AGRICULTURAL RESEARCH FOUNDATION
FINAL REPORT
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TITLE: Development of Vaccine for Johne's Diseases

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SUMMARY: Johne's disease, caused by *Mycobacterium avium* subspecies *paratuberculosis* (MAP), is a severe chronic enteritis which affects large populations of ruminants globally. Prevention strategies to combat the spread of Johne's disease among cattle herds involve adhering to strict calving practices to ensure young susceptible animals do not come in contact with MAP-contaminated colostrum, milk, or fecal material. Unfortunately, the current vaccination options available are associated with high cost and suboptimal efficacy. To more successfully combat the spread of Johne's disease to young calves, an efficient method of protection is needed. In this study, we examined passive immunization as a mode of introducing protective antibodies against MAP to prevent the passage of the bacterium to young animals via colostrum and milk. Utilizing the infectious MAP phenotype developed after bacterial exposure to milk, we demonstrate that *in vitro* opsonization with serum from Johne's-positive cattle results in enhanced translocation across a bovine MDBK polarized epithelial cell monolayer. Furthermore, immune serum opsonization of MAP results in a rapid host cell-mediated killing by bovine macrophages in an oxidative-, nitrosative-, and extracellular DNA trap-independent manner. The use of adult cow antibodies against surface antigens of MAP (following immunization) can trigger the mucosal innate immune response against the pathogen. The bacterial surface antigens were purified and tested in in vitro assays.

OBJECTIVES: Vaccine development for Johne's Disease

PROCEDURES: The studies described above provided basis for the development of the surface proteins vaccine. Genes were cloned and expressed, proteins were purified.

SIGNIFICANT ACCOMPLISHMENTS: Opsonization aids in the uptake of pathogens by host phagocytes and has been demonstrated to enhance killing of intracellular pathogens during infection. Previous investigations have shown that opsonization of plate-grown MAP increases the efficiency at which the bacterium is able to be ingested and increases survival within the intracellular compartment. However, the effect opsonization has on the uptake of MAP by the intestinal mucosa is unknown. To determine the effect of opsonization on the interaction between MAP cultured in milk (hereafter referred to as the infectious phenotype) and the bovine intestinal epithelium, we evaluated how opsonization influenced the invasion of and the translocation across polarized bovine MDBK epithelial cells. The infectious phenotype of MAP was recovered and opsonized with PBS (mock), bovine serum from Johne's negative animals (non-immune serum), or bovine serum from Johne's positive animals (immune serum). Invasion assays indicated that neither non-immune nor immune serum opsonization had any significant effect on the ability of MAP to invade MDBK epithelial cells compared to mock opsonized samples. While the epithelial cell invasion capability of MAP isn't significantly affected by opsonization, the mechanism of uptake may provide a difference in the survival or efficiency of translocation across the epithelium.

To investigate the fate of opsonized MAP after uptake by MDBK epithelial cells, a transwell culture assay was employed. After 4 days in culture, monolayers were determined to be impermeable and intact as demonstrated by both transmembrane resistance measurements and a trypan blue permeability assay performed 4 days after cell seeding. Opsonization with immune serum resulted in enhanced translocation of MAP across an intact polarized MDBK epithelial cell monolayer with 3.1-fold more bacteria being recovered from the basolateral chamber after 6 hours, and a 3.7-fold increase in recovered MAP after 24 hours post-infection compared to non-immune serum opsonized bacteria. These data indicate that opsonization with sera from Johne's infected, immune positive cattle has little effect on uptake of bacteria by MDBK epithelial cells, but results in significantly enhanced translocation of infectious MAP across the polarized epithelial cell monolayer during infection.

Once established that opsonization of MAP increased the level of translocation across a polarized MDBK epithelial cell monolayer, we wanted to address whether enhanced translocation contributes to the virulence of MAP by allowing greater numbers to be taken up by and survive within tissue macrophages, or whether greater translocation results in enhanced uptake and subsequent killing of MAP by macrophages. To assess the MAP-macrophage interaction in this system, we analyzed what effect opsonization had on the uptake of MAP by an immortalized bovine macrophage cell line (BOMAC). Preliminary assays were carried out to determine if our experimental model was able to replicate the effect of macrophage uptake of MAP grown under standard conditions as previously described. Consistent with previous studies, opsonization of broth cultured MAP with serum samples results in a 2-fold increase in uptake of MAP by bovine macrophages after a 15 minute infection. It was also shown that neither mock (PBS), non-immune serum, nor immune serum opsonization treatments of milk-cultured or 7H9-cultured MAP had any detrimental effect on the viability of each pool of bacteria prior to infection (data not shown), suggesting that any observable change is due to the response of the host cell and not due to a difference of the initial inoculum levels or from an impact on viability from serum components, culture, or opsonization treatments occurring before infection.

Prior studies demonstrated the increased ability of the infectious phenotype of MAP to invade MDBK epithelial cells, and our data indicates that the infectious phenotype also has a significantly greater rate of uptake by BOMAC cells after a 15 minutes infection as 8.55 ± 1.15% of MAP expressing the infectious phenotype are ingested by BOMAC cells compared to 3.72 ± 0.32% of 7H9-exposed MAP after infection ($p = 0.0054$). To determine the effect of the infectious phenotype on the interaction between opsonized MAP and BOMAC cells, infection assays were carried out to assess the amount bacteria that were able to be taken up by macrophages. Intracellular bacterial quantification demonstrated that the infectious phenotype resulting from milk exposure results in a 75% decrease in BOMAC uptake of immune serum opsonized samples compared to mock or non-immune serum opsonized MAP populations. This decrease was observed over a variety of infection lengths and sample treatments. Initial 3 hour assays, which included a 1 hour infection followed by 2 hours of

antibiotic treatment, and short-term infections of 15 minutes, 30 minutes and 1 hour in the absence of antibiotic treatment were carried out and result in nearly identical data (other data not shown). Collectively, these data indicate that immune serum opsonization of infectious MAP results in a significant decrease in the amount of bacteria taken up by BOMAC cell cultures during infection over both long- and short-term infections.

Immunoglobulins in the serum, particularly IgG and IgA, are responsible for opsonizing pathogens and toxins to enhance uptake and subsequent killing, or for neutralization and protection from disease, respectively. To ascertain which function the serum antibodies have in our model and the mechanism responsible for the decrease in uptake of immune serum opsonized MAP, the supernatant from each experimental group during BOMAC cell infection was collected and the number of viable bacteria quantified. After 15 minutes of infection approximately equal numbers of MAP were recovered from the supernatant of mock, non-immune, and immune serum opsonized infections. These findings indicate that the decrease in uptake of MAP opsonized with immune serum was not due to a neutralizing effect of the antibodies blocking uptake by macrophages. Rather, the infectious phenotype opsonized with immune serum results in the killing of MAP as there is no difference in the viable amount of bacteria within the supernatants of each treatment. The mechanism used to kill immune serum opsonized bacteria is rapid, as significant changes in viability occur after only a short 15 minute incubation period. In total, these data illustrate that this infectious phenotype of MAP serves as a key mechanism for recognition by immune serum and for the specific response initiated by BOMAC cells during infection.

Innate cellular mechanisms are vital for allowing host cells to react in a rapid and non-specific manner to pathogen associated molecular patterns (PAMPs), as well as antibody coated particles. The phagocytic vacuole is hard-wired to fuse with lysosomes and deliver numerous oxidative stress molecules that eliminate the pathogen within the vacuole. To identify which host cellular mechanism(s) are being employed in the early killing of immune serum opsonized MAP, we inhibited a variety of cellular mechanisms known to be utilized during the process of phagocytosis. Though mycobacteria are well described to inhibit this fusion process, antibody-

mediated phagocytosis may utilize different vacuole trafficking signals and fusion mechanisms which lead to a different outcome of mycobacterial survival. Chemical inhibitors of classical oxidative mechanisms were added to infections to determine if there was an effect on the viability of opsonized and non-opsonized milk-exposed MAP over the course of the 15 minute infection. The inhibition of hydrogen peroxide production by the addition of catalase, and the blockage of superoxide anion radical efficacy by superoxide dismutase (SOD) results in no change in the recovery of immune serum opsonized populations of MAP. As the conversion of superoxide by superoxide dismutase results in the release of oxygen and hydrogen peroxide, to combat both oxidative mechanisms we treated cells with a combination of catalase and superoxide dismutase in tandem, which results in no change from the control groups. The membrane bound NADPH oxidase enzyme complex is responsible for the production of a variety of reactive oxygen species (ROS) including but not limited to peroxides, oxygen radicals, and oxidative stress components that can kill bacteria within the cell. To antagonize the collective production of these toxic products, cells were treated with the NADPH oxidase inhibitor diphenyleneiodonium chloride (DPI). While the overall levels of uptake were lower in DPI treated samples, the overall pattern demonstrating a lower uptake of immune serum opsonized MAP remains the same as with the addition of other inhibitors. It was noted that DPI-treated MAP controls result in a 35% decrease in the viability of the bacteria, while there was a 10% decrease in host cell viability in DPI-treated controls compared to DMSO vehicle treated samples, accounting for the decrease in overall uptake in the DPI-treated assays. All other controls demonstrate negligible changes in viability of either MAP or host cell controls during treatment with inhibitors or their respective vehicle controls (data not shown).

In addition to the stimulation of an oxidative burst upon phagocytosis, host cells can mount a nitrosative burst, specifically by the production of nitric oxide, to aid in the destruction of ingested pathogens. To analyze the role of nitric oxide during phagocytosis of opsonized milk-exposed MAP, treatment was completed with the nitric oxide inhibitor N^G-monomethyl-L-arginine (L-NMMA). Treatment with L-NMMA results in no change in uptake and intracellular bacterial load of immune serum opsonized MAP compared to control treated samples. There

was no effect on viability of the MAP or cell populations upon addition of L-NMMA in controls (data not shown).

To identify if extracellular killing mechanisms were responsible for the rapid killing of immune serum opsonized MAP, we investigated the role of extracellular traps during our infection model. Neutrophil extracellular traps (NETs) have been described as a toxic tool employed upon infection with both gram-negative and gram-positive organisms . Upon exposure to stimuli, cells release a sticky DNA net into the extracellular environment which is characterized by the presence of histones and elastase enzymes. This phenomenon has recently been reported to be used by macrophages as well, resulting in macrophage extracellular traps (METs) in response to TNF-α and a variety of pathogens and toxins. To determine if METs were stimulated in response to infection with our bacterial populations, BOMAC cultures were treated with DNase prior to and during each uptake assay. DNase treatment identifies that the dissolution of DNA complexes outside of the cell does not alter the pattern of selective immune-serum opsonized MAP killing nor does it alleviate the lack of extracellular immune serum opsonized MAP during the assays.

The observation that bovine macrophages were able to more effectively eliminate the infectious phenotype of MAP after opsonization with immune serum in such a rapid manner has not previously been described prior to this study. To understand if the phenomenon was specific to the physiology of bovine macrophages or could be observed in other systems, we completed the opsonization of the infectious phenotype of MAP and infection in a murine model. We first produced antibodies against the infectious phenotype of MAP in C57BL/6 mice. Western blot analysis of infectious MAP lysates and milk protein alone confirm that collected mouse serum contained antibodies which recognize only MAP bacterial lysate proteins and not bovine milk proteins . To identify the effect the collected mouse serum had on the interaction of opsonized infectious MAP with murine RAW 267.4 macrophages, we conducted similar 15 minute macrophage infection assays as done with the bovine model. Murine RAW 264.7 macrophages exhibit a significantly lower level of immune serum opsonized bacteria compared to non-immune serum and mock opsonized samples . Additionally, the decrease in bacterial

uptake by murine macrophages is not an effect of neutralization, as the lack of intracellular MAP in the uptake assays are not recovered from the supernatants of each culture. These data validate that the pattern of rapid host cell mediated killing is consistent between both the bovine and murine *in vitro* model systems, and provides further support that the infectious phenotype of MAP serves as an important and relevant bacterial phenotype for studies involving opsonization, and the resulting rapid phagocyte killing, as a means of preventing infection in multiple species models.

Adult cow vaccine for Johne's disease may address the lack of efficacy of the injectable systemic vaccine. The product is now in development phase in India. Testing is planed for months from now.

BENEFITS & IMPACT: The development of an oral vaccine for Johne's Disease will have very significant impact in the American farms**.**

ADDITIONAL FUNDING RECEIVED DURING PROJECT TERM: Indian Research Institute, Vaccine Center.

FUTURE FUNDING POSSIBILITIES: I have applied to USDA, and although received good review, has not been successful in obtained funding**.**