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13. ABSTRACT *(Maximum 200 words)*

The objective of this program is the development of vaccines which provide lasting immunity with a single administration. To accomplish this, biodegradable polymer microspheres are employed to provide release of the antigen at intervals which mimic conventional booster injections. The in vitro release characteristics of poly-DL-lactide-co-glycilide microspheres were studied using high performance liquid chromatography (HPLC) using a water soluble dye and a commercially available tetanus toxoid C fragment (recombinant). A vaccine was then prepared using type A botulinum toxoid C fragment, and this was used to immunize a series of adult mice. Over a 3 month period, plasma samples were collected permitting the measurement of antibody levels raised to the antigen using enzyme linked immunosorbent assay (ELISA). After 3 months, the immunized animals were supplied to the Army laboratories for an aerosol challenge.

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R. L. W

Robert L. Whalen, Ph.D.
31 October 1995

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Introduction

Although vaccines are one of the most cost effective tools in medicine devised for fighting disease, adult vaccination requirements for the routine immunization of military personnel create financial and logistical problems. This is primarily due to the complex dosage schedule required for many vaccines. Typically, some vaccines require an initial dosage followed by a series of booster injections from 2 weeks to 1 year apart. For example, the vaccine against Bacillus anthracis (anthrax) entails a dosage schedule in which the initial vaccination must be followed by booster shots at 2 weeks, 4 weeks, 6 months, 12 months, and 18 months with additional injections annually to maintain immunity.[1]

Parenteral administration of vaccines also must be given by trained medical personnel. This considerably increases the cost associated with vaccination and creates logistical difficulties, especially for military personnel. Often the prescribed time intervals between individual doses of an immunization series are problematic due to time constraints or other circumstances. For example, all personnel traveling to or deploying to areas with highly endemic disease and deemed to be at risk of contracting a disease related to operational mission or occupational exposure are required to be vaccinated prior to departure.[2] Under these circumstances, an extended immunization series would create considerable delay. This problem is especially acute for members of Alert Forces, both active duty and Reserve Component, which have been designated to be in a state of readiness for immediate deployment to any area outside of the United States.[2]

Physical circumstances also play a role in military vaccination schedules. The next dose in an immunization series is often administered upon opportunity, rather than in strict adherence to a prescribed schedule. At other times, minimum intervals between doses may be reduced upon approval by the appropriate Surgeon General; Commander, Naval Medical Command; or Chief, Coast Guard Office of Health Services, as an operational mission dictates.[2] Variations in the vaccination schedule such as these may reduce the effectiveness of the vaccine.

To alleviate these logistical problems and decrease the overall cost of providing effective immunizations for military personnel, a new approach to vaccine delivery is desirable. Such a vaccine delivery system would provoke an adequate immunological response to establish long-lasting immunity with less frequent parenteral administration of the antigen.

In the Phase I effort of this program, we investigated the approach of incorporating the antigen into a controlled release drug delivery system. This was accomplished by encapsulating the antigen in biodegradable polymer microspheres (poly-DL-lactide-co-glycolide). At the time of vaccination, the drug filled microspheres are suspended in a proper vehicle and injected through an acceptably sized needle in the same manner as current vaccines. The antigen is then released upon the biodegradation of the microspheres, whose characteristics

are adjusted to provide release times corresponding to the recommended booster sequence.

For military personnel, the advantages of this approach are obvious. There are important potential benefits of this technology, however, for civilian healthcare as well. Complicated dosage schedules are costly and time consuming for the physician in private practice; and patient compliance is often a significant problem. The logistics involved in even a small office practice to follow recommended dosage schedules requires reminder notices in patient records, routine questioning of patients by nurses and support staff, and follow-up postcard reminders to patients. While all of this increases the costs associated with vaccination, this process is often unreliable due to human error, lack of patient compliance, and changing patient populations.

The possible benefits of timed release vaccines are further enhanced if applied to worldwide immunization projects. At this point, over 90% of the world incidence of the Plague occurs in Southeast Asia.[3] Likewise, the majority of Anthrax occurs or is transported from Africa, the Middle East and Asia[3]. At this time, efforts to immunize these high risk populations in the aim of worldwide disease control are considerably thwarted by the logistical difficulties of conventional vaccinations.

Therefore, this new approach to vaccine delivery will potentially alleviate many of the problems associated with vaccination dosage schedules, and at the same time lower the overall cost of immunizations. It will thus improve the cost-benefit ratio of worldwide immunization efforts. For these reasons we believe this work is of both commercial and medical significance.

Phase I Methodology

The specific objectives of the Phase I program were: (1) to demonstrate the feasibility of preparing polymeric biodegradable microspheres for the encapsulation of vaccines to provide discrete timed release patterns; (2) to characterize in vitro the release kinetics of the polymer system selected; and (3) to demonstrate the efficacy of a microencapsulated vaccine in animal experiments.

We began the program by encapsulating a readily detectable water soluble dye in polymer microspheres. We then studied the release kinetics of the dye filled microspheres in vitro using high performance liquid chromatography (HPLC) to characterize the polymer systems we had selected. As a parallel effort, we established a methodology for encapsulating a commercially available tetanus toxoid in preparation for preparing a vaccine for Type A botulism to be tested in vivo.

The technique of employing biodegradable microspheres for drug delivery is rapidly evolving. This technology has already been utilized to achieve controlled release of various peptides. For example, injectable microspheres have

been used to deliver luteinizing hormone-releasing hormone analogs such as leuprolide acetate,[4] nafarelin acetate,[5] and [D-Trp[6]]LH-RH.[6] These have been used to treat endocrine-dependent tumors, such as prostate cancer. Similarly, octapeptide analogs of somatostatin have been encapsulated for long-acting formulations in the treatment of endocrine related diseases such as type I diabetes mellitus and diabetic retinopathy.[7] In addition, steroids, such as norethisterone[8] and norethindrone,[9] have been encapsulated to serve as injectable biodegradable contraceptives.

To achieve biocompatability and biodegradability, poly-DL-lactide-co-glycolide (PLGA) was chosen for microencapsulation of the peptides in the above mentioned cases. PLGA and its homopolymers have had substantial use in the past as biodegradable sutures.[10] More recently, these polymers have been established as adequate systems of diffusion and degradation controlled kinetics of compound release. Experiments have shown these microspheres to provide from one month to one year of controlled release [5,6,7,8,11,12], depending on a variety of design factors, such as the components of the polymer and the size of the microspheres. By changing these factors, a variety of microspheres may be produced and combined to achieve peptide release at different predetermined times. In the case of an encapsulated vaccine, this controlled release pattern may mimic the effect of repeated booster injections with the use of one primary injection.

Earlier work has demonstrated that controlled release of drugs from a polymer matrix consisting of poly(DL-lactide co-glycolide) could be attained for periods up to six months.[8] This was based on work utilizing poly(DL-lactide) (DL-PLA), consisting of 100% lactide monomer, in which the polymer was shown to degrade in 12 months. The drug, however, was nearly depleted by 6 months. Further studies based upon this research showed that the biodegradation of the co-polymer was controllable through the manipulation of the molar ratio of the monomers. By increasing the proportion of glycolide, the polymer was shown to degrade at a higher rate. Also, research conducted to approximate the depletion of the drug with the disappearance of the polymer degradation products revealed that the degradation of the co-polymer coincided with the elution rate of the drug.[9] Table 1. summarizes the results of that work.

Table 1. Molar Ratio of Lactide/Glycolide (L/G) vs Elution Period

Ratio L/G	Time (wks.)
96/4	45
92/8	35
87/13	30
74/26	20
50/50	4

Because of the time constraints of Phase I, the animal experiments in this program were limited in duration to 3 months, but the ultimate goal of this effort is to provide immunity for a minimum 1 year period with a single dose system using this technology. In the Phase I in vivo testing, Type A botulinum toxin fragment C (recombinant) supplied to us by the Army was employed to make a microencapsulated vaccine. The vaccine was given to a series of 26 adult mice, and we attempted to measure the antibodies raised to this antigen on a weekly basis using enzyme linked immunosorbent assay (ELISA) tests.

At the conclusion of the Phase I program, the immunized animals were to be supplied to LTC James E. Brown, PH.D., Chief, Department of Immunology and Molecular Biology, Fort Detrick, for a direct challenge with botulinum toxin. As Dr. Brown was no longer in that position when the program was completed, this test was conducted by Dr. Louise Pitt at that facility.

Phase I Work Plan

A detailed summary of our Phase I activities is as follows.

Preparation of Dye-Filled Microspheres

The microencapsulation technique was determined based on the solubility characteristics of the both the polymer and the antigens to be encapsulated (proteins or a water soluble dye). The polymer used was poly(DL-lactide co-glycolide) or PLGA. Because PLGA is not water soluble, it must be dissolved in an organic (water immiscible solvent) solution. On the other hand, the substance used to simulate the drug was a water soluble dye.

In situations such as this, in which the additive to be dispersed is water soluble, a method is used known as a water in oil in water emulsion (W/O/W). This is a multiple emulsion technique consisting of two stages: 1) a water solution of the additive is dispersed into an organic solution of the polymer forming a W/O dispersion, and 2) this W/O dispersion is then emulsified into a non-solvent, generally water, and is mechanically stirred until enough organic solvent has evaporated to harden the spherical droplets. The result is a quantity of individual polymer spheres containing dye.

The W/O/W technique required the preparation of three solutions. First, a polymer solution was prepared to serve as the continuous phase; 5 g of PLGA (50:50; Sigma, St. Louis, MO) was added to 35 g methylene chloride (Fisher Chemical, Pittsburgh, PA) and 1.2 g of a surfactant, Sorbitan Sesquioleate (Arlacel 83; Sigma, St. Louis, MO). This solution was stirred briefly with a small stirrer attachment. Second, the dye solution was prepared to serve as the dispersed phase; 1.2 g of dye (Congo Red; Fisher Scientific, Pittsburgh, PA) was dissolved in 12 ml of distilled water. The dye solution was slowly added to the

polymer solution and stirred on a high setting for 5 minutes. The result was a quasi-emulsion forming the W/O dispersion.

The third solution was prepared from 6 g of polyvinyl alcohol flakes (Airvol 523; Air Products and Chemicals, Inc., Allentown, PA), 0.75g of a surfactant, polyoxyethylenesorbitan monooleate (Tween 80; Sigma, St. Louis, MO), and distilled water up to 1000 ml. This solution was sonicated for approximately 24 hours for complete dissolution. To make the final W/O/W dispersion, the W/O dispersion was added to 550 ml of this solution under vigorous stirring in a 35°C water bath. Mechanical stirring was then continued at a moderate setting for approximately 5 hours to allow the spheres to completely harden by solvent evaporation. The use of a wide mouthed jar and extended stir time provided for quick evaporation rates and subsequently small uniform spheres without clumping.

The resulting spheres were lyophilized which generated 5 g of 50:50 PLGA encapsulated dye. The microencapsulation technique was repeated using PLGA (65:35; Sigma, St. Louis, MO). This generated 5 g of 65:35 PLGA encapsulated dye.

Elution Studies of Polymer Encapsulated Dye

The elution rates of the dye from the two types of microspheres, 50:50 PLGA and 65:35 PLGA, were then characterized to determine the degradation profiles of the different PLGA formulas. 50:50 PLGA was estimated to dissolve in 4 weeks and 65:35 PLGA in 12 weeks. Elution profiles over these time periods were established as the average behavior of three samples of these formulations. Thus, three 0.05 gram portions of each formulation were each added to 50 ml of PBS (Sigma, St. Louis, MO). These samples were placed into a 37°C shaking water bath to raise the solution to body temperature and allow for uniform degradation of the spheres. The concentration of eluted dye in each sample was quantified in periodic sample aliquots with the use of HPLC methods and a UV detector.

The modern isocratic HPLC instrument includes a pump, sample injector, variable wavelength detector, and a recorder. However, the heart of the instrument is the column. The column is a tube, usually a few centimeters long and less than a centimeter wide, packed with particles a few microns in diameter. The vast majority of packing materials in use are based on silica gel and exist in a number of packing states: 1) densely packed columns, 2) loosely packed columns, and 3) open-tubular columns. Conventional HPLC columns are densely packed and employ stainless-steel tubing to withstand the high pressures that will be required to force the mobile phase through the interstices of the packing.[13]

The column performs several functions. During the development of a chromatographic separation, two processes occur simultaneously and to a large extent independently. First, the individual solutes in the sample are moved apart

in the column as a result of their different affinities for the stationary phase. Those solutes that interact strongly with the stationary phase are retained in the column to a greater extent than those solutes that interact more strongly with the mobile phase. Secondly, as the bands are moved apart, they spread or disperse and tend to merge together, blurring the separation that has been obtained. The column, by appropriate design, must minimize this dispersion, so that , having been moved apart and separated, the individual solutes enter the detector as individual bands as far apart as possible but, at the same time, keep each band as narrow as possible.[14]

The most common detector is the UV photometer. It has been used since the early beginnings of liquid chromatography and is very simple in principle. A light source delivers a monochromatic parallel light beam which goes through a cell swept by the column eluent, and falls on a photocell. A signal proportional to the amount of light received is measured and recorded. The light source of the simple UV photometers is often a mercury lamp permitting work at 254 nm. To increase detection selectivity, variable wavelength detectors are often used. They incorporate a deuterium lamp and sometimes a tungsten lamp to extend the wavelength range to the visible domain. A monochromator permits the selection of the desired wavelength and light is detected with a silicon photodiode. The response time is of the order of 50 ms. The noise level has been reduced to the level of *ca.* 5×10^{-5} AU, while flow sensitivity has also been reduced. In current cell design, noise, flow rate and temperature sensitivity originate in the part of the light beam which strikes the cell wall.[15]

Sample aliquots of the eluted dye solution were taken for HPLC analysis at 1, 2, 4, 8, and 24 hours. Thereafter, samples were taken once a week until the period of time around which dissolution of the spheres was expected, 4 weeks for the 50:50 PLGA and 12 weeks for the 65:35 PLGA. At that time, samples were taken approximately every 48 hours to ensure accurate elution rate characterization. Samples were stored and analyzed in groups with the HPLC. Such grouping minimized any discrepancies in calibration.

HPLC analysis was performed with the use of a 3.9 x 150 mm reverse phase c18 column. The mobile phase consisted of 30% acetonitrile and 70% distilled water with an apparent pH of 7.4. A flow rate of 0.5 ml/min was generated with a Waters Solvent Delivery Module (Model 590). Ten microliter (10µl) injections of the sample were introduced through a Waters U 6K injector. The amount of sample present was quantified by means of a Waters 484 Tunable UV Absorbance Detector, set at 254 nm and 0.1 absorbance units full scale (AUF), connected to a Waters Data Module (Model 730). The Data Module is a microprocessor based integrator designed to provide quantitative information for HPLC applications.

Each elution sample was measured two times with the HPLC to ensure a proper reading. These readings were entered into a spreadsheet (Microsoft Excel) and averaged for each sample. The average readings for each of the three samples

were then averaged for each timepoint. The average readings for each sample and each timepoint were converted to concentration values (μg/ml) with the use of a calibration curve. This calibration curve was established at the start of each HPLC analysis session with the use of prepared standards.

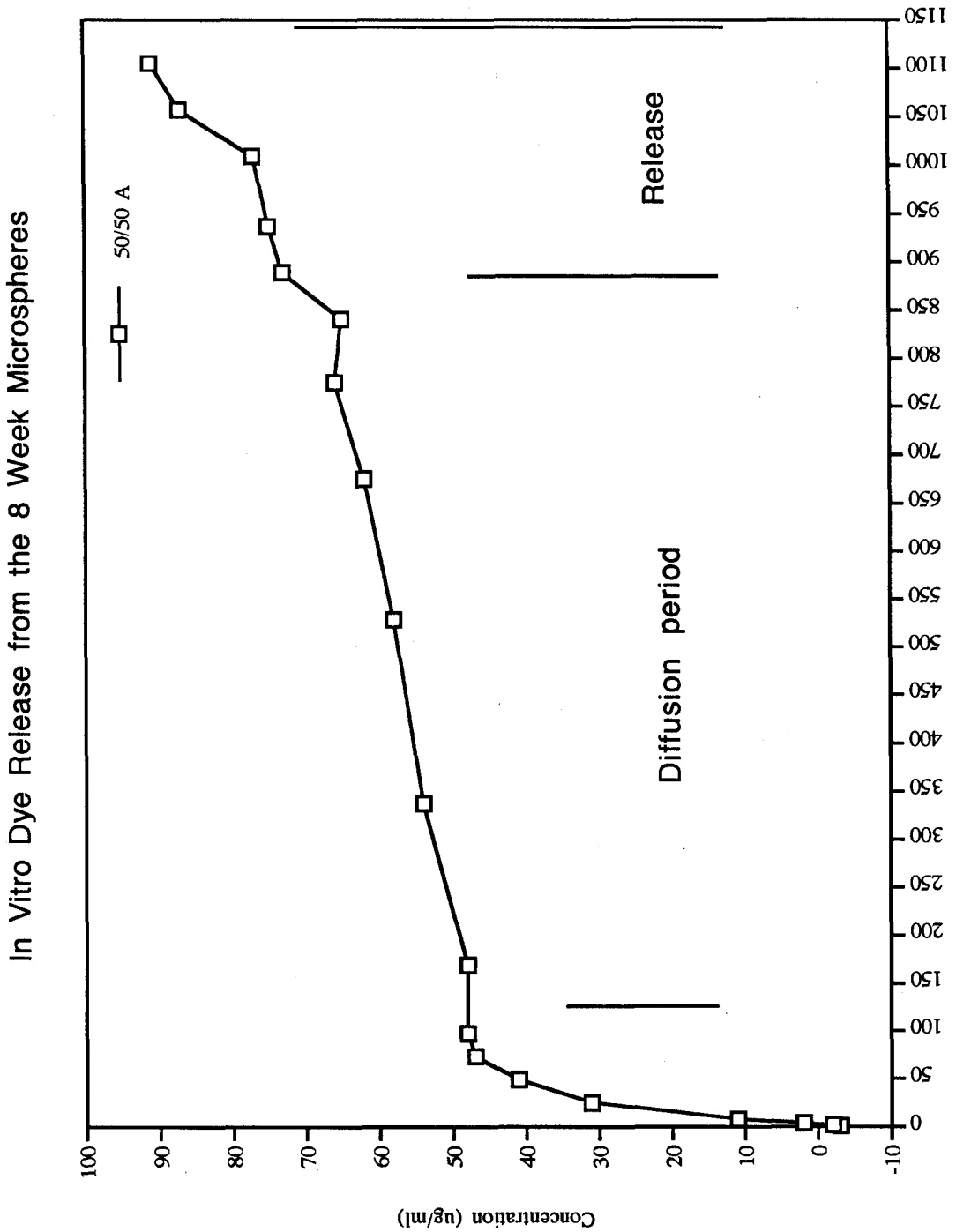
The concentration values were plotted over time to graphically characterize the elution rate of the dye from the two types of microspheres, 50:50 PLGA and 65:35 PLGA. In both cases, the concentration values were plotted over time for each of the three samples individually and as an average curve depicting the general behavior of the polymer formulation.

The HPLC data for the 50/50 formulation microspheres is shown in Figure 1. There is an initial release of dye associated with external wash off of dye adsorbed on the exterior surface of the microspheres (and possibly from any microspheres with wall porosity). The measured dye concentration rapidly levels off to a very low release rate corresponding to diffusion of the dye through the walls of the microspheres. At approximately 850 hours duration, there is an inflection point in the curve where the dye concentration begins to increase more rapidly as the microspheres begin to dissolve.

We measured the maximum elution concentration of dye in the microspheres by dissolving (at elevated temperature) a sample of the filled microspheres. The maximal dye concentration measured was in the range of 80-90 μg/ml, so the dye from the 50/50 formulation microspheres has essentially been completely released by approximately 1.5 weeks after initial dissolution peak was observed. Similar data for the 65:35 (12 week) microspheres is shown in Figure 2. Our in vitro release data suggests that release of the antigen from the microspheres in the in vivo studies will occur at 5-6 weeks and 11-12 weeks respectively for the two formulations.

Statistical analysis of a portion of this data was undertaken to determine the confidence of the values. The 50:50 PLGA average elution curve was used as an example of the data. At each time point, the concentration value was the average concentration of three samples. The standard deviation of these three sample concentrations was calculated for each time point. This information was then used to determine the 95% confidence interval for the mean at each time point. These calculations were performed with the pure HPLC readings and then with the readings converted to concentration values (μg/ml) with the use of the calibration curve, since the latter units are more conventional. It was found that the confidence interval ranged from 0.5 μg/ml to 4 μg/ml, with a mean of 2.2 μg/ml.

To determine the confidence in the HPLC apparatus and measurement technique, seven measurements of the 100 μg/ml calibration standard were taken and compared. These measurements were taken at different times over the course of the sample analysis so variations in run periods would also be included. It was found that the standard had an average of 99 μg/ml with a standard deviation of 4 μg/ml. With two runs per sample, the 95% confidence interval was approximately 5.5 μg/ml. Calculations were repeated with 3, 4, 5, and 10 runs


In Vitro Dye Release from the 8 Week Microspheres
50/50 A
Release
Diffusion period
Concentration (ug/ml)
time (hr)
100
90
80
70
60
50
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30
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10
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-10
1150
1100
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1000
950
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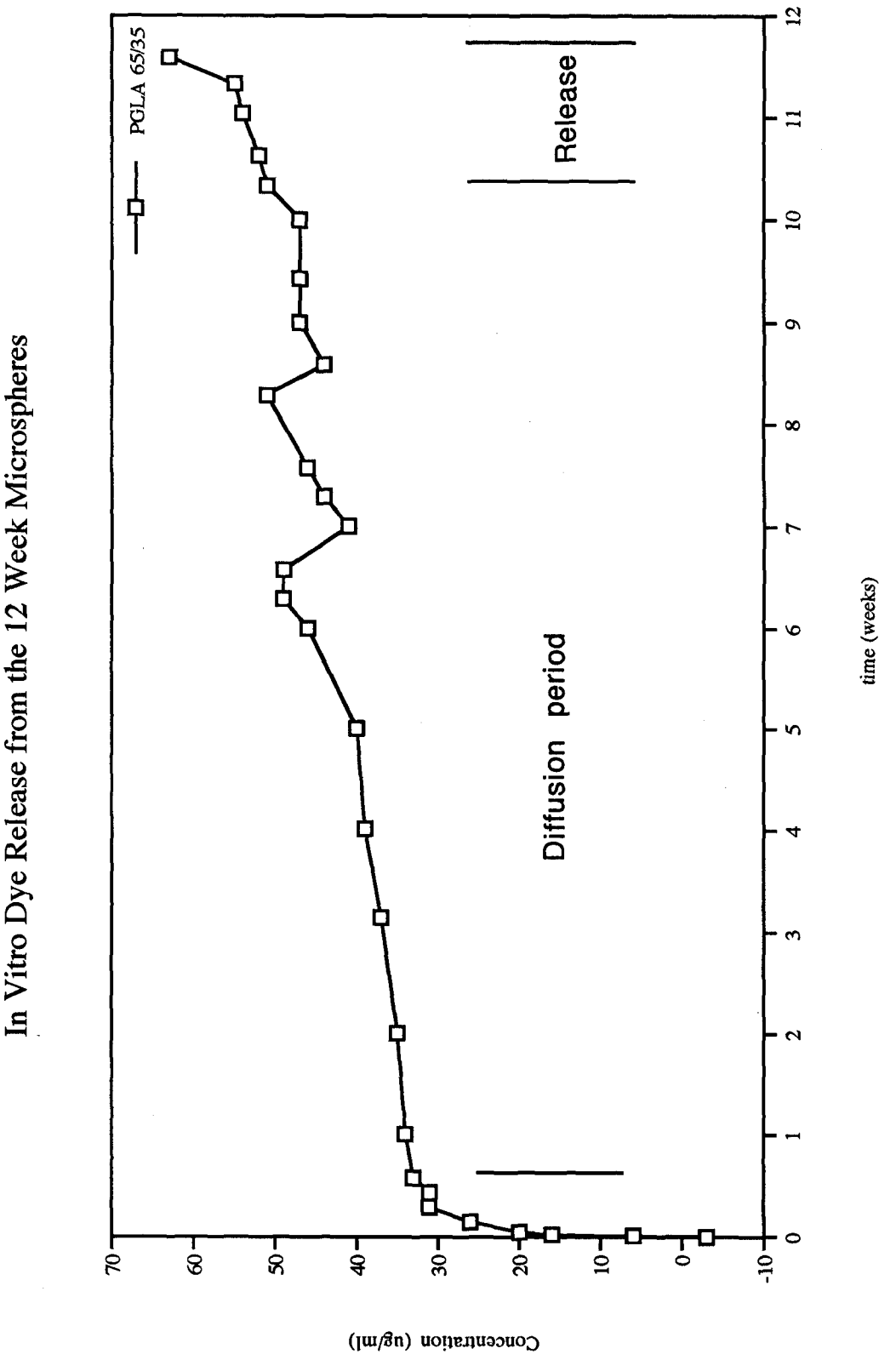
Figure 1


In Vitro Dye Release from the 12 Week Microspheres
PGLA 65/35
Release
Diffusion period
Concentration (ug/ml)
time (weeks)
70
60
50
40
30
20
10
0
-10
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1
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3
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6
7
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11
12

Figure 2

per sample illustrate the reduction in the confidence interval. The results were 4.5, 3.9, 3.5, and 2.5 μg/ml respectively.

Therefore, it was determined that two readings of each sample on the HPLC resulted in a 95% confidence interval of 4.5 μg/ml. This interval is acceptable for two reasons: 1) increase in the number of runs does not appreciably reduce this interval while it does substantially increase the evaluation time, and 2) the HPLC is not sensitive below 5 μg/ml so this is negligible. Similar reasoning can be applied to the number of elution samples. In this study we have used three, which resulted in an average confidence interval of 2.2 μg/ml. This interval is tighter than that found just in the HPLC measurement process, therefore, the number of elution samples is sufficient.

There were two problems which effected these in vitro measurements, however. The first was that the dye used (Congo red) can escape the microspheres by passive diffusion. The comparatively low molecular weight of the dye of approximately 689 Daltons (D) makes this possible. The result was that dye was being lost from the microspheres during the course of the measurements.

The second problem in our in vitro methodology was more significant. Even in the absence of diffusion, it lowered the amount of encapsulated material being released. This resulted because the microspheres tend to form large aggregates in the test solution, since their surfaces become tacky as they begin to dissolve. This aggregation serves to significantly reduce the effective surface area of the microspheres, resulting in a measured release versus time that does not accurately reflect the total content of the microspheres.

This effected our release kinetics data of the tetanus toxoid filled microspheres, bringing the amount of detectable toxoid down to the threshold of our measuring technique. While the high molecular weight of the tetanus toxoid (approximately 50,000 D) eliminated diffusion through the walls of the microspheres, it proved difficult to measure the rate of toxoid eluted because the amount being released was lowered by the formation of aggregates in the samples under test. This was confirmed by mechanically fracturing the aggregates to release the toxoid and dissolving the remaining microspheres.

We believe this problem may be avoided by immobilizing the microspheres in a monolayer to prevent aggregation and loss of surface area in contact with the PBS test medium.

Preparation of Tetanus Toxoid Filled Microspheres

The red dye used in the above mentioned studies was replaced with tetanus toxin C fragment to closer simulate the processing characteristics of the intended vaccine, botulinum toxin C fragment. Native tetanus toxin (from *Clostridium tetani*) consists of a heavy chain of approximately 100 kD and a light chain of approximately 48 kD held together by disulfide bridges. Tetanus toxin C fragment, a 50 kD polypeptide comprising the C-terminal 451 amino acids, is released from the heavy chain by papain digestion. This fragment constitutes the binding portion of native tetanus toxin. However, the tetanus toxin C fragment is

recombinant, containing an N-terminal methionine, so there is no risk of contamination with the hazardous native toxin. A similar recombinant fragment was used for preparing the botulism vaccine; thus no toxic agents were used in conducting the research of this program.

Since the tetanus toxin C fragment is water-soluble, like the previously used dye, the same microencapsulation technique (W/O/W) was used to produce tetanus-filled microspheres. For purposes of in vitro testing, 50:50 PLGA was used to produce spheres that would degrade in 4 weeks time. Thus, the microencapsulation procedure followed that which is stated above with a few minor adjustments. First, the amount of tetanus toxoid to be encapsulated was lowered from the initially high concentration of dye, which is abundantly available, to match the relatively low concentration of the intended vaccine, botulinum toxin C fragment, which is limited in availability. Thus, 500 μg of tetanus toxin C fragment was available for every 1 g of polymer. In addition, the methodology and batch size was adjusted to reduce the size of the microspheres to allow easy passage through a 22-gauge needle.

For the W/O/W technique, three solutions were prepared. First, a polymer solution was prepared to serve as the continuous phase; 2 g of PLGA (50:50; Sigma, St. Louis, MO) was added to 18 g methylene chloride (Fisher Chemical, Pittsburgh, PA) and 0.6 g of a surfactant, Sorbitan Sesquioleate (Arlacel 83; Sigma, St. Louis, MO). This solution was stirred briefly with a small stirrer attachment. Second, the tetanus toxoid solution was prepared to serve as the dispersed phase; 0.001 g of tetanus toxin C fragment, recombinant, (Boehringer Mannheim Corporation, Indianapolis, IN) was added to 6 ml of 0.01 M phosphate buffered solution. The tetanus toxoid solution was slowly added to the polymer solution and stirred on a high setting for 5 minutes. The result was a quasi-emulsion forming the W/O dispersion.

The third solution was prepared from 2.5 g of polyvinyl alcohol flakes (Airvol 523; Air Products and Chemicals, Inc., Allentown, PA), 2.5 g of a surfactant, polyoxyethylenesorbitan monooleate (Tween 80; Sigma, St. Louis, MO), 5 g of methylene chloride, and distilled water up to 250 ml. This solution was sonicated for approximately 24 hours for complete dissolution. To make the final W/O/W dispersion, the W/O dispersion was added to 250 ml of this solution under vigorous stirring in a 35°C water bath. Mechanical stirring was then continued at a moderate setting for approximately 4 hours. At this time, the W/O/W dispersion was diluted with 250 ml of a solution consisting of 2.5 g polyvinyl alcohol flakes, 2.5 g polyoxyethylenesorbitan monooleate surfactant and distilled water up to 250 ml.

To ensure the passage of the microspheres through a 22-gauge needle, the microspheres were sieved through a series of USA Standard Testing Sieves (A.S.T.M.E-11 Specification). Microspheres found between 45 and 150μ were considered acceptable. These microspheres were then lyophilized and 0.56 g of 50:50 PLGA encapsulated tetanus toxin C fragment was generated.

Elution Studies of Polymer Encapsulated Tetanus Toxin C Fragment

The elution rate of the tetanus toxin C fragment from the 50:50 PLGA spheres was characterized to verify a rate profile similar to that of the dye or to document any significant differences in behavior. Again, three 0.05 gram portions of the spheres were added to 50 ml of PBS. These samples were placed into a 37°C shaking water bath to raise the solution to body temperature and allow for uniform degradation of the spheres. The concentration of eluted tetanus toxin fragment in each sample was quantified by periodic sample aliquots with the use of HPLC methods and a UV detector. Samples aliquots were taken once a week until the period of time around which dissolution of the spheres was expected (4 weeks) at which time samples were taken approximately every 48 hours. Samples were stored and analyzed in groups with the HPLC. Such groupings minimized any discrepancies in calibration.

HPLC analysis was performed with the use of a Shodex Protein KW-803 (8 mm x 300 mm) size exclusion column (Waters Corporation; Milford, MA). The mobile phase was composed of filtered phosphate buffered saline, having a concentration of 0.01 M phosphate buffer and 0.137 M sodium chloride. A flow rate of 0.8 ml/min was generated with a Waters Solvent Delivery Module (Model 590). One hundred microliter (100 µl) injections of the sample were introduced through a Waters U 6K injector. The amount of sample present was quantified by means of a Waters 484 Tunable UV Absorbance Detector, set at 214 nm and 0.01 AUFs, connected to a Waters Data Module (Model 730).

The elution samples would give a maximum tetanus toxoid concentration at 100% elution of 25 µg per 50 ml, or 0.5 µg/ml. This concentration was found to be less than the detection level of the HPLC method in use. Consequently, the elution study was repeated with one 0.946 gram portion of the spheres in 10 ml of PBS. This was calculated to yield a theoretical tetanus concentration of 47 µg/ml at 100% elution.

After we had experienced difficulty in measuring the release kinetics because of aggregates in the test solution, the elution study was interrupted to determine the total quantity of tetanus encapsulated in the microspheres. This was accomplished with a two step process. First, the microspheres were ground with a glass rod to break them open and release the tetanus toxoid. The microspheres had already softened during the elution study and agglomerated. Therefore, the mass was repeatedly impinged against the wall and bottom of the flask to disrupt the spheres. The PBS was then decanted and passed through a 0.45 µm filter for later evaluation. Second, the remaining microspheres were dissolved in 3 ml of methylene chloride. After their dissolution, 1 ml of PBS was added and the mixture was shaken. The methylene chloride was removed under vacuum and the PBS suspension remained for analysis.

Solutions collected from both steps of the process were analyzed with HPLC methods to determine the total quantity of tetanus present. Each solution sample was measured two times with the HPLC to ensure a proper reading. These readings were entered into a spreadsheet and averaged for each sample. The average readings were converted to concentration values with the use of a calibration curve. This curve was established with the use of prepared standards.

The first solution, obtained from mechanical disruption of the microspheres, was found to contain approximately 14 μg/ml of tetanus toxoid. Therefore, from the 0.946 gram portion of spheres, 140 μg of tetanus toxoid could be obtained from physically breaking up the spheres. The second solution, obtained from chemically dissolving the spheres, was found to contain 164 μg of tetanus toxoid. Therefore, a total of 304 μg of toxoid were encapsulated in the original 0.946 g portion of microspheres. This represents 64% of the theoretical loading. This information was used to predict the levels of available vaccine in the animal studies.

Preparation of In Vivo Microspheres

The in vivo studies required the production of both vaccine-filled microspheres and saline filled control microspheres. The vaccine to be studied was based on Type A botulinum toxin C fragment. As mentioned, due to the similarity between this toxoid and the tetanus toxin C fragment, the same encapsulation methodology was used for its preparation.

Because of concern about possible deleterious effects on the toxoid resulting from electron beam sterilization, the method which had originally been proposed as a sterilization technique for the finished vaccine, the vaccine was manufactured under sterile conditions in the Class 10,000 clean room at WBI. Had we anticipated manufacturing the vaccine in this way, we would have elected to fabricate specialized fixtures for this purpose to facilitate sterile handling of the reactants and the finished microspheres. We were able to satisfactorily use our existing equipment however.

Three solutions were prepared for the W/O/W encapsulation technique. First, a polymer solution was prepared to serve as the continuous phase; 2 g of PLGA (50:50; Sigma, St. Louis, MO) was added to 18 g methylene chloride (Fisher Chemical, Pittsburgh, PA) and 0.6 g of a surfactant, Sorbitan Sesquioleate (Arlacel 83; Sigma, St. Louis, MO). This solution was stirred briefly with a small stirrer attachment. Second, the botulinum C fragment solution was prepared to serve as the dispersed phase; 0.00132 g (2 ml of 0.66mg/ml solution) of botulinum toxin C fragment (U.S. Army) was added to 4 ml of 0.01 M phosphate buffered solution. The toxoid solution was slowly added to the polymer solution and stirred on a high setting for 5 minutes. The result was a quasi-emulsion forming the W/O dispersion.

The third solution was prepared from 2.5 g of polyvinyl alcohol flakes (Airvol 523; Air Products and Chemicals, Inc., Allentown, PA), 2.5 g of a surfactant, polyoxyethylenesorbitan monooleate (Tween 80; Sigma, St. Louis, MO), 5 g of methylene chloride, and distilled water up to 250 ml. This solution was sonicated for approximately 24 hours for complete dissolution. To make the final W/O/W dispersion, the W/O dispersion was added to 250 ml of this solution under vigorous stirring in a 35°C water bath. Mechanical stirring was then continued at a moderate setting for approximately 4 hours. At this time, the W/O/W dispersion was diluted with 250 ml of a solution consisting of 2.5 g polyvinyl alcohol flakes, 2.5 g polyoxyethylenesorbitan monooleate surfactant and distilled water up to 250 ml.

To ensure the passage of the microspheres through a 22-gauge needle, the resulting microspheres were sieved through a series of USA Standard Testing Sieves (A.S.T.M.E-11 Specification). Microspheres between 45 and 150 micrometers were collected. The resulting microspheres could not be lyophilized without removal from the clean room so the slurry, composed of saline wetted spheres, was weighed; 1.92 g of 50:50 PLGA encapsulated botulinum toxin C fragment were generated.

The actual vaccine formulation used in the in vivo studies consisted of the above described 50:50 PLGA microspheres, similar 65:35 PLGA microspheres, 65:35 PLGA microspheres with a lower concentration of encapsulated vaccine, and free toxoid in solution. This combination would allow for immediate inoculation with the free toxoid and delayed inoculation at varying levels with the encapsulated toxoid.

The 65:35 PLGA microspheres were prepared using the same methodology as the above described 50:50 PLGA microspheres. In one batch, the concentration of encapsulated botulinum toxin C fragment was the same as that in the 50:50 PLGA microspheres, 0.00132 g (2 ml of 0.66 mg/ml). The slurry derived from this batch weighed 2.908 grams. In a second batch, the concentration of encapsulated vaccine was cut in half to 0.00066 g (1 ml of 0.66 mg/ml) and a slurry of 1.06 g was generated.

All three batches (1.92 g of 50:50 PLGA, 2.908 g of 65:35 PLGA, 1.06 g reduced concentration 65:35 PLGA) were combined with the remaining 5 ml (3.3 mg) of botulinum C fragment in a 20 ml vial. The volume was brought up to 20 ml with sterile saline to provide for 40 doses of 0.5 ml each for the in vivo studies.

Empty control microspheres were generated in a similar manner. All methodology was the same with the dispersed phase consisting only of 6 ml of 0.01 M phosphate buffered solution. This resulted in 1 g of 50:50 PLGA spheres. Again, the microspheres were sieved through a series of USA Standard Testing Sieves (A.S.T.M.E-11 Specification) and those found between 45 and 150μ were collected.

In Vivo Testing

The in vivo testing of the microencapsulated vaccine was carried out in the Department of Bioengineering at the Cleveland Clinic Foundation (CCF) under the direction of Hiro Harasaki, M.D., Ph.D., who also conducted the analysis of the serum samples taken during the studies. The program plan called for conducting studies of 3 month duration in Phase I.

1. Materials

The vaccine consists of encapsulated and free botulinum type A Hc fragment (non-toxic). The biodegradable microspheres are made from poly (DL-lactic co-glycolide). The vaccine was encapsulated and supplied by Whalen Biomedical Inc. as a suspension in 20 ml of saline with a net concentration of approximately 6 mg.

The encapsulated botulinum toxin fragment C was received from Whalen Biomedical Inc., in a refrigerated package on July 18, 1995. After receipt, the material was stored in the lab refrigerator until the morning of July 26. The encapsulated vaccine was then transferred to a Styrofoam container with dry ice. While in transit to the 6th floor of the research building, during a 30 minute period of time, the vaccine was found to be completely frozen. The material was thawed by applying body temperature. Although it is not known whether this freeze and thaw process altered the vaccine, this should have been avoided. Retrospectively, it is also clear that the vaccine should have been used **immediately upon receipt, since some aggregation of the microspheres occurred** during the 8 day period of storage at the CCF before it was used.

2. Animals

A total of 26 adult C57BL/6J mice were used in this study. The 26 mice were divided into 3 groups. Groups 1 and 2 were identical study groups injected with the encapsulated Hc protein fragment of botulinus toxin type A and consisted of 10 mice each. Group 3 consisted of 6 mice which were inoculated with saline filled microspheres.

At the end of the 3-month duration, all surviving innoculated mice were delivered to the U.S. Army Medical Research Institute of Infectious Diseases **(USAMRIID) at Fort Detrick for a challenge with the botulinum toxin. This will** demonstrate the efficacy of immunization produced by the vaccine. Group 3 is a negative control group to evaluate the biodegradation and tissue response to the polymers. This group consisted of 6 mice injected with normal saline filled microcapsules.

It is important to note the optimal level of care and maintenance of these **animals. The facility where the animals are housed and where the procedures are**

performed is accredited by the AAALAC and conforms to all federal and local regulations concerning the care and use of laboratory animals. In addition, the animals are handled by qualified animal technicians.

Since the animals are immunocompromised, micro-isolation housing units are used. These micro-isolation housing units consist of autoclavable cages, flasks, feed, and bedding, as well as a wire bar lip and a plastic filter top. All the procedures in this experiment are performed in a HEPA-filtered laminar-flow workbench.

3. Anesthesia

An anesthetic agent was necessary to prevent pain or distress to the animals during the experimental procedure. Sodium pentobarbital (40 mg/kg) was used in this study. Based on the average weight of the mice (approximately 20 g), each mouse was injected intraperitoneally with 0.15 ml of diluted sodium pentobarbital.

Initially, the sodium pentobarbital was diluted with saline in a 1 ml syringe. This method of anesthesia preparation was not found to be satisfactory. Due to the limited size of the syringe, it is believed that preparing the anesthetic in this manner did not produce a homogenous mixture and may have contributed in the three deaths in the study due to unequal distribution of anesthesia. Currently, the sodium pentobarbital is diluted in a 5 ml syringe and mixed prior to withdrawal into a 1 ml syringe for injection. Utilizing a larger syringe not only provided a homogenous mixture, but also a more effective and efficient means of administration.

On July 26, 1 995, a total of 23 mice were anesthetized with 40 mg/kg of sodium pentobarbital intraperitoneally. After a sufficient anesthesia level was obtained, pre-blood samples were obtained from each animal via retro-orbital plexus puncture, collecting two hematocrit capillary tubes (60 microliters each). During this procedure, death occurred in one mouse without clear reason. Asphyxia due to excessive tension to the neck region or idiosyncratic response to anesthetics may have been the cause of death. It was also noted that this particular mouse had a cataract-like white opacity in the eyes. Therefore, a total of 22 mice were injected with the vaccine.

4. Inoculation

The mice were anesthetized with 40 mg/kg sodium pentobarbital as previously described. Groups 1 and 2 were injected with the encapsulated botulinus toxoid. Each mouse was injected with 0.5 ml of the toxoid using an 18 gauge needle subcutaneously in the interscapular area tented by the thumb and forefinger of the administrator. Some difficulty was experienced with obtaining a homogenous suspension as well during the injection procedure. Rapid

sedimentation of beads in the syringe occurred, making the injections impossible even when strong pressure was applied on the plunger of the syringe plunger.

After experiencing these difficulties, several changes in the administration procedure were made. First, the vaccine had to be continually mixed via hand inversion and reversion just prior to injection. Second, it was necessary to place the animal in a vertical position (tail on the ground) as well as inject the animal with the syringe in a vertical manner.

The most likely cause of this difficulty was the excessive storage time between when the vaccine was prepared and its use at the Cleveland Clinic. A period of 8 days elapsed, and while the vaccine was refrigerated, it was suspended in a saline solution. The microspheres thus tended to aggregate, as some dissolution of the polymer did occur in this time.

It was noted also, prior to injection, that the vaccine suspension was frozen while being transferred on dry ice in a Styrofoam container. The suspension was thawed by applying body temperature It is unknown at this time what effect this freeze and thaw process had on the encapsulated vaccine and its membrane properties. It is possible the freeze and thaw process may have contributed to the difficulty experienced during the injection.

Group 3, the control group, was injected with saline filled microcapsules. Although reconstitution of the lyophilized control was somewhat difficult, injection was not. Each mouse was injected with 0.5 ml of the suspended microcapsules using an 22 gauge needle subcutaneously in the same interscapular area as Groups 1 and 2.

5. Blood collection

Blood samples from all the groups were taken from the retro-orbital sinus or plexus using a micro-hematocrit tube. These samples were taken pre-inoculation and then weekly post-inoculation. The weekly collection continued for a 2 month duration and then biweekly until the sacrifice. In addition, when blood is collected on a weekly basis, alternating between the left and right eyes every week is necessary to ensure proper healing for further collection.

The mice are anesthetized with 40 mg/kg Sodium pentobarbital as previously described. The end of the micro-hematocrit tube is introduced at the medial canthus of the orbit. The tip of the microhematocrit tube is advanced slowly with axial rotation towards the rear of the socket until blood flows into the tube. The original micro-hematocrit tube is removed from the orbit and another microhematocrit is then applied for blood sampling. The excess blood from the site is absorbed by a gauze sponge. The animal is observed for recovery from the anesthesia.

The collected blood is centrifuged for 10 minutes. The hematocrit tube is cut at the division of plasma and precipitate. Air from an empty syringe with a 25

gauge needle is used to force the plasma out of the tube and into a 0.5 ml microcentrifuge tube. These microcentrifilge tubes are labeled and then stored at -80°C.

6. ELISA Tests

The development of ELISA methodology for the quantitation of the antibody levels raised against botulinus toxin Fragment C in mice has been difficult. In our first attempt, we developed the assay using the horse anti-bolulinum type A antibody supplied by the Army. Once positive results were achieved, we needed to try our protocol using mouse anti-botulinum antibody in order to quantitate the antibody response to the vaccine in othe vaccinated mice. Unfortunately, mouse anti-botulinum antibody is not commercially available, especially as a monoclonal. We then contacted Dr. George Doellgast at Elcatech Inc (Winston Salem, NC). He supplied our laboratory with a monoclonal mouse anti-botulinum antibody as a hybridoma supernalant (tissue culture fluid).

The next obstacle was to find out what was contributing to non-specific binding in the ELISA tests. When no antigen was present in our wells, we still had a positive, strong signal. After comparing several blocking buffers to the one which we were using, we determined that our blocking buffer (0.25% BSA in 0.05% Tween 20) was the reason for the non-specific binding. For a positive control, we used the horse anti-botulinum, since we knew that a strong positive signal could be achieved. The blocking buffer consisting of 3% gelatin in PBSN (phosphate buffered saline with 0.5% sodium azide) was found to have the least cross-reactivity without inhibiting specific binding

The current obstacle is that the signal is too weak (negative) with the monoclonal mouse anti-botulinum antibody. According to both Army personnel at Fort Detrick and Dr. Doellgast at Elcatech, it is very difficult to bind mouse anti-botulinum antibody to Fragment C. This explains the weak signal problem which was encountered. One possible explanation is that the hybridoma supernatant is not concentrated enough to produce a strong signal. Monoclonal mouse anti-botulinum antibody from ascites fluid would possibly produce a stronger signal, but again this is commercially unavailable and could not be supplied by Dr. Doellgast.

The current protocol is as follows. We coat the wells of our 96 well Immulon plates with 0.1 ml of antigen (fragment C). We used a recommended antigen concentration of 0.7 μg/ml. These plates are incubated overnight at 4°C The wells are then washed three times with 0.2 ml of PBSN to remove unbound antigen. We then block the residual binding capacity of the plate by filling each well with 0.2 ml of 3% gelatin in PBSN and incubating at room temperature for 30 minutes. The plate is washed again using the procedure described earlier.

The antibody is then added to the wells. To each well, 0.1 ml of the mouse anti-botulinum antibody was added with dilutions ranging from 0 to 1/512. We

used this wide range since the actual concentration of the antibody was unknown. The plate was then incubated for two hours at 37°C. Again, the plate was washed and blocked using the procedures described earlier, except that the blocking buffer was incubated for 10 minutes at room temperature. The plates were washed to remove the excess blocking buffer, and then 0.1 ml of our conjugate (antimouse IgG - alkaline phosphatase) was added to each well. The conjugate had dilutions ranging from 1/500 to 10,000 and then incubated for one hour at room temperature. The plates were washed for the last time, and the 0.075 ml of substrate (p-nitrophenyl phosphate) was added to each well. The plates were incubated in the dark for 30 minutes at room temperature. Using a microtiter plate reader with 405nm filter, the plates were read to measure the degree of hydrolysis.

To strengthen the signal, we have tried longer incubation times and more concentrated conjugates but have still been unsuccessful We are considenng an overnight incubation of the antibody and/or more concentrated antigen. In addition, there are some encouraging possibilities for improved ELISA methodolgy thanks to the assistance from Dr. Doellgast. He is currently working on an amplified immunoassay to determine thc mouse anti-botulinum antibody titer. He has developed an extremely sensitive technique (no background or false positives) to quantitate antibody capture to the botulinus toxin and possibly fragment C. Currently, his method is highly effective in quantitating human antibody bound to the botulinum toxin. He is now running preliminary tests using an anti-mouse conjugate and mouse antibodies to see if his method is applicable to this study.

The measured results thus far indicate a lack of sensitivity and specificity for the current ELISA protocol. The antibody levels in the present mice experiment ranged from 0.08 to 2.65 IU/ml. The antibody measuremnts from 5 mice thus far suggest that the animals are definitely producing the antibody against the botulinum toxoid, and that the initial elevation of titers within the first two weeks is followed by a dip in the following 3-6 weeks, with a second elevation at 7-8 weeks. Although at this time the 8 week samples are the longest available, the sampling is being continued to demonstrate the antibody production pattern for at least 3-4 months. The raw antibody titers determined with the current ELISA protocol are given in the Appendix.

In 1988, Siegal et. al. of the USAMRIID reported that after the primary series of 3 immunizations of botulinum pentavalent (ABCDE) toxoid administered at 0, 2, and 12 weeks in humans, 21 of 23 persons tested (91%) had a titer of type A that was greater than or equal to 0.008 IU/ml. Just before the first annual booster, 10 of 28 (48%) lacked a detectable titer for type A. It was noted that at least 8 boosters were required to maintain the titer level greater or equal to 0.25 IU/ml, according to the Centers for Disease Control. The antibody levels in the present mice experiment ranged from 0.08 to 2.65 IU/ml, and would

be fairly compared to those in humans, although the values in the present study should be re-evaluated with a refined assay system with an improved sensitivity.

Doellgast et. al. has recently developed an enzyme-linked immunosorbent assay-enzyme-linked coagulation assay (ELISA-ELCA) for the detection of antibodies to Clostridium botulinum neurotoxins A, B, and E by modifying the commercially available solution-phase complex assay for toxins A, B, and E. The addition of unlabeled polyclonal antibodies to a mixture consisting of toxin with chicken antibody and RVV-XA labeled horse antibody reduced the sensitivity of detection of neurotoxin, and this reduction in sensitivity was shown to be usable as a measure of the specific antibody titer. This newly developed ELISA-ELCA can be used as an alternative to our current ELISA if necessary.

After a 3 month period, the entire group of immunized mice were supplied to the USAMRIID for a direct challenge with botulinum toxin. That will be an unambiguous test of the efficacy of immunization produced by the encapsulated vaccine. Those results are not available at this writing.

Conclusions and Summary of the Phase I Accomplishments

1. Microsphere delivery systems were developed for encapsulating protein antigens to make time release vaccines.
2. The in vitro release kinetics of the microsphere systems were examined in vitro, though aggregation of the microspheres and diffusion of the dye used to make these measurements were complications.
3. A microencapsulated vaccine containing tetanus toxoid was made, and while measurements of the toxoid release in vitro proved difficult, the microspheres were shown to contain significant quantities of the toxoid.
4. Sterile manufacturing techniques were successfully used to prepare a vaccine using botulinum toxin C fragment, and this vaccine was used to immunize a series of adult mice at the Cleveland Clinic Foundation. ELISA measurements of the antibody levels raised in 5 of 18 mice have been carried put to 8 weeks as of this writing. These suggest an initial peak followed by a second 6-8 weeks following inoculation. These measurements are continuing.
5. A group of 20 of the immunized mice were supplied to the Army laboratories at Fort Detrick for a direct challenge with botulinum toxin. Because of a delay in shipment of the toxoid to WBI, the animal experiments were not begun until two months before the completion date of this program, and that test was not completed until after the preparation of this document. There were, however, surviving animals, so we are encouraged that this methodology of microencapsulated vaccines is a promising approach for preparing single dose vaccines.

Overall, we believe the Phase I objectives of this program have been met.

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Appendix

Type A Botulism Antibody Titer Measurements

	Pre IU/ml	1 week IU/ml	2 week IU/ml	3 week IU/ml	4 week IU/ml	5 week IU/ml	6 week IU/ml	7 week IU/ml	8 week IU/ml
Mouse I-1	0.67	1.39	0.08	1.26	0.00	1.30	0.18	0.49	1.47
Mouse II-1	0.00	2.65	0.08	0.20	0.06	0.34	0.00	0.00	0.47
Mouse III-1	1.73	0.39	0.18	0.33	0.08	0.43	0.00	0.60	0.29
Mouse IV-1	1.08	0.17	0.28	0.24	0.68	1.30	0.08	0.78	0.57
Mouse VI-1	0.12	0.33	0.00	0.65	0.03	0.00	0.28	0.37	0.54

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