Understanding the mechanism of immune enhancement and growth increase in chickens using nanotechnology based immune modulators

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I. Abstract/Summary

Compounds that enhance or boost the innate immune system have become of interest to the poultry industry. The use of antibiotics in the industry has come under increasing scrutiny due to the antibiotic resistance and public demand for poultry raised with out antibiotics. Compounds with the ability to stimulate the innate immune system have the potential to replace antibiotics. Unfortunately these compounds tend to be expensive and have a short time of activity. The effective delivery of these compounds will help reduce the amount needed and extend the time of effectiveness.

We have shown that cytidine-phosphate-guanosine oligonucleotides (CpG ODN) is an effective immune stimulant in poultry and can prevent or reduce the severity of systemic bacterial infections in young birds. Other workers have shown that host defense peptides (HDP) are also able to enhance immune responses and provide protection against infection by pathogens in other species.

Carbon nanotubes (CNT) have been shown to be a good carrier for biologically active compounds. In other species they have been used to effectively deliver immune stimulatory compounds.

We hypothesized that low doses of CpG ODN conjugated to CNT would be effective in preventing systemic bacterial disease in poultry and that CNT could also be used to deliver HDP for the same purpose. It was the initial plan to combine both CpG ODN and HDP in a single formulation. The chemistry used to link HDP to the CNT was different than that needed for CpG. This precluded the use of a single formulation that included both CpG ODN and HDP with the CNT. Work done (funded by another research grant) determined that the HDP we intended to use did not provide immune stimulation in poultry. Considerable work done in our laboratory also failed to identify a suitable candidate. Thus the work on HDP was not undertaken and we have concentrated our efforts on the formulation of CpG ODN for enhanced activity.

We have optimized the formulation of CpG ODN with CNT using *in vitro* studies. We have also tested the effect of this combination *in vivo* using a Salmonella challenge model of young chicks. Preliminary work showed that conjugation of CpG ODN to CNT showed promise in lowering the dose of CpG required. However we were unable to repeat these results in subsequent experiments. Because this was the case we elected to test the effect of formulation of CpG ODN with either soluble or particulate polyphosphazenes as these formulations had been effective in other species and had shown promise in poultry. Unfortunately these formulations also did not provide enhancement to the activity of the CpG ODN.

II. Background

The use of antibiotics as growth promoters and as prophylactic treatments to prevent disease early in the rearing period has come under increasing pressure. This is in part due to the issue of antibiotic resistance development in bacteria. Public pressure and subsequent government legislation has further restricted the use of antibiotics. This is a trend that is not likely to be reversed. As such the poultry industry faces the challenge of finding alternatives to the use of antibiotics.

It has been shown that the immune stimulant CpG ODN's can be beneficial in protecting chickens from bacterial infections caused by *E. coli* and Salmonella species. This immune stimulant is effective both in young chicks and in older birds and appears to act by stimulating the innate immune system. Host Defense Peptides (HDPs), also known as Antimicrobial Peptides are naturally anti-bacterial, anti-viral and anti-parasitic, and have been touted as 'natural antibiotics'. HDPs also have activities that correspond to many aspects of innate immunity. HDP have been shown to enhance immune responses and to protect against infection by bacterial pathogens.

The barrier to effective use in the protein industry is three fold. The compounds are expensive, relatively high doses are required, and the effect is relatively short lasting. We believe an effective delivery system could help overcome these barriers. A good delivery system would enhance the efficacy of the product and reduce the cost of vaccination. Ideally the delivery system would be compatible with a number of different compounds and have a good safety profile.

Nanoparticles such as carbon nanotubes (CNT) can be used with a wide variety of immune stimulants including CpG-ODN. Nanoparticles show great promise for biomedical purposes because of their physicochemical properties such as their hydrophobicity, ordered structure, light weight, and high surface area which also allows them to pass through biological barriers to get to intracellular targets. Broadly CNTs come in two major forms, single walled and multi walled nanotubes. In our work we use the multi walled nanotubes. It is possible to link the nanoparticles with other molecules such as CPG ODN and HDP. Not only can other molecules be conjugated to CNT but it has also been shown in short term studies these particles have very low toxicity in the host. These characteristics make nanoparticles excellent delivery systems for immune stimulating particles.

Polyphosphazenes are a group of synthetic polymers consisting of a backbone with alternating phosphorus and nitrogen atoms and organic side groups attached to each phosphorus atoms. This group of compounds has been shown to act as adjuvants to enhance adaptive immunity and when used in formulation with immunotherapeutic agents such as CpG ODN result in enhanced innate immunity. Polyphosphazene polymers can be formulated ether in their soluble state or as microparticles. Poly[di(sodiumcarboxylatoethylphenoxy)phosphazene] (PCEP) has been widely studied and has shown the ability to enhance both Innate and adaptive immunity.

It is our hypothesis that carbon nanoparticles can be used to maintain the biological function of CpG ODN and/or HDPs while enhancing the biological activity of immune stimulant without significantly compromising the host immune response and safety in the chicken model.

III. Methods

A. Linkages of Carbon Nanotube to CpG ODN

Three approaches to linking the CNT to CpG ODN were compared. These include

1. Adsorption chemistry with a linker (CNT-PySE-CpG ODN)
2. Direct Adsorption Chemistry (CNT-AD-CpG ODN)
3. Linking Via Electrostatic Interactions (CNT-PEI-CpG ODN)

The CNT were provided by Cheap Tubes Inc. (USA) and CpG ODN was a gift from Merial Limited. (USA). The CpG-ODN 2007 which is 22 bases in length with the sequence of t**cg**t**cg**ttgt**cg**ttttgt**cg**tt was used. The **cg** sequences are the CpG dinucleotides which stimulate an immune response.

B. Dialysis of CNT-CpG ODN and Determination of CpG ODN concentration.

To purify the CNT-CpG ODN dialysis was performed. Excess reagents (PySE, PEI and CpG ODN) were removed by dialysis for 24 hours at 4^{o}C in PBS (20mM, pH 7.2). The final concentration of the CNT-CpG ODN conjugate was determined by measuring the nucleic acid concentration remaining in the dialysis cassette.

C. In vitro Toxicity Assays

1. Cell Viability

In vitro toxicity of CNT and CNT-PySE-CpG ODN was measured using a CellQuanti-MTT cell viability assay kit (Bioassay Systems, USA) following the manufacturer's protocol. The chicken macrophage (HD11) cells were cultured and treated with a 10-fold dose titration of CNT, CpG ODN or CNT-PySE-CpG ODN starting at 100 µg/ml and ending at 0.001 µg/ml. Cells treated with media alone acted as a negative control. Cells treated with 0.1% Saponin acted as a positive control as Saponin rapidly destroys cellular membranes resulting in cell death. Cell viability was measured by the ability of the living cell to convert MTT, a tetrazolium salt, to formazan.

2. Measurement of Peroxide Production

In vitro peroxide production was measured using a peroxide assay kit (Biomedical research service, USA). HD11 cells were treated with a 10-fold dose titration of 100 µg/ml to 0.001 µg/ml of CNT-PySE-CpG ODN, CNT, or CpG ODN. Concentrations of peroxide from the treated cells were calculated using a colormetric test.

D. Protection of Chickens against Salmonella Infection by treatment with Immune Stimulants

1. Salmonella Typhimurium Culture

A field isolate of Salmonella Typhimurium from a 25-wk-old broiler chicken was used as the challenge strain. Bacteria for use as the challenge were cultured on Brain Heart Infusion Agar for 18–24 hr at 37 ^{o}C from a frozen stock culture. Two to three colonies of bacteria from the agar plate were added to 200 ml of BHI broth in a 1-liter Erlenmeyer flask. The culture was grown at 37 C for 16–18 hr with shaking at 200 rpm. The cultures were further diluted in cold BHI broth to the concentration of bacteria required in the challenge experiments. Birds were challenged with 1 x 10^{8} cfu of Salmonella Typhimurium in a 250 µl volume, subcutaneously in the neck. Serial dilutions of the challenge were cultured on BHI plates, in duplicate for 18–24 hr at 37 ^{o}C to validate the challenge dose.

2. Animal studies

All procedures with animals were done according to the protocol approved by the University of Saskatchewan, Committee on Animal Care. Day-old leghorn chickens hatched from Specific Pathogen Free Eggs were identified individually and were randomly divided into groups of 25. The groups were comingled and housed in the Animal Care housing facilities, Vaccine and Infectious Disease Organization, University of Saskatchewan. Water and commercial broiler ration were provided ad libitum. Air from each room was exhausted through a HEPA filter and replaced with non-recirculated intake air at a rate of 18 changes/hour. Air-pressure differentials and strict sanitation was maintained in this isolation facility.

(a) Treatment with immune stimulants (with or without formulation)

Day-old leghorn chickens were given the selected treatment by subcutaneous injection of a 200 µL volume in the right side of the neck. This treatment was well tolerated and no adverse effects were observed.

(b) Challenge with Salmonella Typhimurium

Birds were challenged with Salmonella Typhimurium by subcutaneous injection of a 250 µL volume in the left side of the neck. Birds were observed twice daily for the first 2 days for clinical signs of disease. After that birds were observed once daily, unless clinical condition of the birds warranted more frequent observations. Daily clinical scores for individual birds were recorded as follows: 0 = normal; 1 = hesitate to move and tire quickly; 2 = unable to stand or forage for food and subsequently euthanatized; 3 = dead. Dead or euthanized birds were necropsied immediately. Gross lesions such as pericarditis, perihepatitis, airsacculitis, and polyserositis were recorded. Bacterial swabs were taken from the air sacs and cultured on MacConkey agar plates

(c) Statistical Analysis

Comparisons of survival patterns and median survivals were done by the log rank (Mantel Cox) test. All data were analyzed by using Prism 5.0 (GraphPad Software Inc., San Diego, Calif.), with a *P* of 0.05 indicating significance

IV. Results

A. Synthesis of various formulations and assessment

1. CNT and CpG

Using the methodologies developed by ourselves and others we have prepare non-covalent or covalent conjugates of CpG ODN. For this work we used CpG ODN 2007 which has been shown to be active in chickens. The first step in using CNTs to aid in the delivery of CpG ODN was the selection of the most effective chemistry to link the two molecules. There is a wide variety of chemistries available to link carbon nanotubes to nucleic acids such as CpG ODN involving direct adsorption with or without a linker, electrostatic linkers and other chemistries. We selected three chemistries based on the chemistry and the effectiveness of linkage. The approaches tested were adsorption chemistry with a PYSE linker (CNT-PySE-CpG ODN), direct adsorption chemistry (CNT-AD-CpG ODN) and linking via electrostatic interaction (CNT-PEI-CpG ODN).

To test which chemistry was the best we first looked at the lymphocyte proliferative response when treated with each of the three chemistry linkages used. Proliferation of B cells in response to proper stimuli is a sign of the generation of an immune response. We isolated lymphocytes from chicken's spleens as this is an important site of lymphocyte conjugation and there is a mix of B lymphocytes, T lymphocytes and other mononuclear cells present. After 48 hours the

proliferative responses were measured and it was found that the cells treated with CNT-PySE-CpG ODN was most effective at lower doses in stimulating proliferation.

We also looked at the up-regulation of some important innate immune genes in HD11 chicken macrophage cells treated with a dose titration of the three formulations. It was observed that certain genes were expressed at higher levels with treatment with CNT-PySE-CpG ODN compared to cells treated with CNT-AD-CpG ODN, CNT-PEI-CpG ODN and free CpG ODN. The genes expressed are vital to immune responses generated by CpG ODN. The observations showed that CpG ODN bound to CNT with the use of PySE as a non-covalent linker is able to stimulate these innate immune genes better then free CpG ODN or any other linking chemistry attempted in this study.

2. CNT and HDP

Preliminary work has shown that it will be necessary to conjugate the HDP using covalent bonds to the CNT. This is in contrast to the linking of CpG which is done using non covalent linkage. This means that it will not be possible to link CpG and HDP to a single CNT. The linkage of HDP to CNT was delayed while we attempted to identify an effective HDP or group of HDP to use in our work (Funded by the Alberta Funding Consortium project 2009F-105R and SCIDF grant).

a. Selection Host Defense Peptides

A cocktail of 6 HDPs consisting of Bovine Myeloid Antimicrobial Peptide (BMAP-27), Indolicidin (Indol),Protegrin-1 (PG-1), Porcine Beta-Defensin-1 (pBF1), Avian Beta-Defensin-2 (aBDF2), and Avian Cathelicidin-3 (aCATHL3), which preliminary data indicated may promote strong immunomodulatory effects on avian immune cells (data not shown) was tested to determine if it had antimicrobial activity. Results indicated that a cocktail consisting of 0.1 µg/ml of each of the six peptides were effective at killing the bacteria. When the antimicrobial activity was assessed using various combinations of the peptides (i.e.BMAP-27 and PG-1 or aBDF2 and aCATHL3 were excluded from the cocktail), the antimicrobial activity decreased significantly. Therefore, we selected BMAP-27, PG-1, aBDF2, aCATHL3, Indol and pBDF1 as our HDP cocktail to use to determine if we could show in vitro activity.

b. Effect of HDP cocktail on infection with *E. coli* of 1-day old or 3- week old broilers.

i. Three-week old broilers

Experiments were performed to assess whether the HDP cocktail could protect three week old birds against *E. coli* infection. We observed that neither10 or 1 µg/ml HDP cocktail provided protection for birds against *E. coli*-related death. Blood from all birds was collected 24 hr post infection and it was determined that there was no significant difference in the number of bacteria. (This work was funded by project 2009F105R and details of the work can be found in that final report.)

ii. One-day old Broilers

The HDP cocktail was given (at either 1 or 10 µg) to 1-day old birds by subcutaneous injection and the next day the birds were challenged with *E. coli* by subcutaneous injection. Clinical signs were monitored and the birds were euthanized when they showed significant signs of illness. The pericardial area of the birds was swabbed and cultured to determine if systemic disease had occurred. The treatment with the HDP cocktail in both concentrations was not effective in reducing the disease caused by *E. coli* (Fig. 1). (Work on the effect of HDP cocktail on infection of the 1-day old birds was funded by the Saskatchewan Chicken Industry Development Fund and only a representative set of data is shown)

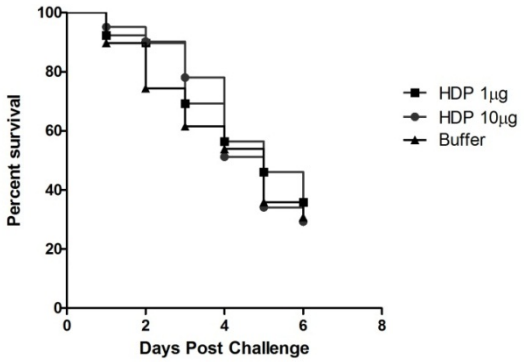

Fig. 1 Effect of treatment of 1-day old birds with a cocktail of HDP on infection by *E. coli* Survival of chickens following treatment by subcutaneous injection with (■) 1µg HDP, (●) 10µg HDP, or (▲) buffer. All birds were challenged with *E. coli* by subcutaneous injection. No protection was observed by treatment with HDP.

We were able to identify several HDP that showed *in vitro* antimicrobial activity. However, none proved effective *in vitro.* For this reason we focused our efforts on the use of formulation to enhance activity by CpG ODN

B. *In vitro* Toxicity

1. Cell Viability

To test the toxicity of free CNT and CpG ODN linked CNT *in vitro* studies were conducted using the chicken macrophage cell line HD11 that were treated for various time points and various doses of free and linked CpG ODN as well as free CNT. Saponin-treated cells were used as a positive control as they almost instantly destroy cells while media treated cells act a negative control. The cells were treated with a 10-fold titration of treatments ranging from100 µg/ml to 0.001 µg/ml. After one day post treatment cells receiving all doses of CpG ODN and CNT-CpG ODN remained near the same level as cells receiving no treatment while cells receiving 100 µg/ml of CNT showed a significant decrease in the percentage of viable cells. After three days post treatment the percent of viable cells receiving doses of CpG ODN remained near the media control level while there was a significant decrease in the percent of viable cells receiving 100 µg/mL of CNT-CpG ODN treatment. There was also a continued decrease in the percent of viable cells receiving 100 µg/mL of free CNT after three days post treatment as well as there was a significant decrease in the percentage of viable cells receiving 10 µg/ml of CNT after this time. After seven days post treatment the cells receiving doses of CpG ODN still remain close to the

media control level while cells receiving 100 µg/ml or 10 µg/ml of either free CNT or CpG ODN linked CNT showed a decrease in the percentage of viable cells to levels found with treatment of Saponin. Cells treated with lower levels of compounds (1µg/ml, 0.1µg/mL, 0.01µg/ml or 0.001 µg/ml.) showed no decrease in viability throughout the experiment.

2. Free Radical Production

To examine if the treatments were causing death of the HD11 cells due to stimulation of free radicals or other means the levels of peroxide were measured following 24 hours post treatment with a 10-fold dose titration of CNT, CpG ODN or CNT-CpG ODN ranging from100µg/ml to10 µg/ml. It was observed that the levels of peroxide being produced by cells treated with CNT-CpG ODN and CpG ODN were at the similar levels and even slightly below levels produced by the control cells treated with media alone. However, chicken macrophage cells receiving treatment of a high dose of CNT at 100µg/ml produced significantly higher levels of peroxide after 24 hours post treatment.

C. Protection Studies

An essential part of developing an effective immunotherapeutic vaccine is the ability of the vaccine to protect the host from infection. A protection study was performed to see the *in vivo* effect CNT-CpG ODN has on the immune system in a chicken model (Trial A). Various doses of CNT-CpG ODN or CpG ODN were injected into one-day old chickens. The chickens were challenged three days later with *S.* Typhimurium 1X 10^8 cfu by subcutaneous injection. CNT alone was not effective in protecting the chickens against infection and therefore any protection provided by CNT-CpG ODN is due to the CpG ODN linked to the CNT (Fig. 2B). The linked CpG ODN did not lose any ability to protect the chickens as high concentrations of CNT-CpG ODN and CpG ODN are just as effective at protecting the chickens (Fig. 2A). It appears that the linking of CpG ODN to the CNT enhanced the activity ten times as unformulated CpG ODN at a concentration of 0.1 µg was not effective in reducing mortality while the same concentration of CpG ODN linked to CNT was effective (Figs. 2A&B) (P=0.0047).

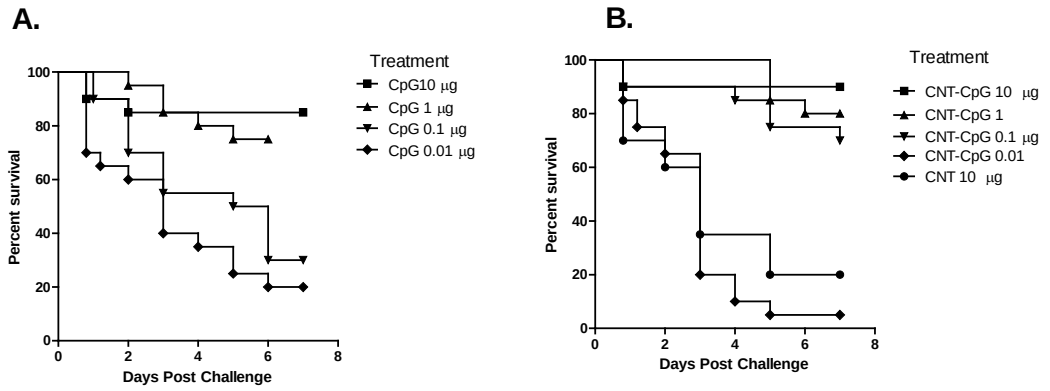

Fig. 2. Mortality in Birds after Treatment with Unformulated CpG and CpG Formulated with CNT and Subsequent Challenge with *Salmonella* Typhimurium (Trial A)
Birds were treated at day of age by subcutaneous injection of the material and challenged with *Salmonella* Typhimurium three days later. Unformulated CpG ODN at 0.1µg did not provide protection while the same concentration of CpG ODN formulated with CNT was protective.

We have repeated this experiment to confirm the results observed above (Trial B). Unfortunately we have been unable to do so. In the subsequent trial the effect of unformulated CpG ODN was the same as we had previously observed. The CpG ODN, at a dose of 10 µg or 1 µg per birds, was effective in preventing disease while CpG ODN at a dose of 0.1 µg was not (Fig 3A). In contrast to our previous results the formulation of 0.1 µg CpG ODN with CNT did not enhance the activity observed. At a dose of 0.1 µg of CpG ODN neither the unformulated nor the formulated treatment provided protection (Fig. 3B; p=O.53). The reason for this contradiction is unclear. It may be that in our original experiment we failed to remove all of the unbound CpG and hence delivered a greater dose than anticipated.

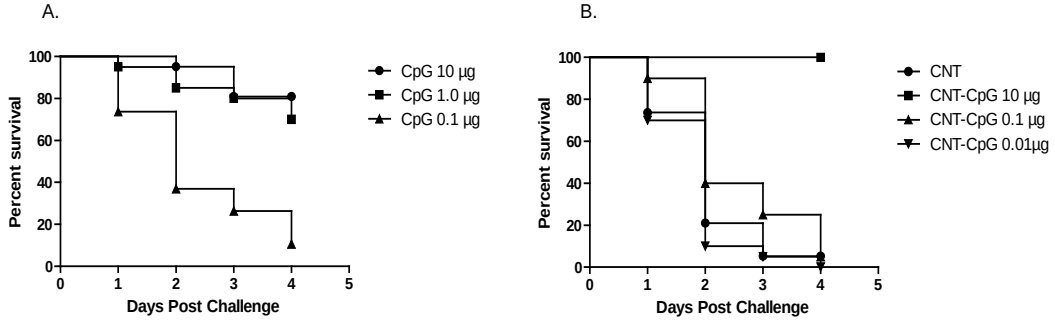

Fig. 3 Mortality in Birds after Treatment with Unformulated CpG and CpG Formulated with CNT and Subsequent Challenge with *Salmonella* Typhimurium (Trial B).
Birds were treated at day of age by subcutaneous injection of the unformulated CpG and CpG formulated with CNT and challenged with *Salmonella* Typhimurium three days later. No enhancement of protection was observed when CpG ODN was formulated with CNT.

In a subsequent trial (Trial C) we were again unable to repeat the effect originally observed (Fig. 4A and B). Once again the treatment with CpG ODN at a concentration of 1.0µg was effective in decreasing mortality from the Salmonella challenge (P= 0.0148) while CpG ODN at a concentration of 0.1µg was not (Fig. 4A). As well the CpG ODN 0.1 µg formulated with CNT was also not effective in reducing mortality due to Salmonella infection (Fig. 4B).

Because of our previous inability to show enhancement of CpG ODN activity by formulation with CNT we also chose to compare formulation with CNT to formulation with soluble polyphosphazenes (PCEP). This adjuvant was chosen as formulation of CpG ODN with PCEP had previously been shown to enhance activity of CpG ODN to provide protection against infection with *E. coli* (Taghavi A, Allan B, Mutwiri G, Foldvari M, Van Kessel A, Willson P, Babiuk L, Potter A, Gomis S. 2009. Enhancement of immunoprotective effect of CpG-ODN by formulation with polyphosphazenes against E. coli septicemia in neonatal chickens. Curr Drug Deliv. :76-82).

PCEP was used at a concentration of 10µg with or without CpG ODN. Like formulation with CNT formulation with PCEP did not enhance the activity of the CpG ODN (Fig. 4B; P = 0.2262). The failure to provide enhanced protection using soluble PCEP may be due to the fact that in the earlier work a dose of 50µg per bird was used. In addition the treatment had been delivered *in ovo* while in this case we delivered the treatment by subcutaneous injection.

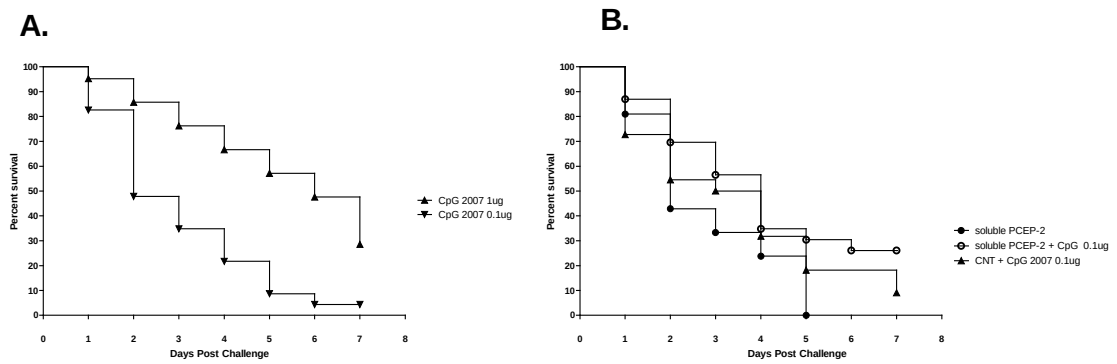

Fig. 4. Mortality in Birds After Treatment with Unformulated CpG and CpG Formulated with CNT and Subsequent Challenge with *Salmonella* Typhimurium (Trial C)
Birds were treated at day of age by subcutaneous injection of the unformulated CpG and CpG formulated with CNT or soluble PCEP-2. On day 4 of age birds were challenged with *Salmonella* Typhimurium . No enhancement of protection was observed when CpG ODN was formulated with CNT or soluble PCEP.

Due to our inability to show reproducible immune enhancement *in vivo* using CNT we revised our research plan and focused on the use of polyphosphazenes as an adjuvant to enhance the activity of CpG ODN. In Trial D (Fig.5) we tested two concentrations of soluble polyphosphazenes (10 µg and 50 µg) in conjunction with CpG ODN. Once again the birds were treated three days before challenge with Salmonella. CpG ODN 1.0 µg provided enhanced protection against challenge with Salmonella when compared to all other treatments (P=0.0001). Interestingly the treatment with PCEP 50 µg in the absence of CpG ODN provided greater protection than treatment with CpG ODN at a concentration of 0.1 µg (Fig 5; P=0.0083). However the combination of CpG ODN with PCEP at either 10 µg or 50 µg did not enhance the effect observed when PCEP 50 µg was used (Fig 5B; P=0.8207). The enhancement of innate immunity by soluble PCEP alone has been observed other species.

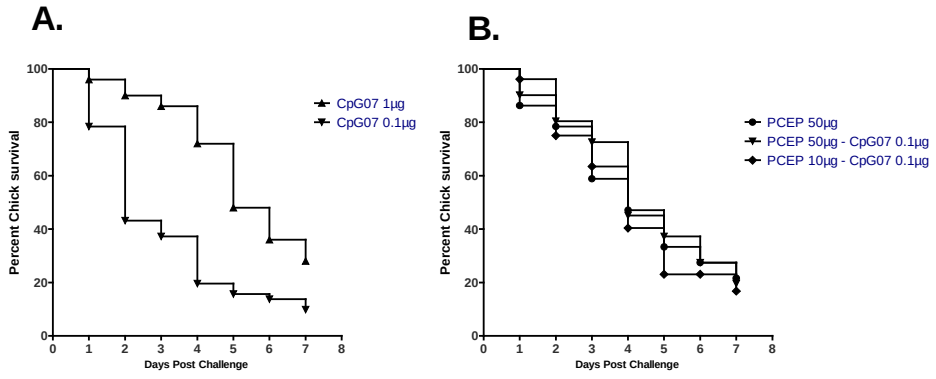

Fig. 5 Mortality in Birds After Treatment with Unformulated CpG and CpG Formulated with soluble PCEP and Subsequent Challenge with *Salmonella* Typhimurium (Trial D)
Birds were treated at day of age by subcutaneous injection of the unformulated CpG and CpG formulated with PCEP-2. On day 4 of age birds were challenged with *Salmonella* Typhimurium . No enhancement of protection was observed when CpG ODN was formulated with soluble PCEP at a concentration of 50µg or 10µg.

In the previous trials the doses of CpG ODN used with and without formulation had differed in concentration by at least a factor of 10. To determine if enhancement of activity by formulation occurred at intervening levels between CpG ODN1 µg and 0.1 µg we carried out another trial (Trial E; AS 2010-138) using two additional concentrations. The results confirmed what had been observed before. Again unformulated CpG ODN 1.0 µg afforded some protection from mortality due to Salmonella while CpG ODN 0.1 µg did not (Fig.6A vs 6D) The difference was not statistically significant (P=0.075) however the median survival day for CpG ODN 1.0 µg was day 6 while for CpG ODN 0.1 µg it was day 3. Once again the soluble PCEP provided some protection alone when compared to the buffer group (P=0.0336). The formulation of CpG ODN with PCEP provided no enhancement in activity over that observed for the unformulated CpG ODN of the same concentration (Fig. 6. A, B, C, D).

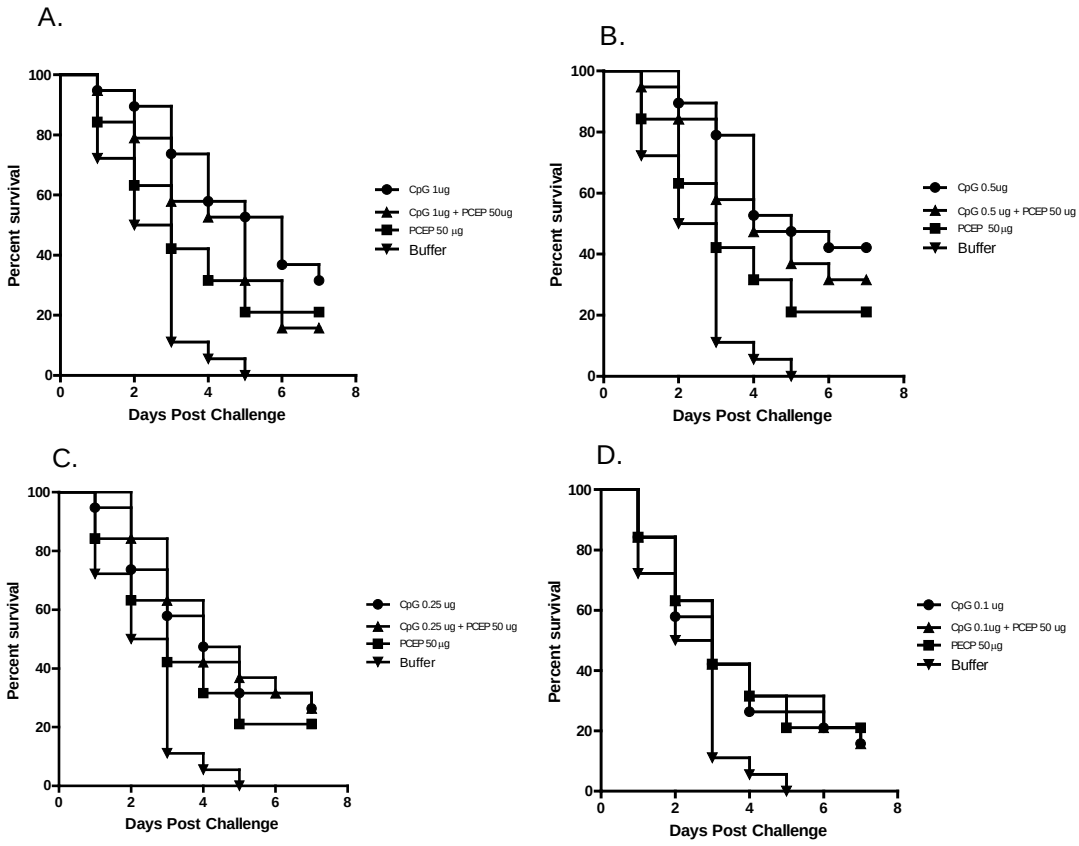

Fig. 6 Mortality in Birds After Treatment with Unformulated CpG and CpG Formulated with soluble PCEP and Subsequent Challenge with Salmonella Typhimurium (Trial E)
Birds were treated at day of age by subcutaneous injection of the unformulated CpG and CpG formulated with PCEP-2. On day 4 of age birds were challenged with *Salmonella* Typhimurium . No enhancement of protection was observed when CpG ODN was formulated with soluble PCEP at a concentration of 50µg although the PCEP alone provided some protection.

In the previous two experiments we had observed no enhancement of activity of CpG ODN by soluble PCEP. In the final animal trial (Trial F; AS2010-139) we tested the ability of PCEP formulated as a micro particle to enhance the activity of CpG ODN. In contrast to the previous trials, in this animal trial there was no significant difference observed among all of the groups

(Fig. 7). Therefore we concluded that PCEP formulated as microparticles did not enhance the activity of the CpG ODN. It also appeared that PCEP formulated as micro particles did not possess the immune stimulatory activity that we observed in the soluble PCEP (Fig. 7)

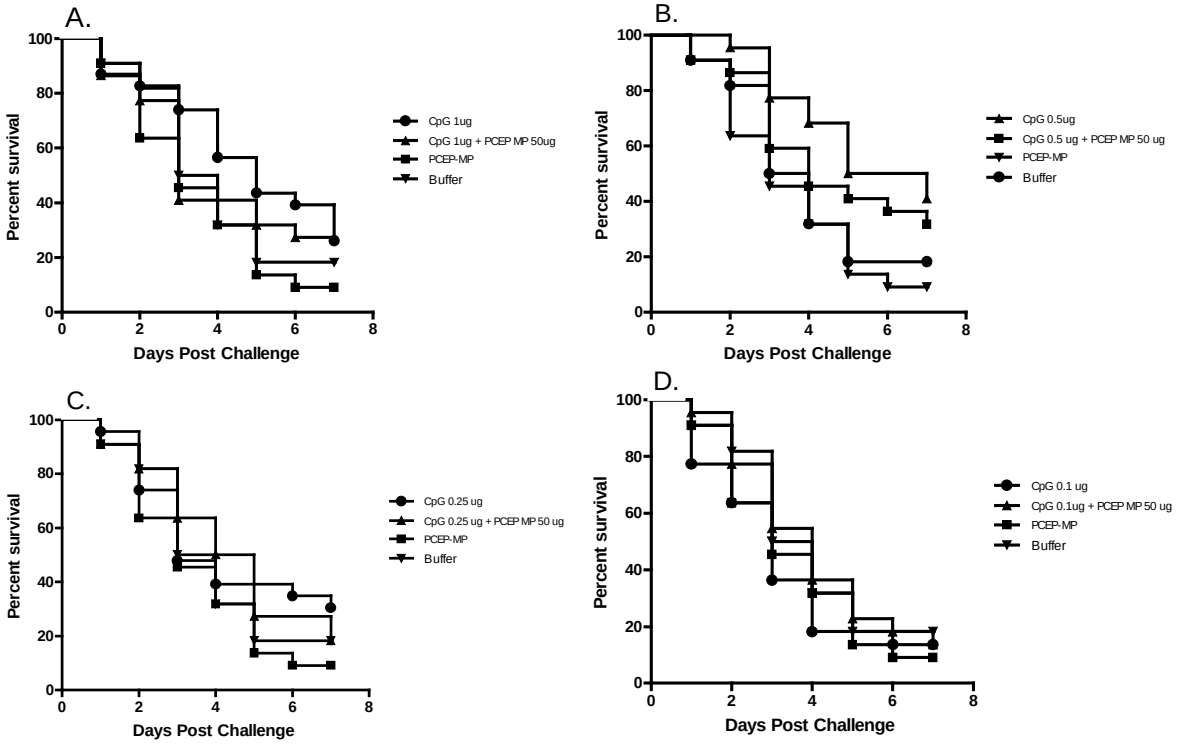

Fig.7. Mortality in Birds After Treatment with Unformulated CpG and CpG Formulated with PCEP-MP and Subsequent Challenge with *Salmonella* Typhimurium (Trial F)
Birds were treated at day of age by subcutaneous injection of the unformulated CpG and CpG formulated with PCEP-microparticles (MP). On day 4 of age birds were challenged with *Salmonella* Typhimurium. No enhancement of protection was observed when CpG ODN was formulated with PCEP-MP at a concentration of 50µg.

V. Conclusions and Recommendations

It was determined that the use of PySE as a non-covalent linker for binding CpG ODN to CNT was most effective

We determined that a cocktail of host defense peptides including Bovine Myeloid Antimicrobial Peptide (BMAP-27), Indolicidin (Indol),Protegrin-1 (PG-1), Porcine Beta-Defensin-1 (pBF1), Avian Beta-Defensin-2 (aBDF2), and Avian Cathelicidin-3 (aCATHL3) had significant antimicrobial activity *in vitro.* However this cocktail when given unformulated to 3-week old or 1 day old broilers was unable to provide protection from septicemia. Because of this observation we did not examine the formulation of this cocktail with CNT

CNT linked to CpG ODN did not provide enhancement of the immune stimulation observed in the unformulated CPG. However as previously observed CpG ODN was able to provide protection against septicemia caused by Salmonella

We have tested the ability of polyphosphazenes as an adjuvant to enhance the activity of CpG ODN. Soluble polyphosphazenes was tested in two trials and no enhancement of immune stimulation by CpG ODN was observed. This was despite the fact that soluble polyphosphazenes by itself is somewhat immune stimulatory. In the final animal trial we used polyphosphazenes that were produced as as microparticles with the CpG ODN. Once again this formulation failed to demonstrate enhancement of the CpG ODN activity.

The food animal industry is under considerable pressure to limit the use of antibiotics as therapeutic agents and as growth promoters. We therefore believe that the continued investigation of immune stimulation of poultry will be a productive field of investigation to provide tools to the industry to replace the use of antibiotic.

VI. Dissemination Strategy

We plan to submit a research paper to a peer reviewed journal (Avian Diseases) on the research described above.

A presentation on this work entitled “Enhanced immunity in young chicks using nanotechnology based immune modulators” was presented at Annual Western Meeting of Avian Clinicians and Pathologists. Lake Louise, Alberta. Sept 2009. This is a meeting of veterinarians who work for government, industry or in private practice and are primarily involved in work with the poultry industry. People attending came from Ontario, Quebec, Saskatchewan, Alberta, British Columbia and the United States

Jason Tomporowski, a graduate student at the University of Saskatchewan, completed the work for his Masters and successfully defended his thesis entitled “Improving the biological activity of CpG ODN by linking to carbon nanotubes.” in the Dept. of Biochemistry, University of Saskatchewan. November 2009