RESEARCH REPORT
SWINE HEALTH

Title: Improving Swine Health: Enhancing Humoral and Cell-Mediated Immunity Using Novel Polymer Adjuvants – **NPB #07-070**

Investigator: Michael J. Wannemuehler

Institution: Iowa State University

Date Submitted: November, 2008

Industry Summary

As commodity markets fluctuate and producer profit margins diminish, economic loss due to infectious disease become even more important to the survivability of operations. Vaccination continues to be the most economic method for controlling infectious disease, especially ones which are difficult to control without prophylactic antibiotics. As consumer acceptance of current livestock practices change (e.g., use of antibiotics), as well as the increase in organic and antibiotic free niche markets, control of disease by preventive vaccination becomes more important. Single dose vaccines have long been sought after in human medicine to improve vaccine efficacy. The same advantage applies to animal health – a single dose vaccine would improve vaccine compliance, reduce labor costs, and, in the end, result in higher producer profits due to prevention of disease. A single dose vaccine could be readily integrated into current livestock management systems. The goal of this project was to evaluate a novel biodegradable polymer adjuvant as single dose vaccine carrier. In many cases, it is impractical in terms of labor and animal stress to immunize more than once. For most vaccines, including swine dysentery, two or three doses of a vaccine administered over several weeks are needed for complete protection. While the disease studied in this case was swine dysentery, the concept could be applied to other infectious disease agents. Using a mouse model of swine dysentery, a single dose microsphere delivered vaccine containing pepsin-digested *Brachyspira hyodysenteriae* antigen (PD) induced immune response to *Brachyspira* antigen and ameliorated inflammatory cytokine production associated with disease. A single dose vaccine containing co-polymers of CPH:SA microspheres encapsulating PD along with some unencapsulated PD was administered to crossbred grower pigs. No tissue reactivity at the injection site of polymer containing vaccine pigs was observed, whereas most of the animals receiving PD antigen incorporated into incomplete Freund's adjuvant (a common mineral oil based carrier) had granulomatous masses at the injection site. While all of the sham-vaccinated pigs developed dysentery, three out of five pigs receiving microsphere based vaccine were protected from developing overt clinical dysentery. Further study is needed to characterize the nature of the immune response (immune regulation and/or inflammatory cytokine profile) of the microsphere vaccine. Partial protection and reduced tissue site reactivity suggest that with further refinement, biodegradable polyanhydride based single dose vaccines will be beneficial/efficacious for use in livestock animals. Principal investigator and contact: Michael J. Wannemuehler, Iowa State University, mjwannem@iastate.edu

Scientific Abstract

Single dose vaccination has long been sought as one of the key hallmarks for increasing vaccine efficacy. Biodegradable polyanhydrides possess many properties that facilitate the development of single dose vaccines, including ability to enhance protein stability, tailorable release kinetics and surface erosion. This study evaluated the use of polyanhydride encapsulated pepsin-digested *Brachyspira hyodysenteriae* antigen (PD) as a vaccine regimen to protect pigs from the development of swine dysentery. In comparison to previously studied microspheres containing a single purified protein antigen, microspheres containing the complex antigen performed as expected with respect to morphology and release kinetics of the encapsulated antigens. Prior to challenge, mice vaccinated with PD encapsulated into microspheres developed demonstrable immune responses, both serum antibody and cellular proliferation, to *B. hyodysenteriae* antigen. Upon challenge with *B. hyodysenteriae* organisms, the cytokine profile of cells recovered from microsphere vaccinated animals and the antibody isotype profile was qualitatively different than those of mice vaccinated with PD or from non-vaccinated *B. hyodysenteriae* infected mice. In swine, animals vaccinated with PD in Freund's incomplete adjuvant (FIA) or PD loaded microspheres showed a reduction in disease severity upon challenge with *B. hyodysenteriae*, 100 % and 60%, respectively. While pigs vaccinated with PD-loaded microspheres exhibited a lower serum antibody titers than the pigs receiving PD in FIA prior to challenge, post-challenge serum antibody titers were equal and greater than that of sham vaccinated pigs indicating immunological priming. Furthermore, lymphocytes recovered from the colonic lymph node of pigs vaccinated with the PD-loaded microspheres exhibited greater in vitro antigen-specific recall responses than cells recovered from pigs receiving the PD-FIA vaccine. In addition, the proliferation of peripheral blood mononuclear cells recovered from the microsphere vaccinated pigs was lower than that for cells recovered from the PD-FIA vaccinated pigs, suggesting differential immune modulation. The results of these studies demonstrate that the use of polyanhydride microspheres is safe, induced no detectable tissue reaction at the site of injection, induced 60 % protection against swine dysentery with a single dose and facilitated immune modulation in vaccinated animals. Taken together, polyanhydride microspheres continue to demonstrate their promise in the pursuit of a single dose vaccine carrier that can be used in livestock species.

Introduction

As commodity markets fluctuate and producer profit margins diminish, economic loss due to infectious disease become even more important to the survivability or operations. Vaccination continues to be an effective method for controlling infectious disease, especially ones such as swine dysentery (SD). The research outlined herein was the first steps in evaluating the use of a novel vaccine adjuvant/delivery system which has application to multiple diseases. Single dose vaccines have long been sought after in human medicine to improve vaccine efficacy and patient compliance. The same advantages apply to animal health – a single dose vaccine would improve vaccine compliance, reduce labor costs, and, in the end, result in higher producer profits due to prevention of disease. A single dose vaccine could be easily integrated into current livestock management systems.

Studies evaluating the use of controlled-release, single dose vaccines in large animals (sheep, mini-pigs, cattle, and horses) have shown promise when employing protein antigens [1-3]. Biodegradable polyanhydride delivery systems for vaccines offer attractive features such as improved adjuvanticity, antigen stabilization, and enhanced immune responses [4-7]. The specific polyanhydrides of interest in this proposal copolymers of 1,6-bis(*p*-carboxyphenoxy)hexane (CPH) and sebasic acid (SA). This class of hydrophobic polymers degrades into biocompatible, water-soluble carboxylic acids which are quickly metabolized, leaving no tissue residue. Studies have shown that polyanhydrides are capable of stabilizing polypeptides and sustaining their release without the inclusion of potentially reactive excipients or stabilizers [8-11]. In addition, polyanhydrides have characteristics that make them suitable carriers for vaccine delivery, including a beneficial environment for protein stabilization by preventing aggregation, enhanced adjuvant effect (controlled by polymer chemistry), and potential immunomodulatory capabilities (Th1/Th2 balance) [8, 15].

There are many different swine diseases where both effective humoral immunity (Th2) and cell-mediated immunity (Th1) are desired [16, 17]. One instance where protection seems to depend on activation of both is swine dysentery (SD) [18]. SD is a severe diarrheal disease of swine and the etiologic agent is an

anaerobic spirochete, *Brachyspira hyodysenteriae*, which colonizes the cecum and colon [19-21]. The disease characterized by the presence of mucohemorrhagic diarrhea, weight loss, dehydration and shedding of spirochetes in the stool. Any age of pig can be affected, however, most severe losses occur during the grower/finisher stage [22]. The acute phase of the disease appears to be driven by leukocytes responding to translocation of luminal commensal bacteria into the lamina propria following epithelial erosion due pathogenic factors secreted by *B. hyodysenteriae* [23]. The chronic phase is mediated by $CD4^+$ T cell infiltrate into the colonic mucosa [23]. The disease is endemic in most pig producing countries, where infection prevalence can be as high as 35% of the swine herds [24, 25]. Efforts to maintain herds free of SD can be complicated by the fact that wild rodents and waterfowl are natural hosts of the bacterium [26, 27]. Control measures include antibiotic therapy, however, recently, antibiotic resistant strains have emerged [28]. With the increase in demand for organic and/or antibiotic free pigs, producers are encountering an increase in incidence in SD. Currently in the United States, there is no licensed vaccine in use for SD. Efforts to produce both recombinant and whole cell vaccines have met with varying success [19, 29-31]. Outer membrane preparations and other recombinant vaccines yielded only partial protection [19, 29-31]. Using a squalene/pluronic acid adjuvant, protection was conferred by a pepsin digest preparation of whole cell *B. hyodysenteriae* [19, 32, 33]. Non-mineral water/oil emulsions such as this are capable in inducing cell-mediated immunity (Th1) with lower dosage of protein than can be induced using an alum based vaccine [34]. The current study was undertaken to provide proof-of-concept that a single dose polyanhydride based vaccine can induce protective immunity in pigs. A single-dose microsphere-based vaccine was compared to an efficacious vaccine regimen for SD in a grower pig model.

Objectives

Specific Goal 1: Evaluate the encapsulation efficiency of a complex antigen, *pepsin-digested B hyodysenteriae antigen* (PD), into polyanhydride microspheres and assess the release kinetics and antigenicity of the encapsulated PD. This will advance our knowledge of antigen-polymer interactions as all studies to date have focused on the encapsulation and release of a single protein (i.e., ovalbumin, tetanus toxoid). Based on prior analysis, PD consists of many small protein fragments 3-10 kDa in size and intact lipooligosaccharide antigen.

Specific Goal 2: Evaluate the efficacy of a single dose SD vaccine based on PD-loaded polyanhydride microspheres in an established murine model. Two formulations of microspheres (20:80 CPH:SA and 50:50 CPH:SA) will be compared in efficacy to PD without adjuvant. Kinetics of the immune response and protection from colitis will be evaluated.

Specific Goal 3: Evaluate the immune response and subsequent protection of a single dose SD vaccine based on PD loaded polyanhydride microspheres in grower pigs. Pigs will be immunized with a single dose of the PD-loaded microsphere formulation yielding the most protection in mice. This novel adjuvant will be compared in efficacy to 2 doses of the mineral oil-adjuvanted PD vaccine previously used [18, 19]. At 30 days post-immunization, peripheral blood mononuclear cells (PBMCs) proliferation and serum antibody will be assessed. The animals will be challenged with *B. hyodysenteriae* and protection from colitis assessed.

Materials & Methods

SG1.1 Pepsin digest preparation

Pepsin digest of *Brachyspira hyodysenteriae* antigen was prepared as described previously in Waters et al [18, 19]. *Brachyspira hyodysenteriae* strain B204 frozen lyophilized bacterial stock was rehydrated with distilled water, briefly sonicated and pH adjusted to 2.0 with 1 N HCl. Lysate was mixed with 0.5 µg Pepsin (Sigma) per mg of dry weight bacteria. The solution was placed on rotary mixer at 37°C for 24 hrs. The pH was then adjusted to 7.2 with 1N NaOH. PD was sterilized by UV irradiation. Protein was quantified by bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL) and re-lyophilized. All measurements were based on dry

weight of lyophilized pepsin digest (PD). Whole cell sonicate (WCS) was prepared from the same lot of frozen lyophilized stock. Briefly, lyophilized bacteria were rehydrated to give 2 mg/ml suspension in sterile PBS, subjected to two freeze thaw cycles and briefly sonicated. WCS was sterilized by UV irradiation and stored at -20 until needed for ELISA or cellular re-stimulation.

SG1.2 Polymer synthesis and Microsphere fabrication

Prepolymers for both CPH and SA were synthesized using a method outlined by Shen et al.[35]. Poly(CPH-SA) (20:80) and poly(CPH-SA) (50:50) were synthesized by melt polycondensation from acetylated prepolymer solutions as described previously [35, 36]. Gel permeation chromatography was performed on a Waters GPC system (Milford, MA) using PL Gel columns (Polymer Laboratories, Amherst, MA). The 20:80 copolymer had an average molecular weight (M_w) of 21,000 and a polydispersity index (PDI) of 2.2. The 50:50 copolymer had an M_w of 13,000 and a PDI of 2.0. Polymers were stored desiccated under dry argon until needed. Microspheres encapsulating PD were fabricated by a solid/oil/oil double emulsion method as reported elsewhere [15, 36]. Polymer (100 mg) was dissolved in methylene chloride (2 ml) and PD (10 mg) was added to the dissolved polymers in a 50 ml centrifuge tube and immediately emulsified by agitation at 30,000 rpm for 20:80 CPH:SA and 20,000 rpm for 50:50 CPH:SA with a handheld homogenizer (Tissue-Tearor^{TM}, Biospec Products Inc., Bartlesville, OK) for one minute. With the homogenizer running at 10,000 rpm, 3 ml of Dow Corning Fluid (silicon oil), saturated with methylene chloride (4 ml), was added drop-wise to form the microspheres. Homogenization at 30,000 rpm was continued for an additional minute. To precipitate the microspheres, the double emulsion was transferred to a 400 ml Berzelius beaker containing 200 ml *n*-heptane on an ice water bath. The heptane was stirred at 300 rpm using a Caframo overhead stirrer (Warrington, Ontario) with a three-inch impeller for two hours to extract the methylene chloride. Heptane was periodically added during the solvent removal to replace the volume lost due to evaporation. The PD-loaded microspheres were isolated by filtration using Whatman #50 filter paper. The beaker and impeller were rinsed several times with fresh heptane to maximize recovery. The microspheres were washed at least three times with 50 ml of heptane to remove residual Dow Corning fluid, and dried for 24 hours under vacuum. This procedure yielded a free-flowing powder with about 80% of the input polymer mass being recovered. Blank and WCS containing microspheres were fabricated by a similar techniques, without the addition of PD or the inclusion of *B. hyodysenteriae* WCS respectively.

SG1.3 Determination of Microsphere Size and Morphology

A JEOL 840A scanning electron microscope (SEM) was used to determine the size and shape of the microspheres. Microspheres were smeared onto carbon stubs, sputter coated with 200 Å of gold, and imaged. Size distribution analysis was performed using Image J software (NIH, Bethesda, MD).

SG1.4 Antigen Release and Antigenicity

Polyanhydride microspheres (10 mg) fabricated by S/O/O were suspended in 1 ml of phosphate buffered saline (PBS, pH 7.4) containing 0.01% sodium azide and placed in an incubator at 37 °C while stirring at 100 rpm. Samples of the buffer were collected at two hours later, then daily for one week, and every other day for 35 days. An aliquot of 750 µL was sampled each time and subsequently replaced with 750 µL of fresh PBS to ensure perfect sink conditions; the microcentrifuge tubes containing the microsphere suspensions were centrifuged before sampling to ensure that no microspheres were removed along with the sampled PBS. In order to quantify the amount of protein released, BCA was performed on each sample, in duplicate, as described by the manufacturer (Pierce, Rockford, IL). The release data is presented as cumulative fraction of protein released, in which the amount of protein released is normalized by the total protein loaded into the microspheres.

To determine the antigenicity of encapsulated antigen, 25 mg of PD or WCS loaded microspheres were added to 3 ml dialysis cassettes (Slide-A-Lyzer Dialysis Cassette, 3,000 MWCO, Pierce, Rockford, IL) and immersed in an excess volume of PBS with 0.5% sodium azide at 37°C. Dialysis buffer was replaced weekly and 2 ml samples taken from the dialysis cassette at 15, 30, 45 and 60 days. Samples were concentrated

(VIVASPIN columns, 3,000 MWCO PES, VivaScience, Hannover, Germany) to 100ul volume. Samples were prepared under reducing conditions by adding 50 µL of SDS-PAGE sample buffer containing 2-mercaptoethanol and SDS to each sample and placing samples on a heating block at 90 °C for 10 minutes. Samples were loaded on 12% sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The gels were run at a constant voltage of 120 V until the dye front was approximately 3 cm from the bottom of the gel. Gel was then fixed in 10% acetic acid, 50% methanol solution for 2 h and stained overnight with Gelcode Blue (Pierce). For western blot analysis, the gels were immediately removed after electrophoresis, placed between buffer soaked filter paper and a PVDF membrane, and placed back in the electrophoresis chamber for 3 hours on ice at a constant current of 70 mA. The PVDF membranes were blocked with 50 ml TBST (Tris buffered saline with 0.05% Tween, pH 7.6) containing 2.5% skim milk overnight. The membranes were rinsed three times with TBST, placed in a 50 ml centrifuge tube, and 10 ml of hyperimmune swine serum diluted (1:1000) was added. The tubes were placed on a rocker platform and incubated for four hours, washed thrice with TBST to remove any unbound antibody, and placed back on the rocker with 15 ml of alkaline phosphatase conjugated goat anti-swine IgG diluted in TBST (1:1000). After two hours, the membranes were removed and rinsed thrice with TBST. A colorimetric detection method using 60 mg napthol phosphate and 30 mg fast red (Sigma) in 40 ml distilled water was used to reveal bands.

SG2.1 Mice

Total of 183 C3H/HeOuJ mice of either sex and 8 to 16 weeks of age were used for these studies. Animals were free of observable clinical signs at the beginning of each experiment. Mice were given autoclaved conventional rodent diet, water, and bedding. During the infection phase of the experiment, mice were house in isolation cages with HEPA filtered positive pressure air. All procedures were approved by the Iowa State University Committee on Animal Care.

SG2.2. Experimental design

In four separate experiments, 35-48 mice were vaccinated with pepsin digested *B. hyodysenteriae* whole cell antigen (PD) loaded microspheres of different formulations with or without soluble antigen, PD alone, whole-cell sonicate (WSC) loaded microspheres, WCS alone, or saline. At 30 days post-vaccination, the majority of the mice were challenged with *B. hyodysenteriae*, and four to seven mice were sham inoculated with broth alone in each experiment. Mice were weighed and observed for any clinical signs of disease every other day during infection. All mice were euthanized by CO_2 asphyxiation 30 days post-infection, tissues were scored for gross lesions, and samples were collected for bacteriological, histopathological, serological, and cell-mediated immune analyses.

SG2.3. Mouse Vaccinations

Mice were immunized with a single vaccination intramuscularly in the right hip with 100 µl pyrogen-free saline containing one of the following: 0.5 µg WCS, 25 µg PD, 0.5 mg WCS-loaded 20:80 CPH:SA microspheres (20:80-WCS), 0.5 mg PD-loaded 20:80 CPH:SA microspheres (20:80-PD), 0.5 mg PD-loaded 50:50 CPH:SA microspheres (50:50-PD), or 0.5 mg PD-loaded microspheres (20:80+PD or 50:50+PD) along with 2.5 µg unencapsulated PD. Suspensions of microspheres were sonicated briefly to disperse aggregates before delivery via 25 gauge hypodermic needle.

SG2.4. Bacterial inoculation

Mice were orally colonized with a bacterial inoculum (0.3 ml, approximately 10^8 cells/ml) of B. *hyodysenteriae* administered by orogastric intubation on two consecutive days. Spirochete infection confirmed bacteriologically at necropsy by demonstration of β-hemolytic spirochetes grown on selective media [37].

SG2.5. Gross inflammatory scores

At necropsy, gross pathological changes to the cecum were scored using a modification of a previously reported scoring system [37, 38]. Lesions were scored from 0 to 6, giving one point for each of the following

parameters when present: cecal blunting, cecal atrophy, cecal emptying (excessive mucus), watery or mucoid cecal contents, enlarged lymphoid aggregates, and observed blood in the cecum.

SG2.6. Histology

Formalin-fixed cecal tissues were embedded in paraffin, sectioned at 5 μm, stained with hematoxylin and eosin (H&E), and scored for microscopic inflammation [37, 38]. Tissue sections were coded and presented blind to the pathologist. Histological scores were based on the severity of mucosal epithelial damage (erosions, edema), degree of lamina propria cellular infiltrate, and architectural distortion (crypt length, cellular hyperplasia).

SG2.7. Antigen-specific serum antibodies

Serum samples were obtained every two weeks via saphenous vein blood collection [39]. Blood was refrigerated overnight and serum separated via centrifugation. Sera samples were stored at -20ºC until assays were performed. Measurement of *B. hyodysenteriae* antibodies in sera was performed by ELISA. Briefly, 96-well plates were coated with 5 μg/ml WCS of *B. hyodysenteriae*. After blocking the plates (2% gelatin with 1% fetal calf serum) for two hours, serial dilutions (1:100 to 1:256,000) was added to each well and incubated at 4 ºC overnight. Then, alkaline phosphatase conjugated goat anti-mouse IgG (H&L), IgG1, or IgG2a (Southern Biotechnology, Birmingham AL) was added and incubated for 2 h at room temperature. Wells were developed using p-nitrophenyl phosphate (Sigma 104) in sodium carbonate/bicarbonate buffer (pH 9.3) at room temperature. Optical densities were measured at 405 nm using a microtiter plate reader.

SG2.8. Lymphocyte Proliferation

In order to asses the cellular response induced by a single vaccination, lymphocytes isolated from the mesenteric lymph nodes and lymph nodes draining the injection site (right side popliteal and inguinal). Single cell suspensions were prepared from each individual animal, cellular debris was removed by settling, and the cells were then washed via centrifugation. The cells were resuspended in complete culture medium (cRPMI) consisting of RPMI 1640 containing L-glutamine and supplemented with 1 % nonessential amino acids, 1 % sodium pyruvate, 2 % essential amino acids, 25 mM HEPES buffer, 100 units/ml penicillin, 0.1 mg/ml streptomycin, 0.05 mg/ml gentamycin, 1 % L-glutamine, 5 x 10^{-5} M 2-mercaptoethanol, and 5 % heat-inactivated FBS. Round-bottomed 96-well microtiter plates were seeded with 2.5 x 10^5 cells in cRPMI at a total volume of 200 μl per well. Wells also contained either concanavalin A (Con A, 0.5 μg/ml, Sigma), *B. hyodysenteriae* WCS (5 or 25 μg/ml), or cRPMI alone (i.e., no stimulation). Plates were then incubated for 3 days at 37 ^{o}C in 5 % CO_2 in air. After 3 days, 0.5 μCi of methyl- [^{3}H]-thymidine (specific activity 6.7 Ci mmole^{-1}) at a concentration of 50 μCi/ml in 10 μl of cRPMI was added to each well, and the plates were incubated for an additional 18 hours. The contents of each well were harvested onto glass fiber filters, and the incorporated radioactivity was measured by liquid scintillation counting. Treatments were run in triplicate and data is presented as mean counts per minute (CPM) or stimulation index (SI) calculated by dividing the CPM of treated wells by the CPM of non-stimulated (background) wells.

SG2.9. Cytokine analysis

Single cell suspensions of lymphocytes were prepared by tissue homogenization from the mesenteric lymph nodes and were incubated in cRPMI at 2.5 x 10^5 cells/well with or without WCS of *B. hyodysenteriae* for 72 h. Cells were stimulated with specific bacterial antigens at a concentration of 25 μg/ml or with Con A (0.5 mg/ml). Cell-free supernatants were harvested and analyzed for the presence of TNF-α, IFN-γ, IL-4, IL-5, IL-6, IL-10 and IL-12 cytokines using a multiplexed flow cytometric assay (FlowMetric System; Luminex, Austin TX).

SG3.1 Pigs

Twenty-seven mixed bred pigs (both genders) were purchased from Northwood Farms, a herd known to be free of dysentery and respiratory mycoplasma disease. Pigs were 10 days of age upon arrival and randomly split

into pens. Pigs were treated with 25 mg EXCENEL (ceftiofur hydrochloride, Pfizer) three times, 24 h apart starting day of arrival and 50 mg Baytril (enterofloxin, Bayer) two times, on days 5 and 7 after arrival. Pigs were fed nursery diet ad libitum for the first two weeks and gradually switched to a standard corn-soybean grower ration (14%) (Heartland Co-op, Des Moines, IA) hand fed once daily. Animals were maintained to preserve the high-health, pathogen free status. Pigs were weighed weekly to monitor growth rate and enrichment provided in the pens. Pigs remained healthy and free of any clinical signs of disease (respiratory or enteric) prior to challenge with *B. hyodysenteriae*. All procedures involving pigs were approved by the Iowa State University Committee on Animal Care.

SG3.2 Experimental Design

At 45 days of age, pigs were randomly assigned to their respective treatment groups. The first group was vaccinated in order to assess the immune response to the vaccine designated NI or non-infected and the second group was vaccinated and challenged with *Brachyspira hyodysenteriae* strain B204, 4 weeks following initial vaccination, designated INF for infected. Pigs were vaccinated once with PD loaded microspheres or blank microspheres on day 0 and a separate group of pigs was immunized twice with PD in incomplete Freund's adjuvant (IFA) on day 0 and day 14. On day 28 and day 29 following initial vaccination, pigs were challenged orally with 100 ml of culture broth containing *B. hyodysenteriae* (10^8 cells/ml). Animals were euthanized on day 42 (14 days post-challenge) via administration of Fatal-Plus followed by exsanguination. All injection sites were examined for presence of granuloma formation or other adverse tissue reactions. Colonic lymph nodes and lymph nodes draining the injection site were excised for evaluation of in vitro lymphocyte responses. Ceca and spiral colon were examined for gross lesions. Tissue samples of colon, cecca and injection site tissue and lymph node were preserved in formalin for histological evaluation.

SG3.3 Vaccination

Vaccines were prepared and administered as follows: PD vaccinated pigs: 1.25 mg dry weight PD (40% protein) dissolved in 1 ml pyrogen-free saline was then emulsified with 1 ml IFA. Total volume of 2 ml delivered intramuscularly (IM) into neck muscle with 20 gauge hypodermic needle on days 0 and 14. MS vaccinated pigs: 7 mg 50:50 CPH:SA PD-loaded (10 % w/w), 7 mg 20:80 CPH:SA PD-loaded (10% w/w) and 0.8 mg dry weight PD (40% protein) were suspended in 2 ml of saline, sonicated briefly to suspend the microspheres and injected IM with 20 gauge hypodermic needle on day 0. Non-loaded (i.e., blank) microsphere vaccinated pigs: 7 mg 50:50 CPH:SA microspheres and 7 mg 20:80 CPH:SA microspheres were suspended in 2 ml of saline, sonicated briefly to suspend particles and were injected IM using a 20 gauge hypodermic needle on day 0.

SG3.4 PBMC Proliferation Assay

To evaluate the in vitro antigen-specific recall response induced by vaccination, peripheral blood was collected at days 0, 14, 28 and 35 from the jugular vein into heprinated vaccutainer tubes. Peripheral blood mononuclear cells (PBMC) were isolated by diluting peripheral blood 1:3 in phosphate buffered saline (PBS, pH 7.2). Diluted blood was layered over Lymphocyte Separation Medium (density 1.077) (Mediatech Inc, Manassas VA), and centrifuged at 500 x g for 40 minutes. PBMC were obtained from the medium/plasma interface (buffy coat), washed three times in sterile PBS, and the cells were enumerated for use in proliferation assays.

The cells were resuspended in culture medium (cRPMI) consisting of RPMI 1640 containing L-glutamine and supplemented with 1% nonessential amino acids (Mediatech), 1% sodium pyruvate, 2% essential amino acids, 25 mM HEPES buffer, 100 units/ml penicillin, 0.1 mg/ml streptomycin, 0.05 mg/ml gentimycin, 1% L-glutamine, 5×10^{-5} M 2-mercaptoethanol, and 10% heat-inactivated FBS. Flat-bottomed 96-well microtiter plates were seeded with 2.5×10^5 cells in cRPMI at a total volume of 200 µl per well. Wells also contained either Con A (5 µg/ml, Sigma), 5 or 25 µg/ml *B. hyodysenteriae* WCS antigen or cRPMI alone (i.e., no stimulation/background). Plates were then incubated for 3 days as described above. Treatments were run in triplicate and data are presented as mean counts per minute (CPM) ± SEM.

SG3.5 Lymphocyte proliferative Response of Lymph Nodes from the Injection Site and Colonic Lymph Nodes

Injection site lymph nodes were identified by administering diluted India ink in PBS to a test pig in the same site and manner as vaccines were administered eight hours prior to sacrifice. Upon necropsy, muscles and tissue were carefully dissected until lymph nodes containing the ink were found. Two small lymph nodes located between the ear and shoulder, deep to the trapezius and longissimus muscles were removed from each pig. Additionally, colonic lymph nodes located within the fold of the spiral colon were analyzed. Lymph nodes were homogenized and single cell suspensions were passed through a 40 µm mesh cell filter (BD) to remove fibrous material and washed twice by centrifugation. Flat-bottomed 96-well microtiter plates were seeded with 5×10^5 cells in cRPMI at a total volume of 200 µl per well. Wells also contained either Con A (5 µg/ml, Sigma), 5 or 25 µg/ml *B. hyodysenteriae* WCS antigen or cRPMI alone (i.e., no stimulation/background) and were cultured as described above. Stimulation indices were calculated by dividing the CPM of treated wells by the CPM of non-stimulated (background) wells.

SG3.6 PD-specific enzyme-linked-immunosorbent-assay (ELISA)

Ninety-six well microtiter plates (Costar high protein binding) were coated overnight with 100 µl PBS containing 5 µg/ml PD-WCS. To remove unbound PD, plates were washed with PBS containing 0.05% Tween 20 (PBST) and then blocked for two hours at room temperature with PBST containing 2% gelatin and 2% fetal calf serum (FCS). Serum samples (100 µl/well) from individual pigs were serially diluted in PBST supplemented with 1% FCS (PBST-FCS). The plates were then incubated overnight (18 h) at room temperature (25°C). The plates were again washed three times with PBST followed by addition of 100 µl of PBST-FCS containing alkaline phosphatase-conjugated goat anti-swine IgG (H&L) (0.5 mg/ml diluted 1:1000) (KPL, Gaithersburg, MD). After a two hour incubation period, the plates were washed three times with PBST followed by the addition of 100 µl of sodium carbonate buffer (pH 9.3) containing phosphatase substrate (Sigma 104, Sigma-Aldrich, St. Louis, MO) at a concentration of 1 mg/ml and allowed to react for 1 hour at room temperature. The optical density (OD) of the reaction was measured at 405 nm using a Spectramax 190 Plate Reader (Molecular Devices, Sunnyvale CA). Serum titers are reported as the reciprocal of the highest dilution giving an OD equal to or greater to a value calculated from the average of all background (d0) wells plus one standard deviation.

Statistical Analysis:

Where appropriate, one-way analysis of variance (ANOVA) was conducted on group data with the aid of various computer software packages. Pair-wise comparisons were done for significant ANOVA using Bonferroni's Correction for multiple comparisons.

Results

*Results **Specific Goal 1**: Encapsulation and Characterization of PD loaded Microspheres*

Pepsin digest (PD) whole cell lysate of *Brachyspira hyodysenteriae* was encapsulated into either 20:80 CPH:SA or 50:50 CPH:SA microspheres. Scanning electron microscopy revealed spherical particles between 5 and 10 µm in diameter as seen in Figure 1. Resulting microspheres were a gray free-flowing powder which suspended in PBS following brief sonication. The data in Figure 2 depicts the in vitro release kinetics of the encapsulated protein. The large initial burst was consistent with previous reports using ovalbumin which clustered near the outer surface of the microsphere during solid-oil-oil solvent emulsification [11]. For microspheres made form 20:80 CPH:SA formulation, 50% of the material was released within the first two hours, with over 80% of the total protein encapsulated released in approximately 12 days. The release profile of 50:50 CPH:SA formulations was more consistent with previous results evaluating the release of ovalbumin [11, 40] or tetanus toxoid [15], the release profile indicated that 40 to 60 % of the material was released within 20 days and material continued to be released for at least 35 days (the length of this release kinetic study). SDS-PAGE gel analysis of proteins released after encapsulation into and release from polyanhydride microspheres

had similar molecular size to non-encapsulated protein preparations (data not shown). The immunoblot analysis of released proteins indicates that the materials released from the microspheres maintained antigenicity during encapsulation and release from the polyanhydride microspheres (data not shown).

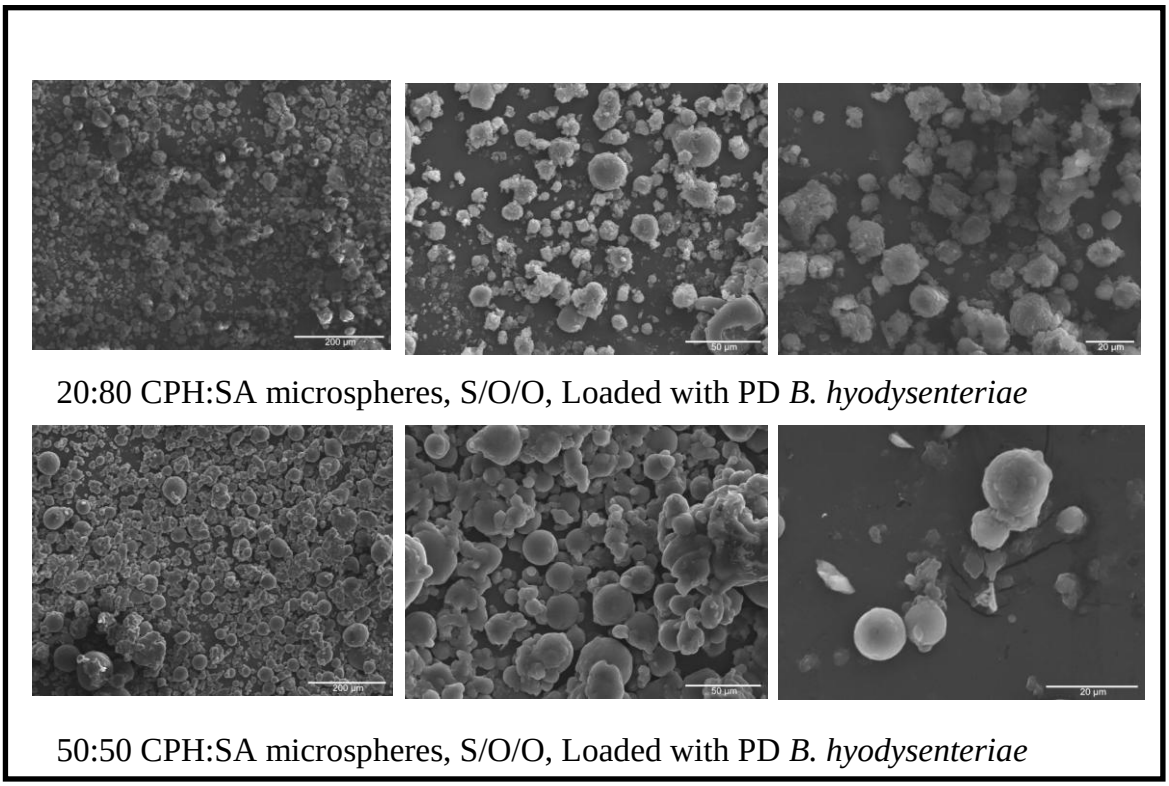

Figure 1. Scanning electron microscopy (SEM) of pepsin-digest loaded microspheres. Photomicrographs are increasing in magnification, as shown by the scale bar in the bottom right of each image. Top panel depicts 20:80 CPH:SA formulation, and the bottom panel depicts the 50:50 CPH:SA formulation. Microspheres were spherical in shape, non-aggregated and had a size distribution between 5 and 10 µm.

*Results **Specific Goal 2**: Evaluate the efficacy of a single dose SD vaccine based on PD-loaded polyanhydride microspheres in an established murine model*

In order to asses the immune response and efficacy of pepsin digest-loaded microspheres, C3H/HeOuJ mice were immunized with various microsphere formulations. Four independent experiments were performed. Results presented are from a single replicate but are representative of trends present in each independent experiment.

Rarely do mice exhibit clinical signs of chronic *B. hyodysenteriae* infection. In order to more closely monitor the overall health status, mice were weighed every two days during the infection period. Infection with *B. hyodysenteriae* alone did not cause a significant decrease in weight as compared to non-infected mice (Figure 3). Vaccination had no discernible impact on weight gain. Four weeks following infection, mice were euthanized and evaluated for protection or attenuation of the typhlocolitis induced by *B. hyodysenteriae* infection of mice. Unlike the swine, vaccination in mice does not protect from the acute phase of disease but does facilitate recovery from the chronic or immune mediated phase of infection [41]. Gross cecal scores between the individual vaccinated groups were not significantly different (Figure 4), but there was a trend toward less severe cecal lesion scores in the mice receiving either the single dose of PD or PD-loaded 50:50 microspheres administered along with a small bolus (0.5 µg) of free PD. In previous studies in our lab, mice received two doses of PD given 10 to 14 days apart.

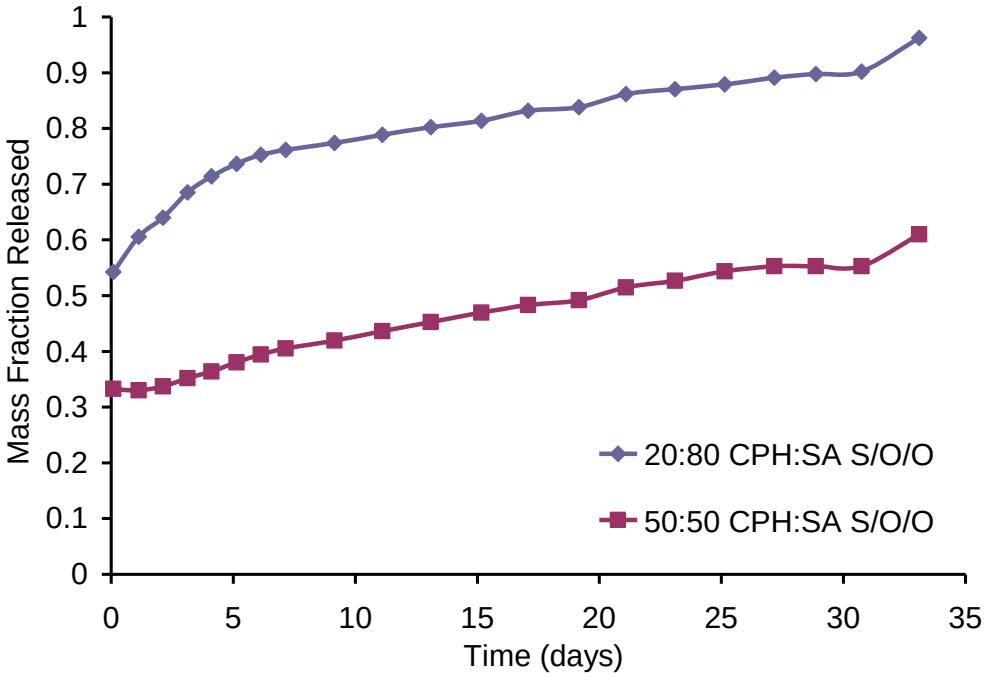

Figure 2. In vitro release kinetics of encapsulated *B. hyodysenteriae* pepsin digests from polyanhydride microspheres. Following encapsulation, microspheres were incubated in buffer for up to 35 days. Samples of the released materials were analyzed for protein content. Data depicts the accumulated fractional release of the protein from the microspheres. The *B. hyodysenteriae* PD antigen preparation was encapsulated into one of two polyanhydride formulations: 20:80 CPH:SA or 50:50 CPH:SA.

PD was considered successful if 60% of the mice were protected. In the current study, all mice received only a single dose of PD, thus confirming the need for multiple antigen exposures to generate a protective immune response. When the ceca were evaluated microscopically for lesions (Figure 5), there was a trend toward less severe epithelial erosions, restitution of the crypt architecture, and less inflammatory cell infiltrate even though statistical difference between vaccinated groups was not observed.

Despite lack of protection from typhlocolitis, the PD-loaded microspheres either primed the mice or induced antigen-specific immune response prior to infection. As seen in Figure 6, the serum antibody response mice vaccinated with microspheres (20:80 or 50:50) with or without free PD greater than that of non-vaccinated mice. The highest antibody responses were observed in mice receiving the PD-loaded 50:50 CPH:SA plus free PD, and PD alone groups. Post-challenge, all challenged mice demonstrated nearly the same level of serum antibody response (Figure 7). There was a trend for a greater antibody response in the 50:50 microsphere vaccinated group, indicating that the mice were primed by the antigen-loaded microspheres prior to challenge. However, the only statistical difference was observed between the non-infected mice and the challenged groups as a whole. Antibodies can be induced to switch to different IgG isotypes depending on immune modulating signals from the $CD4^+$ T cells. To evaluate the ability of the microspheres to modulate the antibody response, the induction of antigen-specific IgG1 (Th2) and IgG2a (Th1) antibody were measured. Mice immunized with 20:80 CPH:SA microspheres, PD or WCS, with or without bolus, exhibited an antigen-specific antibody response suggesting a dominant Th2 immune response (IgG1:IgG2a ratio greater than one, Figure 8). A ratio less than one would indicate the presence of higher amounts of IgG2a which is indicative of a dominant Th1 immune response. The differential IgG1:IgG2a ratios induced by vaccination with the PD-loaded microspheres supports the immune modulation observed with other antigens [15]. To evaluate the cellular immune response induced by microsphere and PD vaccination, lymph nodes draining the injection site (popliteal and inguinal) were removed and evaluated for proliferation in response to in vitro stimulation with WCS antigen. While there

was demonstrable proliferation of cells recovered from all of the challenged groups, Figure 9, the highest amount of proliferation was the group receiving 50:50 CPH:SA microspheres (significantly different from *B. hyodysenteriae* challenged only and Non-infected). In contrast, proliferation from the mesenteric lymph node (Figure 10) was elevated in all challenge groups with no significant difference between them. To further analyze the in vitro recall response of the cells recovered from the mesenteric lymph node, culture supernatants were harvested and assessed for the presence of cytokines. Levels of IL-4, IL-5, IL-6, IL-10, IL-12, IFNγ and TNFα in the culture supernatants were evaluated in order to assess whether there was a biased activation of either Th1 or Th2 pathways. There was no evidence that there was a measurable in vitro cytokine response following antigenic stimulation. However, the 20:80 + PD regimen appeared to modulate the IgG response towards a Th2 response (Fig. 8).

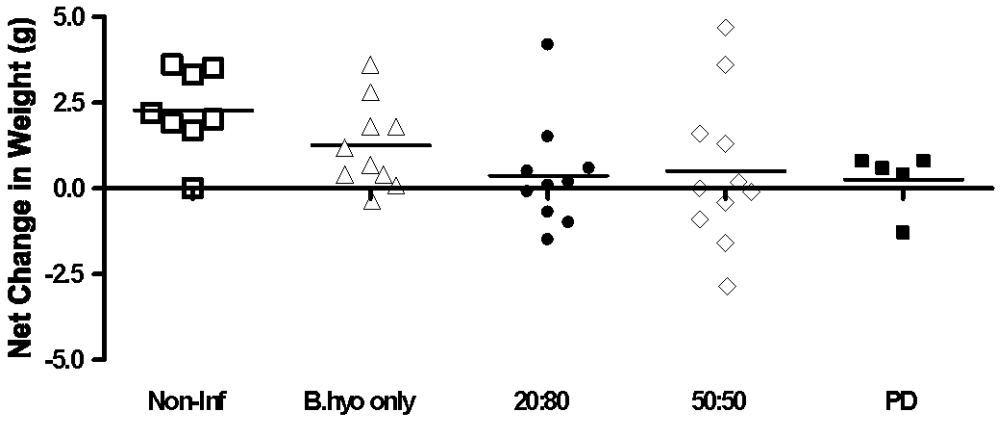

Figure 3. C3H mice were vaccinated with pepsin digest (PD), 50:50 CPH:SA loaded with PD, 20:80 CPH:SA loaded microspheres and challenged with *Brachyspira hyodysenteriae*. Weight changes during the four week infection period were monitored. The horizontal bar represents the group mean.

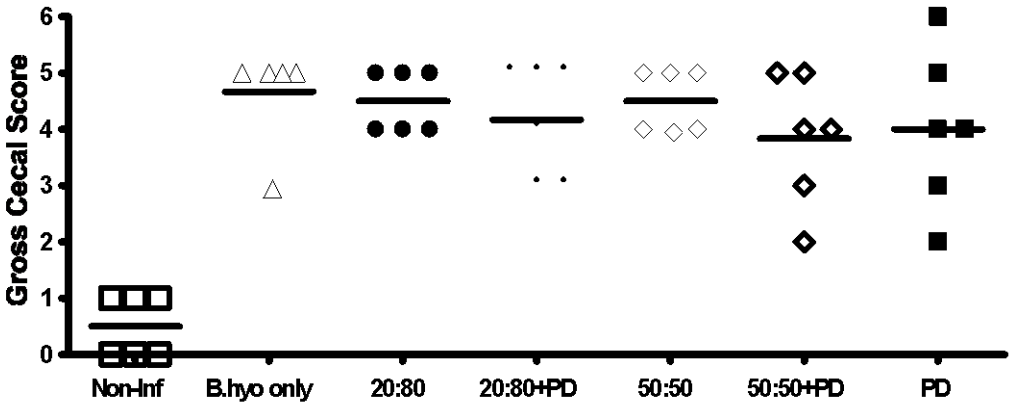

Figure 4. Four weeks following infection with *B. hyodysenteriae*, cecal lesions were evaluated using a modification of a previously established scoring system. Lesions scores of non-infected mice were significantly different ($p \leq 0.05$) from all other groups but none of the vaccinated groups were significantly different from each other.

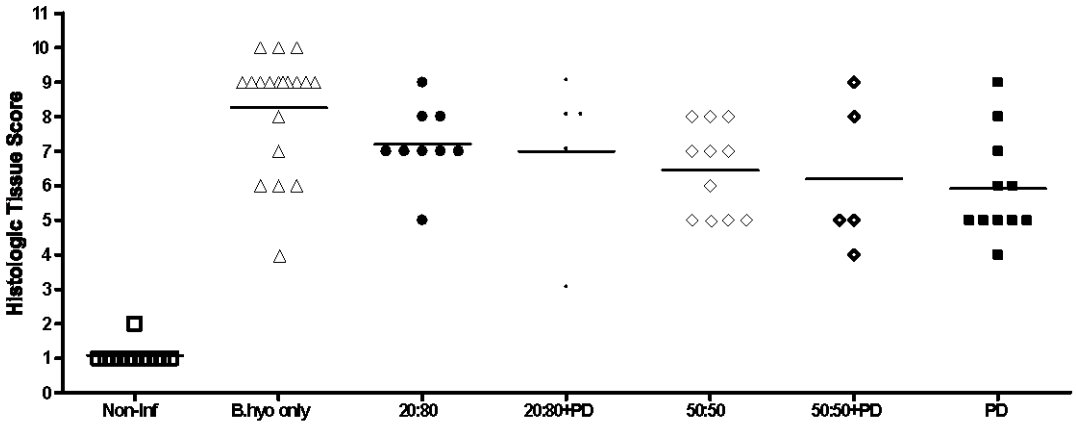

Figure 5. Four weeks following infection with *B. hyodysenteriae*, ceca were placed in formalin, paraffin embedded, sectioned and stained with hematoxylin and eosin. Slides were evaluated in a blinded fashion by a pathologist for epithelial erosion, inflammatory cell infiltrate, and changes in cecal tissue architecture. Tissue sections from non-infected mice were significantly different from all other groups ($P<0.05$) and *B. hyodysenteriae* infected only group was significantly different from 50:50 and PD vaccinated groups.

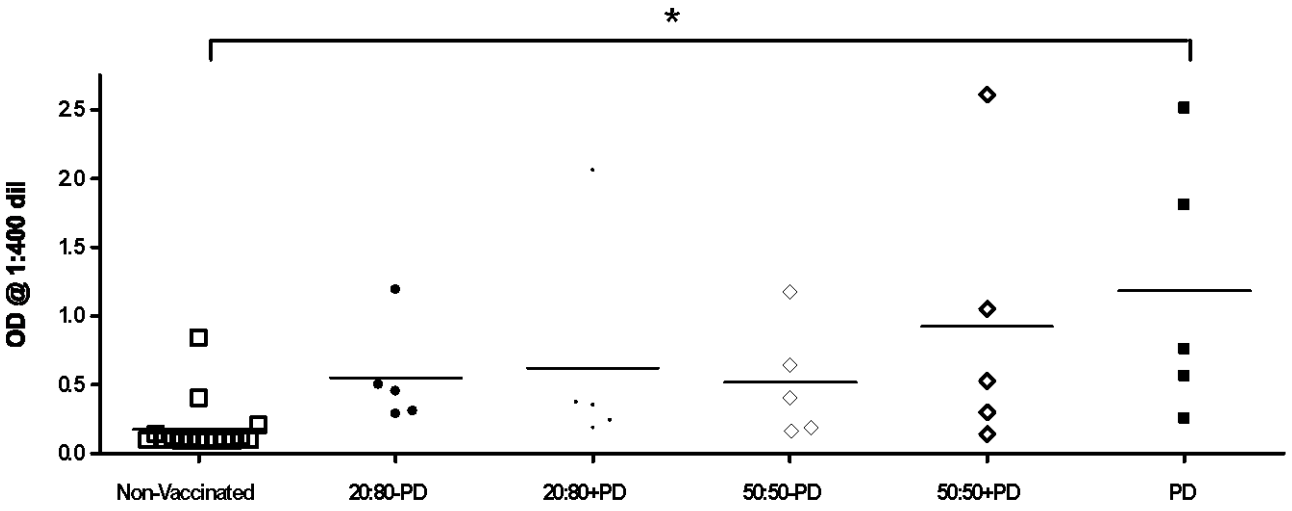

Figure 6. Analysis of serum antibody responses (total IgG) following a single immunization of mice. Mice were immunized as described in materials and methods. Data depicted is the ELISA results four weeks after immunization, but before *B. hyodysenteriae* challenge. Asterisk indicates that the antibody response of the PD vaccinated group was significantly different only from non-vaccinated mice ($P < 0.05$)

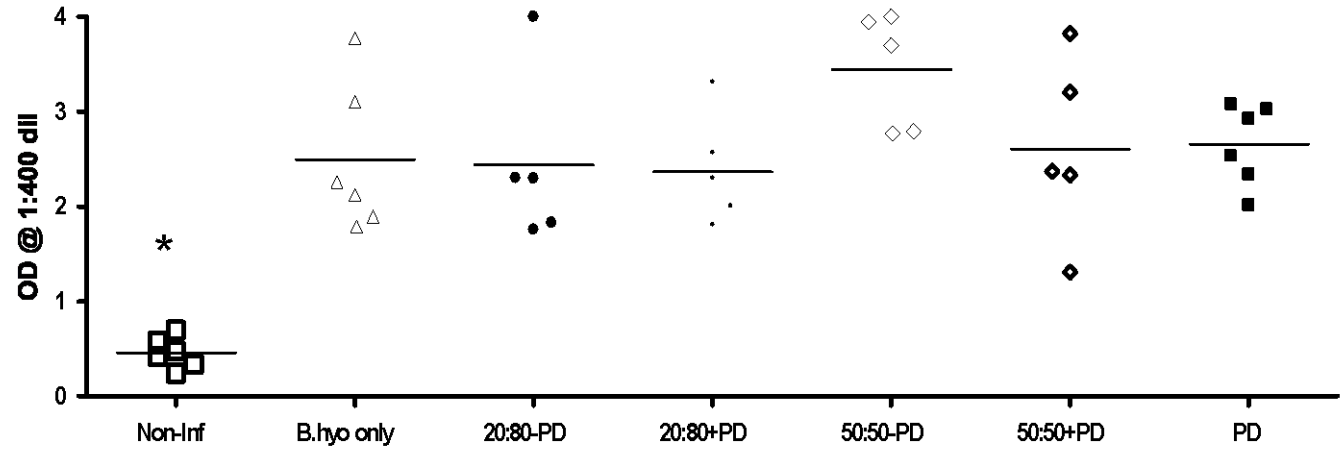

Figure 7. Analysis of serum antibody responses (total IgG) following challenge with *B. hyodysenteriae*. Mice were immunized as described in materials and methods. Serum antibody responses in mice vaccinated with PD-loaded 20:80 and 50:50 microspheres (with and without free antigen bolus) increased compared to controls and to pre-challenge serum antibody responses (Figure 6) indicating that the lower serum antibody responses observed prior to challenge were not due to the induction of immune tolerance. The asterisk indicates that the antibody response of the non-infected group was significantly different ($P < 0.05$) from all other groups.

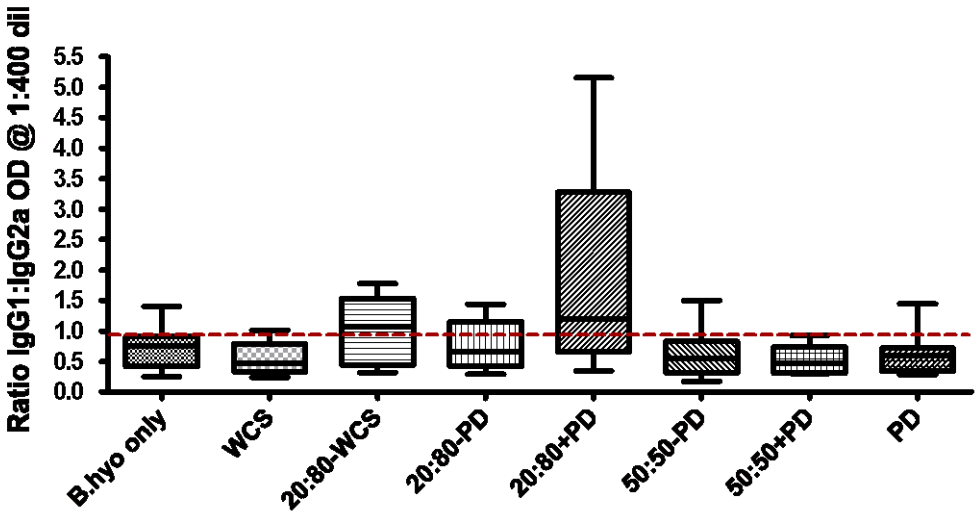

Figure 8: Assessment of the immunological balance in the IgG isotype response induced by vaccination with the PD-loaded microspheres followed by challenge with *B. hyodysenteriae*. A ratio of one would suggest a similar amount of IgG1 and IgG2a were induced against *B. hyodysenteriae*. Ratios below one would suggest a Th1-bias in the antibody response while ratios above one would suggest a Th2-bias in the antibody response.

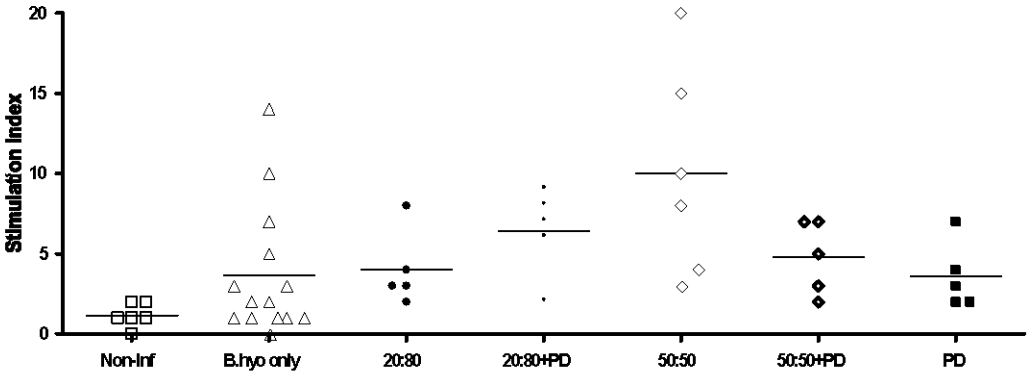

Figure 9: Antigen-specific proliferation of lymphocytes recovered from lymph nodes draining the injection. Mice were immunized once as described in materials and methods. Four weeks after challenge, mice were euthanized and lymph nodes draining the injection site were collected and analyzed for antigen-specific recall responses. Enhanced responses induced by 50:50 PD-loaded microspheres suggests that the persistent release of antigen has a differential effect on lymphocyte proliferation.

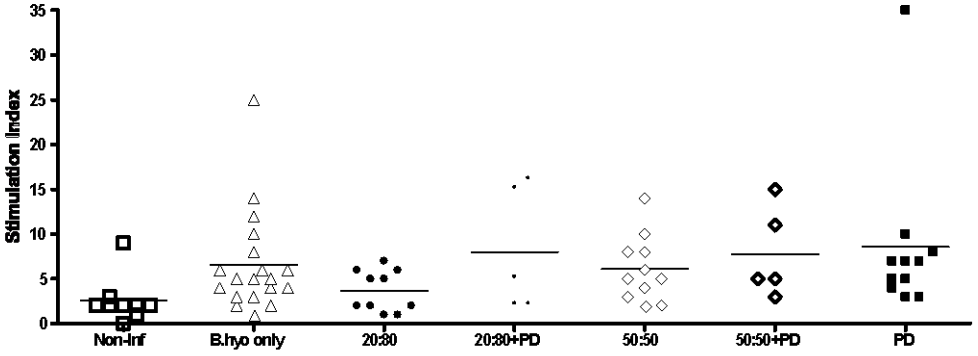

Figure 10: Lymphocyte proliferation from mesenteric lymph node cells following restimulation with *B. hyodysenteriae* WCS. Mice were immunized and challenged as described in materials and methods. Four weeks after challenge, mice were euthanized and the antigen-specific proliferation of mesenteric lymph node cells was analyzed.

*Results **Specific Goal 3**: Evaluate the immune response and subsequent protection of a single dose SD vaccine based on PD loaded polyanhydride microspheres in grower pigs.*

In order to evaluate the efficacy of a single dose vaccine based on polyanhydride microspheres, a model of swine dysentery (SD) was chosen. The investigators have had success developing a vaccine for SD, and many years of experience with the model. 27 crossbred pigs were purchased at 10 days of age from a farm known to be free of both swine dysentery and mycoplasma respiratory diseases. Upon arrival at ISU, pigs were given several rounds of long lasting, broad spectrum antibiotics and housed to prevent acquisition of respiratory pathogens. When animals reached 45 days of age, vaccination for evaluation of microsphere based vaccines was initiated. A time line of the study is depicted in Figure 11.

Pigs were weighed weekly in order to monitor growth. Growth rate is also an economically important clinical sign of *B. hyodysenteriae* infection. Prior to challenge, vaccination had no effect on growth rate. Upon challenge, vaccination had a definite effect on growth rate indicating protection from disease. The growth rate (average daily gain, ADG) for the group receiving two doses of pepsin digest *B. hyodysenteriae* antigen in incomplete Freund's adjuvant (IFA), a common mineral oil vaccine adjuvant, was significantly higher ($p \leq 0.05$) than the group receiving the sham vaccine (Fig. 12). The variability of ADG in the pigs receiving the PD-loaded microspheres correlated with the severity of clinical disease of the animals in the group. For example, pigs presenting with little or no disease had an ADG of 1.6 to 1.8 pounds/day compared to pigs presenting with severe disease (ADG 0 to 0.4).

Reduction of carcass loss due to adverse reactions and granuloma formation at the injection site is of economic importance to producers as well as important for adjuvants with potential use in human as well as livestock vaccines. At necropsy, injection sites were examined for any gross signs of adverse reactions. As summarized in Table 1, 6 of 8 pigs receiving PD in IFA developed granulomas in the muscle tissue of the injection site. The abscesses were sterile (no bacterial growth from the exudate) and contained mostly inflammatory cells. In the pigs receiving either the blank and PD-loaded microspheres there was no evidence of tissue reactivity, granuloma formation or other adverse reactions. This is consistent with other observations from mice and sheep vaccinated with the polyanhydride microspheres in that there is little to no local inflammatory reaction or granuloma formation in response to microsphere vaccination.

Pigs were observed daily beginning day of challenge and evaluated for any clinical signs of dysentery including loose or watery stools, presence of blood and/or mucus, inappetence, and lethargic behavior. Observations were recorded for each individual pig and total number of diarrhea days for each individual are reported (Figure 13). The PD-IFA vaccinated group remained free of any clinical signs of dysentery. While there was no significant difference between the PD-microsphere vaccinated group and the sham (blank) vaccinated groups, there was animal-to-animal variability. All five of the animals in the sham vaccinated group displayed clinical signs of dysentery whereas one of the microsphere vaccinated pigs remained completely healthy. In contrast to all previous studies in this laboratory with the swine dysentery model, four out of 5 pigs in the sham vaccinated group spontaneously recovered from disease by 10 days following challenge. Likewise, two of the animals with disease symptoms in the PD-microsphere vaccinated group recovered between days 7 to 10 post-challenge. The spontaneous resolution of clinical signs was not due to clearance of *B. hyodysenteriae*. As shown in Table 1, there were animals in each group that were positive for β-hemolytic anaerobic spirochetes at time of necropsy.

In order to assess the immune response induced by PD-loaded microspheres, peripheral blood was collected at day 0 (pre-vaccination), day 14 following vaccination, day 28 (prior to challenge) and day 38 (10 days post-challenge). Serum antibody responses to *B. hyodysenteriae* antigen were measured by ELISA. No group showed any appreciable antibody on day 14, but on day 28 the IFA-PD vaccinated groups had a serum

antibody titer of 12800 and 6400 for the infected and non-infected groups (Figure 14). Following infection, the median titer for the IFA-PD vaccinated group increased from 12800 on day 28 to 25600 on day 38. The group receiving PD-loaded microspheres did not exhibit any appreciable antibody until day 38 and only those pigs that were challenged with *B. hyodysenteriae*. No appreciable antibody titer was observed after infection in the pigs receiving the blank microspheres, indicating that the PD-loaded microspheres primed these pigs for a secondary immune response.

On days 0, 14, 28 and 38 mononuclear cells were isolated from peripheral blood and stimulated with *B. hyodysenteriae* antigen. The proliferation of these PBMC is depicted for the challenged groups in Figure 15. The IFA-PD vaccinated group's proliferative response increased after day 14, after the second PD immunization, and again between days 28 and 38, increasing after challenge. Similarly, IFA-PD vaccinated but non-challenged pigs proliferative response increased from day 0 to 14 and again days 14 to 28 (data not shown). Peripheral blood mononuclear cells recovered from pigs vaccinated with the blank microsphere did not exhibit appreciable proliferation until after challenge (day 38). In contrast, the PD-loaded microsphere vaccinated group did not show an increase their proliferative response following challenge. This failure to increase proliferation in the PD-loaded microsphere vaccinated group in contrast to the blank microsphere group further indicates immunological priming and differential immunological response induced by the microsphere delivered vaccine from PD delivered in Freund's incomplete adjuvant or the immune response induced by infection alone (blank microsphere group).

At necropsy, lymph nodes draining the injection site were excised, single cell suspensions were prepared, and stimulated in vitro with *B. hyodysenteriae* antigen. Very little antigen-specific proliferation was observed and was not statistically different between vaccination groups (data not shown). Unlike the vaccinated mice, antigen-specific cells were not detected in the lymph nodes adjacent to the injection site four weeks following vaccination. In addition, antigen-specific proliferative responses were also evaluated for cells recovered from the colonic lymph nodes. A robust proliferative response was observed in the colonic lymph node cell cultures from challenged pigs receiving the IFA-PD and PD-loaded microspheres (Figure 16). A significant proliferative response was also observed in non-challenged animals receiving the PD-loaded microspheres. In contrast, colonic lymphocyte proliferation to *B. hyodysenteriae* antigen was not observed in infected only (blank microsphere vaccinated) animals. Colonic lymph node proliferation is in contrast to the PBMC response further indicating a different immune response induced by the two vaccine regimens.

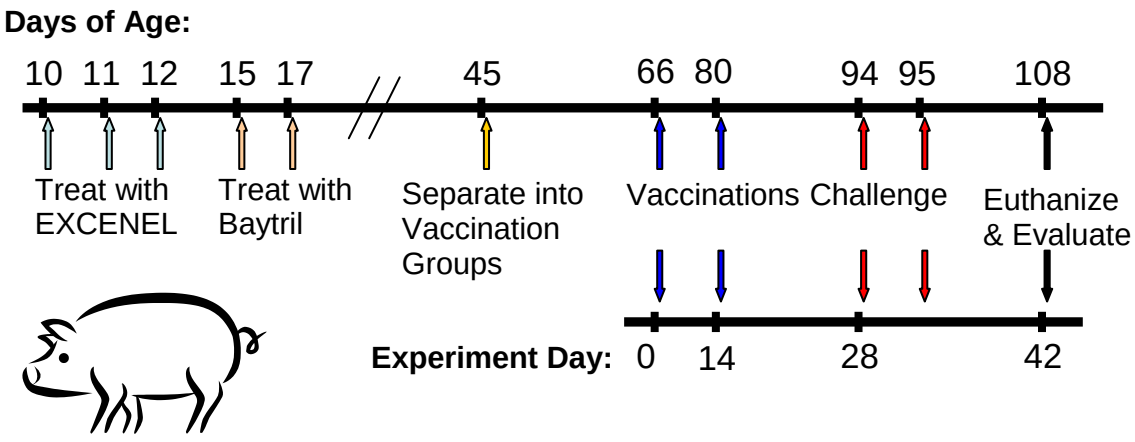

Figure 11: Timeline depicting experimental design for vaccinating and challenging pigs. Animals were given antibiotics upon arrival, at 45 days of age the pigs were separated into their respective vaccination groups, at day 66 (experimental day 0) all pigs were injected with vaccine or received a sham treatment (IFA-PD, PD-MS and blank), and at day 80 (experiment day 14) the IFA-PD group received the second vaccination. Pigs were challenged with *Brachyspira hyodysenteriae* on two consecutive days (28 and 29).

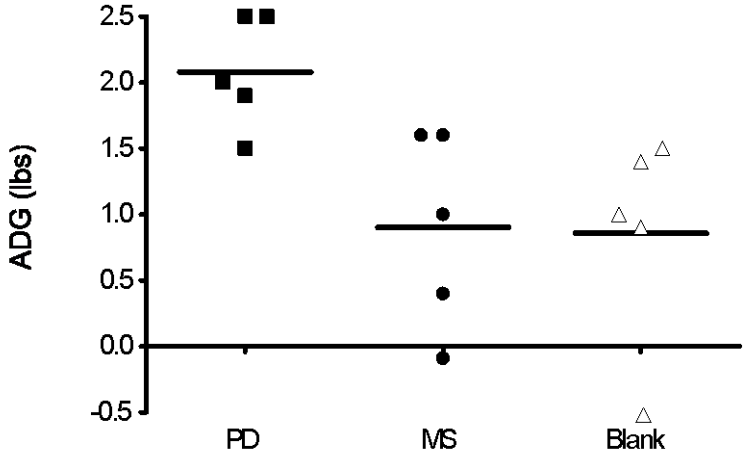

Figure 12: Average daily gain (ADG) of pigs following infection with *B. hyodysenteriae*. The ADG for the group receiving two doses of the IFA-PD vaccine was significantly different ($P<0.5$, $\alpha=0.05$) from the group receiving blank microspheres but neither was significant from the group receiving the singe dose of PD-loaded microspheres (MS) (n=5). Non-infected (control) animals were housed in a separate room and had an ADG of 1.7 lbs (n=12).

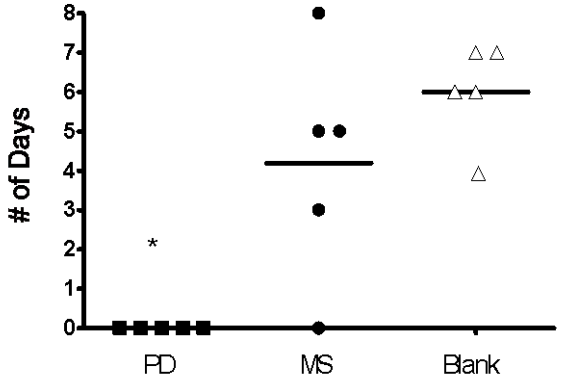

Figure 13: Total number of days that clinical signs of dysentery were observed in pigs infected with *B. hyodysenteriae*. Animals were observed daily during the 14 day infection period. Signs of dysentery included loose or watery stools, presence of blood or mucus in the stools, lethargy and reduced appetite. The group receiving two doses of IFA-PD (PD) was significantly different (* $P < 0.05$) from either group receiving blank microspheres or the group receiving a singe dose of the pepsin digest loaded microsphere vaccine (MS) (n=5).

Table 1: Summary of clinical observations of infected and non-infected pigs following vaccination.

Treatment Group	No. pigs with Colonic Lesions[a]	Positive for β-hemolytic spirochetes[b]	No. pigs with clinical signs of dysentery[c]	No. pigs with injection site reactions[d]
IFA-PD Vaccinated	0/5	2/5	0/5	6/8
PD-loaded Microspheres	3/5	3/5	4/5	0/8
Blank Microspheres	1/5	3/5	5/5	0/8

Animals received either two doses of pepsin digested *B. hyodysenteriae* antigen in IFA (PD), a single dose of PD-loaded microspheres (MS) or a single injection of blank microspheres containing no antigen. (Blank).

[a]gross lesions at necropsy included mild to severe hyperemia, hemorrhage, mucus, or fibrin deposition.

[b]culture of colonic samples for β-hemolytic spirochetes on selective media.

[c]presence of clinical signs of dysentery during the 14 days post-challenge.

[d]presence of granulomatous reactions at the injection site at time of necropsy. Observations of injection site reactivity also include vaccinated non-challenged animals.

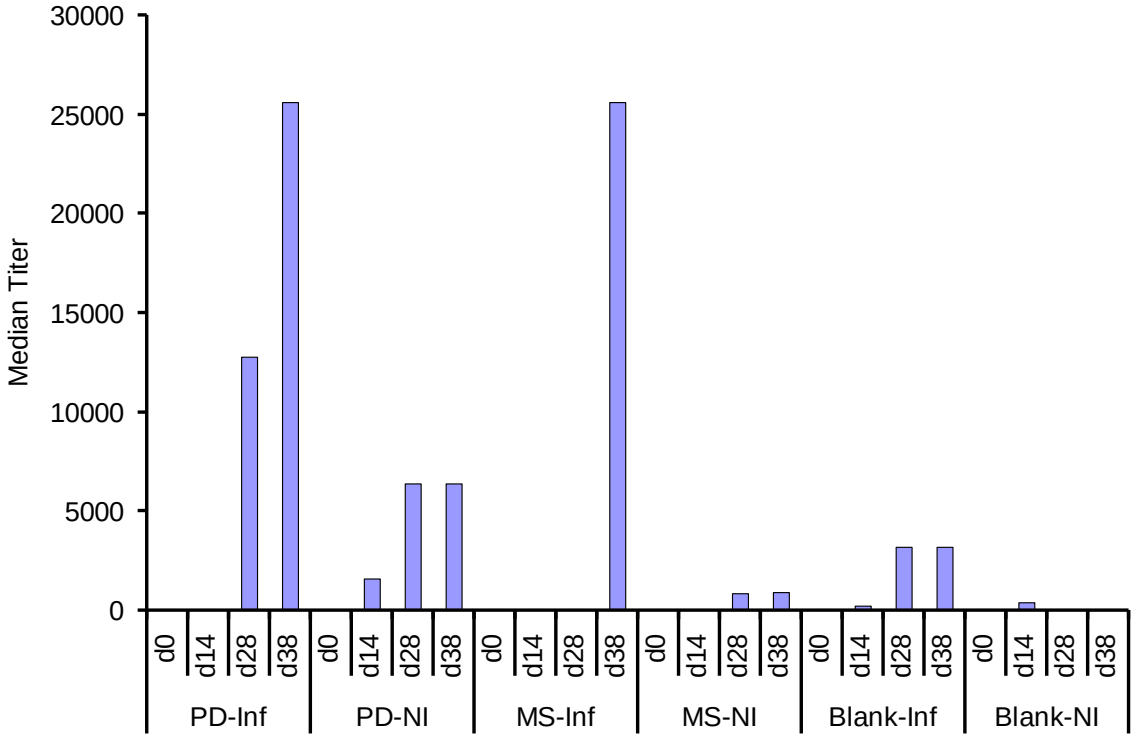

Figure 14: Serum antibody (IgG H&L) responses to B. *hyodysenteriae* antigen. Peripheral blood was collected prior to vaccination (d0), at day 14 after initial vaccination, day 28 (prior to challenge) and day 38 (10 days after challenge). Data depicted is group median titer that was determined as described in materials and methods. Pigs were vaccinated with either two doses of pepsin digested *B. hyodysenteriae* antigen in incomplete Freund's adjuvant (PD), a single dose of pepsin digest loaded microspheres (MS) or a single injection of blank

microspheres containing no antigen (Blank). On day 28, some animals (n =15) were challenged with *B. hyodysenteriae* (-Inf), and the other animals (n = 4) remained unchallenged (-NI). While no antibody was detected in MS vaccinated animals prior to challenge, the sharp increase in the serum antibody titer following infection indicates that these pigs were systemically primed for a subsequent secondary immune response.

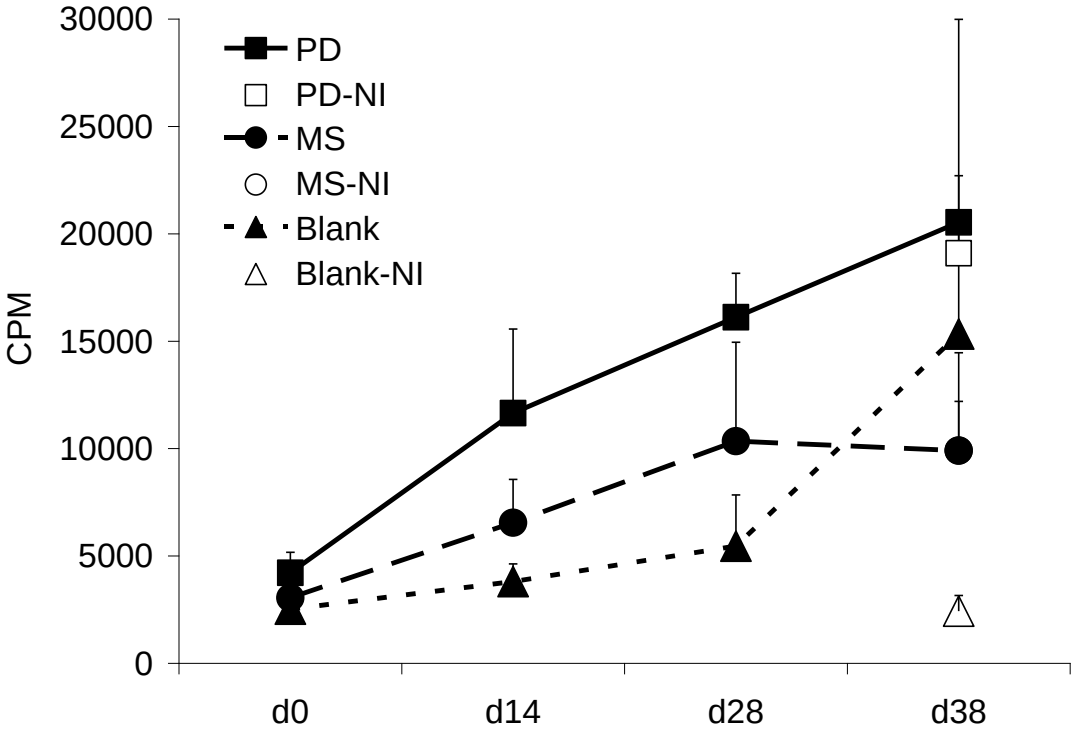

Figure 15: Peripheral blood mononuclear cell (PBMC) proliferative recall response to *B. hyodysenteriae* antigen. Peripheral blood was collected prior to vaccination (d0), at day 14, day 28 (prior to challenge), and day 38 (10 days after challenge). PBMC were isolated and stimulated as described in materials and methods. Pigs were vaccinated and challenged as described in materials and methods. Data points PBMC proliferation at day 38 for the corresponding non-infected pigs are represented by open symbols: IFA-PD (open square, n =5), PD-MS (open circle, n = 4 – superimposed with the closed circle) and blank-MS (open triangle, n = 3).

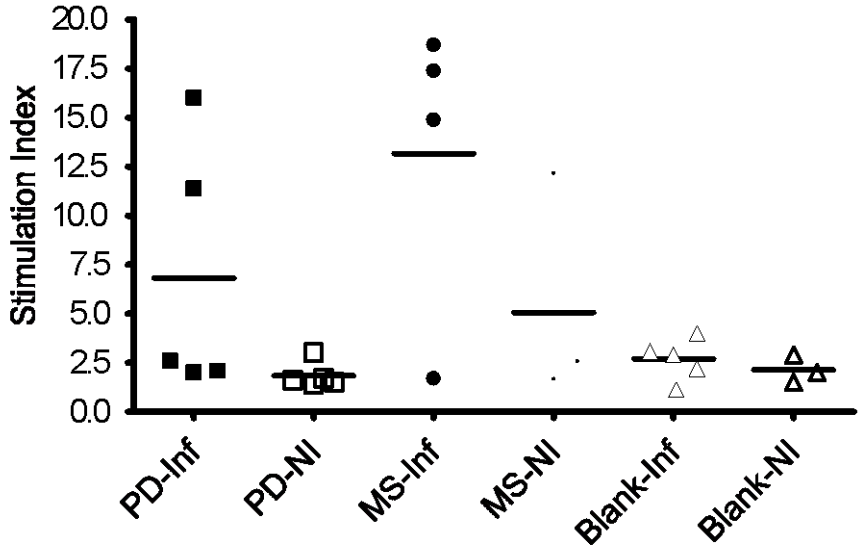

Figure 16: Antigen-specific proliferative response of cells recovered from colonic lymph nodes. At necropsy, colonic lymph nodes were excised and single cell suspensions were prepared as described in materials and methods. Stimulation indices were calculated by dividing the counts per minute of antigen stimulated wells with the counts per minute from non-stimulated (background) wells. Statistical differences were found between PD-NI group and MS-Inf, and between MS-Inf and Blank-Inf but no other groups.

Discussion

To date, a majority of commercial vaccines used to induce protection against bacterial-induced diseases of swine employ two or more vaccinations increasing the burden on personnel needs. Additionally, these vaccines are often whole bacterins and there is concern that adverse reactions at the injection site can affect carcass quality. Previous studies from this laboratory had shown that the intramuscular administration to pigs of an enzymatic digest of *B. hyodysenteriae* (PD) incorporated into IFA induced protection from swine dysentery. As was shown in the current study, two doses of the IFA-PD vaccine induced protection in 100 % of the pigs challenged with *B. hyodysenteriae.* Consistent with previous observations, the IFA containing vaccine induced demonstrable antigen-specific serum antibody and proliferation of PBMCs that was accompanied by a characteristic granulomatous reaction at the injection site. In contrast all of the sham vaccinated pigs developed clinical swine dysentery following challenge. The majority of the pigs (4 of 5) the single dose vaccine containing the PD-loaded polyanhydride microspheres (PD-MS) also developed swine dysentery. Relative to the sham vaccinated pigs, the PD-MS vaccinated pigs had slightly fewer diarrhea days (Fig. 13) and were primed for the induction of a serum antibody response after challenge (Fig. 14, day 38). There is little evidence that a serum antibody response alone provides protection from swine dysentery. However, these results suggest that pigs immunized with a single dose vaccine formulated with antigen-loaded MS would induce significant serum antibody following infectious challenge.

As cell-mediated responses may also be an important component of protective immunity, the results of these studies indicate that lymphocytes recovered from lymph nodes (LN) draining site of infection of vaccinated pigs proliferated better than those from the sham immunized pigs (Fig 16). In addition, the antigen-specific proliferative response of colonic LN cells recovered from the PD-MS proliferated more than cells recovered from the IFA-PD group. The nature of this response (i.e., type of T cells proliferating or cytokines produced) was not evaluated. However, prior experience by the authors indicated that the clinical signs of swine dysentery were often exacerbated (earlier onset) by the induction of an inappropriate immune response. In this regard, clinical signs of swine dysentery appeared one to two days earlier in the PD-MS vaccinated group in

comparison to the sham vaccinated group suggesting that vaccination with the PD-MS exacerbated disease induction. However, there were fewer total number of days with clinical disease in the PD-MS group compared to the sham vaccinated pigs (Fig. 13).

While the mouse studies did not provide evidence that the antigen-loaded MS induced protection from disease, there was evidence that the PD-loaded 50:50 microspheres enhanced antigen-specific antibody responses prior to and after challenge with *B. hyodysenteriae.* While the results were not statistically significant, there was also a trend toward less severe typhlocolitis and histopathological lesions in mice receiving the PD-loaded 50:50 polyanhydride microspheres.

In conclusion, an enzymatic digestion of a whole cell antigen preparation was encapsulated into polyanhydride microspheres. The antigen was released from the MS with the expected kinetic rate and the released material was antigenic. In addition, administration of the PD-loaded MS to both pigs and mice induced antigen-specific immune responses; however, the PD-MS formulations used in these studies did not induce protection from clinical dysentery. Future studies will be required to optimize the immunization regimen or to evaluate the ability of antigen-loaded MS to induce protection against a systemic bacterial or viral infection of pigs. Taken together, these results indicate that single dose vaccination using biodegradable polyanhydrides is a viable approach and, with refinement, will become an efficacious vaccine carrier beneficial to livestock health.

Acknowledgements: The investigators would like to deeply thank Dr. Jesse Hostetter for evaluating the histology, Brenda Carrillo for preparing the microspheres, SEMs and performing the release kinetics studies, Dr. F.C. Minion for kindly collaborating on this study, Drs Erin Strait and Alex Ramirez for their expertise with the pig study and Michael Drey, Jennie Walker, Meghan Wymore, and Ryan Schafer for their assistance in the mouse and pig studies.

References:

[1] O'Brien CN, Guidry AJ, Douglass LW, Westhoff DC. Immunization with *Staphylococcus aureus* lysate incorporated into microspheres. J Dairy Sci 2001 Aug;84(8):1791-9.

[2] Munoz PM, Estevan M, Marin CM, Jesus De Miguel M, Jesus Grillo M, Barberan M, et al. *Brucella* outer membrane complex-loaded microparticles as a vaccine against *Brucella ovis* in rams. Vaccine 2006 Mar 10;24(11):1897-905.

[3] Singh M, Briones M, O'Hagan DT. A novel bioadhesive intranasal delivery system for inactivated influenza vaccines. J Control Release 2001 Feb 23;70(3):267-76.

[4] Schwendeman SP, Costantino HR, Gupta RK, Tobio M, Chang AC, Alonso MJ, et al. Strategies for stabilizing tetanus toxoid towards the development of a single-dose tetanus vaccine. In: Brown F, editor. New Approaches to Stabilization of Vaccines Potency. Karger: Basel, 1996: 293-306.

[5] Tamada J, Langer R. The development of polyanhydrides for drug delivery applications. Biomater Sci Polym Ed 1992;3(4):315-53.

[6] Narasimhan B, Kipper MJ. Surface-Erodible Biomaterials for Drug Delivery. Advances in Chemical Engineering 2004;29:169-218.

[7] Leong KW, Amore PD, Marletta M, Langer R. Bioerodible polyanhydrides as drug-carrier matrices. 2. Biocompatibility and chemical reactivity. Journal of Biomedical Materials Research 1986;20:51-64.

[8] Torres MP, Determan AS, Anderson GL, Mallapragada SK, Narasimhan B. Amphiphilic polyanhydrides for protein stabilization and release. Biomaterials 2007 Jan;28(1):108-16.

[9] Ron E, Turek T, Mathiowitz E, Chasin M, Hageman M, Langer R. Controlled release of polypeptides from polyanhydrides. Proc. Natl Acad Sci USA 1993;90:4176-80.

[10] Tabata Y, Gutta S, Langer R. Controlled Delivery Systems for Proteins Using Polyanhydride Microspheres. Pharm Res 1993;10(4):487-96.

[11] Determan AS, Trewyn BG, Lin VS, Nilsen-Hamilton M, Narasimhan B. Encapsulation, stabilization, and release of BSA-FITC from polyanhydride microspheres. J Control Release 2004 Nov 5;100(1):97-109.

[12] Waeckerle-Men Y, Allmen EU, Gander B, Scandella E, Schlosser E, Schmidtke G, et al. Encapsulation of proteins and peptides into biodegradable poly(d,l-lactide-co-glycolide) microspheres prolongs and enhances antigen presentation by human dendritic cells. Vaccine 2006 Mar 10;24(11):1847-57.

[13] Thai R, Moine G, Desmadril M, Servent D, Tarride JL, Menez A, et al. Antigen stability controls antigen presentation. J Biol Chem 2004 Sep 10;279:50257-66.

[14] Mine Y, Zhang JW. Comparative studies on antigenicity and allergenicity of native and denatured egg white proteins. J Agric Food Chem 2002 Apr 24;50(9):2679-83.

[15] Kipper MJ, Wilson JH, Wannemuehler MJ, Narasimhan B. Single dose vaccine based on biodegradable polyanhydride microspheres can modulate immune response mechanism. J Biomed Mater Res A 2006 Mar 15;76(4):798-810.

[16] Bowersock TL, Martin S. Vaccine delivery to animals. Adv Drug Deliv Rev 1999 Jul 26;38(2):167-94.

[17] Charerntantanakul W, Platt R, Johnson W, Roof M, Vaughn E, Roth JA. Immune responses and protection by vaccine and various vaccine adjuvant candidates to virulent porcine reproductive and respiratory syndrome virus. Vet Immunol Immunopathol 2006 Jan 15;109(1-2):99-115.

[18] Waters WR, Hontecillas R, Sacco RE, Zuckermann FA, Harkins KR, Bassaganya-Riera J, et al. Antigen-specific proliferation of porcine CD8$\alpha\alpha$ cells to an extracellular bacterial pathogen. Immunology 2000 Nov;101(3):333-41.

[19] Waters WR, Pesch BA, Hontecillas R, Sacco RE, Zuckermann FA, Wannemuehler MJ. Cellular immune responses of pigs induced by vaccination with either a whole cell sonicate or pepsin-digested *Brachyspira (Serpulina) hyodysenteriae* bacterin. Vaccine 1999 Nov 12;18(7-8):711-9.

[20] Trott DJ, Alt DP, Zuerner RL, Wannemuehler MJ, Stanton TB. The search for *Brachyspira* outer membrane proteins that interact with the host. Anim Health Res Rev 2001 Jun;2(1):19-30.

[21] Rosey EL, Kennedy MJ, Yancey RJ, Jr. Dual flaA1 flaB1 mutant of *Serpulina hyodysenteriae* expressing periplasmic flagella is severely attenuated in a murine model of swine dysentery. Infect Immun 1996 Oct;64(10):4154-62.
[22] ThePigSite Pig Health Swine Dysentery (SD). [cited 2006 11/24/2006]; Available from: http://www.thepigsite.com/pighealth/article/361/swine-dysentery-sd
[23] Hontecillas R, Bassaganya-Riera J, Wilson J, Hutto DL, Wannemuehler MJ. $CD4^+$ T-cell responses and distribution at the colonic mucosa during *Brachyspira hyodysenteriae*-induced colitis in pigs. Immunology 2005 May;115(1):127-35.
[24] Carvajal A, de Arriba ML, Rodriguez H, Vidal AB, Duhamel GE, Rubio P. Prevalence of *Brachyspira* species in pigs with diarrhoea in Spain. Vet Rec 2006 May 20;158(20):700-1.
[25] Suh DK, Song JC. Prevalence of *Lawsonia intracellularis*, *Brachyspira hyodysenteriae* and *Salmonella* in swine herds. J Vet Sci 2005 Dec;6(4):289-93.
[26] Jansson DS, Johansson KE, Olofsson T, Rasback T, Vagsholm I, Pettersson B, et al. *Brachyspira hyodysenteriae* and other strongly beta-haemolytic and indole-positive spirochaetes isolated from mallards (Anas platyrhynchos). J Med Microbiol 2004 Apr;53(Pt 4):293-300.
[27] Joens LA. Experimental transmission of *Treponema hyodysenteriae* from mice to pigs. Am J Vet Res 1980 Aug;41(8):1225-6.
[28] Asano T, Adachi Y. Effects of griseoviridin and viridogrisein against swine dysentery in experimental infection by using mice and pigs. J Vet Med Sci 2006 Jun;68(6):555-60.
[29] La T, Phillips ND, Reichel MP, Hampson DJ. Protection of pigs from swine dysentery by vaccination with recombinant BmpB, a 29.7 kDa outer-membrane lipoprotein of *Brachyspira hyodysenteriae*. Vet Microbiol 2004 Aug 19;102(1-2):97-109.
[30] Wannemuehler MJ, Hubbard RD, Greer JM. Characterization of the major outer membrane antigens of *Treponema hyodysenteriae*. Infect Immun 1988 Dec;56(12):3032-9.
[31] Diego R, Lanza I, Carvajal A, Rubio P, Carmenes P. Serpulina hyodysenteriae challenge of fattening pigs vaccinated with an adjuvanted bivalent bacterin against swine dysentery. Vaccine 1995 May;13(7):663-7.
[32] Waters WR, Sacco RE, Dorn AD, Hontecillas R, Zuckermann FA, Wannemuehler MJ. Systemic and mucosal immune responses of pigs to parenteral immunization with a pepsin-digested S*erpulina hyodysenteriae* bacterin. Vet Immunol Immunopathol 1999 Jul 1;69(1):75-87.
[33] Bassaganya-Riera J, Hontecillas R, Zimmerman DR, Wannemuehler MJ. Long-term influence of lipid nutrition on the induction of $CD8^+$ responses to viral or bacterial antigens. Vaccine 2002 Jan 31;20(9-10):1435-44.
[34] Aucouturier J, Dupuis L, Ganne V. Adjuvants designed for veterinary and human vaccines. Vaccine 2001 Mar 21;19(17-19):2666-72.
[35] Shen E, Pizsczek R, Dziadul B, Narasimhan B. Microphase separation in bioerodible copolymers for drug delivery. Biomaterials 2001 Feb;22(3):201-10.
[36] Kipper M, Shen EE, Determan AJ, Narasimhan B. Design of an injectable system based on bioerodible polyanhydride microspheres for sustained drug delivery. Biomaterials 2002;23:4405-12.
[37] Nibbelink SK, Wannemuehler MJ. An enhanced murine model for studies of *Serpulina (Treponema) hyodysenteriae* pathogenesis. Infect Immun 1992 Aug;60(8):3433-6.
[38] Jergens AE, Dorn A, Wilson J, Dingbaum K, Henderson A, Liu Z, et al. Induction of differential immune reactivity to members of the flora of gnotobiotic mice following colonization with *Helicobacter bilis* or *Brachyspira hyodysenteriae*. Microbes Infect 2006 May;8(6):1602-10.
[39] Hem A, Smith AJ, Solberg P. Saphenous vein puncture for blood sampling of the mouse, rat, hamster, gerbil, guinea pig, ferret and mink. Lab. Animals 1998;32:364-8.
[40] Determan AS, Wilson JH, Kipper MJ, Wannemuehler MJ, Narasimhan B. Protein stability in the presence of polymer degradation products: consequences for controlled release formulations. Biomaterials 2006 Jun;27(17):3312-20.
[41] Khalifeh MS. The role of T-cell mediated immune response in the development and resolution of colitis induced by S*erpulina hyodysenteriae* [Master of Science Thesis]. Ames, Iowa: Iowa State University; 1999.