

UNCLASSIFIED

AD NUMBER

ADB164360

LIMITATION CHANGES

TO:

Approved for public release; distribution is unlimited. Document partially illegible.

FROM:

Distribution authorized to DoD only; Proprietary Information; 11 MAY 1992. Other requests shall be referred to Army Medical Research and Development Command, ATTN: SGRD-RMI-S, Fort Detrick, MD 21702-5012. Document partially illegible.

AUTHORITY

Ft Detrick/SGRD-RM1-S (70-ly) memo dtd 25 Jul 1994

THIS PAGE IS UNCLASSIFIED

AD-B164 360
[Barcode]

AD _____

L
②

ULTRASENSITIVE DETECTION OF TOXINS
USING IMMUNOASSAY AMPLIFICATION

PHASE I FINAL REPORT

GEORGE DOELLGAST

FEBRUARY 1, 1992

DTIC
ELECTE
JUN 10 1992
S B D

Supported by

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21702-5012

Contract No. DAMD17-91-C-1103

Elcotech, Inc.
4291 Lantern Drive
Winston-Salem, North Carolina 27106

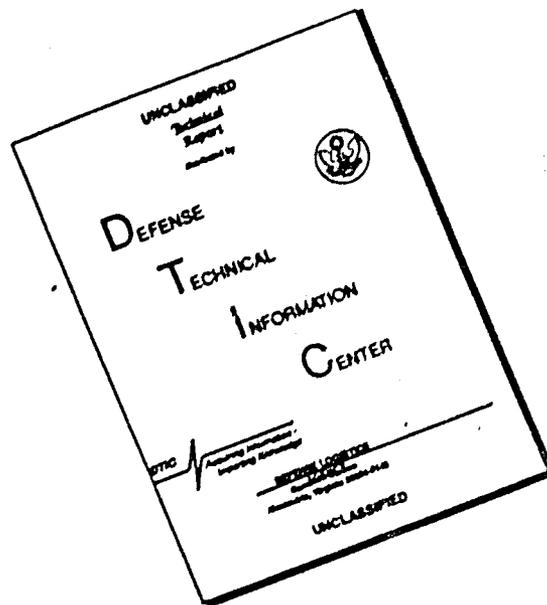
Distribution authorized to DOD Components only, 11 May 1992.
Other requests shall be referred to the U.S. Army Medical
Research and Development Command, ATTN: SGRD-RMI-S, Fort
Detrick, Frederick, MD 21702-5012. PROPRIETARY INFORMATION

The findings in this report are not to be construed as an
official Department of the Army position unless so designated
by other authorized documents

92 6 10 088

92-15244
[Barcode]

DISCLAIMER NOTICE



THIS DOCUMENT IS BEST QUALITY AVAILABLE. THE COPY FURNISHED TO DTIC CONTAINED A SIGNIFICANT NUMBER OF PAGES WHICH DO NOT REPRODUCE LEGIBLY.

REPORT DOCUMENTATION PAGE			Form Approved OMB No. 0704-0188	
<small>Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden, to Washington Headquarters Service, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503.</small>				
1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE February 1, 1992	3. REPORT TYPE AND DATES COVERED Phase I Final 15 Jul 91 - 15 Jan 92		
4. TITLE AND SUBTITLE Ultrasensitive Detection of Toxins Using Immunoassay Amplification			5. FUNDING NUMBERS DAMD17-91-C-1103 65502A ✓ 3P665502M802 AA DA335926	
6. AUTHOR(S) George Doellgast				
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Elcotech, Inc. 4291 Lantern Drive Winston-Salem, North Carolina 27106			8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research & Development Command Fort Detrick Frederick, Maryland 21702-5012			10. SPONSORING / MONITORING AGENCY REPORT NUMBER	
11. SUPPLEMENTARY NOTES				
12a. DISTRIBUTION / AVAILABILITY STATEMENT Distribution authorized to DOD Components only. 11 May 1992.			12b. DISTRIBUTION CODE	
13. ABSTRACT (Maximum 200 words)				
14. SUBJECT TERMS RA 1; Botulinum toxin; Detection; Assay; Amplification			15. NUMBER OF PAGES	
			16. PRICE CODE	
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited	

Table of Contents

A. INTRODUCTION 1

B. TECHNICAL ISSUES 1

C. APPROACH USED AND PRELIMINARY DATA. 2

1. Assays using 'First Flight' antiserum exclusively. 2
2. Assays using biotinylated 'First Flight' antibody to measure Ab titer 4
3. Assays using whole late-course antisera. 5
4. Logic for isolating reagents for the assay by affinity chromatography on toxin-agarose columns. 6
5. Preparation of absorbents from WAKO toxins. 7
6. Test of affinity-purified antibodies on food samples and at Fort Detrick. 8
7. Use of 'First Flight' antibody as a capture reagent. 9
8. Preparation of neurotoxin E absorbent and use for antibody isolation. 9
9. Neurotoxin E-specific assays using the eluate from Detrick neurotoxin E. 9
10. ELCA substrates; preparation of clotting factors. 10

D. CONCLUSIONS AND SUMMARY 11



Accession For	
NTIS GRA&I	<input type="checkbox"/>
DTIC TAB	<input checked="" type="checkbox"/>
Unannounced	<input type="checkbox"/>
Justification	
By _____	
Distribution/	
Availability Codes	
Dist.	Avail and/or Special
E-4	



A. INTRODUCTION

The objective of this Phase I effort, was to develop an amplified immunoassay which could detect botulinal neurotoxins A, B, E and F at concentrations equivalent to the mouse toxicity assay (i.e. 5 pg/ml). Reasons for doing this included the likelihood that such an assay would be much shorter than the mouse toxicity (hours rather than days), could be developed for field use, and would be substantially more economical than mouse bioassay.

The approach which was to be applied to this problem, was the amplified immunoassay system developed by Elcotech. This system is based on the ultra-sensitive measurement of coagulation-activating processes by generation of a solid-phase enzyme-labeled fibrin matrix (enzyme-linked coagulation assay, or ELCA). When a coagulation-activating protease is attached to an antigen or antibody, then the very sensitive detection of this labeled conjugate permits the very sensitive detection of the immune complexes formed in various assay protocols (ELISA-ELCA). The general principle of this immunoassay is reflected in the Figure below.

In this case, we see that the attachment of the Factor X-activating enzyme obtained from the venom of the Russell's viper (Russell's viper venom factor X activator, or RVV-XA) to an immune complex and its subsequent detection by ELCA permits the detection of immune complex formation by enzyme-linked fibrin deposition. The limit of detection of immune complex by this technology, is documented to be at a level of less than 50 femtograms per ml of RVV-XA, or a concentration of 5×10^{-16} M. By comparison, the mouse test can detect as little as 5 picograms/ml of neurotoxin, which is a concentration of 3.6×10^{-14} M. If we could devise an immunoassay which delivered as little as 1% of the neurotoxin as immune complex with RVV-XA-labeled reagents, then it would be reasonable to expect that we could detect this neurotoxin at concentrations equivalent to the mouse test. Demonstrating the feasibility of this was the objective of the Phase I effort.

B. TECHNICAL ISSUES

The technical issues related to the accomplishment of this goal can be divided into the following categories:

1. Choice of antisera. There are 7 known neurotoxins, i.e. A,B,C,D,E,F and G, which are immunochemically distinguishable. Of these, toxins A,B,E and F have been involved in human disease, but any of the toxins could potentially be a threat if produced in sufficiently large amounts. USAMRIID made a commitment to producing large amounts of antibody to each of these toxins in horses in December of 1990. Since toxoid immunization is essential for the primary response, the booster immunization of horses with purified neurotoxin has only recently begun. Production of high-affinity (i.e. late-course) antibodies from these horses which are strongly reactive and specific for individual neurotoxins is essential for the development of a potent, specific immunoassay. On the other hand, there is a horse antiserum available which is from a horse immunized over 8 years ago (the so-called 'First Flight' horse) which has potent antibody against all the neurotoxins A-G. This high-affinity antibody has broad potential in development of a highly specific assay, when combined with the specific labeled antibody from the Detrick horses immunized with specific individual neurotoxins and now producing high-affinity antibodies.
2. Assay protocol. Ideally, the assay should be simple to perform, and the reagents should be stable enough to be practical for field use. The assay system is colorimetric, so the possibility of producing a 'color test' for botulinal toxins is central to the potential simplicity of the

system (no need for instrumentation). We must also consider a way of producing test kits which need minimal manipulation and have simplified instructions so that it can be brought out of the laboratory if possible.

3. Serum samples. Since one of the USAMRIID priorities is to have the capability of measuring toxin in serum samples from toxin-infected patients, we would wish to design an assay which could measure the toxin levels in serum. The assay is based on measurement of coagulation, so that means that the 'background' activity of serum proteases must be eliminated in the assay.
4. Toxin standards. Neurotoxin is a part of the complex which *Clostridium botulinum* releases into the medium; there is also a hemagglutinin component. At the start of these studies, we were assured the availability of good, potent antisera which were toxin-specific. Since there were commercially-available toxins available, and several sources for the pure and food-contaminating toxins, we assumed that the assay design would not be compromised by the standardization of the assay against pure toxin. This was a problem in assay development, which we now have under reasonable control.

C. APPROACH USED AND PRELIMINARY DATA.

Of the protocols tried, we excerpt several relevant experiments which illustrate the problems encountered and the success obtained to date. They will be presented in historical sequence.

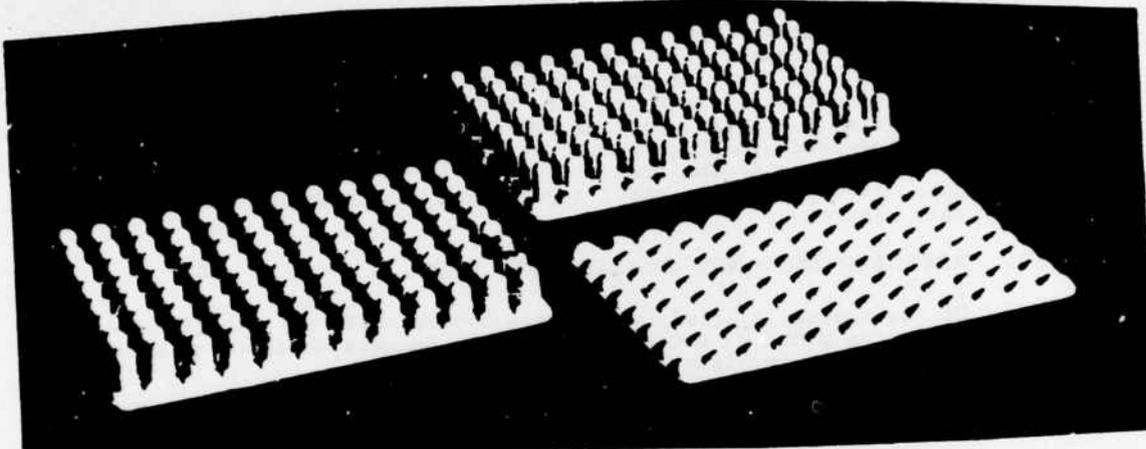
1. Assays using 'First Flight' antiserum exclusively.

The use of 'First Flight' antiserum was thought to have the best chance of a universal botulin toxin test, since it had reactivity against all the neurotoxins of interest. The high affinity to be expected from this hyperimmunized animal had great potential for generating a sufficiently sensitive assay. The objective is to measure low concentrations of antigen, so affinity was regarded as the most important property for an antiserum preparation.

The protocol we adopted for these studies, was based on the idea that small amounts of complex would be detectable using this antiserum if the plate could be coated with a higher concentration of the polyclonal-polyvalent antibody, and if the unbound labeled antibody could be washed out efficiently. To accomplish this, we coated the plate with 'First Flight' antibody using an attachment protocol based on binding to polylysine-glutaraldehyde. We have found this to be superior to 'passive' coating in other experiments. This was also tested for Botulin toxin assay, and found to be the case. In order to optimize the washing of the plate, we used the preparation of biotin-labeled 'First Flight' (Fab)₂ as the labeled antibody in solution. This reagent was used rather than the intact IgG molecule or the RVV-XA-conjugated antibody, because in our experience the larger conjugates are more difficult to wash from the plate, and we wished to use the labeled antibody in excess. Finally, the snake venom enzyme-labeled streptavidin (RVV-XA-Streptavidin) was incubated with the plate with the Ab-Toxin-biotin-Ab complex, and the washed plate was assayed for bound RVV-XA-streptavidin. The steps in this reaction sequence are as follows: (note that washing is required between each step).

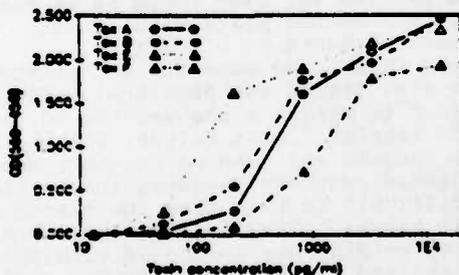
1. Plate-'First Flight' Ab + Toxin + Biotinyl-'First Flight' Ab>
Plate-Ab*Toxin*Biot-Ab (complexes Biotinyl-Ab to plate via toxin.)
2. Plate-Ab*Toxin*Biot-Ab + RVV-XA-Streptavidin>
Plate-Ab*Toxin*Biot-Ab*RVV-XA-Str (RVV-XA-Str binds to complex.)
3. Plate-Ab*Toxin*Biot-Ab*RVV-XA-Str + ELCA subst + 'FG-nubs'>
Alkaline phosphatase-fibrin is bound to 'nubs'
4. 'nubs' are placed in alkaline phosphatase substrate; color develops.

The 'nubs' alluded to above were developed for the purpose of servicing this assay. As seen in the Figure below, they are silicone rubber protrusions which are fixed in the same format as the microtiter plate. They are coated with fibrinogen and then lyophilized. When they are placed into the microtiter plate along with the substrate for RVV-XA (consisting of factors II, X, V and alkaline phosphatase-fibrinogen in a calcium buffer) the alkaline phosphate-labeled fibrin deposited on the 'nubs' is correlated with the amount of toxin complex formed.

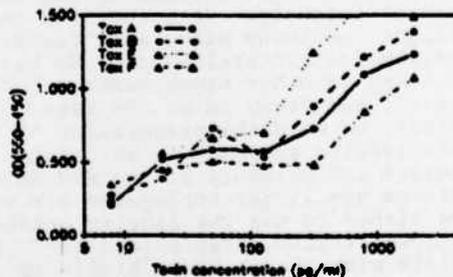


The Figures below demonstrate the results of applying this protocol for the measurement of toxins A, B, E and F using the 'First Flight' reagents. It appeared that there was a high degree of specificity and reasonable sensitivity. Given that these assays had a limit of detection of as little as 50 pg/ml of the WAKO toxins, for which 33-50 pg was the MLD₅₀, the goal of assaying the toxin at appropriate levels seemed straightforward.

Assay of toxins A-B-E-F using ELISA-ELCA. Strains: Ab and capture Ab were both 'First Flight', bleeding of January 1991. Toxins used were WAKO toxins. Exp Feb 12, 1991.



Assay of toxins A-B-E-F using ELISA-ELCA. Strains: Ab and capture Ab were both 'First Flight', bleeding of January 1991. Toxins used were WAKO toxins.



Having this assay working, we then attempted to apply it to the measurement of food samples contaminated with toxin, in collaboration with Kraft General Foods. We found that the presence of toxin was detectable in all the contaminated food samples derived from challenge studies. However, we encountered 1/24 food samples in which there was no toxin by the 'mouse test', no *C. botulinum* was inoculated into the sample before processing, and there was the highest assay result by this immunoassay. The only logical conclusion from this work, was that there was another contaminant in the food sample against which the 'First Flight' horse had raised antibodies. Thus, although the sensitivity seemed to be nearly as good as

the 'mouse test', the specificity derived from this whole antiserum did not appear to be adequate for the purposes of developing a specific assay which could be used for toxin detection without ambiguity.

Another potential problem with the 'First Flight' horse, was the fact that it was immunized with all the toxins. It was therefore unlikely that it could be the basis of toxin-specific assays. To resolve both of these problems, we assumed that the use of the horses begun on immunization regimens with individual neurotoxoids in December 1990 would yield useful discriminatory reagents, and optimally would have some antisera which did not show reactivity in common with the 'First Flight' horse with food contaminants. Also, we considered that we might need to develop assays based on the purification of the antibodies on neurotoxin columns, since this would likely give the appropriate specificity for the assay.

2. Assays using biotinylated 'First Flight' antibody to measure Ab titer

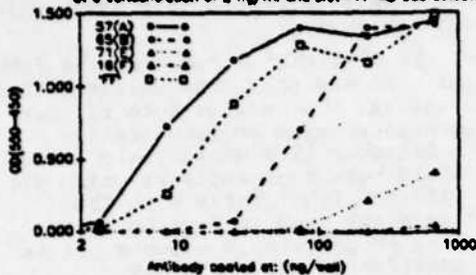
When the first bleedings were obtained from the horses immunized in December, and they were found to have produced highly potent neutralizing antisera, achieving titers in the thousands for some horses immunized with toxin A. These results strongly suggested that they would be the ideal animals to use for assay development. One reason for hoping that they would react with distinct environmental antigens and so would be usable for these assays was that the horses were kept in a completely different environment than the 'First Flight' horse.

The first assay we needed, was one in which we could compare the ELCA-based measurement of toxins for the different antisera obtained. In March, a collection of bleedings were made of the immunized horses, and samples of these were sent to us to use in assay development. Since we had the biotinylated 'First Flight' antibody (Fab')₂, we set up the assay to use the purified antibodies bound to the plate, and determined toxin-specific reactivity by adding toxin and Biotinylated 'First Flight' antibody, then proceeding exactly as described in the protocol above. In this case we used variable concentrations of antibody coating the plate, and a single concentration of each toxin (2 ng/ml). The table below shows the neutralization titers for the antisera used; the figures show the comparable titers by ELISA-ELCA, for four of the specific antibodies as well as the 'First Flight' antibody. Note that these data are with antibodies purified by ion-exchange chromatography from each antiserum, so that the concentration of antibody in coating is not only specific antibody. Detection of as little as 10 ng/well of antibody means that the antibody was diluted approximately 100,000-fold with 100 μ l of diluted IgG.

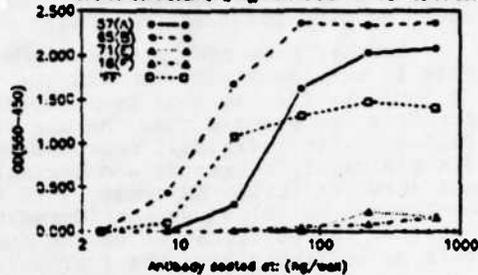
Neutralization titers for 'First Flight' antibody as well as four monovalent antisera. 'First Flight' was obtained after a booster injection in January; monovalent antisera were bleedings of March 1991.

Antiserum	Tox A	Tox B	Tox E	Tox F
'First Flight'	2620	146	1425	81
Monovalent As (Horse #)	2032 (57)	138 (65)	205 (71)	397 (16)

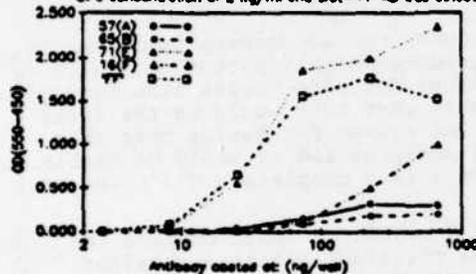
Assay of antibodies to Bot toxins A,B,E,F tested against toxin A. Plate was coated with the indicated concentration of Ab from horses 18,37,65,71 and 'First Flight'. Toxin A was added at a concentration of 2 ng/ml and biotin-PP Ab was detector.



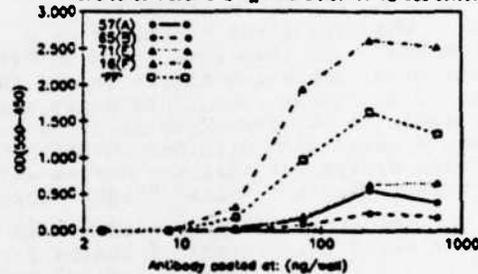
Assay of antibodies to Bot toxins A,B,E,F tested against toxin B. Plate was coated with the indicated concentration of Ab from horses 18,37,65,71 and 'First Flight'. Toxin B was added at a concentration of 2 ng/ml and biotin-PP Ab was detector.



Assay of antibodies to Bot toxins A,B,E,F tested against toxin E. Plate was coated with the indicated concentration of Ab from horses 18,37,65,71 and 'First Flight'. Toxin E was added at a concentration of 2 ng/ml and biotin-PP Ab was detector.



Assay of antibodies to Bot toxins A,B,E,F tested against toxin F. Plate was coated with the indicated concentration of Ab from horses 18,37,65,71 and 'First Flight'. Toxin F was added at a concentration of 2 ng/ml and biotin-PP Ab was detector.



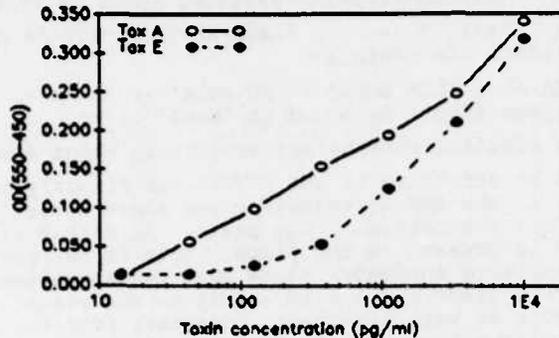
The data is interesting when the 'First Flight' antibody is compared with the rest of the antisera. The titer of the 'First Flight' antibody for neutralization was equivalent for toxins A and B, higher for toxin E, and lower for toxin F. The titer was roughly equivalent or slightly less when compared by ¹²⁵I-LISA-ELCA. Note that 2 ng/ml of antigen was used in these assays, which is about 2 orders of magnitude higher than the 'target range' of 5-50 pg/ml for an amplified ELISA using this system.

This was a convenient range for the assay since it gave a high signal at low antibody concentrations, but it may have hidden the fact that the toxin concentrations which could be measured would be relatively high using these early bleedings. In fact, we worked for some time varying conditions to try to get a specific assay developed using these reagents, and were uniformly unsuccessful. We were able to get usable assays for the range >200 pg/ml, but were unable to get higher sensitivity assays.

3. Assays using whole late-course antisera.

We obtained later-course antisera in late summer 1991; these antibodies proved to be of use in amplified assays for toxins, as seen in the Figure below. The protocol used in this assay was identical to that shown above for the 'First Flight' antibody, except that the relevant antibody was used both for 'capture' as well as biotinylated for detection.

Assay of toxins A-B-E-F using ELISA-ELCA. Biotinyl-Ab and capture Ab were both monovalent antibodies obtained in Aug 1991. Toxins used were WAKO toxins. Expt Sept 28, 1991.



As can be seen, the Toxin A assay approached the level of 10 pg/ml of whole toxin, or 1.5 pg/well in this assay. At this point, we did not discriminate between toxin and hemagglutinin reactivity. WAKO listed its toxicity as 2×10^7 MLD₅₀/mg, or an MLD₅₀ at .50 pg. This is consistent with about 10-20% of the WAKO toxins being neurotoxin. If we wanted to present the best possible interpretation of these results, shifting the scale of this assay one order of magnitude down would not have been unreasonable, if we wanted to adjust the scale to the MLD₅₀. However, we note that the horses were not all immunized with pure neurotoxin, so the antigen concentration is accurately reflected on the graph. As we work to make the assay neurotoxin-specific, we can assume that we will have to achieve a sensitivity of 5-10 pg/ml for antigen, so protocols must deliver this sensitivity. Note that the toxin E reactivity was somewhat lower in this assay, so the use of these hyperimmune (<1 year) antisera did not appear able to achieve the desired sensitivity.

4. Logic for isolating reagents for the assay by affinity chromatography on toxin-agarose columns.

We concluded that it would be necessary to purify the antibodies used for the assay by immunoabsorption, since we were not able to achieve the desired sensitivity by using the whole antisera. Our original attempt to get high sensitivity by using the amplification system with whole antisera was extremely optimistic, given the situation with botulinum toxin immunization.

Animals immunized against botulinum toxin are not 'boosted' with the pure toxin until some time after receiving the initial immunization. They are therefore immunized with a protein (toxoid) which resembles the pure toxin, but antibodies raised against it will likely be only 'cross-reactive' with the neurotoxin. This cross-reactivity may be adequate to bind to the toxin when it is injected along with the antibody at a toxin concentration of 50-100 pg/ml into a mouse, and thereby prevent its distribution to the nervous system. It may not be adequate at early stages after immunization to effectively bind the antigen to a microtiter plate at a concentration of 10 pg/ml.

Since antigen-antibody complexes are reversible and there are several washing steps in the protocol for measurement of toxin by ELISA-ELCA, reversibility of the complex would effectively eliminate it from the plate if the antibody is of low affinity. Note that the use of streptavidin-RVV-XA, in particular, would lead to dissociation during the 30 minute incubation time needed to bind this complex to the biotinyl-antibody, followed by additional washing steps. The objective in purifying the specific antibody was therefore not only to increase the amount of specific reagents which could bind the antigen, but also to make possible an

alternative protocol using RVV-XA-labeled antibody which had some potential in improving the retention of the complex on the plate because reduced washing would be required. This alternative protocol would be as follows:

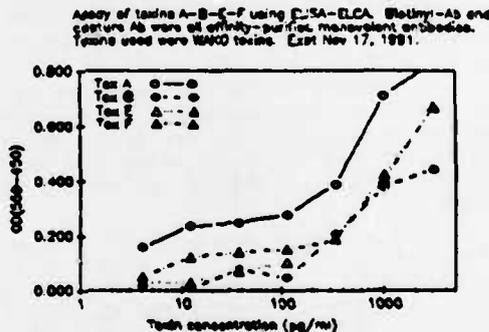
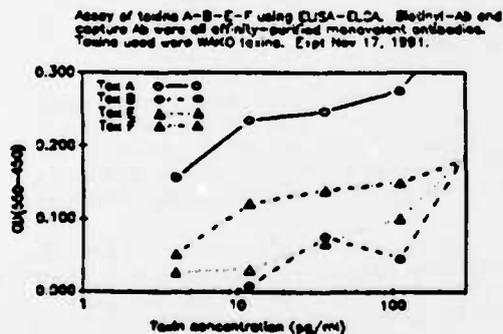
1. Plate-Ab + Toxin + RVV-XA-Ab -----> Plate-Ab*Toxin*RVV-XA-Ab (complexes RVV-XA-Ab to plate via toxin.)
2. Plate-Ab*Toxin*RVV-XA-Ab + ELCA subst + 'FG-nubs'-----> Alkaline phosphatase-fibrin is bound to 'nubs'
3. 'nubs' are placed in alkaline phosphatase substrate; color develops.

This protocol should be effective if the RVV-XA was directly conjugated to the antibody, or if the RVV-Streptavidin was added directly to the biotinylated antibody before incubation in the plate. In either case, only labeled specific antibody is present in the plate. This is not possible by directly labeling whole antisera antibody, since high concentrations of large complexes would have a tendency to bind avidly to the plate, and it would be much more difficult to wash irrelevant complexes from the plate. It should have the particular advantage for the assay of eliminating a washing step and reducing the background activity of the assay.

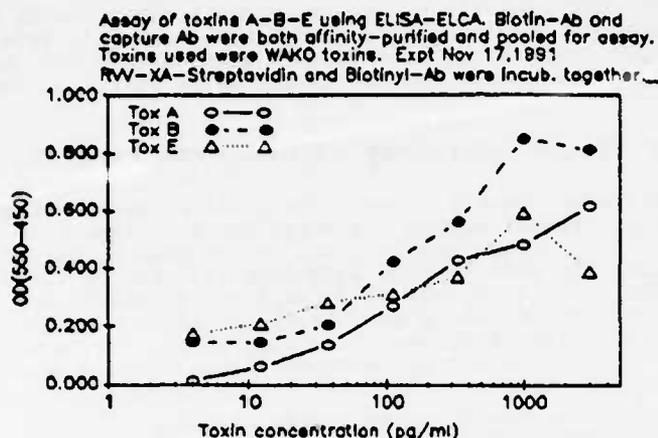
5. Preparation of absorbents from WAKO toxins.

We purchased toxins A, B, E and F from WAKO. These preparations are known to contain hemagglutinin as well as neurotoxin. 1 mg of each was attached to a type of column which allows little 'leaching' of bound protein (Pierce Chemical Company 'Aminolink' gel), and antibody was purified from each of the late-course antisera obtained from Detrick.

First, we used these purified antibodies in an assay for toxin, using a protocol similar to that above, i.e. binding of biotinyl antibody, washing, binding of RVV-XA-Streptavidin, assay ELCA. When this was done, the data in the Figures below was obtained. A plateau of readings in the 10-100 pg/ml range seemed to apply to all the purified antibody-based assays, with higher values in the >200 pg/ml range.



These data suggested that the assay could deliver higher sensitivity than the unabsorbed antisera. On the strength of this, we tried the alternative protocol of the RVV-XA-Streptavidin*Biotinyl Ab complex added together rather than sequentially. In this case, we were able to get highly sensitive assays for toxins A-B-E. These are represented in the Figure below.



6. Test of affinity-purified antibodies on food samples and at Fort Detrick.

On the strength of this, we went first to Kraft General Foods, to determine whether this assay could detect contaminated food samples in challenge studies. It was able to do this, with 24 samples half of which were contaminated with *C. botulinum* in challenge studies; at dilutions as high as 100-fold, these samples showed the exact pattern expected. 12 samples which were positive by mouse test, were positive by ELISA-ELCA assay; twelve samples which were negative by the mouse test were negative by ELISA-ELCA. The experience of our using the 'First Flight' antibody - i.e. the appearance of the single false positive sample, was not repeated in this series.

This result was very encouraging, and on the strength of it, we went to Fort Detrick to attempt to run the assay there, in December 1991. An auxiliary issue which we especially wanted to resolve, was the question of the correlation of the assay with the presence of neurotoxin, since all our standardization was with the 'WAKO' toxin preparations. WAKO toxin standards were known to consist of neurotoxin and hemagglutinin, and so we did not know whether the antibodies we purified were reactive with both proteins, or preferentially reactive with one of them. The result of this visit, was that we demonstrated a strong preference of reaction with the hemagglutinin component of toxins A, B and E. Pure neurotoxin was not reactive in these assays, but the 'crude' toxin was. For toxin F, we were able to get an assay to work, but it was less sensitive in this first experiment than we had found it to be at Elcotech. This was of less concern to us than the toxin specificity issue at that point. (Sensitivity could have been related to instability of reagents to transport, first setup in a different lab, etc.)

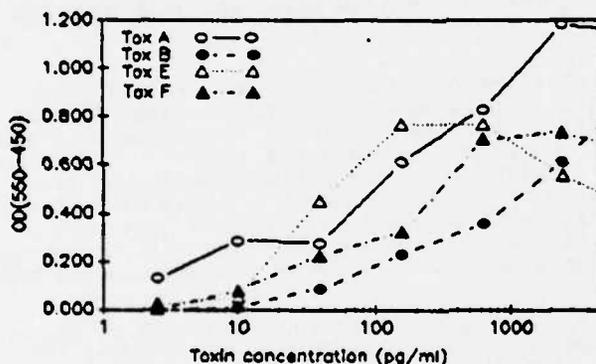
We noted that the toxin F obtained from WAKO had a molecular weight of 140,000, which was appropriate for neurotoxin, while the toxins A, B, and E were MW 500,000. This difference indicated that the toxin F was purified neurotoxin, while toxins A, B and E were complex. Based on neurotoxin reactivity (MLD₅₀ - 5-10 pg), absorbents made from the complex would contain at most about 200 micrograms of toxin in 1 mg of complex. The antibody purification would not remove most of the neurotoxin-specific antibody, and would likely contain a preponderance of hemagglutinin-specific antibody. The F toxin, being all neurotoxin, was able to purify more of the specific antibody, so the assay worked using this affinity-purified antibody. A further confirmation of this interpretation, was the finding that we were able to measure 'crude' toxins in this assay while at Detrick.

In our view, the most sensible way to solve the problem was to purify the antibody to be used in the assay on neurotoxin columns. In this case, hemagglutinin reactivity would be eliminated during the absorption, and the isolated antibody should be usable for amplified assays if it was from late-course antisera.

7. Use of 'First Flight' antibody as a capture reagent.

We wished to determine whether it would be possible to have a more efficient (i.e. less expensive) way to 'capture' the complexes than use of affinity-purified antibody. For this purpose, we returned to the 'First Flight' antibody, which was able to bind with high affinity to all the neurotoxins of interest. We used this as a 'capture' antibody, and tested the binding of the WAKO toxins, using affinity-purified, RVV-XA-labeled antibody (not biotinyl-Ab*RVV-XA-Streptavidin complexes). We found that this combination was able to detect low concentrations of these toxin-hemagglutinin (WAKO toxins) complexes, as seen in the Figure below.

Assay of toxins A-B-E-F using ELISA-ELCA RVV-XA-Ab was affinity-purified; capture antibody was 'First Flight' antibody. Toxins used were WAKO toxins. Expt Feb 6, 1982.



While this was not a neurotoxin-specific assay, it did demonstrate that the appropriate sensitivity assay could be developed using such a combination (high-affinity antiserum IgG for capture, affinity-purified antibody labeled in solution).

8. Preparation of neurotoxin E absorbent and use for antibody isolation.

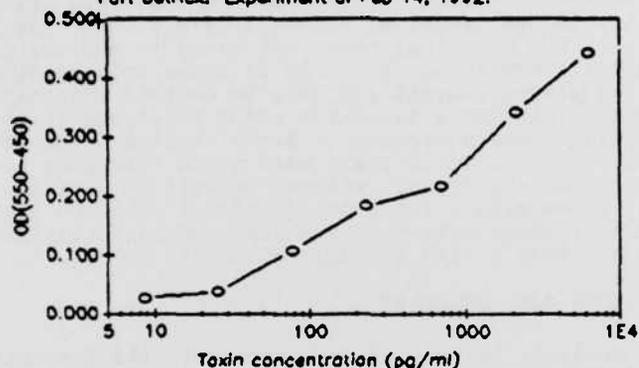
In order to test the other component of the solution to assay development, i.e. the purification of neurotoxin-specific antibody, we went to Detrick in January, to use 1 mg of neurotoxin in preparation of a specific absorbent column. This column was tested at Detrick for the separation of neurotoxin-specific antibodies, using standard ELISA protocols on toxin-coated plates, and was found to purify the specific antibody, although it did not efficiently remove all of it from antiserum. This column was brought back to Elcotech, and used to purify more anti-toxin E IgG, which was separated on G-200 to eliminate any complexes or IgM from the antibody. It was biotinylated, and used in several toxin-specific assays.

9. Neurotoxin E-specific assays using the eluate from Detrick neurotoxin E.

First, we attempted to use the eluate from the Detrick Toxin E column in an assay of a freshly-prepared dilution of toxin E. For this purpose, we used the two-stage assay described in the protocol above, in which the

toxin is bound to the biotinylated antibody, excess antibody is washed off, and RVV-XA-Streptavidin is added to the plate for 30 minutes, washed and ELCA assay is performed. The toxin we used was obtained from DasGupta at the University of Wisconsin, stabilized at 0.625 mg/ml in ammonium sulfate suspension at 0°C. This toxin was diluted just before use to the indicated concentrations, and assayed on plates coated with 'First Flight' antibody. The results clearly showed that the assay was able to detect toxin E at concentrations as low as 10 pg/ml from this freshly-prepared material.

Assay of toxin E using ELISA-ELCA. Toxin used was freshly-diluted toxin E obtained from Das Gupta. Biotin-Ab was purified on a column of toxin E-agarose obtained from Fort Detrick. Experiment of Feb 14, 1992.



The 'raw' data obtained in this experiment, was as follows:

Tox E concn (pg/ml)	6250	2083	694	231	77	25.7	8.6	0
OD ₅₅₀ -OD ₄₅₀	.442	.342	.217	.184	.108	.039	.028	.005

Controls included use of neurotoxin F, which did not react, and a companion experiment using biotinylated affinity-purified anti-toxin F, which did not react with the toxin E preparation. This result suggests that the assay was effective in measuring toxin at the appropriate concentrations in freshly-diluted samples of toxin. By inference, this suggests that denaturation of toxin used in generation of standard curves was a confounding and hitherto uncontrolled variable. Some work will be required to determine optimal conditions for storage of toxin standards to be used for amplified immunoassay.

10. ELCA substrates; preparation of clotting factors.

At the start of this project, the only clotting factors we had prepared were from human plasma, and these were used for all the initial assay on botulin toxin. The labeling enzyme used in this work is Russell's viper venom factor X activator (RVV-XA), which is able to activate the factor X to Xa from any mammalian and avian species tested. This stands in contrast to the known physiological activation, since human factor Xa is very inefficient in activation of coagulation in avian species.

There are several reasons for not using human clotting factors in assays for botulin toxin in human patient samples. First, the use of these factor preparations with serum samples has the potential for spurious activation of clotting unrelated to the presence of botulin toxin. Second, there is the potential for contamination of the plasma with human viruses, and their persistence after purification. Third, there is the expense of human plasma and the special handling requirements to purify clotting factors from it and include them in test kits.

For these reasons, we attempted to set up the assay using purified bovine and duck clotting factors. Plasma used for starting material for both of these was obtained from local slaughterhouses. In the case of the duck plasma, it took some time to collect enough blood to prepare plasma, and the starting material showed some contamination with activated clotting factors. In the case of bovine plasma, blood collection was much more rapid, and we were able to completely prevent the activation of coagulation by rapidly cooling the blood and adding high concentrations of appropriate inhibitors. For this reason, the bovine blood yielded clotting factor preparations which had no background activity.

By use of the appropriate controls, all three sources were able to yield substrates for ELCA assay which could measure RVV-XA at concentrations of 50-150 femtograms/ml (.05-.15 pg/ml). At this level of sensitivity, if 1% of the botulinum toxin was bound to a microtiter plate in the form of a toxin*RVV-XA-Ab, it would be detectable by ELCA assay. Since all three of the plasma sources are able to deliver this sensitivity, it will be possible to develop a sensitive assay which showed no activation by human clotting factors present in serum samples (duck or chicken clotting factor mix) or which would yield more rapid assays by use of high concentrations of clotting factors without background activity (bovine clotting factors). If we make a later preparation of chicken clotting factors which show less background activity, the appropriate specificity and rapidity should be achievable with a single substrate source.

D. CONCLUSIONS AND SUMMARY

In this project, we set out to demonstrate the feasibility of the ELISA-ELCA system for measurement of *C. botulinum* toxins at concentrations equivalent to the mouse test. At this point, we have demonstrated this sensitivity in selected cases, using labeled affinity-purified antibodies and high-affinity antisera for 'capture' of complexes. We have also shown that the assay can be used with relatively crude samples, i.e. those obtained from food processing challenge studies.

We are committed to the application of this assay system to measurement of *C. botulinum* toxins, and plan to bring this through AOAC (Association of Official Analytical Chemists) approval for general use in botulinum testing. During this effort, the goal will be standardization of the procedure with respect to protocol, preparation of reagents in 'kit' form, and application to antiserum testing for affinity and specificity.