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13. ABSTRACT *(Maximum 200*

This SBIR Phase I research project focuses on the development of an orally administered microencapsulated vaccine against staphylococcal enterotoxin A (SEA). The research is centered around using a known bioadhesive, vitelline protein B (vpB), to increase contact time with the gut and enhance absorption of the microspheres by the Peyer's patches of the gut-associated lymphoid tissues. Results clearly show that orally administered SEA containing microspheres produce a greater immune response than orally administered non-encapsulated SEA. When vpB is coencapsulated with SEA into BSA microspheres the resulting immune response to SEA is less than microspheres that contain only SEA and BSA. However, when vpB is coencapsulated with SEA into poly(DL-lactide-*co*-glycolide) (PLG) microspheres the resulting immune response to SEA is greater than microspheres that contain only SEA and PLG. Whether vpB is acting as a true bioadhesive when included in PLG microspheres is not clear. It is first necessary to determine how the presence of vpB affects release rate from and surface hydrophobicity of PLG microspheres since both of these parameters can greatly affect immune response to SEA.

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G D Witt

Principal Investigator's Signature

9/13/96

Date

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1. Introduction

Vaccines have tremendous dual use potential for both military and large scale civilian immunization programs such as the Children's Vaccine Initiative (CVI) sponsored by the World Health Organization, Unicef, United Nations Development Fund, Rockefeller Foundation, and World Bank[1]. The ideal vaccine would: i) be stable enough to be stored at room temperature, ii) provide lifelong protection with a single dose, iii) and be orally administered.

Microencapsulation of vaccine antigen is a promising method to achieve all three of the above criteria. Since most vaccine antigens are proteinaceous, microencapsulation would stabilize the antigen against a variety of degradative reactions that typically plague protein preparations including deamidation of asparagines and glutamines, oxidation of tryptophans, methionines and disulfides, hydrolysis of the peptide backbone, and many others. Therefore, antigen protection by microencapsulation would increase shelf life and possibly eliminate the cold chain requirement of most vaccine preparation[2]. Microencapsulation of antigen can also eliminate the need for booster vaccine administration since microcapsules can be prepared to provide a timed-release of antigen over a period of months. The exposure of the immune system to a constant low dose of antigen will frequently generate a potent and long lasting immune response. Finally, if the microspheres were bioadherent and of the appropriate size (< 10 μm) they would "stick" to the intestinal lining and be phagocytized by M cells in the Peyer's patches and ultimately induce both a mucosal and systemic immune response[3,4]. This dual immune response would provide a primary and secondary immune defense against pathogens and toxins that invade the body via mucosal surfaces[2,3]. In addition, orally administered vaccines can overcome the problems associated with parenteral vaccines, such as infection and the required administration by trained medical personnel[5].

This research focuses on the development of a biodegradable, bioadherent, orally administered vaccine against staphylococcal enterotoxin A (SEA). SEA is one of seven distinct pre-formed enterotoxins produced by *Staphylococcus aureus*[6]. It is frequently implicated in many of the 1.2 million cases of food poisonings in the United States annually[7] and is a potential biological warfare agent. As little as 0.1 μg of enterotoxin can produce the clinical symptoms of staphylococcal food poisoning which include nausea, retching, vomiting and diarrhea[8].

In this research SEA is encapsulated in biodegradable proteinaceous and polyester microspheres. The protein-based microspheres consist of bovine serum albumin (BSA) and recombinant vitelline protein B (vpB) from the liver fluke *Fasciola hepatica* which is a known bioadhesive[9-11]. The polyester-based microspheres consist of poly(DL-lactide-*co*-glycolide) and vpB. The encapsulation methods are the classical, well described water in oil technique for

the preparation of protein-based microspheres [12] and the solvent extraction technique for the polyester-based microspheres[13]. The encapsulated SEA is orally administered to Balb/c mice by gavage and antibody titers in both sera and saliva are measured.

2. Materials and Methods

2.1. Materials

vpB is expressed in *Pichia* yeast and isolated and purified as described below. SEA was purchased from Toxin Technologies. The primary monoclonal was purchased from Sigma, and secondary polyclonal conjugated to horseradish peroxidase, from Biogenesis, Inc.. Corning 96 well ELISA microtiter plates were purchased from Fisher. 2,2'-azinobis(3-ethylbenzothiazoline)-6-sulfonic acid (ABTS) was purchased from Pierce. BSA, 25% glutaraldehyde in 1 ml ampoules, poly(lactide-*co*-glycolide) (PLG), poly(vinylalcohol) (PVA), olive oil, castor oil, hexadecane, Tween-20, buffers and other miscellaneous items were purchased from Sigma.

2.2. Expression, Isolation, and Purification of vpB

A 100 ml flask culture (MMY media) was inoculated with a recombinant *Pichia* yeast clone containing the vpB gene integrated at the alcohol oxidase (AOX) locus in the *Pichia* genome. The culture was grown at 30°C for approximately 48 hours until an optical density (OD_{650}) of 3.0 was reached. The culture was then used to inoculate a 2 liter fermentor containing 1.75 liters of MMY media. The culture was grown at 30°C for 72 to 84 hours until the culture reached an OD_{650} of 3.0. The OD_{650} was measured every 4 hours until a plateau in the OD_{650} was achieved. This ensured that all glycerol substrate had been consumed from the media; while glycerol is a very good carbon source for growth it directly inhibits the transcription from the AOX locus and must be depleted prior to protein induction with methanol. Following glycerol depletion, methanol was added to a final concentration of 0.5% each day for 10 days. At the end of the ten day induction, cells were pelleted from the culture at 5,000xg and the supernatant harvested.

Protein was purified and concentrated from bulk culture media by ammonium sulfate precipitation. Ammonium sulfate was added initially at a level to achieve 20% saturation, stirred on ice for 30 minutes and centrifuged at 10,000xg for 10 minutes at 4°C. The supernatant was collected and ammonium sulfate was added to achieve a 40% saturation, allowed to stir for 30 minutes and centrifuged at 10,000xg for 10 minutes at 4°C. The pellet which contained the majority of the vpB was resuspended in a small volume of PBS, pH 7.2, exhaustively dialyzed against PBS and concentrated by Centricon filtration. The protein (~200 mg/liter of culture) was then ready to use for microencapsulation.

2.3. Preparation of BSA-Based Microspheres

Most of the literature that describe the preparation of protein microspheres use the water in oil emulsion polymerization technique with either heat or chemical crosslinking[12]. Heat

crosslinking is frequently used when the encapsulated agent is usually a drug which is resistant to the high temperatures used to crosslink the microspheres (usually >105°C). The high temperatures almost certainly denature or at least considerably disrupt the three dimensional structure of the encapsulating protein (either albumin, fibrinogen, collagen, etc.) but this is of little consequence since the main purpose of the encapsulating protein is to form a biodegradable microsphere to trap and slowly release drug.

However, when the encapsulated agent is itself proteinaceous (such as most vaccine antigens), chemical crosslinking is preferred. This crosslinking method uses compounds such as glutaraldehyde and formaldehyde which indiscriminately target all accessible primary amino groups[14] resulting in intermolecular links between proteins in the microsphere. Therefore, chemical crosslinking is expected to modify the surface of encapsulating agent (BSA) and encapsulated agent (antigen) but it is not expected to significantly destabilize the protein's overall three dimensional structure. Chemical crosslinking is relatively mild to proteins compared to the high temperature crosslinking method which almost certainly adversely affects the primary, secondary and tertiary structure of all proteins in the microsphere.

All BSA-based microspheres prepared during the Phase I project were prepared by the water in oil emulsion technique using glutaraldehyde crosslinking. Briefly, 100 ml of oil is stirred at 1,200 rpm for 30 minutes. Then 2 ml of a 150 mg/ml protein solution containing BSA, vpB, and SEA is added to the oil and stirring is continued for 30 minutes. The aqueous droplets in the emulsion are solidified by adding 100 µl of an aqueous glutaraldehyde solution (1 to 25% glutaraldehyde). The emulsion is stirred for an additional 15 minutes. Then 50 ml of acetone is added and the emulsion is stirred for an additional 10 minutes. The microspheres are then collected by centrifugation at 3,000 rpm for 10 minutes. The pelleted microspheres are resuspended in 50 ml acetone to remove residual oil. The centrifugation and acetone washes are repeated twice more. The microspheres are allowed to air dry overnight at room temperature to allow residual acetone to evaporate. Yields of the free flowing microspheres ranged from 240 to 288 mg of microspheres from 300 mg of starting protein (i.e 80 to 96%).

2.4. Preparation of PLG-Based Microspheres

All PLG-based microspheres prepared during the Phase I project were prepared by the solvent extraction technique. Briefly, 5 ml of 10 % PLG (50:50, Mw = 40 to 75 kD) in methylene chloride was emulsified with 0.5 ml of an aqueous protein solution (either vpB, SEA, or both) at 10,000 rpm for 3 minutes. The emulsified solution was then added to 100 ml of 8% poly(vinylalcohol) (PVA) in water and allowed to stir at 5,000 rpm for 15 minutes. The PVA solution was then added to 1L of water and stirred for 3 hours at 1,200 rpm. PLG microspheres were collected by centrifugation and washed three times with distilled water and finally

lyophilized to dryness. Yields of the free flowing microspheres ranged from 420 to 490 mg of microspheres from 500 mg of starting protein (i.e. 84 to 98%).

2.5. Determination Microsphere Size and Surface Morphology

The size and surface morphology of the microspheres were determined by a Jeol T330A scanning electron microscope at the Electron Microscopy Center on the campus of Texas A&M University. The approximate average diameter of dry microspheres was determined qualitatively for each batch by visual inspection of the micrographs. Surface morphology was also determined qualitatively.

2.6. Determination of *In Vitro* Release Kinetics

The *in vitro* release assay was performed in duplicate for each sample as follows: 7 mg of microspheres were suspended in 70 µl of acetone and 630 µl of 30 mM HCl (pH ~1.8) and shaken in a rotary shaker at 37°C for 2 hours. The microspheres were pelleted by centrifugation at 3,000 rpm for 5 minutes. All of the supernatant was removed, saved and replaced with an equal volume of 10 mM PBS, pH 7.4. At various time points the microspheres were again pelleted to collect the supernatant and it was replaced with an equal volume of fresh PBS. The protein concentration of each supernatant aliquot was determined by the BCA protein assay (Pierce) and the total amount of protein released from the microspheres to that time was determined.

2.7. Oral Administration of Formulations to Mice

Ten-week-old Balb/c female mice weighing ~ 18 g were orally administered formulations in 0.5 ml 10 mM PBS, pH 7.4 using a curved tube style 1.5 inch, 20 gauge feeding needle attached to a 3 ml Becton Dickinson syringe with Leur-Lok tip and 0.1 ml graduations.

2.8. Determination of *In Vivo* Antibody Response to SEA

100 µl of blood was collected from the tail vein of all mice at the designated time. Blood from all mice in the same group was pooled, centrifuged and collected. The sera was analyzed for the presence of SEA antibodies by the following ELISA:

96 well polystyrene microtiter plates were coated with 100 µl/well of 10 µg/ml SEA diluted in 0.05 M carbonate buffer pH 9.6 (1 µg SEA/well). The plates were incubated overnight in a humid chamber with shaking at room temperature to allow SEA to bind to the plates. The SEA solution was discarded and the plates were washed four times with PBS containing 0.05% Tween-20 pH 7.4. The plates were inverted and tapped to remove excess PBS between each wash. The serum samples collected from the mice were initially diluted 1:4 with PBS containing

0.05% Tween-20 followed by a two-fold serial dilution in the wells of the microtiter plate across 11 wells resulting in a maximum dilution ratio of 1: 8,192 ($4 \times 2^{11} = 8,192$). The plates were incubated with the diluted sera (containing 1°AB) for two hours at 37°C. The diluted sera was discarded and the plates were washed twice with PBS containing 0.05% Tween-20. Then goat anti-mouse polyvalent immunoglobulins (IgG, IgA, and IgM, whole molecule, diluted 1:1000 with PBS/Tween) horseradish peroxidase conjugate were added to the wells and incubated for one hour at 37°C. The 2° AB solution was discarded and the plates were washed five times with PBS containing 0.05% Tween-20. The plates were inverted and tapped to remove excess buffer between each wash. Then 100 µl of ABTS was added in 0.05 M phosphate/citrate buffer (pH 5.0) containing 0.03% sodium perborate. The resulting color development was determined after 30 minutes at 410 nm. Data was then corrected by subtracting the negative control absorbance (normal mouse sera). Since the antibody titers were low for all samples measured, Figures 4, 5 and 6 plot absorbance of the 1:4 dilution. The antibody titer was not determined for each mouse in each group but for the pooled sera from each group and the data plotted in Figures 4, 5 and 6 represent a single determination for each time point.

To estimate the statistical significance of the antibody responses to SEA, the standard deviation of the negative control absorbances between assays was calculated at 0.064. Therefore absorbances less than 0.064 in Figures 4, 5 or 6 are considered statistically insignificant since they fall within a single standard deviation of the negative control.

3. Results

3.1. Microsphere Size and Surface Morphology

BSA-based microsphere preparations were made to determine how microsphere size was affected by impeller stirring speed and the type of emulsifying oil. BSA microspheres were prepared in hexadecane, olive oil, and castor oil at a variety of stirrer speeds ranging from 300 to 4000 rpm. The results are plotted in Figure 1. The lowest stirrer speed tested for each of the three oils was the minimum speed necessary to form an emulsion. The average microsphere diameter was determined by measuring the diameter of ~20 microspheres under magnification using a bench-top light microscope. The conclusion from Figure 1 is that BSA-based microspheres ranging in size from 200 (±60) μm to less than 10 μm can be prepared using the current microsphere preparation method. That the olive and castor oil curves appear to approach an asymptote of 10 μm is most likely artificial. Microspheres smaller than 3 μm are difficult to detect using a bench-top light microscope, therefore the asymptote is a reflection of the limitations of the optical sizing method and not of the microsphere preparation method. Subsequent scanning electron micrographs of BSA microspheres prepared in castor oil at 4,000 rpm revealed microsphere diameters ranging from 0.6 to 6 μm. Attempts to make submicron microspheres were avoided since many literature references state that the optimum microsphere size for uptake by Peyer's patches is ~ 10 μm. Therefore subsequent microspheres were prepared in olive oil at 1,200 rpm.

It was also observed that microsphere size was dependent on the temperature of the emulsion. Microspheres prepared in olive oil at 1,200 rpm were considerably larger when prepared at 58°C (~150 μm) than at room temperature (~10 μm). That temperature can affect microcapsule size is not surprising because higher temperatures can adversely affect protein stability and strengthen intermolecular forces. However, olive oil viscosity is inversely proportional to temperature (~ 24 centipoise at 58°C and ~ 84 centipoise at 23°C), therefore a more likely explanation is the lower viscosity oil (58°C) results in a lower surface tension at the water / oil interface, producing larger microspheres.

The surface morphology of the BSA microspheres was strongly dependent on the concentration of vpB in the preparation and the concentration of glutaraldehyde used to crosslink the microspheres. The scanning electron micrographs in Figure 2 were taken at the Electron Microscopy Center on the campus of Texas A&M University. It is apparent from Figure 2 that higher glutaraldehyde concentration yields microspheres with a smoother surface texture and presumably smaller pores, Figure 2. A and B, respectively. This presumption is corroborated by the *in vitro* release data (described below) which shows that microspheres crosslinked with a higher concentration of glutaraldehyde have slower release kinetics.

In addition microspheres containing 4% vpB have a very textured surface compared to 100% BSA microspheres, Figure 2. C and A, respectively. Cremers and coworkers[15] observed extensive surface texture of albumin/heparin microspheres. They attributed this observation to "... matrix inhomogeneity which can be caused by a phase separation of the polymer solution, favoring the presence of albumin at the oil / water interface". This conclusion is based on the reasonable assumption that albumin is more hydrophobic than heparin (a polyanionic saccharide). It is possible that the textured surface of vpB/BSA microspheres is also due to "matrix inhomogeneity" since surface texture is directly proportional to percent vpB in the microspheres (SEMs of 8 and 26% vpB microspheres not shown) however it is difficult to conclude which protein preferentially partitions at the oil / water interface.

Figure 2 also shows a scanning electron micrograph of a PLG microsphere containing 2% SEA (panel D). This microsphere appears to have a similar texture as the 100% BSA microsphere crosslinked with 1% glutaraldehyde. Other varieties of PLG-based microspheres have not been prepared.

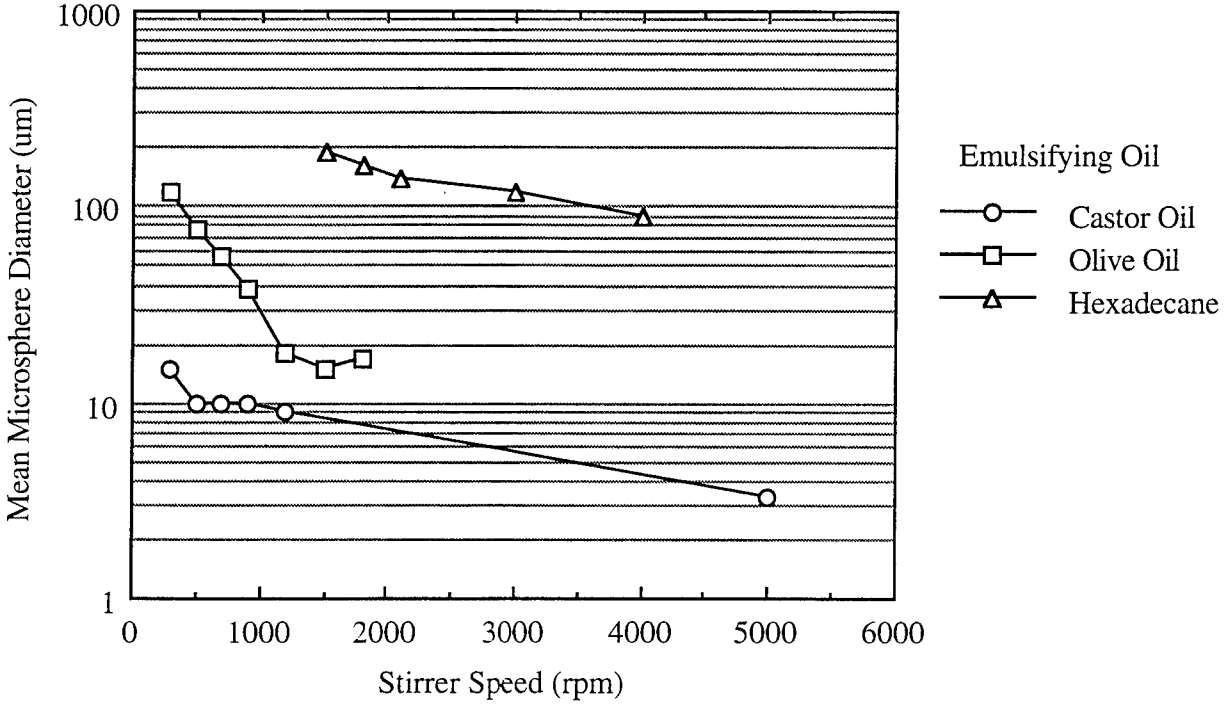


Figure 1. Microsphere Size Dependence on Stirrer Speed and Emulsifying Oil.

3.2. *In Vitro* Release Kinetics of BSA Microspheres

Microsphere preparations were made to determine how the concentration of glutaraldehyde affects *in vitro* release kinetics. The *in vitro* release experiments were performed to estimate release rates *in vivo*. Limitations of this model will be discussed later.

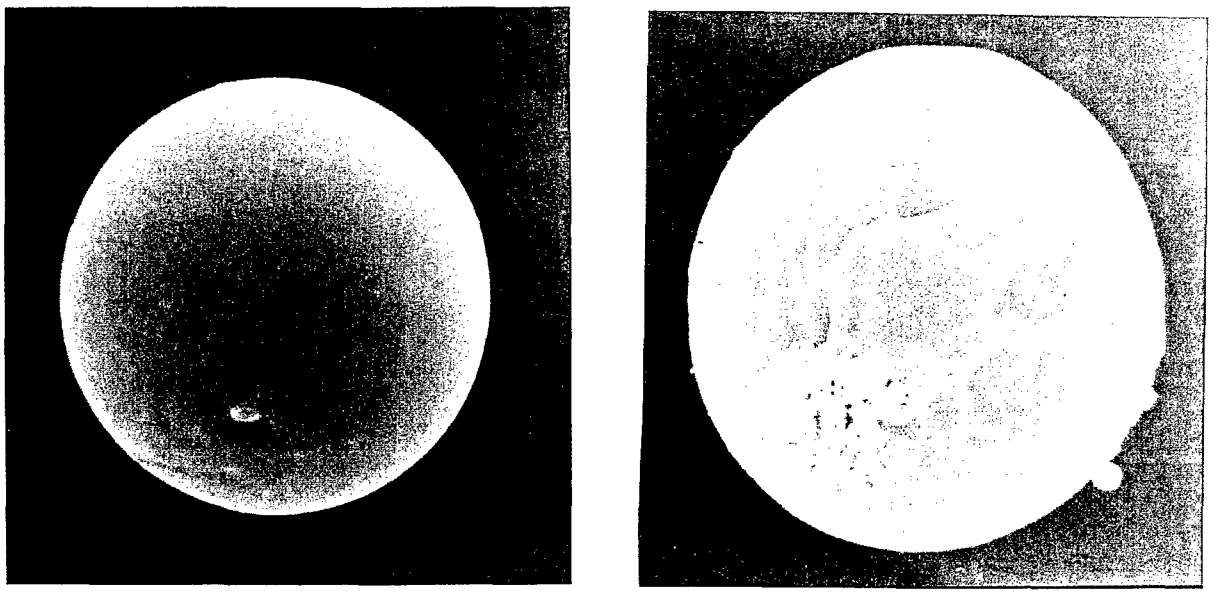
It was assumed that the *in vitro* release profile of SEA formulated in BSA microspheres would be similar to the release profile of bulk BSA from the microspheres. This assumption is based on two reasons:

1. BSA and SEA are similar in size. Assuming both molecules are spherical and have the same density, the diameter of BSA is only one-third larger than that of SEA. (The ratio of molecular weights is 69 kD / 28 kD = 2.46, and the cube root of 2.46 is 1.35). Since the release rate of proteins from the microsphere is dependent on molecular size, the release rate of each protein from the microsphere is assumed to be similar.
2. BSA and SEA have similar lysine content. The main target of glutaraldehyde crosslinking is the side chain of lysine residues (Lys) in the protein. The amino acid sequence of BSA contains 9.9% Lys (60 Lys residues in the complete 607 amino acid sequence). The amino acid sequence of SEA contains 9.4% Lys (22 Lys residues in the complete 233 amino acid sequence)[16]. This small difference in Lys content is not expected to result in preferential crosslinking of BSA over SEA.

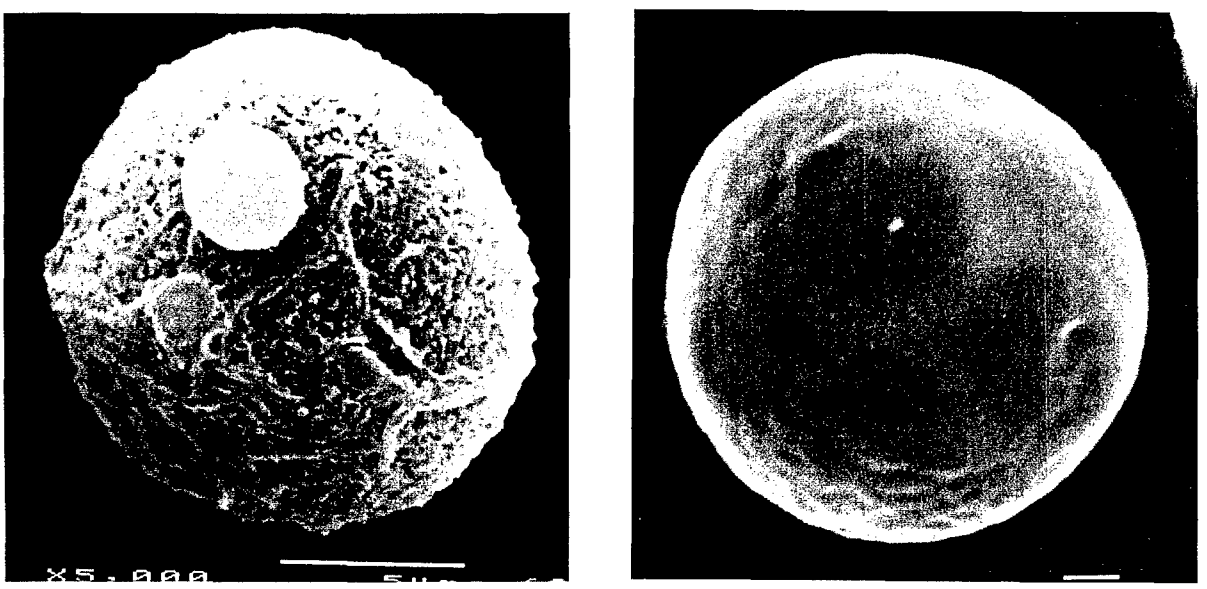
In addition, since 300 mg of total protein was frequently used for each microsphere preparation, a single 2% SEA preparation requires $1,350 worth of toxin (at $225/mg SEA). As a result of the prohibitive expense of including SEA in every microsphere preparation, SEA was used exclusively in preparations intended for *in vivo* experiments and for ELISA. Therefore, in release rate experiments, the bulk protein release rate was determined with time and it was assumed that the SEA release profile would be similar.

In the following release rate experiments, BSA microspheres were prepared in olive oil at 1,200 rpm and were crosslinked with various amounts of glutaraldehyde. Microspheres for all preparations were similar in size (from 5 to 20 μm). The amount of BSA released from seven microsphere preparations at various time points is plotted in Figure 3. The first observation from the plot reveals that protein release rate is *inversely* proportional to the percent glutaraldehyde used to crosslink the microspheres. At 1% glutaraldehyde crosslinking, approximately 20% of the protein is release after two days, while microspheres crosslinked with more than 1.5% glutaraldehyde had not yet released detectable amounts of BSA.

That the release does not continue to increase at a uniform rate towards 100% but appears to approach an asymptote considerably less than 100% is anticipated. The release rate profile from crosslinked BSA microspheres is expected to be biphasic. The initial "burst" phase results from the release of BSA monomers and low molecular weight polymers from the surface



A) B)



C) D)

Figure 2. Scanning electron micrographs of A) a 100% BSA microsphere crosslinked with 25% glutaraldehyde, B) a 100% BSA microsphere crosslinked with 1% glutaraldehyde, C) a 96% BSA / 4% vpB microsphere crosslinked with 1% glutaraldehyde, and D) a 98% PLG / 2% SEA microsphere. Microsphere diameters are 17.5, 11.4, 12.8, and 5.1 μm, respectively.

and pores of the microsphere. The second "slow" phase is considerably slower because it requires breaking of covalent bonds to completely solubilized and release the remaining contents of the microsphere.

The *in vitro* release rate model should be fairly accurate for approximating *in vivo* release during the burst phase but probably not during the slow phase. The slow phase dissolution of the microsphere requires hydrolysis of the BSA peptide backbone which is thermodynamically favorable but kinetically unfavorable *in vitro*. However proteinases exist *in vivo* which catalyze peptide bond hydrolysis. Therefore, *in vivo*, the release rate during the slow phase will be much faster than predicted by the *in vitro* model. *In vitro* slow phase release data was considered relatively uninformative and was not collected.

It was also noted that the color of the microspheres was proportional to the amount of glutaraldehyde used to crosslink the microspheres. Microspheres crosslinked with 1% glutaraldehyde were a very light tan color (almost white) while those crosslinked with 25% glutaraldehyde were brown.

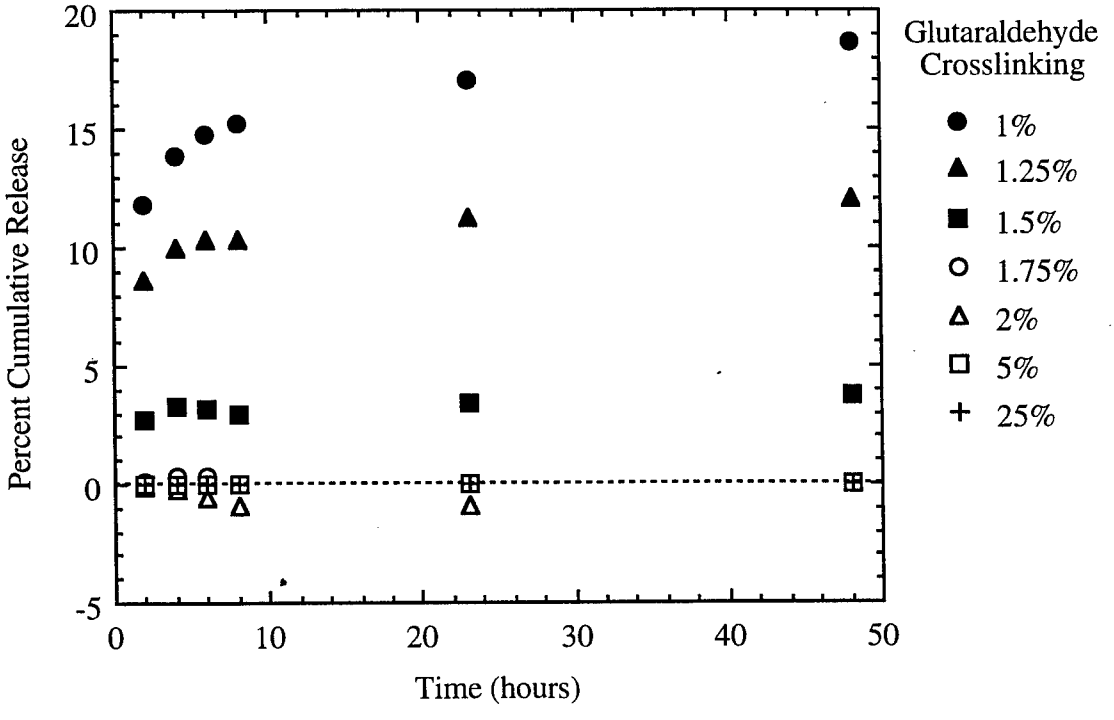


Figure 3. BSA Microsphere Release Rate Dependence on Percent Glutaraldehyde Crosslinking.

3.3. *In Vivo* Immune Response to Orally Administered SEA Loaded Microspheres

The *in vivo* immune response to orally administered SEA was monitored for three experiments. The details of the three experiments (Exp. # 1, Exp. # 2, and Exp. # 3) including evaluation of results are listed in the following sections.

3.3.1. Exp. # 1: Dose Dependent Response to Orally Administered SEA

The first experiment, Exp. # 1, was primarily performed to verify that the intended dose of orally administered SEA toxin (not toxoid) was not lethal to the mice. Typical toxoiding procedures reported in the literature require at least a month to prepare[17]. Because of the limited time available in a six month Phase I study, SEA was not toxoided. Also, apparent ambiguity exists in the literature concerning the LD_{50} of SEA toxin in mice. Edwin and coworkers[13] describe interperitoneal administering 50 μg of SEA to each ten-week-old Balb/c female mouse (2,800 μg/kg) to generate an immune response to SEA. However the emetic response in humans and other primates is ~ 1 μg/kg[8].

To resolve this ambiguity toxin was orally administered to twenty-eight Balb/c female mice to estimate lethality of orally administered doses of SEA toxin. Table 1 lists the groups of mice with the corresponding SEA dose and formulation (two mice per group). The mice were monitored for seven days and although some mice did die during this period (data not shown), the deaths did not correlate to administered SEA dose.

This experiment also provided the opportunity to practice various techniques such as mouse gavaging and bleeding and to provide sera to optimize the SEA immunoassay before beginning the major animal experiment of the Phase I, Exp. # 2. The immune response to SEA in Exp. # 1 was monitored in these animals and are reported in Figures 4 and 5. The only formulation that provided a significant immune response was 3MS. However, all of the immune response data from the formulations in Exp. # 1 (Figures 4 and 5) should be interpreted cautiously since each group contains only one or two animals.

Table 1. Exp. # 1: Dose dependent response to orally administered SEA.

Group	SEA (μg)	Formulation
1	50	SEA
2	10	"
3	1	"
4	0.5	"
5	0.1	"
6	0.01	"
7	0.001	"
1MS	50	Microspheres: 0.2% SEA, 99.8% PLG
2MS	10	"
3MS	1	"
4MS	0.5	"
5MS	0.1	"
6MS	0.01	"
7MS	0.001	"

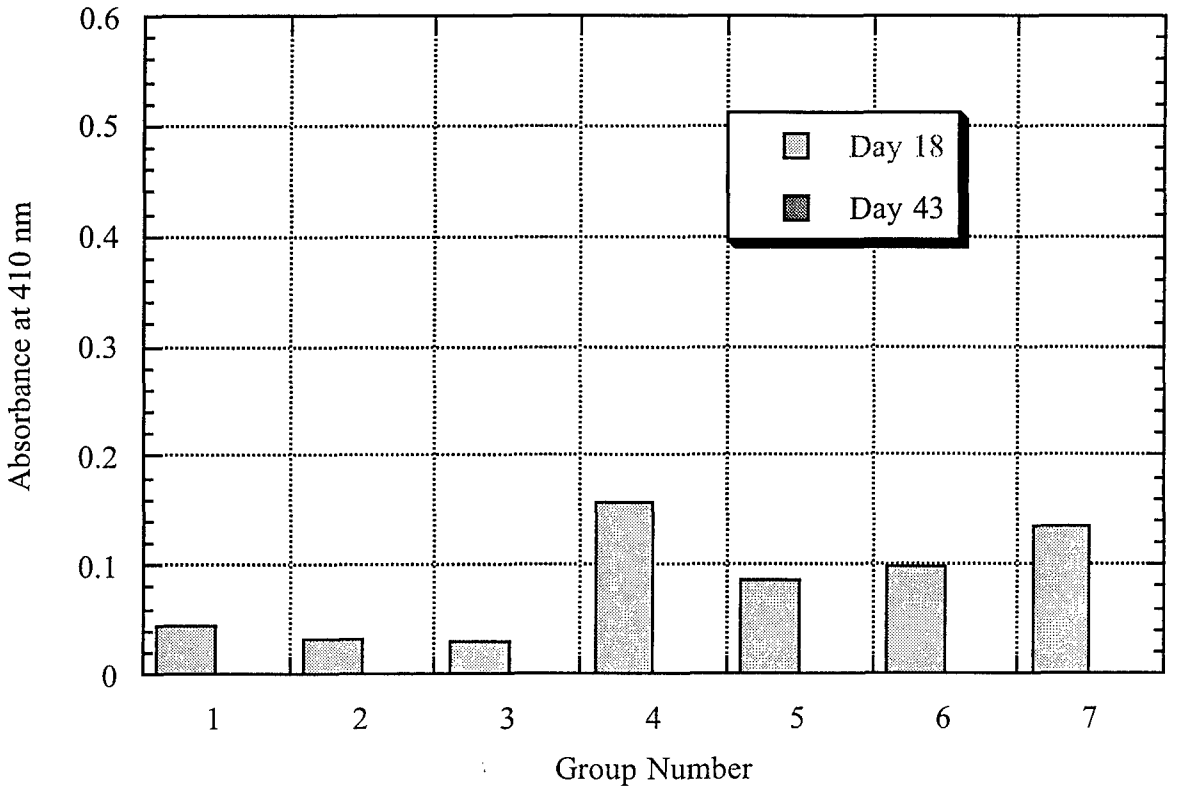


Figure 4. Exp. # 1: Dose dependent response to orally administered *soluble* SEA.

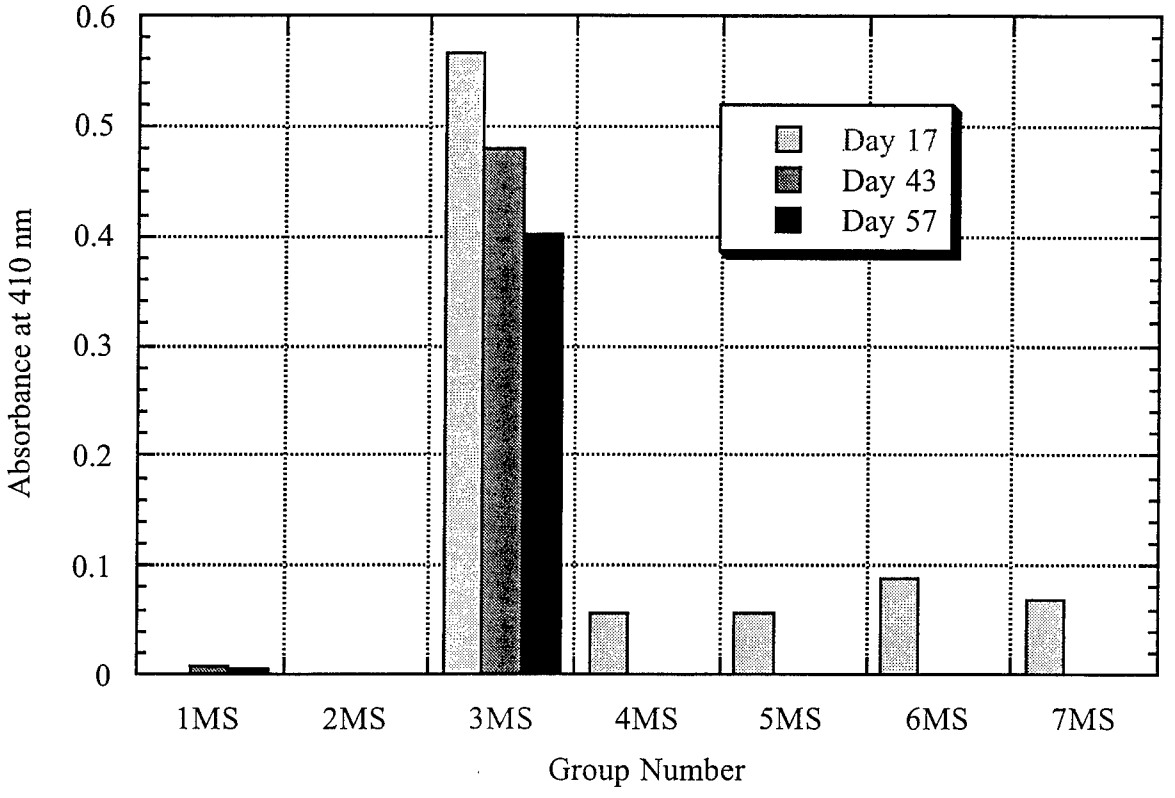


Figure 5. Exp. # 1: Dose dependent response to orally administered *microencapsulated* SEA.

3.3.2. Exp. # 2: Formulation Dependent Response to Orally Administered SEA

The animal experiment designated Exp. # 2 was the main animal experiment in this Phase I project and is outlined in Table 2. One hundred and twenty ten-week-old Balb/c female mice were used (twelve groups of ten mice per group) to evaluate various microsphere formulations containing either BSA or PLG with and without vpB and two different SEA doses. The results from Exp. # 2 are shown in Figure 6 and categorically discussed below.

Controls

Groups 1, 3 and 7 received no SEA, therefore the sera from these mice should show no anti-SEA response. Inspection of Figure 6 reveals that the absorbance for groups 1, 3, & 7 is below the standard deviation of the negative controls and therefore not considered significant.

Groups 2, 4 and 8 received 50 μg of free SEA in either PBS alone (Group 2), co-injected with 4% vpB / 96% BSA microspheres (Group 4), or co-injected with 4% vpB / 96% PLG microspheres (Group 8). This set of data was also expected to give no significant anti-SEA titer. Although Group 4 showed no response, Group 8 displayed a response at day 48. This value has yet to be confirmed with a subsequent bleed but this result may suggest that SEA is associating with the microsphere through non-covalent interactions and slowly releasing to generate a 48 day response.

An electrostatic interaction between PLG microspheres and SEA is plausible at physiological pH if the overall charged states of the PLG microsphere and SEA are considered. Although poly(DL-lactide-*co*-glycolide) polymers are considered "neutral" they do have a free carboxyl group at the end of each polymer chain. Therefore the overall charge of a PLG microsphere at physiological pH (~7.4) is expected to be negative which is confirmed by zeta potential measurements of the surface of PLG microspheres[18]. The isoelectric point (pI) of SEA is ~ 7.3[19] and is therefore considered "neutral" at physiological pH. However, the pI is a measure of the overall charge of the molecule and fails to consider the distribution of those charges over the surface of the molecule. A non-uniform distribution of charges over the surface of SEA could result in a "cluster" of positive charges that can interact with the negatively charged PLG microsphere. Also PLG microspheres degrade by base catalyzed hydrolysis of the ester linkages in the polymer which yield an alcohol and a carboxylic acid. Therefore the negative charge on the microsphere increases as it degrades which could amplify both attractive and repulsive interactions between the microsphere and SEA. Ultimately the accumulation of negative charges in the microsphere results in microsphere disintegration by repulsive forces.

Table 2. Exp. # 2: Microsphere formulation dependent response to orally administered SEA.

Group	SEA (μg)	Formulation
1 (control)	0	
2 (control)	50	100 μg/ml SEA
3 (control)	0	Microspheres: 4% vpB 96% BSA
4 (control)	50	Microspheres: 4% vpB 96% BSA in 100 μg/ml SEA
5	50	Microspheres: 2% SEA 4% vpB 94% BSA
6	125	Microspheres: 2% SEA 4% vpB 94% BSA
7 (control)	0	Microspheres: 4% vpB 96% PLG
8 (control)	50	Microspheres: 4% vpB 96% PLG in 100 μg/ml SEA
9	50	Microspheres: 2% SEA 4% vpB 94% PLG
10	125	Microspheres: 2% SEA 4% vpB 94% PLG
11	50	Microspheres: 2% SEA 98% BSA
12	50	Microspheres: 2% SEA 98% PLG

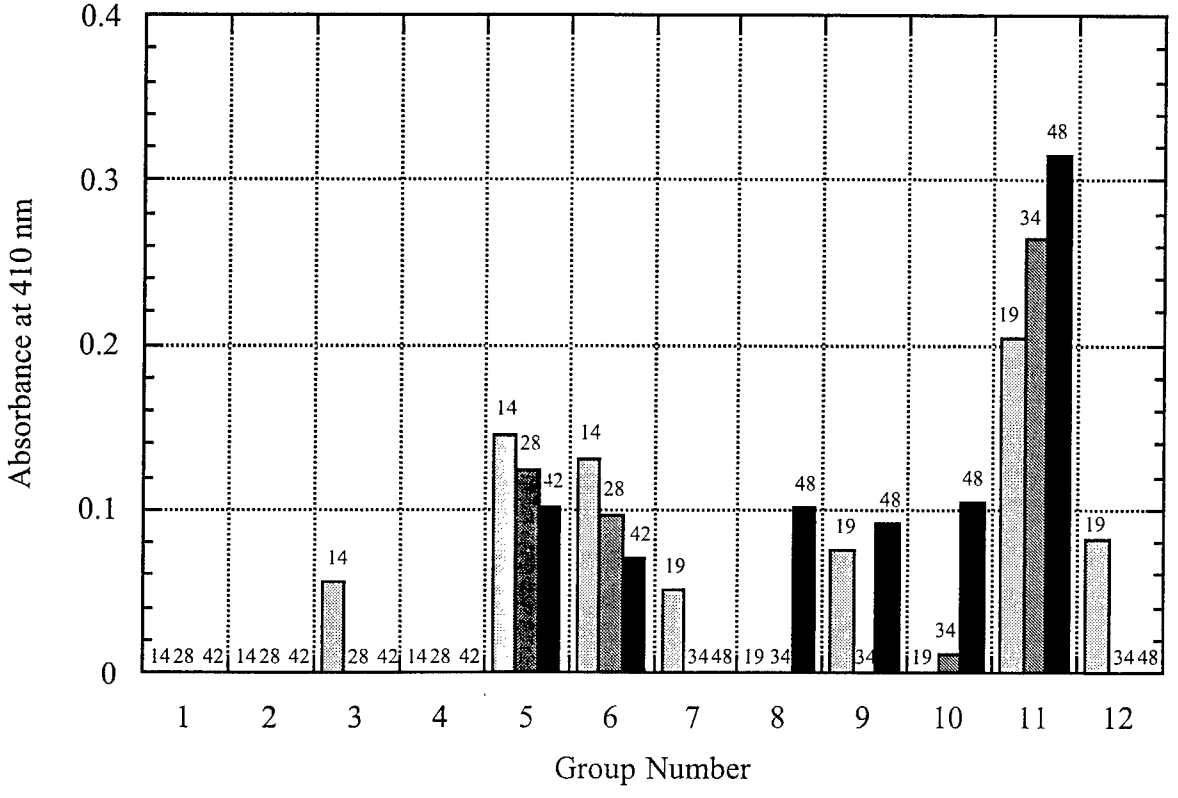


Figure 6. Exp. # 2: Microsphere formulation dependent response to orally administered SEA. Only absorbances greater than 0.064 are considered statistically significant.

Microencapsulated SEA Formulations Versus Free SEA

From the BSA based formulations, it is evident that orally administered microencapsulated SEA (Groups 5, 6 and 11) illicits an immune response while free SEA (Group 2) and SEA coadministered with microspheres that contain no SEA (Group 4) do not. Group 4 was included as an additional control to insure that observed immune response to encapsulated SEA (Groups 5, 6, and 11) was not because of SEA in the presence of microspheres but exclusive because of encapsulated SEA. That SEA encapsulated in BSA microspheres generates an immune response, while free SEA does not, is presumably because microspheres can transport the SEA to the Peyer's patches for adsorption into the mucosal associated lymphoid tissue. The microspheres are then transported to other lymphatic areas where they begin to induce the humoral arm of the immune system[3]. However, free SEA is most likely hydrolyzed by proteolytic enzymes in the digestive tract and never reaches the appropriate cells to illicit a response from the humoral arm of the immune system.

From the PLG based formulations, encapsulated SEA (Groups 9, 10, and 12) does illicit an immune response while free SEA (Group 2) does not. However, free SEA coadministered with PLG microspheres that contain no SEA does provide a comparable immune response to encapsulated SEA. This may be a result of non-covalent interactions between the protein and the microsphere, as previously described in the **Controls** section.

BSA-Based Versus PLG-Based Microsphere Formulations

Groups 11 and 12 received equivalent amounts of SEA (50 μg) in two different formulations. Group 11 received a BSA-based formulation and Group 12 received a PLG-based formulation. Neither formulation contained vpB. It is obvious from the responses in Figure 6 that the BSA formulation provided a greater immune response than the PLG formulation, although direct comparison of the formulations is difficult. Currently it is not understood why the BSA microspheres are better than PLG microspheres. Eldridge and coworkers[17] monitored the absorption of microspheres of various composition by the Peyer's patches of the gut-associated lymphoid tissue. They concluded that absorption by the Peyer's patches was directly proportional to microsphere hydrophobicity. One possible explanation is that BSA microspheres may be more hydrophobic than PLG microspheres. At first this may seem unlikely since PLG is soluble in methylene chloride and not in water, while BSA is soluble in water and not methylene chloride. Because PLG is more hydrophobic than BSA does not necessarily imply that PLG microspheres are more hydrophobic than BSA microspheres. This is because the emulsifying agent may determine the microspheres' surface hydrophobicity. PLG microspheres are prepared by an oil in water emulsification technique which may preferentially partition the most hydrophilic parts of the polymer (i.e. the terminal carboxyl groups of each

polymer) on the surface of the microsphere. Conversely, BSA microspheres are prepared by a water in oil emulsification technique which may preferentially partition the most hydrophobic parts of the protein on the surface of the microsphere. In addition, BSA in the presence of a very hydrophobic substance (i.e. oil at the water/oil interface) will most certainly denature exposing the very hydrophobic core of the protein. These microsphere preparation methods can result in BSA microspheres being more hydrophobic than PLG microspheres.

Also, release rate will certainly play an important role in stimulating immune response. Comparison of release rates from the PLG- and BSA-based microspheres is essential before direct comparisons between formulations can be made.

Presence or Absence of vpB in Microsphere Formulations

Groups 5 and 11 provide comparison of BSA formulations with and without vpB. The two differences between the antibody responses displayed in Figure 6 are the magnitude of the response and the direction of the response with time. The formulation without vpB (Group 11) generated a considerably larger response than that with vpB (Group 5) and the antibody response in Group 11 increases with time while the antibody response of Group 5 decreases with time. Groups 9 and 12 provide comparison of PLG formulations with and without vpB. Both formulations show the same 19 day response but Group 9 shows a 48 day response that Group 12 fails to show. Why vpB enhances the antibody response when present in the PLG formulation but diminishes the antibody response in the BSA formulation is not currently known. Further studies are necessary to determine how the presence of vpB influences hydrophobicity (and therefore absorption by Peyer's patches) and release rate in both PLG- and BSA-based microspheres.

Two Different Microsphere Dose Levels

Groups 5 and 6 received different amounts of the same 2% SEA / 4% vpB / 94% BSA formulation. Group 5 received 2.5 mg of microspheres (50 μg SEA) and Group 6 received 6.25 mg of microspheres (125 μg SEA). The antibody response to SEA is detectable when these formulations are orally administered to mice. In addition, these data show a typical antibody response over time starting out high and decreasing with time. This suggests that the SEA was released early and that not a high enough amount of SEA is being released to sustain high antibody titers. Also, the data from Figure 6 show similar results for both groups suggesting that antibody response to SEA is independent of antigen dose in this concentration range.

Groups 9 and 10 also received different amounts of the same 2% SEA / 4% vpB / 94% PLG formulation. Group 9 received 2.5 mg of microspheres (50 μg SEA) and Group 10 received 6.25 mg of microspheres (125 μg SEA). It is interesting to note that the 48 day responses of

Groups 9 and 10 are similar to each other and perhaps once again reflects the independence of antigen dose in this concentration range. Although the half-life in animals of PLG microspheres prepared from 50:50 copolymer is approximately one week[20], the microspheres administered to Groups 9 and 10 have provided a "late" immune response. The 48 day response needs to be validated by a subsequent sera sample from the Groups. Why Group 9 does not show a 34 day response is not understood and casts serious doubt on the validity of the 19 day response.

3.3.3. Exp. # 3: Formulation Dependent Response to 1μg of Orally Administered SEA

After Exp. # 2 had begun, immune responses from Exp. # 1 suggested that the optimal SEA dose to stimulate an immune response was 1 μg SEA (Figure 5, Group 3MS) and not 50 μg which was the dose most mice received in Exp. # 2. Therefore a third animal experiment was begun, Exp. # 3, to determine the formulation dependent response to 1 μg of orally administered SEA. The experiment consisted of three groups with five animals per group. The formulations orally administered to each group are shown in Table 3.

No detectable immune response was measured in either of the three groups at 18, 32 or 46 days. That no response was detected in Groups 2 and 3 perhaps suggest that 1 μg of SEA is simply is not enough antigen to stimulate a measurable immune response in these formulations. However Group 1 of Exp. # 3 is a repeat of Group 3MS of Exp. # 1 and a reproducible immune response is not observed. Greater significance must be placed on the results from Exp. # 3 because Exp. # 3 contained five animals per group while Group 3MS of Exp. # 1 contained a single mouse (the other mouse died a few days after gavaging).

Table 3. Exp. # 3: Microsphere formulation dependent response to 1μg of orally administered SEA.

Group	SEA (μg)	Formulation
1	1	Microspheres: 0.2% SEA 99.8% PLG
2	1	Microspheres: 2% SEA 98% BSA
3	1	Microspheres: 2% SEA 4% vpB 94% BSA

4. Discussion

The first step involved the preparation of microencapsulated SEA. SEA was encapsulated in BSA microspheres by the water-in-oil polymerization technique. Microspheres ranging from 8 to 200 μm were prepared using this technique and it was demonstrated that microsphere size was *directly* proportional to the temperature of the emulsion and *inversely* proportional to both stirrer speed and the viscosity of the emulsifying oil (suspension medium). The latter two observations are consistent with the following proportionality, which is a qualitative guide for controlling the size of microspheres produced by the water-in-oil polymerization technique[21].

$$\bar{d} \propto K \frac{D_v R v_d \gamma}{D_s N v_m C_s}$$

where $\bar{d}$ is the average droplet (particle) size; K symbolizes parameters such as vessel design and type of stirrer; D_v is the diameter of the vessel; D_s is the diameter of the stirrer; R is the volume ratio of the droplet phase to suspension medium; N is the stirring speed; v_d is the viscosity of the droplet phase; v_m is the viscosity of the suspension medium; γ is the surface tension between the two immiscible phases; and C_s is the stabilizer concentration. Although the temperature of the emulsion does not appear in the above proportionality, it is expected to have a strong influence on particle size because parameters such as v_d, v_m, and γ are temperature dependent.

The surface texture of the BSA microspheres was also strongly dependent on the amount of vpB in the microspheres and the extent to which they were crosslinked. Higher percentages of vpB in the microsphere resulted in greater surface texture. This observation is consistent with previously reported textured surfaces of albumin/heparin microspheres[15]. The textured surface may be a result of preferential partitioning of either BSA or vpB at the oil / water interface in the emulsion droplet resulting in a nonhomogeneous distribution of protein in the microsphere. It was also noted that microspheres prepared with a higher percentage of glutaraldehyde had a much smoother surface. Scanning electron micrographs of BSA microspheres crosslinked with 25% glutaraldehyde exhibited virtually no surface bumps, pits or distortions at 5,000 X magnification while microspheres crosslinked with only 1% glutaraldehyde exhibited significant surface distortions at an equivalent magnification.

It was also demonstrated that the release rate of BSA microspheres can be manipulated by varying the glutaraldehyde crosslinking concentration. As expected, *in vitro* release rates from the BSA microspheres was *inversely* proportional to the percent glutaraldehyde used to crosslink

the microspheres. Microspheres crosslinked with greater than 1.5% glutaraldehyde released no detectable protein during the first two days.

Following oral administration of SEA containing microspheres of various formulations to Balb/c mice, the immune response was measured over time by quantitating antibodies against SEA in sera. It is anticipated that vaccination of a mucosal surface would most likely result in a greater antibody titer in mucosal fluids than in sera; however collection of blood from the tail was simpler and far less invasive than gut lavage or saliva collection illicited by interperitoneal injections of pilocarpine or other salivating agents. Furthermore, a successful orally administered vaccine must illicit a circulatory as well as a secretory immune response.

The *in vivo* results showed that orally administered microspheres containing SEA does produce an immune response while orally administered free SEA does not. The immune response is greater in the mice that received the BSA preparations as compared to those that received the PLG preparations. This discrepancy can be the result of different SEA release rates from the microspheres and/or preferential absorption of BSA microspheres by Peyer's patches. Further work that includes determining the release rates of SEA from PLG microspheres and quantifying the number of BSA versus PLG microspheres present in excised Peyer's patches can begin to explain the observed difference.

It is evident from the data in Figure 6 that the presence of vpB in the microspheres affects the immune response. In BSA microspheres it diminishes the immune response while in PLG microspheres it enhances the response. At this stage it is difficult to determine how vpB is exerting its influence. The presence of vpB in the microspheres may affect release rate as well as surface hydrophobicity of the microspheres which can indirectly affect immune response. Future research can eliminate the question of different release rates by manipulating microsphere preparation conditions to prepare vpB/PLG and vpB/BSA microspheres with very similar release profiles. Additional work can also determine the relative hydrophobicities of the microspheres. Only then can it be determined whether vpB is exhibiting truly bioadhesive characteristics.

The immune responses observed in this report should be compared to those reported by Eldridge and coworkers who orally immunized mice against staphylococcal enterotoxin B (SEB)[17]. They observed anti-SEB end point titers of ~ 1:1024 for orally administered SEB encapsulated in PLG microspheres 20 days post-immunization. Although the single mouse in Groups 3MS from Exp. # 1 produced a 17 day end-point titer of 1:2048, a repeat of that formulation in five mice (Group 1, Exp. # 3) produced no detectable immune response. In addition, no significant immune response was observed at 19 days for Exp. # 2 Group 12, who received SEA encapsulated in PLG. Although SEA and SEB are homologous (36% amino acid identity according to the alignment provided by reference 21), the formulations in this experiment

encapsulated free SEA toxin into PLG microspheres, while the formulations prepared by Eldridge and coworkers encapsulated SEB toxoid into PLG microspheres. The toxoiding procedure used by Eldridge polymerizes SEB to high molecular weight, water soluble complexes[22]. It is well established for a number of soluble protein antigens that aggregation of polymerization markedly enhances the immunogenicity of the proteins[23-25]. Therefore the higher immune response observed by Eldridge can be, at least partly and perhaps entirely, attributed to the toxoiding procedure. This also suggests an explanation for the higher immune response observed for BSA versus PLG formulations in this research. SEA is released from PLG microspheres as monomers since no crosslinking occurs in preparation of PLG microspheres, however SEA is released from BSA microspheres in a variety of predominantly heteropolymeric states with BSA because of the crosslinking action of glutaraldehyde used in the preparation of BSA microspheres.

5. Conclusions

During this Phase I Small Business Innovation Research Project, the feasibility of an orally administered, microencapsulated vaccine against staphylococcal enterotoxin A (SEA) was demonstrated in Balb/c mice. Significant conclusions from the research are listed below:

1. Orally administered, microencapsulated SEA stimulated the production SEA specific antibodies in sera; orally administered, soluble SEA did not.

2. SEA encapsulated in bovine serum albumin microspheres produced a greater and longer lasting immune response than SEA encapsulated in poly(DL-lactide-*co*-glycolide) microspheres.

3. Coencapsulating recombinant vitelline protein B in poly(DL-lactide-*co*-glycolide) microspheres enhanced the immune response to SEA, while coencapsulating recombinant vitelline protein B in bovine serum albumin microspheres diminished the immune response to SEA.

Although more work is necessary to optimize the microsphere formulations, the data presented in this report demonstrate the feasibility of an orally administered vaccine against SEA.

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