*, 1982; Vick and Wiles, 1975). Sublethal doses can exhibit a variety of problems such as a delayed, unusually strong hemolytic effect (Habermann *et al*, 1981), skin irritation (Wiles *et al*, 1974; Vick and Wiles, 1975) and non-TPA type tumor promoter activity (Kano et al., 1987; Fujiki et al., 1986). Some of these effects may be reversible if the therapy were immediately available.

A rapid, sensitive, specific and robust assay for palytoxin that could be easily used in the field to detect exposure would be highly desirable. It would enable: 1) the avoidance of exposure to additional personnel, and 2) the possible treatment of any sublethal toxin exposure related medical conditions. The quantitative palytoxin assay could then be used to assess the levels in biological fluids or water sources.

Background:

The only published report of an immunoassay for palytoxin is the radioimmunoassay (RIA) developed by Levine *et al*, 1988. The RIA could detect 0.04 pmoles of palytoxin, very good sensitivity. The specificity of the polyclonal rabbit antiserum was also good as shown in Table II.

Table II. Specificity of I-125 Palytoxin-anti palytoxin Immune System (Levine *et al*,1988).

Inhibitor	IC 50*
Palytoxin (P. turberculosa)	0.27
Palytoxin (P. caribaeorum)	0.30
Maitotoxin	a (2.9 nmoles)
Teleocidin	b (22.8 nmoles)
Okadaic Acid	c (10.0 nmoles)
Debromoaplysiatoxin	d (14.7 nmoles)

*pmoles required for 50% inhibition of binding
a-d = 0 inhibition with quantities in parentheses

One of the disadvantages of this method was that the double antibody separation method required an overnight incubation at 4 °C., making the total assay time very long (20-24 hours). The RIA also required incubations at two temperatures 35 °C and 4 °C. Another disadvantage of the RIA is the ^{125}I-labelled palytoxin tracer with its: 1) high energy level and short radioactive half life of 60 days, and 2) need for a gamma counter to detect the assay signal.

Rationale:

For field use, a different assay method is required which will provide a rapid, sensitive, specific and robust assay with limited access to controlled laboratory conditions. An immunoassay as a general method has many

advantages over other analytical methods for this application and these advantages have been commonly applied to the analysis of low level hormones in the home-test-kit market.

During Phase I, an enzyme immunoassay of immobilized antigen version for palytoxin has been generated which already has demonstrated excellent sensitivity and an assay time of approximately one hour. During Phase II, a dipstick method will be developed that will serve the need for a non-instrumented screening method to be used in the field. The quantitative EIA will be further developed and optimized to provide a quantitative assay. For both versions increasing reagent stability and assay simplicity will be emphasized during Phase II to provide a very high probability of meeting the goals of this project.

Standard Scientifics, Inc. has developed a proprietary technology, the ferritin-linked immunoassay (FLIA™), which is capable of RIA sensitivity using a non-enzymatic, non-isotopic label. The ferritin can be read colorimetrically by a color change from red to blue with increasing amount of ferritin label bound to the antibody. Unbound ferritin is washed away prior to the liberation of the iron from the ferritin for detection. Ferritin is a very stable molecule and is well-suited to the analysis of ultra-low analytes using a non-instrumented or colorimetric detection. During Phase I, the current FLIA™ method has demonstrated sufficient sensitivity to analyze palytoxin. The major problem is the time to generate the signal. This must be reduced to 10 minutes or less for the method to offer any advantage over the EIA. During Phase II, in connection with another project, the optimization of the FLIA™ will occur along a parallel path. When the assay time is acceptable, it will be substituted for the EIA.

Results:

Antigen-Coated EIA:

During Phase I, methods were developed to immobilize palytoxin on polystyrene microtiter wells. After coating the microtiter wells were washed thoroughly with distilled water and 0.05% Tween 20/PBS. Both palytoxin standard over a range of concentration from 0.4 to 300 ng/ml (60 µl)(or sample) and diluted crude palytoxin antibody (60µl) were added and incubated for 40 minutes at room temperature. The wells were again washed as described above and diluted goat anti-rabbit horseradish peroxidase (Sigma Chemical Co., St. Louis, MO) was added and incubated for 15 minutes at room temperature. Wells were again washed and the substrate added. After 5-10 minutes at room temperature, HCl (1N) was added to stop the reaction and the absorbance was read at 450 nm.

Several substrates were evaluated in the assay, the goal being to select the most stable reagent. The initial substrate used was OPD (ortho-phenylenediamine) in citrate phosphate buffer with 0.012% hydrogen peroxide, pH 5.0. This reagent was not stable and required preparation immediately before use. ABTS (2,2'-azino-di-(3-ethylbenzthiazine-6-sulfonic acid) was evaluated as an alternative. This substrate provided less signal/sensitivity; it was therefore abandoned. TMB (3,3',5,5',-tetramethylbenzidine) peroxidase substrate (Kirkegaard and Perry Laboratories, Inc.) was also evaluated. This substrate provided the same sensitivity as OPD, but the reagents were more stable (the solution is stable for 1 yr stored at 4 °C.) and required no preparation other than dilution. The TMB system is then the current substrate of choice. There are reports that a local company has under development a substrate for HRP with ten-fold enhanced sensitivity and better stability. When those reagents are commercially available, they will be evaluated for the palytoxin EIA.

The assay buffer used to dilute the palytoxin antibody and goat anti-rabbit horseradish peroxidase was optimized for minimum background, since background signal is a factor that limits sensitivity. PBS and TRIS buffers were evaluated with BSA supplementation ranging from 0.1 to 1%. The best buffer for maximum signal/minimum background was determined to be PBS with 0.1% BSA. Figure 1 presents the results of an experiment using three levels of BSA in

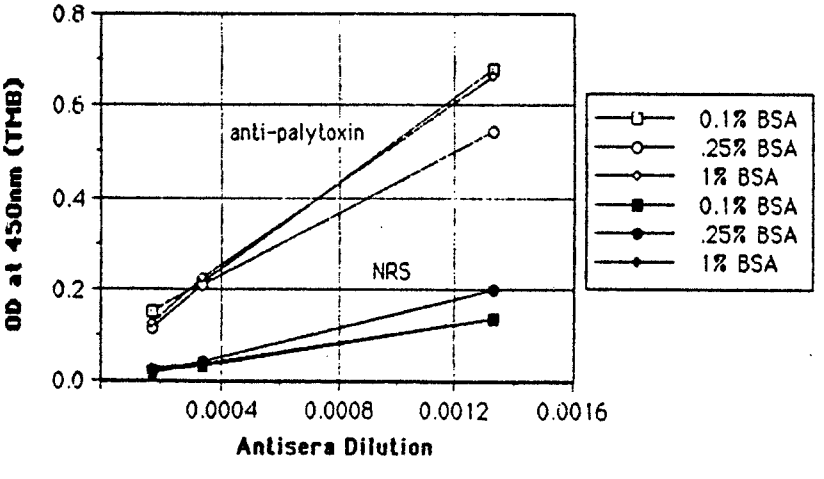

Figure 1. Effect of BSA in PBS Buffer System.

PBS with three levels of anti-palytoxin. Normal rabbit serum (NRS) controls were included to evaluate the background. The three curves in the lower portion of the figure illustrate the signal generated by NRS and the three curves

in the upper portion of the figure show the signal generated by anti-palytoxin bound to solid phased palytoxin. These data clearly show that 0.1% BSA is better than higher concentrations.

Another variable in the EIA which was optimized was the concentration of biotinylated palytoxin used to bind to avidin coated microtiter wells. It was determined that increasing the concentration provided us with higher signal and hence improved signal:noise ratio in the assay. The biotinylation reaction was evaluated. The biotinylated palytoxin was found to be stable for several months stored at 4 °C. No significant differences in the EIA were detectable using different batches of biotinylated palytoxin using this procedure.

The effect of serum and urine on the assay were preliminarily evaluated by spiking the fluids with palytoxin and testing them in the assay. Serum increased non-specific binding but did not alter the slope of the standard curve. This is a very positive finding. The serum did not reduce the assay to zero net signal. Therefore, the increase in non-specific binding is minor. The urine results were less clearcut and require further study, however, if serum did not interfere with the assay, urine should not interfere when the system is further optimized. A trio of standard curves with the palytoxin in buffer, serum and urine are presented in Figure 2

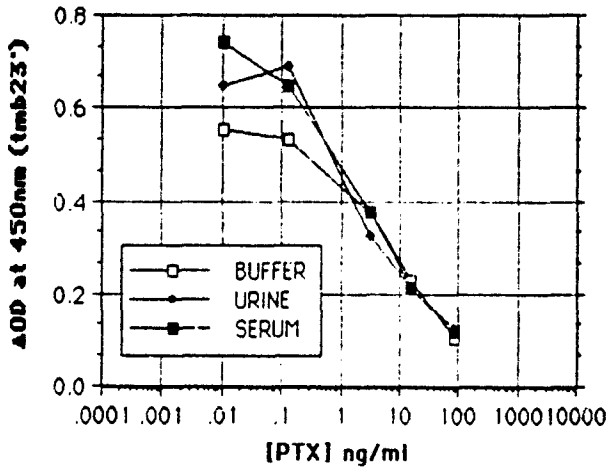

FIGURE 2. Effect of Serum and Urine on the Ptx Immunoassay.

A preliminary evaluation of reagent stability indicates that all EIA components are stable for at least 25 days at 4 °C. The data for the goat anti-rabbit-horseradish peroxidase, which should be the least stable component of the EIA is presented in Figure 3. Far more extensive stability studies and optimization of formulations will occur in Phase II.

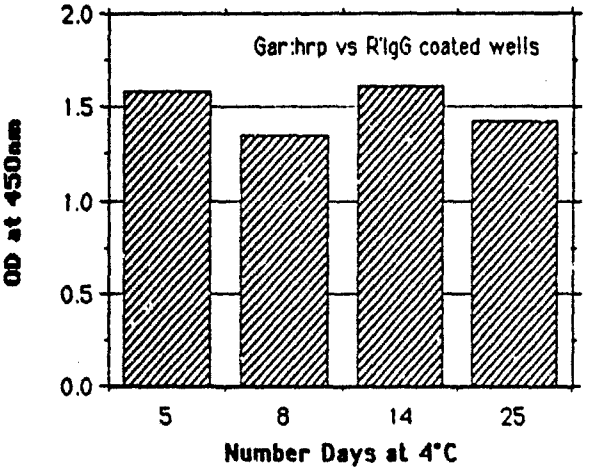

FIGURE 3. Stability Study--Goat Anti-Rabbit HRP Conjugates.

The data for the solid phase immunoassay for palytoxin that was developed during Phase I is presented in Figure 4. It has RIA sensitivity and

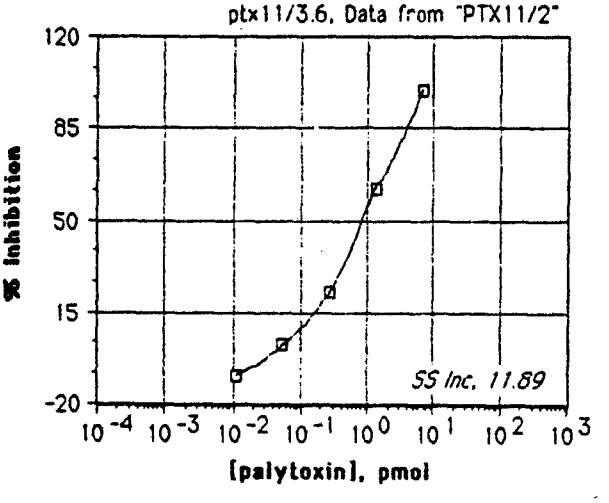

FIGURE 4. A SOLID-PHASE IMMUNOASSAY FOR PALYTOXIN--AN ANTIGEN BOUND VERSION. Palytoxin was bound to treated microtiter wells. After washing the wells, 60 µl of free palytoxin antigen in concentrations ranging from 0.4 to 300 ng/ml were added to the wells. Crude palytoxin antibody diluted 1:1,000 (60 µl, for an effective concentration of 1:2,000 in the assay) was added to each well. The bound palytoxin and the free palytoxin competed for binding sites on the palytoxin antibody during the incubation time (40 minutes). The wells were washed and Goat anti-rabbit horseradish peroxidase diluted 1:1,000 was added to each well for 15 minutes incubation time. The wells were washed and the assay was completed with the addition of Tetramethylbenzidine (TMB) Peroxidase Substrate (Kirkegaard and Perry

Laboratories, Inc. No. 50-76-00). The kinetic reaction was monitored at 650 nm by a Vmax microtitre reader (Molecular Device Corp, CA) and, end-point readings at 450 nm were determined after the addition of 1 N HCl. The data points are the mean of triplicate samples.

can detect 0.05 pmoles of palytoxin. The assay has a much shorter assay time (approx. 1 hour) than a conventional RIA (20 or more hours) and can be performed entirely at room temperature. With further optimization during Phase II, we expect to significantly improve the assay for field use by: increasing sensitivy, shortening assay time and reconfiguring the assay for non-instrumented field use (it will be deatailed in our Phase II Proposal).

Antibody-coated FLIA™:

Effort on the FLIA™ was focused on the antibody-coated microtiter well approach. The initial experiments used microtiter wells passively coated with crude polyclonal antiserum obtained from Dr. Lawrence Levine (Brandeis University). Preliminary results indicated that a dose response to palytoxin could be achieved using a 1:5K dilution of the antiserum as shown in Figure 5. The only problem at present with the FLIA™ was that the signal generation took much longer than that required for the EIA. For the sake of expediency, it was decided to work on improving the signal generation reaction of the FLIA™ off-line and focus resources on the EIA during Phase I.

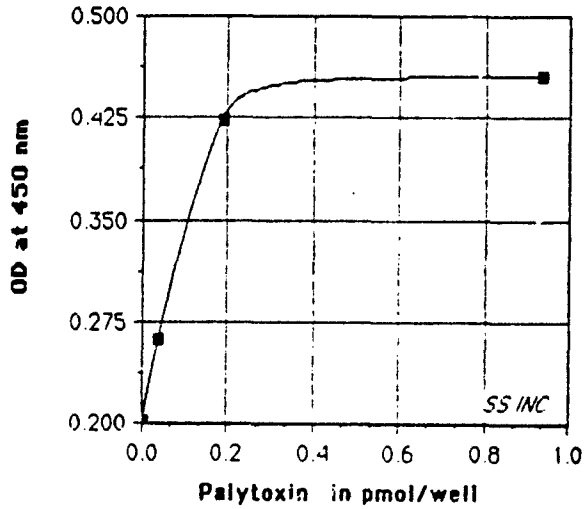

FIGURE 5. Dose Response of Biotinylated Palytoxin with Palytoxin Antibody Coated Wells.

For the remainder of the Phase I period, work proceeded to affinity

purify the polyclonal antibody for use in both antibody and antigen coated well configurations for both EIA and FLIA™. The IgG fraction was isolated using Protein A affinity column chromatography (BioRad Affi-Gel Econopac Protein A Kit ; Cat no. 732-2020). The rabbit anti-palytoxin antiserum was diluted 1:2 with PBS and applied to the column. The eluted IgG fractions (including rabbit IgG not specific for palytoxin) accounted for approximately 5% of the total starting protein based on UV absorption at 280 nm.

The purification process was carried one step further with the removal of anti-BSA antibodies to decrease the potential for non-specific binding interference in the assay. This was accomplished using Pierce Reacti-Gel (6X) Agarose beads to which BSA was covalently bound. The IgG fraction obtained from the Protein A column was applied to the column containing BSA-agarose beads. The anti-BSA antibodies bound to the column, while the palytoxin specific antibodies flowed through the column and were collected. Since time did not permit a thorough evaluation of this purified antiserum in Phase I, it was carefully stored for use early in Phase II.

Discussion:

Our original plan was to develop an EIA as a reference method and then develop the FLIA™ in the two forms. Since an EIA method was being used for another project and since the early experiments applying the analogous method to palytoxin were so promising, it was decided that this method would be more efficient in terms of time and final product. That decision was confirmed by the fact that the palytoxin EIA is working very well. It has excellent sensitivity, takes a much shorter time to run, and should be easier to transform into a dipstick method than a RIA method would have been.

Phase I was very successful. We have developed a rudimentary palytoxin EIA with excellent sensitivity (0.05 pmol). The assay can be completed in approximately 1 hour. The polyclonal palytoxin antibody was purified and will be used for the dipstick version of the assay.

The FLIA™ has produced comparable sensitivity, but takes much longer to perform at present. Work on the FLIA™ is proceeding on a parallel path with the effort focused on changing the method of generating the final signal since that is the portion of the assay responsible for the increased assay time. Another colorimetric iron detection method, electrochemical (Ferrochem II Analyzer, Bedford, MA) or potentiometric (iron sensitive biosensor) methods are under evaluation. In connection with another project, the proposal to develope an iron biosensor is underway. It should be available to test in the palytoxin assay in Phase III.

We feel confident that during Phase II, a sensitive, rapid and robust method will be produced that will fulfill the needs of a portable, field test to detect palytoxin exposure.

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