AD NUMBER

ADB206724

NEW LIMITATION CHANGE

TO

Approved for public release, distribution unlimited

FROM

Distribution authorized to DoD only; Specific Authority; 6 Feb 96. Other requests shall be referred to US Army Medical Research and Material Command, Ft. Detrick, MD 21702-5012.

AUTHORITY

U.S. Army Medical Research and Materiel Command ltr., dtd January 21, 2000.

AD_____

CONTRACT NUMBER: DAMD17-95-C-5048

TITLE: Biodegradable Bioadherent Microcapsules for Orally Administered Sustained Release Vaccines

PRINCIPAL INVESTIGATOR: Cynthia L. Sheffield, Ph.D.

CONTRACTING ORGANIZATION: Lynntech, Inc.
College Station, TX 77840

REPORT DATE: 23 Oct 95

TYPE OF REPORT: Final, Phase I

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

B206724

Of 21

AD NUMBER: B206724
FIELDS AND GROUPS: 6/5, 6/13, 6/15
CORPORATE AUTHOR: LYNNTECH INC COLLEGE STATION TX
UNCLASSIFIED TITLE: BIODEGRADABLE BIOADHERENT MICROCAPSULES FOR ORALLY ADMINISTERED SUSTAINED RELEASE VACCINES. PHASE 1.
DESCRIPTIVE NOTE: ~~ANNUAL~~ REPT. 24 APR-23 SEP 95,
Final, Phase I,

PERSONAL AUTHORS: SHEFFIELD, CYNTHIA L.
REPORT DATE: OCT 23, 1995
PAGINATION: 34P MEDIA COST: $ 6.00 PRICE CODE: AA
CONTRACT NUMBER: DAMD17-95-C-5048
MONITOR ACRONYM: XA
MONITOR SERIES: USAMRMC
REPORT CLASSIFICATION: UNCLASSIFIED
LIMITATIONS (ALPHA): DISTRIBUTION AUTHORIZED TO DOD ONLY; SPECIFIC AUTHORITY; 6 FEB 96. OTHER REQUESTS SHALL BE REFERRED TO US ARMY MEDICAL RESEARCH AND MATERIAL COMMAND, FT. DETRICK, MD 21702-5012.
LIMITATION CODES: 4

Errata

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	23 Oct 95	Final, Phase I, 24 Mar 95 - 23 Sep 95

4. TITLE AND SUBTITLE
Biodegradable Bioadherent Microcapsules for Orally Administered Sustained Release Vaccines

5. FUNDING NUMBERS
DAMD17-95-C-5048

6. AUTHOR(S)
Cynthia L. Sheffield, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Lynntech, Inc.
College Station, TX 77840

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, MD 21702-5012

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION/AVAILABILITY STATEMENT
Distribution authorized to DoD Components only, Specific Authority. Other requests shall be referred to Commander, U.S. Army Medical Research and Materiel Command, ATTN: MCMR-RMI-S, Fort Detrick, Frederick, MD 21701-5012.

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*

Traditional vaccines have many characteristics which pose logistical problems for both military and civilian vaccinations programs. Currently, vaccines require several booster injections to obtain maximum protection, they are often effective against only a single organism and most require refrigeration. Biodegradable microspheres for vaccine encapsulation could eliminate many of these problems. Microencapsulated vaccines offer the promise of extended antigen presentation, thus eliminating the need for booster injections. Microencapsulation also allows incorporation of multiple antigens in a single dose. This report describes the production and *in vivo* evaluation of a new microsphere vaccine approach. Several microsphere tetanus and botulinum vaccine formulations were examined *in vivo* using a BALB/c mouse model. Several formulations of microencapsulated vaccines provided both an elevated and prolonged serum IgG response. At 9 weeks post injection, the microsphere vaccine evidenced 47% of its initial (2 week) serum IgG levels, without a booster injection. This study has demonstrated that this biodegradable microsphere antigen delivery system has the potential for prolonged protection. The vaccine delivery system described in this report, if successfully developed, will benefit military and civilian health efforts and could be marketed around the world.

14. SUBJECT TERMS
Vaccines, Microencapsulation, Oral Administration, Biodegradable, Bioadherent, Fasciola, Recombinant

15. NUMBER OF PAGES
33

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Limited

GENERAL INSTRUCTIONS FOR COMPLETING SF 298

The Report Documentation Page (RDP) is used in announcing and cataloging reports. It is important that this information be consistent with the rest of the report, particularly the cover and title page. Instructions for filling in each block of the form follow. It is important to ***stay within the lines*** to meet ***optical scanning requirements***.

Block 1. Agency Use Only *(Leave blank)*.

Block 2. Report Date. Full publication date including day, month, and year, if available (e.g. 1 Jan 88). Must cite at least the year.

Block 3. Type of Report and Dates Covered. State whether report is interim, final, etc. If applicable, enter inclusive report dates (e.g. 10 Jun 87 - 30 Jun 88).

Block 4. Title and Subtitle. A title is taken from the part of the report that provides the most meaningful and complete information. When a report is prepared in more than one volume, repeat the primary title, add volume number, and include subtitle for the specific volume. On classified documents enter the title classification in parentheses.

Block 5. Funding Numbers. To include contract and grant numbers; may include program element number(s), project number(s), task number(s), and work unit number(s). Use the following labels:

C	- Contract	**PR**	- Project
G	- Grant	**TA**	- Task
PE	- Program Element	**WU**	- Work Unit Accession No.

Block 6. Author(s). Name(s) of person(s) responsible for writing the report, performing the research, or credited with the content of the report. If editor or compiler, this should follow the name(s).

Block 7. Performing Organization Name(s) and Address(es). Self-explanatory.

Block 8. Performing Organization Report Number. Enter the unique alphanumeric report number(s) assigned by the organization performing the report.

Block 9. Sponsoring/Monitoring Agency Name(s) and Address(es). Self-explanatory.

Block 10. Sponsoring/Monitoring Agency Report Number. *(If known)*

Block 11. Supplementary Notes. Enter information not included elsewhere such as: Prepared in cooperation with...; Trans. of...; To be published in.... When a report is revised, include a statement whether the new report supersedes or supplements the older report.

Block 12a. Distribution/Availability Statement. Denotes public availability or limitations. Cite any availability to the public. Enter additional limitations or special markings in all capitals (e.g. NOFORN, REL, ITAR).

- **DOD** - See DoDD 5230.24, "Distribution Statements on Technical Documents."
- **DOE** - See authorities.
- **NASA** - See Handbook NHB 2200.2.
- **NTIS** - Leave blank.

Block 12b. Distribution Code.

- **DOD** - Leave blank.
- **DOE** - Enter DOE distribution categories from the Standard Distribution for Unclassified Scientific and Technical Reports.
- **NASA** - Leave blank.
- **NTIS** - Leave blank.

Block 13. Abstract. Include a brief *(Maximum 200 words)* factual summary of the most significant information contained in the report.

Block 14. Subject Terms. Keywords or phrases identifying major subjects in the report.

Block 15. Number of Pages. Enter the total number of pages.

Block 16. Price Code. Enter appropriate price code *(NTIS only)*.

Blocks 17. - 19. Security Classifications. Self-explanatory. Enter U.S. Security Classification in accordance with U.S. Security Regulations (i.e., UNCLASSIFIED). If form contains classified information, stamp classification on the top and bottom of the page.

Block 20. Limitation of Abstract. This block must be completed to assign a limitation to the abstract. Enter either UL (unlimited) or SAR (same as report). An entry in this block is necessary if the abstract is to be limited. If blank, the abstract is assumed to be unlimited.

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the US Army.

(N/A) Where copyrighted material is quoted, permission has been obtained to use such material.

(N/A) Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

(X) Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

( ) In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

( ) For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

( ) In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

( ) In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

(X) In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

9/30/95

PI - Signature Date

TABLE OF CONTENTS

SECTION I
INTRODUCTION

A. Objectives

The aim of the SBIR Phase I was to determine the feasibility of using a recombinant *Fasciola* protein in conjunction with microencapsulation techniques, as the basis for a biodegradable, subcutaneously administered vaccine which would provide extended antigen release characteristics.

B. Background

1. Microencapsulation of Vaccines

Traditional parenteral vaccines have many limitations which pose problems for both military and civilian vaccination programs. One of the major limitations of current vaccines is that in order to obtain maximum protection repeated booster injections are generally required. Further, most vaccine formulations have a short shelf-life and require refrigeration. New vaccine delivery systems are needed which alleviate these problems. Development of a vaccine delivery system which can provide controlled prolonged antigen presentation to the immune system, thus eliminating the need for repeated booster injections would eliminate many of the logistical problems currently faced by the military. Additionally, a vaccine that could be administered via several different routes would add to the flexibility required by both military and large scale civilian vaccination programs.

Biodegradable microencapsulated vaccines have tremendous potential for both military and large scale civilian immunization programs such as the Children's Vaccine Initiative (CVI) sponsored by the WHO, Unicef, United Nations Development Fund, the Rockefeller Foundation, and the World Bank.[1] The military and the CVI are both seeking affordable vaccines that require fewer doses (i.e. exhibit extended antigen presentation characteristics), can be combined in novel ways to incorporate more antigens in a single dose (multivalent) and will remain stable at tropical temperatures.[1]

Subcutaneous administration of microencapsulated antigens activates the humoral arm of the immune system resulting in increased levels of serum IgG. Additional studies have shown that sustained antigen release from non-biodegradable implants resulted in an enhanced immune response for over 6 months.[2] The major drawback of the implant system is that both the required implantation and the subsequent removal of the implant are highly invasive. Biodegradable microencapsulated systems that can be subcutaneously administered have at least two advantages over these non-biodegradable implants: a) they eliminate the need for invasive implantation and removal, and b) they can be more readily designed to release antigen in peaks or bursts over a 1 year interval, providing a more 'natural' sustained release pattern than that obtained by implants.[2] Microencapsulation has been shown to reduce the dose of antigen that is required to achieve high level humoral immunity.[3]

Further, the microencapsulation of antigens stabilizes the vaccine, resulting in prolonged shelf life and a reduced dependence on refrigeration.

Microcarriers (microcapsules or microspheres) are described as a core material (i.e., antigens) encased or entrapped in a specialized coating. The technology of microencapsulation has found widespread application especially in the pharmaceutical area, such as coating drugs to extend or delay their release, or to target their release to a specific area of the body.[4]

Among the proteins routinely used for microencapsulation are albumin and gelatin. [4] Although each has been widely used, neither is naturally produced as a microencapsulating agent. The materials which we have chosen for capsule formation in this project are recombinant proteins representing natural composites produced by the helminth *Fasciola hepatica* for biological microencapsulation. The natural microcapsule can withstand treatment with strong acids and bases, heat, light, desiccation and numerous proteases; in addition, the shell material and its precursors are poorly antigenic.

2. *Fasciola hepatica* Eggshell Proteins

Fasciola hepatica is a digenetic trematode which encapsulates its eggs in a proteinaceous shell. The shells are a prototypical microencapsulating system that protects *Fasciola* eggs from the host natural defenses while allowing uptake of essential nutrients and release of metabolic products. The long-term stability of this natural composite material results from crosslinks formed by the quinone tanning or sclerotization[5] of specialized eggshell proteins in which tyrosine residues have been post-translationally modified to 3,4-dihydroxyphenylalanine (DOPA).[6-16] DOPA proteins occur rarely in nature, the most studied of all these materials is the muscle adhesive protein secreted by the common blue mussel *Mytilus edulis* [17-21]. Figure 1 shows the adhesive precursor, a 130,000 mw glycoprotein which contains 75 to 80 repeated decapeptide sequences in its primary structure. The repeating decapeptide is Ala-Lys-Pro-Ser-Tyr-Hyp-Hyp-Thr-Dopa-Lys [17-21].

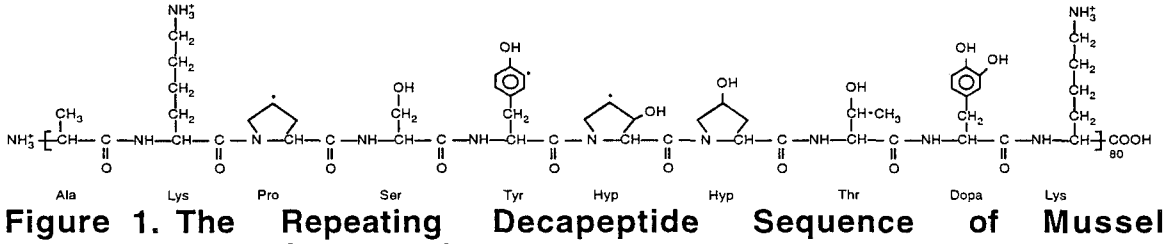

Figure 1. The Repeating Decapeptide Sequence of Mussel Adhesive Protein

The three major protein constituents of the *Fasciola* eggshell are vitelline protein A, B, and C, which are 17, 31 and 70 kDa in size respectively. All three are rich in glycine and DOPA. The 31kDa vitelline protein B (vpB), which will be used in the present microencapsulation studies, has been purified and characterized in detail [5-16]. The protein has a isoelectric point (pI) of 9.0 and constitutes approximately 7% of the protein in adult *Fasciola hepatica*. Ten per cent of the amino acid residues of the protein are DOPA residues which are

further modified during shell crosslinking *in vivo* or upon treatment with mushroom polyphenol oxidase *in vitro*. All three major protein precursors of the shell, vitelline proteins A, B and C (vpA, vpB, vpC)[5-16] have been purified and characterized; in addition cDNAs encoding two variants of the major eggshell component have been sequenced and expressed as recombinant proteins. The proteins encoded consist of highly degenerate repeats of a hexapeptide enriched in glycine and containing clusters of basic amino acid residues as well as clusters of acidic residues.[6]

These vitelline proteins are very similar to the DOPA proteins found in the common blue mussel *Mytilus edulis* .[17-21] The unique composition of these proteins facilitates the spread of these materials over interface surfaces. Further

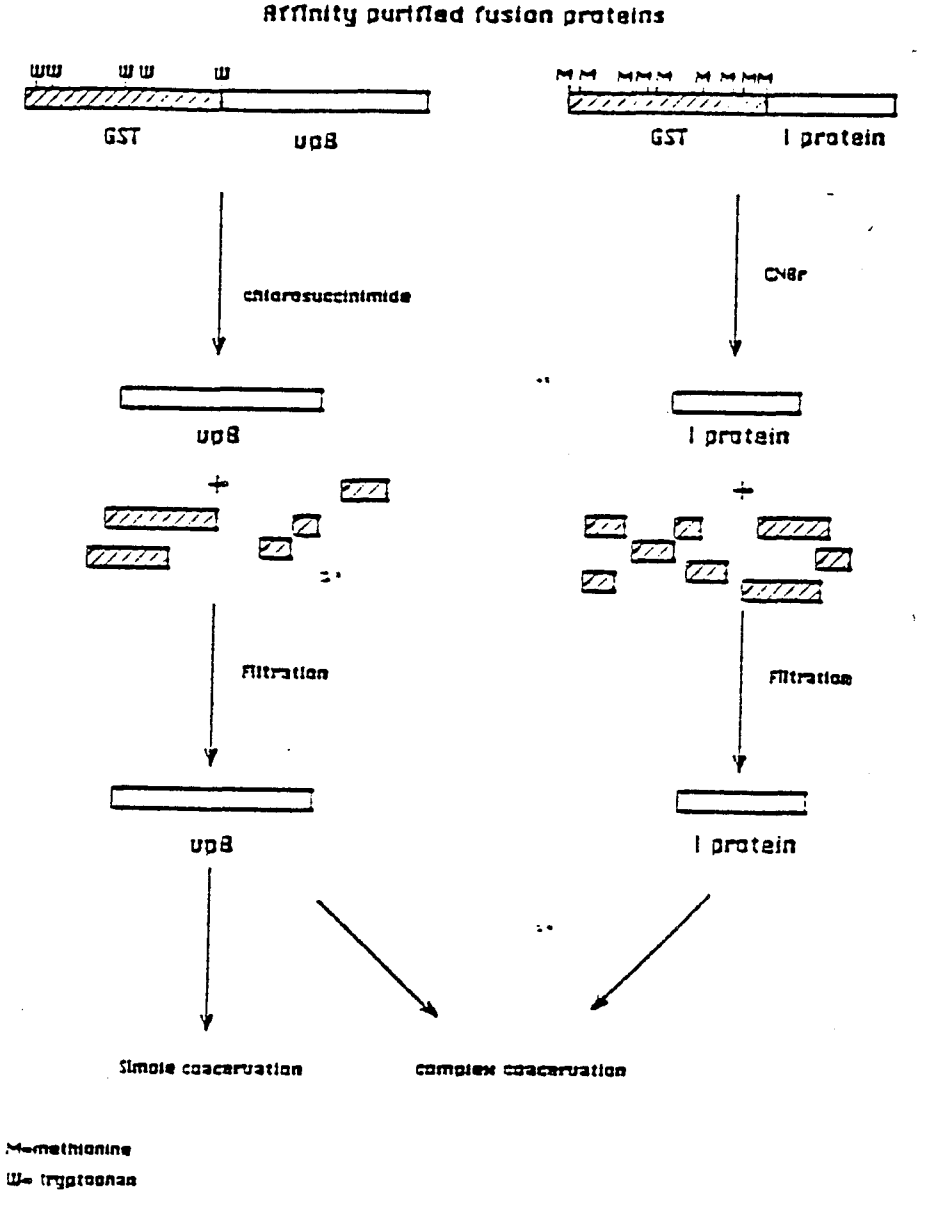

Figure 2. Cleavage and filtration of affinity purified *Fasciola* recombinant fusion proteins.

these proteins are capable of a large number and range of chemical interactions with other materials as well as within the protein itself (i.e., covalent crosslinking)[17-21].

The eggshell protein vitelline B (vpB) has been produced in *E. coli* from cloned *F. hepatica* genes. The vpB, the most abundant structural protein of the eggshell has a very basic isoelectric point (pI=9.0), combining it with a protein with an acidic isoelectric point such as bovine serum albumin (pI-5.4) it could drive coacervation through charge neutralization.

The gene encoding the vpB protein has been introduced into the pGEX expression vector described by Smith and Johnson [22] which facilitates protein production and purification. This vector allows production of a (glutathione-S-transferase(GST))-*Fasciola* protein fusion product with a proteolytic cleavage site at the junction of the two (Figure 2). The product will be affinity purified on glutathione beads and N-chlorosuccinimide[22] will be used to produce quantitative removal and degradation of GST from the fusion protein; addition of methionine to the reaction mixture will prevent the conversion of methionine in the protein to methionine sulfoxide. The use of filtration based on size will remove all organic reagents as well as the contaminating GST degradation products. The vpB recombinant protein has been produced and purified at a level of 9 mg/liter of *E. coli* culture.

An alternative approach to the production and purification of the recombinant *Fasciola* proteins would be to transfer the vpB gene into the newly available *Pichia* yeast expression vector system (Invitrogen, Inc., San Diego, CA). This yeast system has several advantages over the *E. coli* expression system. Production by this vector has been reported to exceed *E. coli* expression system production levels by as much as 1000 fold. Further, the protein of interest is secreted directly into the media eliminating the need for time consuming and expensive purification scheme required by the GST–*E. coli* expression system described above. In addition, the yeast system is well-suited for scale-up and thus can take advantage of large batch fermentation technology.

3. Methods for Microencapsulation

Numerous methods exist for the production of microcapsules and microspheres. Some of the more common techniques include: solvent evaporation or emulsion polymerization; phase-separation coacervation; spray-drying; film-casting and air-suspension coating.[2, 23-28] The low antigen loading required by vaccines, generally <1% by weight, is easily achieved by many of these techniques.

Phase separations are commonly induced by: a) manipulation of temperature, pH, salt or alcohol concentrations; b) addition of incompatible polymers; c) liquid coacervate formation; and d) congealing or denaturation in oil emulsions.

The phase separation coacervation techniques utilize solubilized polymers and antigens which are mixed with salts, non-solvents or with an incompatible second polymer resulting in a separation of the first polymer phase engulfing the antigen particles (Figure 3). This approach is limited to antigens that remain insoluble in the organic solvent used to dissolve the first polymer. A higher degree of antigen loading can be achieved using this technique, and larger particles can be formed than are possible with the solvent evaporation technique.[23-25]

The air-suspension coating technique utilizes reservoir microcapsules formed by coating particles or droplets of antigen in copolymer while they are suspended in an air stream. This technique is limited to the production of microcapsules that are >50μm in size; particles smaller than this adhere together in the air flow.[128] All of these techniques have advantages for different applications; however, for this work, the techniques of emulsion polymerization and phase separation were applied.

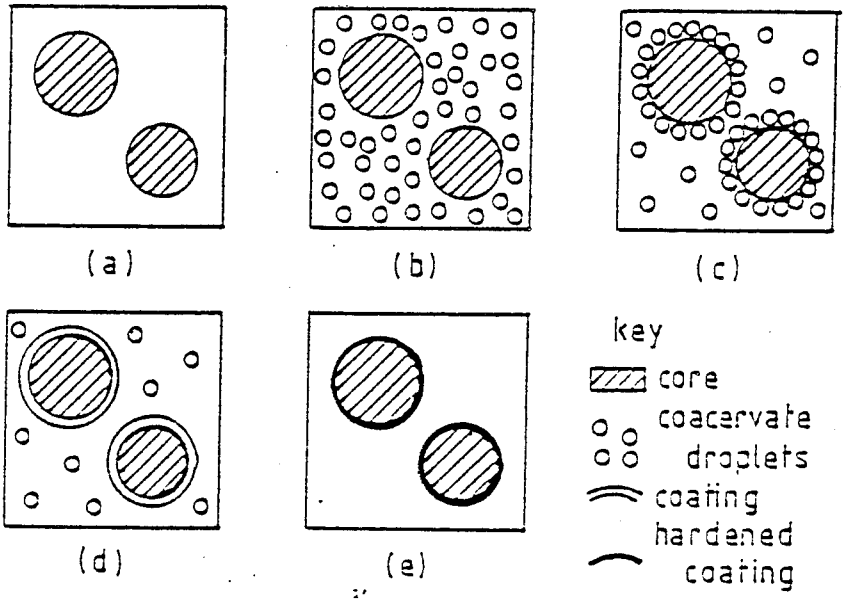

Figure 3 Typical steps in a coacervation method of microencapsulation. (a) Core particles dispersed in solution of polymer by agitation. (b) Coacervation visible as droplets of colloid-rich phase induced by one or more agents. (c) Deposition of coacervation droplets on surface of core particles. (d) Mergence of coacervation droplets to form the coating. (e) Shrinkage and crosslinking of the coating to stabilize it as necessary.

Proteins have enjoyed extensive use as encapsulating agents, [23] and so there exist numerous protein specific protocols exist which may be applied to vitelline protein microencapsulation. Many of these approaches involve the induction of a phase separation in a mixture of core material (liquid or solid) and

encapsulating agent (polymethyl 1 methacaylate) such that the core is efficiently engulfed by the encapsulating agent, usually followed by stabilization of the microcapsule walls by low molecular weight cross-linking agents such as glutaraldehyde (Figure 3).

The emulsion polymerization method for the production of microcarriers utilizes low levels of harsh solvents (i.e., acetone, chloroform, and toluene), and should therefore prove more acceptable in the current regulatory climate. In the emulsion polymerization method, the microcarriers will be prepared by the formation of an emulsion containing the microcarrier constituent protein and a vegetable oil. This emulsion will be formed by stirring the two components (protein and oil) together at speeds ranging from 1000-2000 rpm. The other parameters involved with this method which effect the overall quality of microcarriers produced include: duration of emulsion mixing time, ratio of constituent proteins, antigen loading level, duration and concentration of stabilization step. Another major factor that effects the quality and size of microcarriers produced by this system is the viscosity of the oil used to form the emulsion. Three varieties of vegetable oil (olive, soybean, and peanut) which exhibit high, medium and low viscosity, respectively, will be tested.[29]

C. Scope

The long term goal of this project is the development of a supervaccine that will offer prolonged protection against several etiologic agents with a single dose. In order to produce such a supervaccine, several obstacles must be overcome. The most important task will be the identification of a vaccine delivery system that provides sequestration of antigen along with long term antigen presentation and enhanced recruitment of the antigen presenting cells of the immune system.

Numerous issues must be resolved before this supervaccine can be developed. The fundamental questions involved in this research include: how best can the *in vivo* sequestration of antigen be accomplished; what type of vaccine system can provide slow biodegradation, extended antigen release, exhibit biocompatible characteristics and offer a carrier with low antigenic properties; and finally, can this system be used to incorporate multiple antigens in a single product?

Microencapsulation offers great promise as a means of resolving many of these issues. Current efforts involving development of microencapsulated vaccines have concentrated on adapting biocompatible synthetic polymers, many of which were originally developed for purposes, other than vaccine delivery[2]. Many microencapsulation techniques have utilized various proteins (i.e., bovine and human serum albumin) as the basis for making microcarriers however, these efforts have not yet resulted in a vaccine delivery system that exhibits the desired characteristics[1,2].

This project has taken a new approach to the development of a vaccine delivery system capable of providing satisfactory solutions to the fundamental issues involved in the production of a supervaccine. At the core of this new

approach is the recombinant form of a *Fasciola* eggshell protein, which is a natural microencapsulation agent used by the helminth to protect its eggs within a hostile environment. It offers several advantages over microencapsulating materials in current use, such as synthetic polymers and gelatin (see Table 1).

For these reasons, this material was selected as the basis for the development of a microcarrier that would provide the characteristics deemed critical to the production of a supervaccine. In order to enhance the production of *Fasciola* eggshell material for encapsulation, the vpB gene was transferred to a newly available *Pichia* yeast expression vector system (Invitrogen, Inc., San Diego, CA). The yeast system is superior to the current bacterial expression system in several ways. Yields reported for this vector exceed current production levels in bacteria by 100-1000 fold and the product is secreted into the media obviating the need for extensive affinity purification. In addition, the yeast system is well-suited for scale-up and thus can take advantage of large batch fermentation technology.

Table 1. Benefits of Recombinant Eggshell Protein as Encapsulating Agent

- This protein offers a slow rate of biodegradation, bioadherent capabilities, and responsiveness to gentle crosslinking techniques.
- As recombinant proteins they can be obtained in bulk with uniform and defined characteristics.
- The protein compositions can be genetically engineered to vary the density of tyrosine/DOPA residues, and so control the cross-linking characteristics and the resultant porosity and stability of microcapsules.
- The cross-linking agent is already integrated into the protein and only needs to be oxidized, either spontaneously or with easily separable reagents, to initiate curing.
- The vitelline proteins are poorly antigenic.
- Though durable, cured protein is (ultimately) biodegradable and non-toxic.

The objectives of the SBIR Phase I were to determine the feasibility of using a recombinant *Fasciola* protein in conjunction with microencapsulation to produce a vaccine for subcutaneous administration. A highly focused *in vivo* evaluation approach was used to rapidly assess key issues such as : a) is the recombinant *Fasciola* protein a suitable microcarrier material; b) will microcarriers composed of *Fasciola* protein and antigen be biocompatible; c) will these microcarriers have the ability to sequester antigens *in vivo*; and, d) will the microcarriers produce a favorable antibody response. The results of this study are given in the following sections.

As a result of the progress made during Phase I, a clear pathway for expanding the scope of this technology has been established and specific

technical objectives have been identified to enable scale up production and validation of immune response characteristics. This report describes the research results and the proposed direction of future research and development.

SECTION II
METHODS

A. Production; Purification; And, Quantitation of the Recombinant *Fasciola* Protein from the *Pichia* Yeast Expression System

The vpB vitelline protein cDNA was transferred into three *E. coli* shuttle vectors, in order to determine which vector would yield the optimal protein expression and secretion characteristics. The vpB cDNA sequence was inserted in each case into the EcoRI site of the respective vector, transformed into the *E. coli* strain TOP10F' and all constructs were sequenced to determine proper reading frame and orientation.

Figure 4 outlines the transfer of vpB constructs into the *Pichia* vectors, this produced thousands of vpB-producing yeast colonies. Each correct construct was transferred to *Pichia* by first linearizing the plasmid DNA and then transformed into the yeast by electroporation at 0.75 kV for 8 milliseconds. The constructs were integrated into the genome based on homology with the yeast alcohol oxidase (AOX) or histidine (his 4) loci and also upon the site of linearization of the plasmid since DNA ends promote recombination in yeast. Each plasmid was integrated at two genomic locations and protein production in yeast determined. Yeast containing pPIC9 inserted at the his 4 locus in one case and at the AOX locus in a second case were identified by plating on selective media. Genomic integrity for each clone, of which there were several hundred, was verified by using a combination of polymerase chain reaction (PCR) amplification followed by electrophoretic sequence analysis. Yeast constructs containing phil-S1 integrated at the two locations were similarly selected and verified.

The selected yeast constructs were cultured in Buffered Methanol-complex Medium (BMMY) (*Pichia* Expression Kit, Invitrogen Corp., San Diego, CA) at 24°C on a rotary shaker to facilitate fermentation. Methanol was added to a final concentration of 0.5% enhance alcohol regulated protein expression. The cultures were assayed over a 5 day time course to determine the peak amounts of vpB protein secreted into the surrounding media. The vpB protein was concentrated through ammonium sulfate fractionation and centrifugal assisted membrane filtration. Protein concentration was determined using a BCA (Pierce Inc., Rockford, IL) colorimetric protein assay. Prior to use as a microcarrier constituent, the vpB was dialyzed against PBS-7 and concentrated using centrifugal assisted membrane filtration.

B. Production of Microcarriers

An emulsion polymerization technique was used to prepare all microcarriers.[29] All mixing procedures were conducted inside of a laminar flow

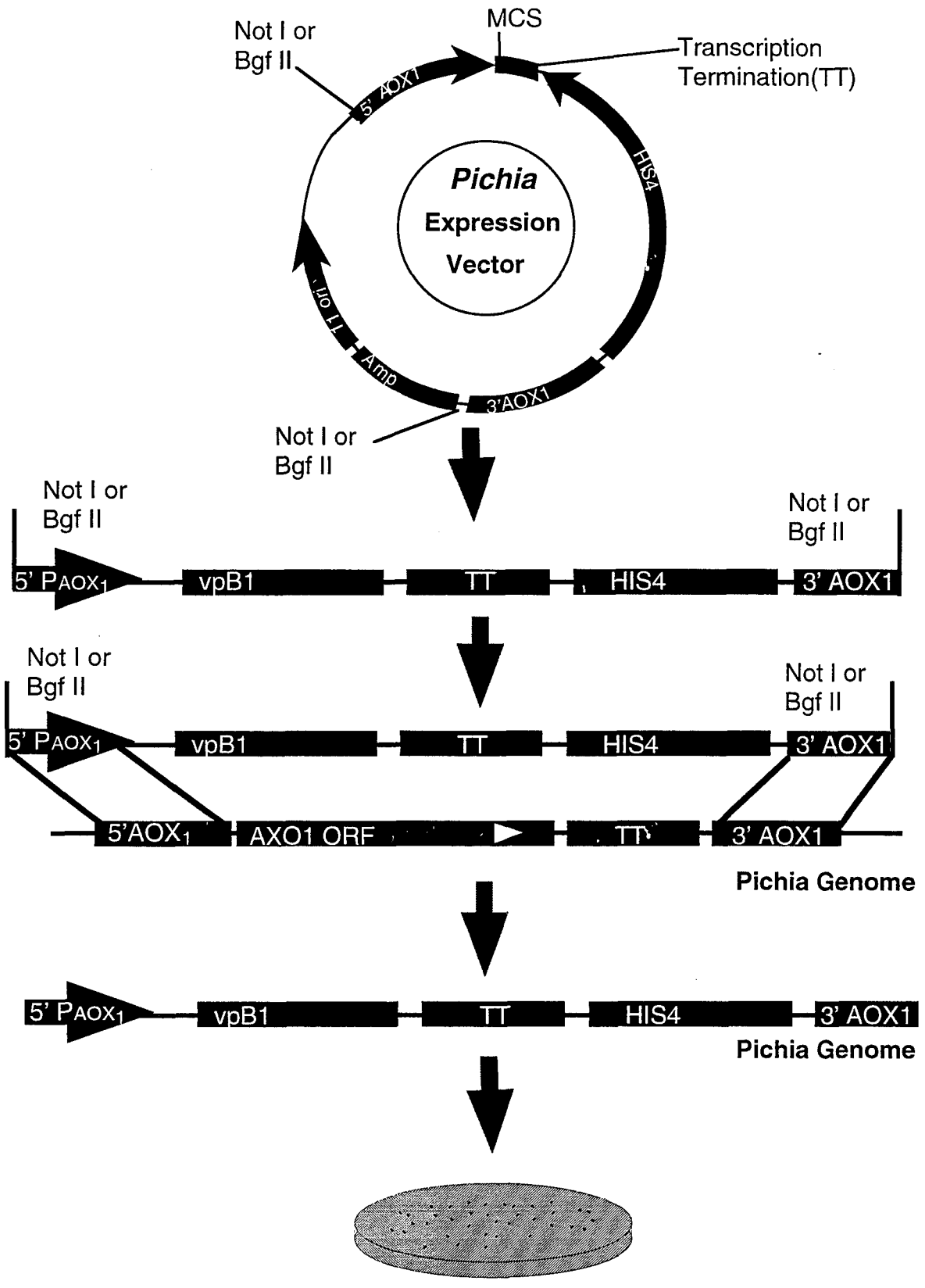

Figure 4. Transfer of vpB Construct into the *Pichia* Expression Vector.

hood. All glassware, spatulas, and the mixing propeller were baked for 2 hours at 180°C, and all dilution solutions were filtered at 0.45 µm to reduce potential contamination. Phosphate buffered saline pH 7.0 (PBS-7) was used to dilute or suspend all proteins used in the preparation of the microcarriers. The emulsions were prepared using a variable speed mixer (Barant Model 700-5400, Barant Company, Barrington, IL) equipped with a four blade fixed angle shaft mounted propeller.

Briefly, 100 ml of oil was mixed at 1200 ± 100 rpm in a 300 ml tall form beaker for 30 minutes prior to the addition of the protein solution. The oil : protein solution ratio was 100 ml : 2 ml. The concentration of the protein solution was 125 mg/ml. The protein solution was added drop wise to the mixing oil. When addition of the protein solution was completed, the emulsion was mixed at the same speed for a further 30 minutes. The microcarriers were stabilized by the addition of 100 µl of a 25% aqueous glutaraldehyde (electron microscopy grade, Sigma Chemical Comp., Inc., St. Louis, MO) yielding a final concentration 0.025%. After addition of the glutaraldehyde, the emulsion was mixed for an additional 15 minutes. When mixing was complete, the microcarrier–oil emulsion was poured into two sterile 50 ml conical bottom disposable centrifuge tubes, and centrifuged at 2000 X g for 5 minutes. The oil was decanted and the microcarrier pellet was resuspended in 40 ml of diethyl ether (Sigma Chemical Comp., Inc., St. Louis, MO). This microcarrier ether suspension was vortexed at maximum speed until the pellet was completely dispersed. The microcarrier suspension was allowed to settle for 2 minutes, then the oil–ether layer was aspirated. The microcarriers were washed in this manner a minimum of 3 and a maximum of 5 times. The microcarriers were air dried for a minimum of 48 hours prior to resuspension in PBS-7. The microcarriers were sonicated in PBS-7 for 20 minutes to facilitate resuspension.

The effect of oil viscosity and composition on microcarrier configuration was examined by employing a high viscosity olive oil (Sigma Chemical Comp., Inc., St. Louis, MO), a medium-high viscosity soybean oil (Kroger Co., Inc., Cincinnati, OH), a medium viscosity rapeseed oil (Lou Ana Foods, Inc., Opelousas, LA) and a low viscosity peanut oil (Lou Ana Foods, Inc., Opelousas, LA) as well as a tissue culture grade mineral oil (Sigma Chemical Comp., Inc., St. Louis, MO) for the emulsion polymerization technique.

Five antigen containing and one non-antigen containing microcarrier formulations were prepared for both physical characterization and *in vivo* testing. Microcarrier formulations were composed of several combinations of either recombinant tetanus toxin C-fragment (TT) (Boehringer Mannheim Biochemicals, Inc., Indianapolis, IN or Calbiochem-Novabiochem Corp., San Diego, CA) or recombinant botulinum toxin C-fragment (Bot) (USAMRIID), and bovine serum albumin fraction V (Sigma Chemical Comp., Inc., St. Louis, MO), and recombinant vpB *Fasciola* protein (prepared for this project by Dr. Allison Rice-Ficht, TAMU). As a control, 0.300 mg of each antigen (TT or Bot) was crosslinked by the addition of 100 µl of 25 % aqueous glutaraldehyde (electron microscopy grade, Sigma Chemical Comp., Inc., St. Louis, MO). The excess glutaraldehyde was removed by dialysis against PBS-7 prior to injection.

Table 2 gives the respective amounts of the protein constituents expressed as mg/100 ml of oil for the microcarrier formulations evaluated. Formulation I (vpB–BSA–TT) contained the recombinant *Fasciola* protein, bovine serum albumin and recombinant tetanus toxin C-fragment. Formulation 2 (BSA–TT) was composed of only bovine serum albumin and recombinant tetanus toxin C-fragment. Formulation 3 (vpB–BSA–Bot) was composed of the recombinant *Fasciola* protein, bovine serum albumin and recombinant botulinum toxin C-fragment. Formulation 4 (BSA–Bot) was composed of bovine serum albumin and recombinant botulinum toxin C-fragment. Formulation 5 (vpB–Bot) was composed of recombinant *Fasciola* protein and recombinant botulinum toxin C-fragment. Formulation 6 (vpB–BSA) was composed of only recombinant *Fasciola* protein and recombinant botulinum toxin C-fragment.

Table 2. Composition of Microcarrier Formulations.

Tetanus Microcarriers

		Formulation (mg/100ml of oil)		
		vpB	**BSA**	**Antigen**
Formulation I	(vpB-BSA-TT)	32.0	218.0	0.30
Formulation II	(BSA-TT)	–	250.0	0.30

Botulinum Microcarriers

		Formulations (mg/100ml of oil)		
		vpB	**BSA**	**Antigen**
Formulation 3	(vpB-BSA-Bot)	32.0	218.0	0.30
Formulation 4	(BSA-Bot)	–	250.0	0.30
Formulation 5	(vpB-Bot)	250	–	0.30

Non-Antigen Containing Microcarriers

		Formulation (mg/100ml of oil)		
		vpB	**BSA**	**Antigen**
Formulation 6	(vpB-BSA)	32.0	218.0	–

C. *In Vivo* Evaluations

Male BALB/c mice were purchased through Texas A & M Laboratory Animal Research Resources (LARR) and housed at this facility. Mice were housed 5 per cage, given ready access to standard laboratory mouse chow and water. All animals were allowed to acclimate to their surrounding for a minimum of 1 week prior to treatment. Five animals per test group were used for all test series. Each animal was given a subcutaneous injection distal to the head just between the shoulder blades, using a 26 G 1/2" needle attached to a 1 cc tuberculin syringe. Animals receiving microcarriers were given a 100 µl injection which contained 0.3 µg of antigen. Each control animal received a single 50 µl injection which contained 60 µg of crosslinked antigen either recombinant tetanus or botulinum toxin C-fragment.

Mice were bled via the tail at two week post-injection intervals. The tail of the mice were rinsed thoroughly with alcohol, then a small portion of the distal end of the tail was removed. The tail was then "milked" and the droplets of blood were collected into heparinized microcapillary tubes. The blood collected was used to monitor the animals hematocrit and the serum from the sample was used for determining antibody titers, both of these procedures are explained in detail in the sections below.

D. Enzyme Linked Immunosorbent Assay (ELISA)

Ninety-six well microtiter plates (Nuncelon, Inc.) were coated with either 2.5 µg/well of recombinant tetanus toxin C-fragment or 2.2 µg/well of recombinant botulinum toxin C-fragment. Antigens were diluted in carbonate buffer (pH 9.6). Plates containing antigen were incubated at 37 °C for one hour to facilitate binding. Upon completion of binding, the antigen solution was discarded and plates were washed (5X) with phosphate buffered saline (pH 9.0) (PBS-9). Washed plates were inverted and tapped on absorbent toweling to remove excess PBS-9.

Nonspecific binding sites on the well surfaces were blocked by the addition of a 3% nonfat dry milk (NFDM) in carbonate buffer solution. The plates were incubated with blocking solution at room temperature for 1.5 hours. The blocking solution was then discarded and the plates were washed as previously described.

Serum samples collected from test animals (1° AB) were diluted with carbonate buffer initially 1:10, then a 2-fold serial dilution was performed in the wells of the microtiter plate across 10 wells resulting in a maximum dilution ratio of 1:10,240. Plates containing the 1° AB dilution were incubated at 37 °C for one hour. The 1° AB solution was then discarded and the plates were washed as before.

A goat anti-mouse IgG (whole molecule) alkaline phosphatase conjugate (Sigma Chemical Company, Inc.) diluted 1:10,000 with assay buffer (0.03% NFDM in PBS-7) was used as the secondary antibody (2° AB). One hundred microliters of 2° AB was added to each well of the microtiter plate, and

incubated at 37 °C for 1 hour. The 2° AB solution was discarded and plates were washed (5X) in a 0.5% TWEEN-20 (Sigma Chemical Company, Inc.) distilled water solution and then given a single distilled water rinse. Plates were dried as described above.

The substrate p-Nitrophenyl Phosphate was chosen for the detection of alkaline phosphatase activity. Each well received 100 µL of p-NPP substrate (Sigma Chemical Company, Inc.) prepared according to package instructions. The alkaline phosphate substrate color development reaction proceeded at room temperature for 45 minutes. Absorbence was then measured at 405 nm using a BioRad Model 3550 microplate reader interfaced with a Power Mac.

E. Histology of Injection Site Tissue

At 3 days post treatment, a tissue sample was collected from the injection site of an animal which received a subcutaneous vpB-BSA-TT injection. The tissue samples were fixed in 10% neutralized buffered formalin for 24 hours. Tissues were processed and embedded using standard histological methods. Sections were cut at a thickness of 5µm and stained with Hematoxylin® Mayer's formulation (Sigma Chemical Company, Inc.) and counter stained with Eosin Y aqueous (Sigma Chemical Company, Inc.). Stained sections were examined microscopically and representative photomicrographs were taken.

F. Hematology — Hematocrit

Heparinized microcapillary tubes were used to collect blood samples (120 µl) via tail bleeding at 2, 3, 5, 9, and 11 weeks post treatment. Microcapillary tubes were centrifuged at 11,500 rpm for 5 minutes using an Autocrit® Ultra 3 centrifuge. The hematocrit value was determined using a Spiracrit® capillary tube reader.

SECTION III
RESULTS

A. Production; Purification; and, Quantitation of the Recombinant *Fasciola* Protein from the *Pichia* Yeast Expression System

Transfer and integration of the vitelline protein gene containing plasmid into the *Pichia* yeast genome produced thousands of potential vpB producing yeast colonies. Each of these colonies were analyzed for the presence of the integrated vpB gene. In addition the protein expression level of each vpB producing yeast colony was assayed over a 5-day time course. Three constructs that secreted acceptable levels of vpB were identified. The phil-SI construct inserted at the AOX locus was found to secrete vpB at an average of 50 mg / liter of yeast culture. The phoA construct inserted at the histidine locus secreted vpB at a level of 100 mg / liter of yeast culture. A third construct pPIC9 secreted vpB at 250.0 mg / liter of yeast culture. The single band in lanes 2 and 4 of the polyacrylamide gel shown in Figure 5 clearly demonstrates the extreme purity of the vpB used in the microcarriers.

B. Production of Microcarriers

Typical BSA microcarriers produced using olive (*Olea europaea sativa*), soybean (*Glycine soja*) and peanut (*Arachis hypogaea*) oils to form the emulsion are seen in Figure 6a-c. These photomicrographs, taken at a magnification of 1000X, clearly show that the microcarriers formed in olive oil (6a) have a denser wall structure than evidenced by the microcarriers formed in either soybean (6b) or peanut (6c) oil. In addition, the wall of the microcarriers formed in peanut oil (6c) appear to have buckled, therefore, these microcarriers were unable to withstand the extensive ether wash process.

While there is relatively little difference in the density of these three oils, there is a major difference in their composition. Of the oils tested, peanut oil was the most highly unsaturated, while olive oil was the least highly unsaturated. In peanut oil oleic acid represents 56% of the unsaturated fatty acids, more importantly, oleic acid represents 82% of the unsaturated fatty acids found in olive oil. The combination of a lower level of unsaturated bonds combined with the increased acid value of the high oleic acid content may account for the ability of the olive oil to facilitate the formation of microcarriers with a denser wall structure. Based on these observations, the olive oil was used for microcarrier production for the duration of the research.

The photomicrographs in Figure 7 a-c show microcarriers at a magnification of 1000X. Figure 7a shows microcarriers produced using Formulation 2. These microcarriers were microscopically indistinguishable form the microcarriers yielded by Formulation 4 (not shown). Additionally, the microcarriers seen in Figure 6a were microscopically identical to the non-antigen containing microcarriers shown in Figure 6a. This demonstrates that the addition of antigen does not affect the basic structure of the microcarriers.

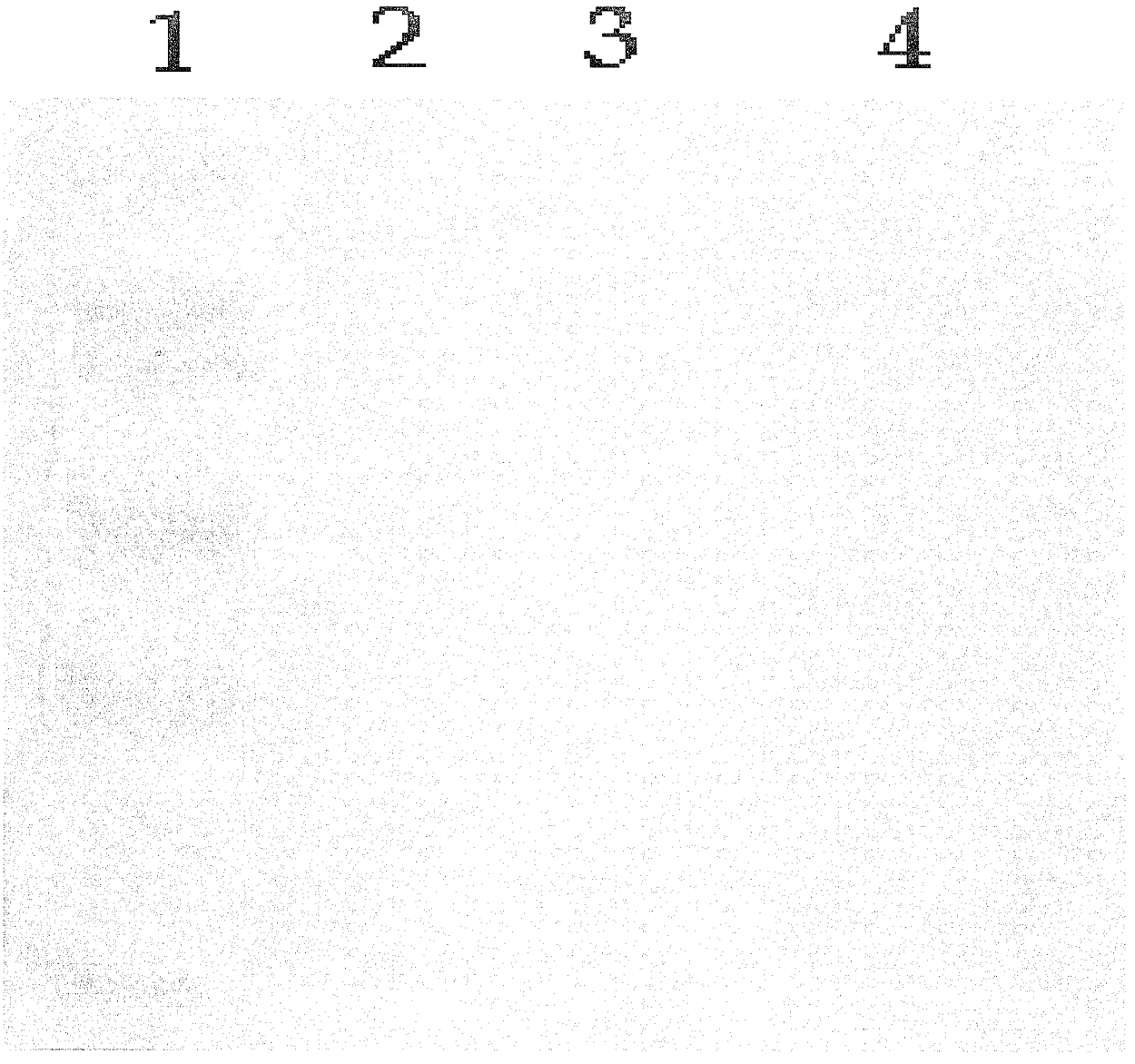

Figure 5. Polyacrylamide Gel Electrophoretic Analysis Of Purified vpB From Yeast Constructs, Stained With Coomassie Brilliant Blue R. Lane 1 — Molecular Weight Size Standards, Lane 2 — Purified vpB (2 μg), Lane 3 — No material, Lane 4 — Purified vpB (4 μg).

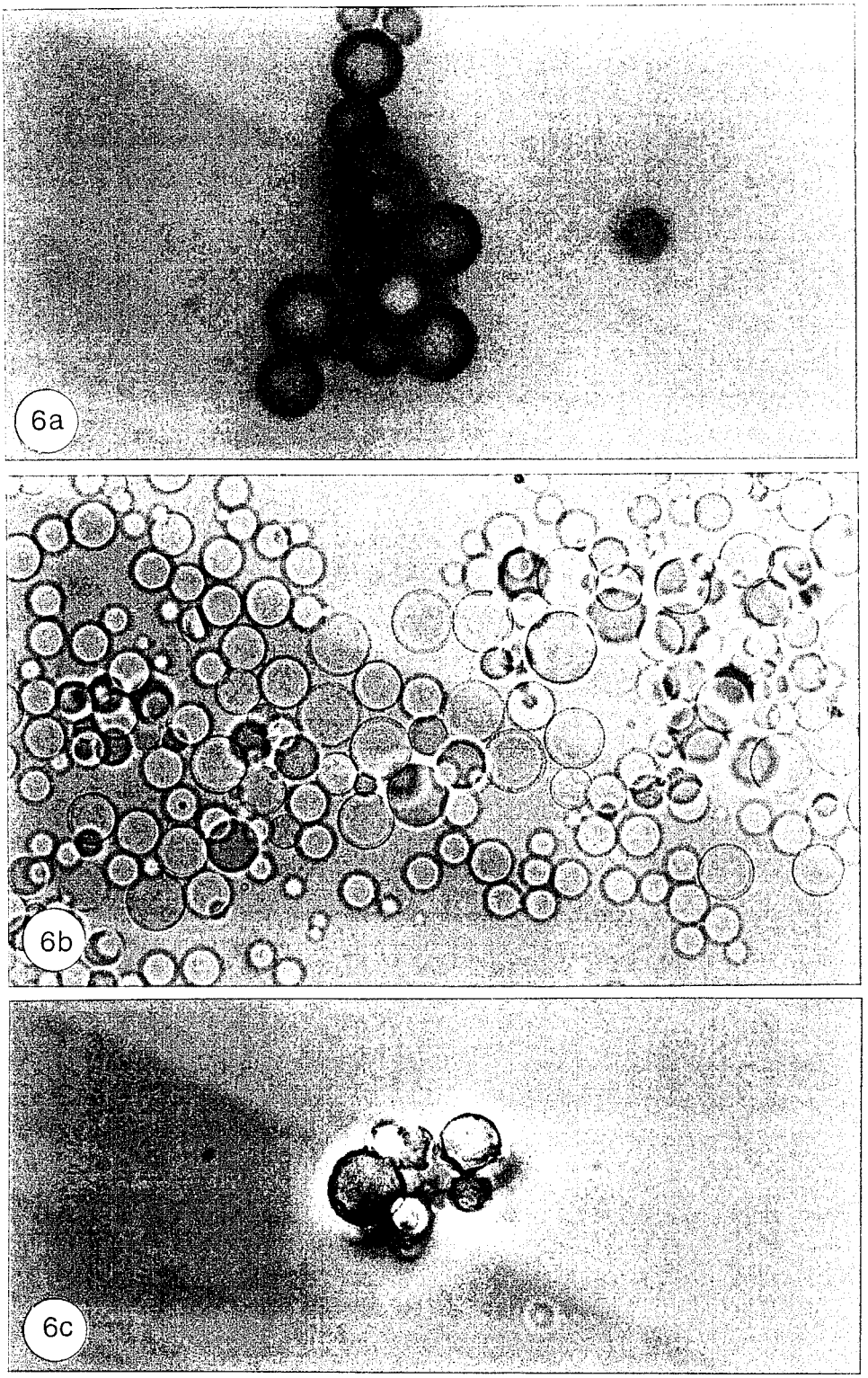

Figure 6. Photomicrographs Of BSA Microcarriers Produced Using: a) Olive; b) Soybean; and c) Peanut Oil.

Figure 7b shows microcarriers resulting from Formulation 1, these microcarriers were indistinguishable from those yielded by Formulation 3 (not shown). More importantly, this data clearly demonstrate that the addition of vpB alters the physical structure of the microcarrier, resulting in the appearance of a granular almost net-like matrix with the microcarrier (7b). The BSA appears to bind the particulate vpB facilitating the formation of a granular sphere.

Formulation 5 resulted in "microcarriers" that do not, in fact, exhibit any recognizable shape. As shown in Figure 7c, this formulation appears as a cohesive stroma like matrix. This failure of Formulation 5 to form microcarriers is most probably a result of the propensity of the vpB to precipitate out of solution at the pH level employed for these studies.

The ability of the vpB to interact with other proteins to form composite microcarriers offers tremendous possibilities for customizing microcarriers in future efforts. This basic compatibility also promises to enhance the capability of adding specific immunostimulating compounds to future microcarrier formulations.

C. Tetanus Containing Microcarriers: Antibody (IgG) Titers

The results presented in this section were obtained from analyzing the pooled sera collected from the various tetanus vaccination test groups. The serum samples were analyzed using the ELISA described in the methods section of this report. The level of antibody response at a 1:160 dilution was compared over the course of the experiments. All values were expressed as the percentage of the two week antibody level.

The typical antibody response profile shows an initial lag phase of about 7 to 10 days followed by a peak in antibody levels at 14 days post injection then a drop-off in antibody levels to almost baseline at 21 days. The profile for tetanus as an antigen has a somewhat extended drop-off phase, for this reason, tetanus is considered somewhat of a superantigen, and in some cases has been used as an adjuvant in it s own right.

Since the goal of any vaccination is to effectively prime the immune system and provide an adequate level of circulating IgG, it was decided that only the IgG levels would be examined. Therefore, no attempt was made to examine the initial levels of IgM at or prior to the 14 day sample. Figure 8 is a graphical representation of the temporal change in circulating IgG levels resulting from a single injection either Formulation 1 (3 μg tetanus toxin / dose), Formulation 2 (3 μg tetanus toxin / dose), or crosslinked tetanus toxin (60 μg / dose). This data clearly shows that the tetanus toxin microcarriers containing both BSA and vpB (Formulation 1) offered the most sustained antibody response of any formulation. At 11 weeks post injection, the antibody response was equal to 30% of the initial titer. The presence of the vpB granules within Formulation 1 served to extend the period of time required for biodegradation and

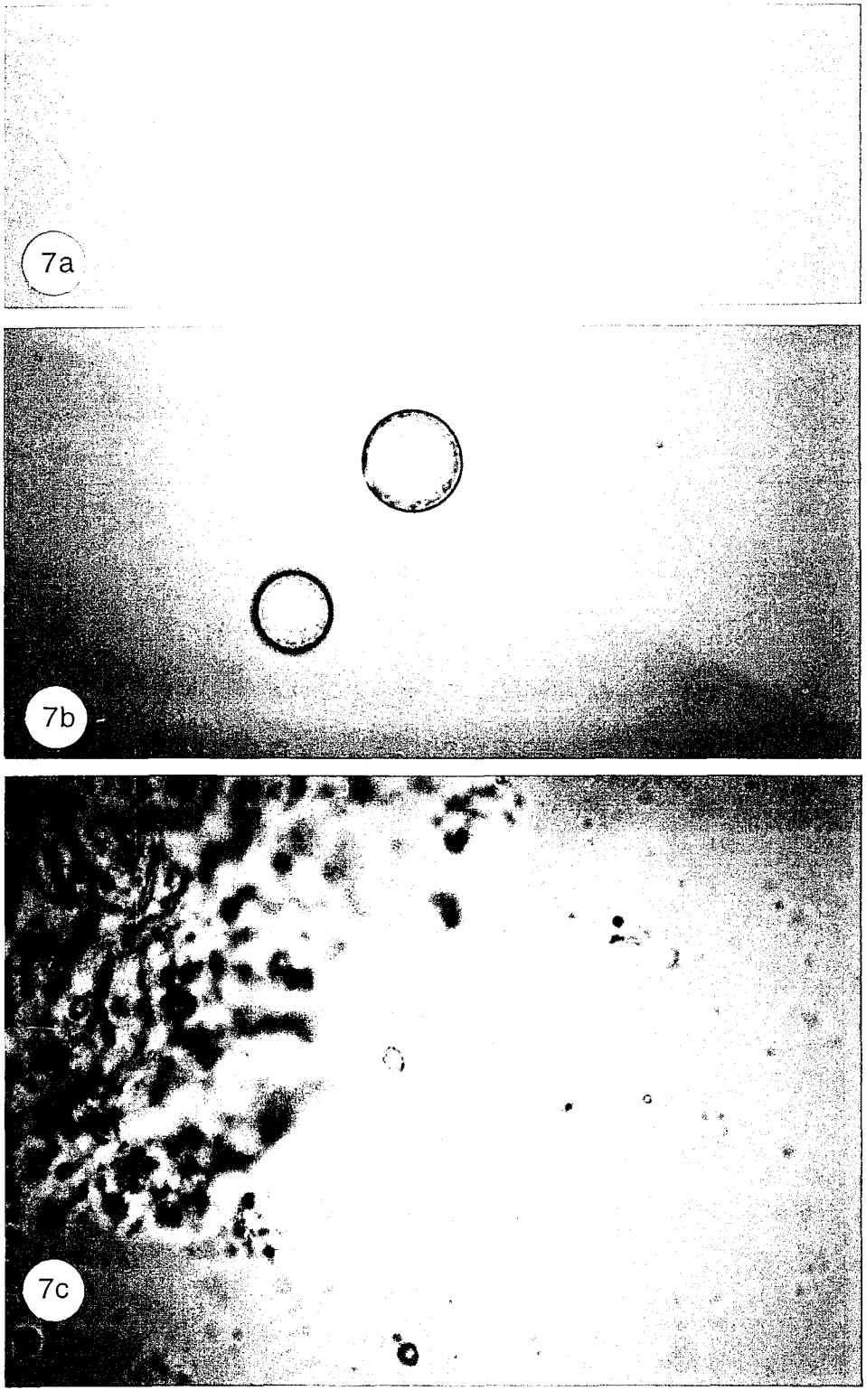

Figure 7. Photomicrographs Of Microcarriers Composed Of: a) BSA + Antigen; b) vpB + BSA + Antigen; and c) vpB + Antigen.

presentation of the antigen to the cells of the immune system. In contrast, the microcarriers containing only BSA and tetanus toxin (Formulation 2) maintained only 5.6% of their initial titer. The crosslinked tetanus toxin was administered at an approximately 20X higher dosage, than the Formulation 1 microcarriers, yet only 22% of it's initial titer level was sustained at 11 weeks.

D. Botulinum Containing Microcarriers: Antibody (IgG) Titers

The results presented in this section were obtained from analyzing the pooled sera collected from the various botulinum vaccination test groups. The serum samples were analyzed using the ELISA described in the methods section of this report. For the purposes of this report, endpoint titer will be defined as the largest dilution at which an optical density reading could be obtained at 405nm.

The antigen dosage level for all microcarrier formulations was approximated to be 3 μg per mouse, while the dosage level of the crosslinked toxin was approximated to be 60 μg per mouse. This disparity in antigen dosage level was primarily a result of the physical administration constraints associated with these initial microcarrier formulations.

Figure 9 depicts the temporal change in endpoint titers resulting from a single injection of each of the three botulinum microcarrier formulations and the crosslinked botulinum toxin. This data clearly demonstrated once again that the highest sustained antibody response resulted from the microcarriers containing BSA, vpB, and botulinum toxin (Formulation 3). At 5 weeks post injection Formulation 3 had a sustained antibody response which represented 100% of the initial (2 week) antibody response. Additionally, the 3 week antibody response for Formulation 3 had doubled from that seen initially.

Both the crosslinked botulinum toxin (Bot) and the BSA - botulinum microcarriers (Formulation 4) were only capable of sustaining 12.5% of their initial 2 week antibody levels by the 5 week mark. The vpB-botulinum toxin microcarriers, Formulation 5, while doubling in antibody titer at 3 weeks, yielded the lowest 5 week antibody response with only 6.25% of the 2 week response still present. The results of the botulinum evaluations exhibit the same overall trends as did the tetanus evaluations. Additionally, the botulinum Formulation 3, which contained both vpB and BSA exhibited an even better ability to maintain high levels of antibody production with a 5 week level equal to levels seen at 2 weeks.

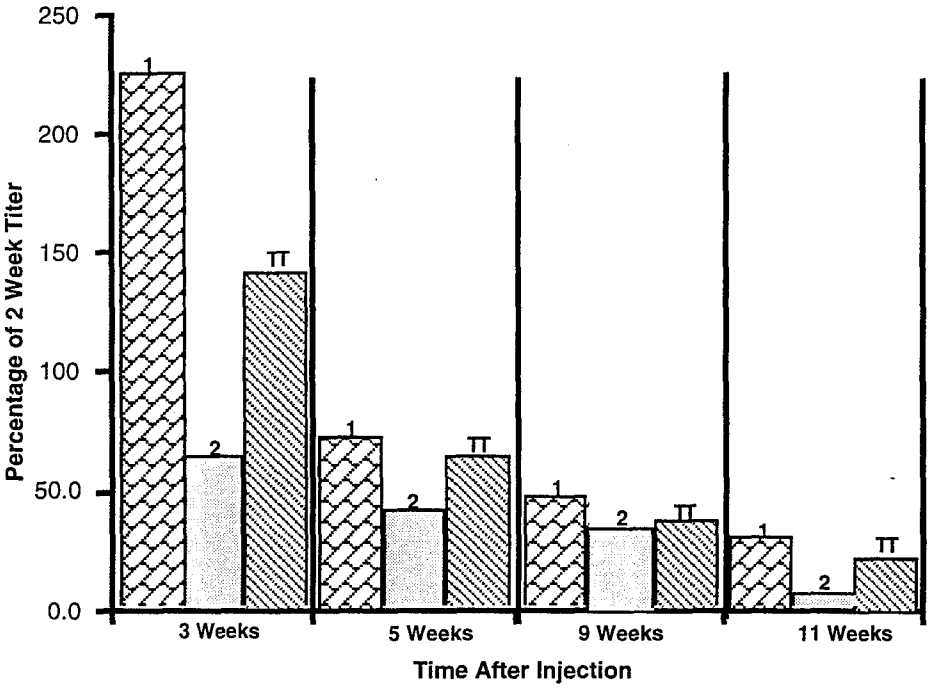

Figure 8. Temporal Changes In The Antibody Titers Resulting From Subcutaneous Injection of Tetanus Containing Microcarriers. TT — Crosslinked Tetanus Toxin, 1 — Formulation 1, and 2 — Formulation 2.

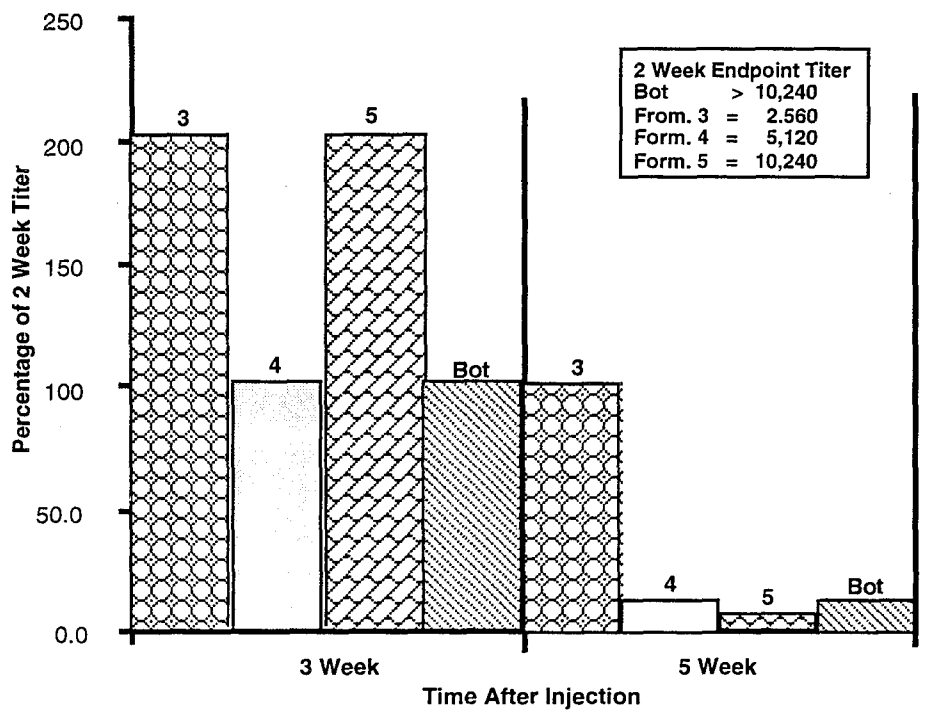

Figure 9. Temporal Changes In The Antibody Titers Resulting From Subcutaneous Injection of Botulinum Containing Microcarriers. Bot — Crosslinked Botulinum Toxin, 3 — Formulation 3, 4 — Formulation 4, and 5 — Formulation 5.

E. Histology of Injection Site Tissue

The photomicrographs of injection site tissue samples seen in Figure 10a-c show the relative distribution and physical condition of the microcarriers at 3 days post injection. Figure 10a at a magnification of 125X, clearly shows that the majority of the microcarriers, seen in these photomicrographs as bright pink spheres, can still be found concentrated at the site of injection just below the subcutaneous fat layer and above the muscle.

In Figure 10b at a magnification of 250X, it is clear that many of the microcarriers can be found in close association with the infiltrating cells of the immune system, represented in these photomicrographs by their dark blue-purple stained nuclei. With a magnification level of 500X, Figure 10c clearly shows that the microcarriers in the closest association with the cells of the immune system have begun to loose their characteristic spherical shape and appear to be slowly disintegrating. It is further clear from this data that the microcarriers are being isolated by the cells of the immune system granuloma formation has begun. Granuloma formation serves to sequester the antigen containing microcarriers thus providing a central depot for the localization of cell of the immune system. These cells are then available to process and present the antigenic component of the microcarriers resulting in a prolonged stimulation of the immune response.

F. Hematology

The blood samples which were collected over the course of this work clearly showed that neither the vaccination process nor the periodic bleeding caused any appreciable decrease in hematocrit (HCT). The average starting HCTs of the tetanus and botulinum experimental groups were 51% and 54%, respectively, while the final HCTs of 50% and 53% for the tetanus and botulinum experimental groups respectively. Additionally, routine observation of the buffy coat and serum in the samples evidenced no marked deviation either component.

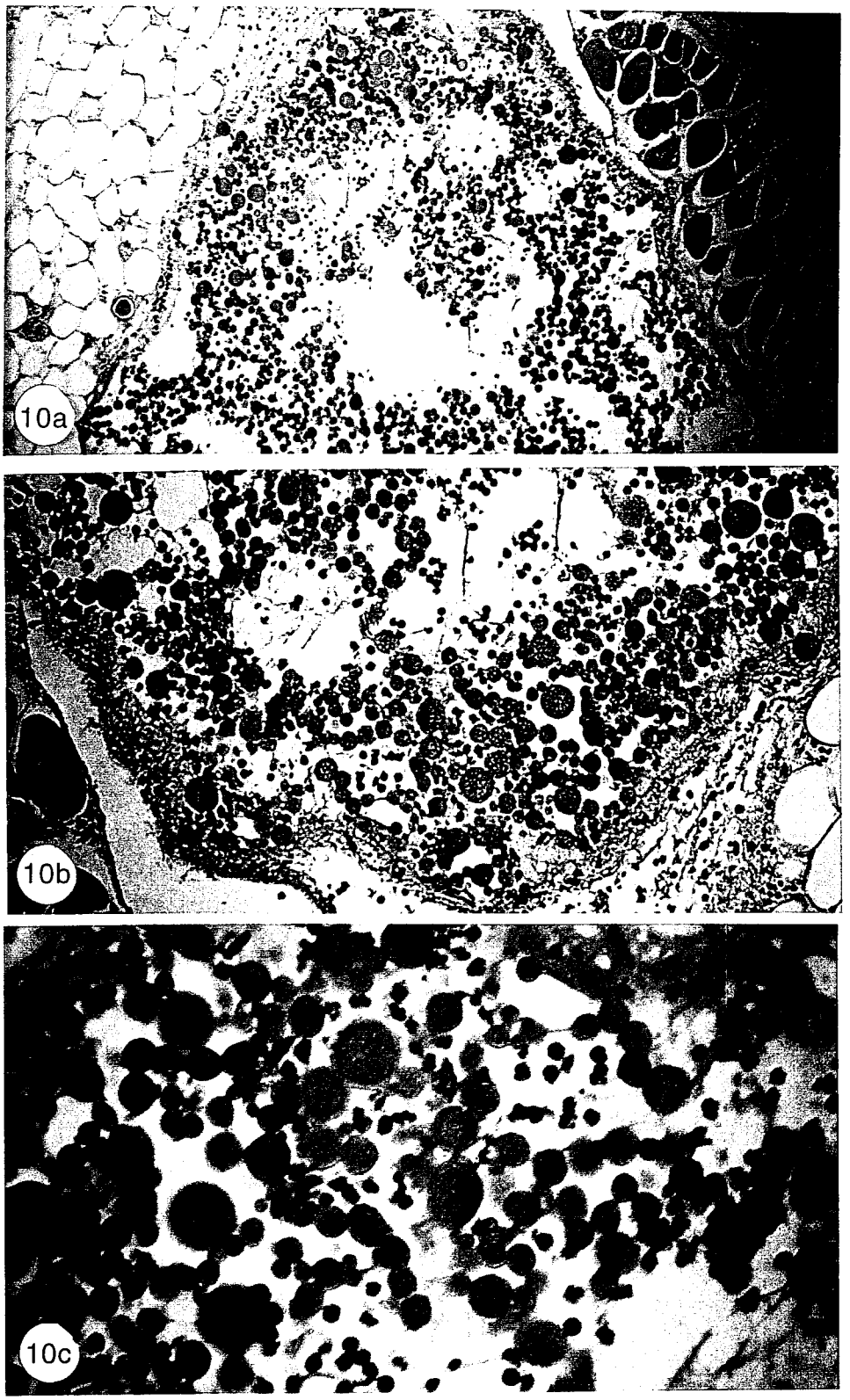

Figure 10. Photomicrographs Of Injection Site Tissue At 3 Days Post Injection, At A Magnification of : a) 125X; b) 250X; and c) 500X.

SECTION IV
DISCUSSION

The results from Phase I clearly demonstrate the feasibility of using the recombinant *Fasciola* eggshell protein, vpB, as the basis of a vaccine delivery system which provides both enhanced and extended antigen presentation characteristics. A single subcutaneous injection of the vpB vaccine delivery system containing either tetanus or botulinum toxin resulted in elevated serum antibody levels for periods of up to 11 weeks. Further, preliminary research has shown that the vpB is effective in combination with two different antigens, thus opening the door for the development of a polyvalent vaccine. The vpB was also found to be compatible with other proteins (e.g. bovine serum albumin) for the production of hybrid microcarriers. These hybrid (vpB / albumin) microcarriers exhibited far superior adjuvant characteristics than were observed when either protein, (BSA or vpB), was used individually to form microcarriers.

Cremers[30] similarly observed that the drug loading and release characteristics of hybrid albumin / heparin microspheres were superior to those found with single protein microspheres.[31] The ability of vpB to combine with other proteins to form hybrid microcarriers offers several technically feasible possibilities for tailoring the physical characteristics of this vaccine delivery system. Additionally, the generally compatible nature of the vpB suggests that a multifunctional hybrid could be formed by the selected addition of known immunostimulating compounds such as cytokines to even further enhance the adjuvant characteristics of the vpB vaccine delivery system. The vpB was shown to be biocompatible, yet still capable of offering excellent adjuvant characteristics. Finally, the results show that even though the vpB acts as an adjuvant, it does not appear to illicit an antibody response. This latter consideration is of great technical significance because it offers the potential for the use of these microcarriers as multipurpose delivery vehicles.

In order to achieve the long term goal of developing a vaccine which will produce elevated antibody levels for up to 1 year with a single vaccination several key issues must be resolved. Examples of these issues are: a) the effect of vpB concentration on the physical characteristics of the microcarrier; b) concentration and exposure time needed to achieve optimum crosslinking; and, c) the effect of incorporation of additional recombinant *Fasciola* eggshell proteins on the resulting microcarriers.

The vpB microcarrier vaccine delivery system holds tremendous promise as a means of providing complete protection against toxins within 2-3 weeks post immunization, as well as, offering high level protection for up to 1 year with a single subcutaneous injection. The medical importance of the "single dose long term immunocompetence capability for vaccines has been clearly illustrated by Tseng[31] in his work with *Staphlococcous* enterotoxin B. High levels of circulating antibodies are critical for the prevention of toxicosis following exposure to lethal doses of aerosolized *Staphlococcous* enterotoxin B.

Lynntech's Phase I study clearly demonstrated that a single subcutaneous injection of the vpB based microcarrier vaccine delivery system resulted in high levels of circulating antibodies within 2-3 weeks post immunization, and the circulating antibody levels remained elevated for up to 11 weeks post immunization. Such preliminary results offer great promise that this system once refined and optimized will provide the high level prolonged protection sought by the military.

Another facet of the vpB microcarrier vaccine delivery system is it's potential for oral administration. The recombinant *Fasciola* protein, vpB, has several core features that would be essential to the successful development of an oral vaccine delivery system. For example, in nature the vpB is used by the helminth *Fasciola hepatica* for biological microencapsulation of it s eggs to provide protection within the hostile environment of the mammalian gastrointestinal system. This is exactly the environment an oral vaccine would have to survive in order to produce a successful immune response. Another technical advantage is that, the vpB microcarriers can be enzymatically or chemically modified to exhibit extreme bioadherence. This extreme bioadherence would enhance their ability to remain within the gastrointestinal system in close association with the gut associated lymphoid tissue.

One of the foremost issues in the vaccine research arena is the development of a new adjuvant that can amplify both the humoral and cell-mediated immune response to an antigen. The only adjuvant currently approved for human vaccines in the United States is aluminum salt. The aluminum salt adjuvants have several limitation: a) they preclude lyophyilization or freezing of the vaccines; b) they are not effective with all antigens; and, c) they do not offer any stimulation of cell-mediated immunity.

Based on the technical understanding developed during this study relative to adjuvant characteristics and requirements, there is a high potential for bypassing the present limitations of aluminum salt adjuvants by using the vpB based microcarrier vaccine delivery system.

Current research into the development of alternative adjuvants has focused on: a) oil-based emulsions which contain biodegradable materials, and b) nonionic block copolymer surfactants. To date, none of these approaches has led to an acceptable adjuvant. Some of these compounds have been too toxic for use in humans, causing tissue damage at the site of injection, or they have been implicated as the cause of delayed reactions like arthritis and anterior uveitis.[32]

The evaluation of block copolymers as a potential vaccine adjuvant has been ongoing since as early as 1979.[2] Despite the potential of block copolymer microencapsulated vaccines, the degree to which different antigens can be incorporated into these materials and the safety concerns about residual solvents integrated into the block copolymer microspheres remains a problem.[2]

The vpB microcarrier vaccine delivery system offers a new approach to the development of an acceptable adjuvant that is free of many of the problems associated with the vaccine adjuvant approaches discussed above. The vpB is nonantigenic, no evidence currently exists that would suggest any antigen compatibility problems. Further, unlike the block copolymer microspheres, no solvent is required to form the vpB microcarriers so the risk associated with solvent incorporation into the vaccine is eliminated.

In summary, the technical feasibility for development and use of vpB based microcarriers for one-time administration of antigen for imparting *in vivo* antibody protection was successfully demonstrated. There is a high potential for wide ranging health and disease protection benefits. Key medical advantages, logistic factors and cost benefits can be accrued for both military and civilian purposes. Currently available techniques can be employed for scale up production at cost effective levels. A clear research and development pathway for expanding the scope of this technology development has been established. As a result of the Phase I study specific technical objectives have been identified to enable scaleup production. The final technical objective of the Phase II will be the validation of immune response characteristics through extensive *in vivo* evaluations in mice. These studies will set the stage for the preclinical trials to be conducted as part of future Phase III research efforts.

SECTION V
CONCLUSIONS

1. The recombinant *Fasciola* eggshell protein vpB is suitable for use as a key component in the production of a microcarrier vaccine delivery system.

2. Microcarriers composed of vpB and albumin in addition to the antigen, resulted in the highest sustained circulating antibody response.

3. Different antigens can successfully be incorporated into the vpB-albumin microcarrier vaccine delivery system.

4. The recombinant *Fasciola* eggshell protein vpB is both nonantigenic and biocompatible.

5. The recombinant *Fasciola* eggshell protein vpB can be produced in a yeast vector system, providing an economical means of producing large quantities of this protein using currently available biofermentation technology.

6. A single subcutaneous injection of the vpB-albumin microcarrier vaccine resulted in a continuous elevation in circulating antibody levels for 11 weeks post immunization.

SECTION VI
REFERENCES

1. Christensen, D. (1994) Sci. News **145**:344.

2. Morris, W., Steinhoff, M.C., and Russell, P.K. (1994) Vaccine **12**:5.

3. Theis, C. (1979) "Yearbook of Science and Technology" McGraw-Hill Inc. N. Y. p12.

4. Deasy, P. B. (1984) "Microencapsulation and Related Drug Processes" Marcel Dekker, Inc. New York.

5. Pryor, M.G.M. (1940) Proc Roy Soc London B **128**:378.

6. Waite, J.H., Rice-Ficht, A.C. (1987) Biochemistry **26**: 7819.

7. Schecter, Y., Patchorik, A., and Burstein, Y. (1976) Biochemistry **15**:5071.

8. Waite, J.H. (1987) Int. J. Adhesion & Adhesives **7**:9.

9. Waite, J.H. (1987) Chemtech Nov:692.

10. Allen, L.W. (1989) J. Adhesion **21**:261.

11. Kaleem, K., Chertok, F., and Erhan, S. (1987) Angew. Makromol. Chemie **155**: 31.

12. Soriaga, M.P., and Hubbard, A.T. (1982) J. Am. Chem. Soc. **104**:2735.

13. Waite, J.H., Rice-Ficht, A.C. (1989) Biochemistry **28**: 610.

14. Waite, J.H., Rice-Ficht, A.C. (1992) Mol. Biochem. Parasitol. **54**: 143.

15. Rice-Ficht, A.C., Waite, J.H. (1992) Mol. Biochem. Parasitol. **54**: 127.

16. Rice-Ficht, A.C. (1992) in: "Results and Problems in Cell Differentiation 19, Biopolymers", Steven T. Case, Ed. , Springer Verlag, Heidelberg, pp. 77.

17. Comyn, J. in: "Developments in Adhesives" Volume 2, Kinlock, A.J., Ed., Applied Science Publishers, Barking, UK, 279 (1981).

18. Brown, C.H. (1952) Quart. J. Microsc. Sci. **93**:487.

19. Yonge, C.M. (1962) J. Mar. Biol. Assoc. UK, **42**:113 .

20. Cook, M. (1970) in: "Adhesion in Biological Systems" Manley, Ed., Academic Press, New York, 137.

21. Yonge, C.M. in: "Pathways in Malacology" van der Spoel, S., van Bruggen, A.C., and Lever, J., Eds., W. Junk, the Hague, (1979).

22. Smith, D. B., and Johnson, K. S. (1988) Gene **67**, 31. 23.

23. Watanabe, A., and Hayashi, T. (1976) in: "Microencapsulation" Nixon, JR, Ed., Marcel Dekker, New York, Chapter 2.

24. Phares, R.E., and Sperandio, G.J. (1964) J. Pharm. Science **53**: 515.

25. Luzzi, L.A., and Gerraughty, R.J. (1964) J. Pharm. Sci. **53**:429.

26. Gross, E. (1967) Methods in Enzymology **11**:238.

27. Watts, P.J., Davies, M.C., and Melia, C.D. (1990) Crit. Rev. Ther. Drug Carrier Syst. **7**:235.

28. Deasy, P.B. (1988) Crit. Rev. Ther. Drug Carrier Syst. S:99.

29. Tomlinson, E., Burger J.J. (1985) Methods of Enzymology **112**:27.

30. Cremers, H.F.M., Kwon, G., Bae, Y.H., Kim, S.W., Verrijk, R., Noteborn, H.P.J.M., and Feijen, J. (1994) Biomaterials **15**:38.

31. Tseng, J., Komisar, J.L., Chen, J.Y., Hunt, R.E., Johnson, A.J., Pitt, L., Rivera, J., Rublf, D.L., Trout, R., and Vega, A. (1993) Infection and Immunity **61**:391.

32. Robinovich, N.R., McInnes, P., Klein, O.L., and Hall, B.F. (1994) Science **265**:1401.

Received 2/8/2000

DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
504 SCOTT STREET
FORT DETRICK, MARYLAND 21702-5012

REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y) 21 Jan 00

MEMORANDUM FOR Administrator, Defense Technical Information Center, ATTN: DTIC-OCA, 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports written for the attached Awards. Request the limited distribution statements for Accession Document Numbers listed be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Virginia Miller at DSN 343-7327 or by email at virginia.miller@det.amedd.army.mil.

FOR THE COMMANDER:

Phylis Rinehart

Encl
as

PHYLIS M. RINEHART
Deputy Chief of Staff for
Information Management