ABSTRACT

SOMODY, ROSE F. Immunoglobulin A Dynamics in Selenium-Fed and Reovirus-Challenged Broiler Chickens. (Under the direction of Dr. Frank W. Edens).

Avian reoviruses (ARV) are icosahedral, nonenveloped viruses with double-protein capsid shells and genomes consisting of ten double-stranded RNA segments. They cause significant economic losses in the poultry industry due to the diseases with which they are associated. Natural ARV infections occur via the fecal-oral route, with infection mainly via oral entry with initial replication in the intestine and bursa of Fabricius. The small intestine is the most important site for ARV infection regardless of the route of inoculation, with the spread of infection beginning in the villus and crypt epithelium. Nutrients such as selenium could play a role in the chicken’s ability to resist or regain function after ARV infection. The dietary treatments in this virus (ARV-CU98) study consisted of supplemental Se in the organic (Sel-Plex) or inorganic (sodium selenite) forms, or a control diet which did not contain supplemental Se. Morphological alteration in the ileal villi of selenium-fed and ARV-challenged broiler chickens was quantified in this study. The improved profile of the intestinal villus was not evident until 16 days post-infection (p.i.) when organic selenium-fed broiler intestines were possibly recovering at a faster pace than inorganic Se-fed broilers because of the greater villus height: crypt depth ratios. Mucosal surfaces are important locations for the entry of viruses into the body, and secretory immunoglobulin A (sIgA) is the primary antibody involved in antigen-antibody interactions in the mucosal immune system, providing the first line of defense against pathogens. Polymeric IgA, secreted by plasma cells, is taken up by polymeric immunoglobulin receptor (pIgR) and is then transported to the apical membrane and secreted into the intestinal lumen. The output of sIgA in the intestinal tract of chickens on the different Se diets and subjected to challenge with ARV-CU98 was evaluated using an enzyme-linked immunosorbent assay. Early in the post challenge period, intestinal sIgA levels were highest in inorganic Se-fed birds, followed by organic selenium-fed birds, and lowest in the control group which was not given supplemental
selenium. By the end of the study, a significant effect was seen with infected, organic Se-fed birds having higher ileal IgA than the non-challenged, organic Se-fed birds in one trial. The average biliary sIgA was often highest in organic Se-fed birds by three weeks of age, regardless of ARV infection status. The rate of IgA transcytosis across epithelial and endocrine cells may depend on the level of pIgR expression with one molecule of pIgR needed for each molecule of IgA that is transported. Quantitative real-time PCR was conducted to determine if dietary Se and ARV challenge may have influenced the expression of pIgR in broiler chickens since recent evidence has shown that Se has a positive influence on resistance and recovery from double stranded RNA viruses. Throughout the infection period in this investigation, inorganic Se-supplemented birds showed overall higher expression than organic Se-supplemented birds. By 16 days p.i., once the virus had ran its course, those infected birds that had been given organic Se were often showing decreased levels of expression. Possibly, the response was low for this group due to sufficient early recovery from the virus challenge. Overall, selenium appears to have an effect on the dynamics of secretory IgA and its receptor, pIgR. The improved integrity of the villi as demonstrated through histomorphometric analysis may be related to the mucosal immune response. The results of this research at times support the concept that selenium improves intestinal immunocompetence, but more studies are necessary.
DEDICATION

To my Dad, Mom, and siblings (John, Rebecca, Christine and Melissa)-Thank you all for
the love and encouragement throughout this challenging time and always!
BIOGRAPHY

Rose Frances Somody was born in Greensboro, North Carolina on August 13, 1985. She grew up in Oak Ridge, North Carolina with her older brother and three younger sisters. After graduating from Northwest Guilford High School in 2003, she moved to Raleigh, NC to pursue a B.S. in Biological Sciences at North Carolina State University. She also minored in Microbiology, Genetics, Health, and Spanish while completing her undergraduate coursework. After graduating in December 2006, she decided to stay at NCSU and complete a Master of Physiology degree, under the guidance of Dr. Malcolm Roberts, with plans to possibly attend medical school afterwards. Her fellow graduate students in the Department of Poultry Science encouraged her to be involved in research projects in order to decide if a thesis was the route she wanted to take. After assisting with data collection for several projects and learning about research opportunities with her Masters advisor, Dr. Frank Edens, she decided to switch to a Master of Science in the Physiology Program. After working on her research and completing coursework that included a minor in Biotechnology, she realized that a career in research was something she very much wanted.
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I am very thankful for the guidance of my advisor, Dr. Edens, throughout this degree. He was always eager to answer my questions and encourage me when there were difficulties with the research. I also would like to thank Dr. Koci for allowing me to use his laboratory and for the time and effort he put into helping me to prepare the reovirus and understand results. Also, the deep discussions about physiological processes were extremely beneficial. I can never thank Debbie Ort enough for her assistance with the bird trials and with the analysis in the laboratory. Also, her friendship throughout these years has meant so much to me on both a professional and personal level! I also am very grateful to Rizwana Ali. She was always so helpful with the cell culture portion of this project, and her advice throughout my research and thesis-writing was very much appreciated. Dr. Ashwell’s assistance with the gene expression portion of this thesis was very helpful, and I couldn’t have done it without him. I really appreciated Dr. Black’s advice, specifically with the histology work. I am very grateful to Alltech for funding this research. I would like to thank my family and friends for being there for me throughout the past few years, and for the wonderful love and support.
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CHAPTER 1: LITERATURE REVIEW

The Avian Enteric Immune System

Introduction

Branches of the Immune System

The avian and mammalian immune systems function similarly (Sharma, 1991), but there is still much to be learned in the field of immunology for both groups of animals. Immune responses are categorized as either adaptive or innate, with adaptive occurring during the lifetime as the animal adapts to infection by a pathogen and innate being the response present at all times in an animal (Janeway et al., 2005). There are complex interactions between the adaptive and innate immune responses, with the innate initially encountering and destroying pathogens, and in many cases, other events including recruitment of immune components and induction and modulation of the adaptive immune system follow (Davison et al., 2008). Humoral and cellular immunity make up the adaptive immune response, with humoral immunity being characterized by antibodies secreted by B lymphocytes. B cell development is under the control of the bursa of Fabricius in the avian immune system, while mammals lack a comparable structure (Sharma, 1991). Instead, B lymphocytes mature in the bone marrow (Janeway et al., 2005). The cellular immune system is under the control of the thymus which is essential for the maturation of T lymphocytes. T lymphocytes are the principle cells of cellular immunity, but others include macrophages, dendritic cells, natural killer cells and effector cells of antibody dependent cellular cytotoxicity (Sharma, 1991).
Humoral Immunity

The extracellular spaces are protected by the humoral immune response in which antibodies produced by B cells cause the destruction of extracellular microorganisms and prevent the spread of infections within cells. B cells are activated by antigen and differentiate into antibody-secreting plasma cells and memory B cells. Helper T cells help with this B cell activation and differentiation (Janeway et al., 2005).

The humoral immune response begins when B cells bind to antigen and are signaled by helper T cells or by the antigen alone. B cells interact with T cells, leading to the production of antibodies, affinity maturation of the antibody response, isotype switching for functional diversity of immunoglobulins, and the generation of memory B cells. Once B cells are activated, some migrate to follicles and proliferate to ultimately form a germinal center. The germinal center is composed mainly of proliferating B cells, but a small amount of antigen-specific T cells are present to assist B cells. The reaction here allows for an effective later response (Janeway et al., 2005).

The interaction of an antigen-binding B cell with a helper T cell leads to the expression of CD40 ligand on the helper T cell surface and the secretion of B-cell stimulatory cytokines. These drive proliferation and differentiation of the B cell into plasma cells which secrete antibody (Janeway et al., 2005).

The DNA rearrangements that are necessary for isotype switching and therefore functional diversity of the humoral immune response are directed by cytokines, especially those released by helper T cells. Different cytokines induce switching to different isotypes, leading to antibody types IgA, IgE, IgG (IgY in birds), IgD and IgM (Janeway et al., 2005).
Immunoglobulins

Immunoglobulins are the form of lymphocyte antigen receptors on B cells, being the means by which lymphocytes sense antigens in the environment. The surface immunoglobulin, which serves as the B-cell antigen receptor, transmits signals to the interior of the B cell upon antigen binding and delivers antigen to intracellular sites to be degraded and recognized by helper T cells. Somatic hypermutation and switching to certain immunoglobulin isotypes depends on the interaction of antigen-stimulated B cells and helper T cells (Janeway et al., 2005). Antibodies of different isotypes operate in distinct areas and have different functions. Through isotype switching, the progeny of a B cell can produce antibodies that have the same specificity but protect different body compartments.

IgM is the first antibody produced in the humoral immune response because it does not require isotype switching. IgM antibodies tend to be of low affinity because they are produced before B cells have undergone somatic hypermutation. IgM molecules form pentamers, so they can bind simultaneously to multivalent antigens, which compensates for the low affinity of IgM monomers. IgM is large as a pentamer, therefore being found mainly in the blood and in the lymph. IgM is produced rapidly and activates the complement system to control infection in the bloodstream. IgM is also produced in secondary and subsequent responses, although other isotypes are more prevalent in the later antibody responses. IgM antibodies also are produced in the peritoneal cavity and pleural spaces (Janeway et al., 2005). Chicken IgM is structurally and functionally homologous to mammalian IgM, being the predominant B-cell receptor during embryonic development and the first expressed after initial exposure to an antigen. The response is usually transient in both groups of animals but can also remain active longer (Davison et al., 2008).
IgG, IgA, IgE and IgD antibodies are smaller than IgM and diffuse easily into the tissues from the bloodstream. IgA can form dimers, but IgG, IgE and IgD are always monomeric. It is therefore critical that there is a strong affinity of the antigen-binding site for the antigen in order for these antibodies to be effective (Janeway et al., 2005).

IgG is the principle isotype in blood and extracellular fluid, while IgA is the principle isotype in secretions (Underdown and Schiff, 1986; Kerr 1990), especially those of the epithelium lining the respiratory and intestinal tracts. IgG opsonizes pathogens by engulfment by phagocytes and activates the complement system since it operates mainly in body tissues where the necessary accessory cells and molecules reside. IgA, however, is a less potent opsonin and a weak activator of complement since it operates where complement and phagocytes are lacking. Therefore, IgA functions mainly as a neutralizing antibody (Janeway et al., 2005). IgY is the avian homologue of mammalian IgG and is similar to mammalian IgG and IgE. It appears to be like the evolutionary predecessor of both IgG and IgE. It is the main isotype produced in the secondary response and is found mainly in sera. Chicken IgY and the mammalian homologue are different in that chicken IgY has a longer H chain (Davison et al., 2008).

IgE is present at low levels in blood and extracellular fluid. It is bound by mast cells that are under the skin and mucosa, and along blood vessels in connective tissue. When antigen binds to cell-associated IgE, the mast cells are triggered to release chemical mediators that induce reactions to expel the agents (Janeway et al., 2005). There is not an avian homologue of IgE (Davison et al., 2008).

IgD is expressed along with IgM on the surface of most mature B cells, although it is secreted in small amounts and its function is unknown (Janeway et al., 2005). There were early studies reporting a chicken homologue of IgD on lymphocytes, but now it is generally accepted that there is no avian homologue of IgD (Davison et al., 2008).
IgA

The mucosal membranes in the conjunctiva and in respiratory, intestinal, and genital tracts cover much of the body’s surface and are in contact constantly with the environment. Therefore, the mucosal immune system represents the first line of immunological defense against pathogens which encounter the mucosal surfaces. Secretory IgA (sIgA) is the primary antibody involved in antigen-antibody interactions in the mucosal immune system (Harriman et al., 1999). When pathogens establish contact with a host at a mucosal surface, the adaptive immune defense at the site is initiated by lymphocytes and secretion of secretory sIgA (Reese et al., 2006). The sIgA functions in immune exclusion by binding to bacterial and viral antigens, therefore interfering with attachment and colonization (Muir et al., 2000). Secretory IgA may prevent contact of pathogens with the mucosal surface by helping to entrap pathogens in mucus followed by peristaltic or ciliary clearance. IgA may directly block or sterically hinder the attachment proteins that mediate epithelial attachment. It may intercept pathogens within cell vesicular compartments (Silvey et al., 2001).

IgA is the predominant form of antibody in bodily secretions (Underdown and Schiff, 1986; Kerr 1990). It binds to the polymeric immunoglobulin receptor (pIgR) on the apical surface of epithelial cells and the receptor becomes integrated into IgA as the secretory component (SC). This complex is transported through the epithelial cell and secreted into the lumen of the organ collecting tubules or into the intestinal tract. SC promotes the adhesion of IgA to the epithelial surface and protects it from degradation by proteases within the cells (Avian Immunology, 110-111). Chicken IgA is often larger than IgA found in mammals, which suggests it is a trimer instead of a dimer (Davison et al., 2008).

IgM, IgA and IgG (IgY) plasma cells are found in the villi, but mainly cells producing IgA are in the lamina propria of the GI tract. IgM and IgY plasma cells are
mostly located between the crypts, while IgA plasma cells are found scattered from the crypts to villi tips (Jeurissen et al., 1989). Therefore, IgA has distinct antigenic differences from IgM and IgG and is specifically associated with the intestinal tract, as seen in cells in the lamina propria of the intestinal mucosa for all segments (duodenum, jejunum, ileum, caecum, caecal tonsils) stained for IgA (Lebacq-Verheyden et al., 1972).

There is evidence that IgG can prevent mucosal infection, and that secretory IgA might not be essential. Using IgA knockout mice, it has been shown that IgA is not necessary for protection against certain pathogens (Silvey et al., 2001), but similar work has not been conducted with chickens. Still, the presence of the large quantities of sIgA in the secretions implies that IgA must play a large role in the regulation of the mucosal immune response.

Certain factors are important for the secretory immune system to effectively clear pathogens. Evidence shows that certain intestinal bacteria are important for maintaining intestinal IgA levels. A study using germ-free mice showed that bacteria including segmented filamentous and four particular clostridial bacteria are important for developing the IgA and secretory immune system (Ohashi et al., 2006).

History

Immunoglobulins Found in Chicken Secretions

In 1971, Leslie and colleagues first confirmed the presence of immunoglobulins in chicken secretions after examination of seminal plasma, tracheobronchial, crop and duodenal washes and feces (Leslie et al., 1971a). The first two immunoglobulin classes
identified in chickens were found in their serum and, based on the antigenic differences in their heavy chains, were comparable to mammalian IgG and IgM. The homology between chicken “macroglobulin” IgM and mammalian IgM was well established early on, but some preferred to refer to chicken IgG (molecular weight ~7S) as IgY since it differed in several important respects from mammalian IgG and IgA and therefore its phylogenetic status was still somewhat uncertain. In mammals, IgA was known to be the predominant immunoglobulin in secretions. It was then understood that chickens, like many mammals, have immunoglobulins in their gastrointestinal, respiratory and reproductive tracts (Leslie and Clem, 1969; Leslie et al., 1971a). However, evidence was still lacking for the distribution of immunoglobulin classes in chickens.

Immunofluorescent and histological studies on the distribution of immunoglobulin-producing cells and classes associated with secretory immunoglobulin function were conducted. In the gastrointestinal tract, large numbers of Ig-containing cells were at first located in the lamina propria of the duodenal mucosa and cecal tonsils, but few were observed in the colon. The estimate of the types of plasma cells in the GI tract was that there were at least three times as many IgY-containing cells as IgM-containing cells (Leslie et al., 1971b). This was different than observations by Lebacq-Verheyden and colleagues, and was likely due a lack of the ability to ascertain the absence of reactions with chicken IgA (Lebacq-Verheyden et al., 1972). At this point, the presence of a secretory system in the chicken was thought to be highly likely due to the presence of immunoglobulins in secretions, the presence of specific antibodies in secretions after local immunization and the presence of immunoglobulin-containing cells below mucosal surfaces (Bang and Bang, 1968; Leslie et al., 1971a; Leslie et al., 1971b). However, the persistent problem related to establishing similarities between secretory IgA in mammals and chicken IgY (Leslie et al., 1971b).
Immunoglobulin A Discovered in Chickens

An IgA-like immunoglobulin was originally described in the chicken by Orlans and Rose (1972). Cecal contents were examined by immuno-electrophoresis and the protein mobility was shown to be different from that of the Fab and Fc fragments of IgG. The specific intimate association of the presumed IgA with the intestinal tract and its abundance in exocrine secretions was finally satisfied (Orlans and Rose, 1972). Lebarcq-Verheyden et al. (1972) reported a possible homologue of mammalian IgA which was found to be the major immunoglobulin in chicken bile and present in intestinal secretions. The homologue was shown to differ from IgY (IgG) and IgM by its inability to react with certain antisera and its antigenic determinants absent from these other antibodies. The high secretion: serum concentration ratio in mammals had only been seen for IgA, further suggesting this to be a homologue to mammalian IgA (Lebarcq-Verheyden et al., 1972). Demonstration that IgA in chickens is similar to that in mammals, through its detection and purification, stressed that there is an avian secretory immunologic system (Lebarcq-Verheyden et al., 1972). Identification of the immunoglobulin as chicken IgA was verified by demonstration that the chicken immunoglobulin was capable of associating with secretory component of human origin (Bienenstock et al., 1973). All evidence that supported the existence of chicken immunoglobulin A similar to that in mammals (Lebarcq-Verheyden et al., 1972) contradicted the previous belief that IgY is the major immunoglobulin in chicken secretions.

Although serologic evidence indicated the presence of IgA in chickens (Orlans and Rose, 1972; Lebarcq-Verheyden et al., 1972), a structural and evolutionary relationship between the chicken IgA-like isotype and mammalian IgA still remained unsettled (Mansikka 1992). IgA was later shown to be present as polymeric immunoglobulin in serum and in several external secretions (Wantanabe et al., 1975).
The chicken Ca gene was cloned, and this provided structural data allowing definite designation of the gene as the avian homologue for the mammalian αH chain of immunoglobulin IgA (Mansikka 1992).

**Secretory Component in Chicken Proposed**

After immunoglobulin M and Y had been characterized in chickens, IgA was demonstrated in chicken serum and secretions, but little was known about its molecular structure and possible possession of secretory component in secretions like that of mammalian IgA (Watanabe and Kobayashi, 1974). The existence of avian sIgA, found to be structurally similar to mammalian IgA, supported the hypothesis of a secretory immune system in chickens (Wantanabe et al., 1975).

Research that led to a better understanding of the enteric immune system in chickens involved examination of several different external secretions in order to detect secretory component and immunoglobulins. The secretory immune system in mammals, which plays the first line of defense against antigens on the mucosal surface using mainly IgA, was well understood before studying the system in chickens (Wantanabe et al., 1975). It was known that sIgA in mammals exists primarily as a dimer and possesses secretory component, and that secretory component (SC) protects the immunoglobulin from enzymatic digestion in the intestinal tract (Watanabe and Kobayashi, 1974).

Immunochemical and physiochemical data first indicated that chicken biliary IgA is the polymeric serum type IgA, lacking SC and in the form of a tetramer or pentamer like serum IgM. It also indicated that intestinal IgA in chickens has a similar structure to mammalian sIgA even though it differed from IgA in the bile (Watanabe and Kobayashi, 1974; Wantanabe et al., 1975). Studies both proved and disproved the presence of SC in chicken IgA from bile. Two types of IgA were identified- biliary IgA (tetramer or
pentamer) lacking in SC and intestinal IgA mainly in the dimeric form associating with SC. Free SC in chicken intestinal secretions was found to be antigenically deficient compared to bound SC. Possible reasons for this are that the antiserum used was prepared by immunization with intact intestinal IgA and that some portion of free SC may be digested by proteolytic enzymes. Although not detected in this early study, free SC was later detected in chicken bile, tracheobronchial mucus and egg white (Wantanabe et al., 1975). Reports also described a possible equivalent of mammalian secretory component associated with chicken IgA of the intestines (Watanabe and Kobayashi, 1974).

It was shown that a J-chain is associated with chicken IgA using immunological cross-reactivity with antiserum specific for the human J chain (Kobayashi et al., 1973). However, studies with antisera specific for human α-chain failed to demonstrate cross-reactions with chicken IgA and the homology with mammalian α-chains had not been reported. One study provided further information on the characteristics of chicken sIgA with evidence for an analogue of mammalian secretory component. Although true immunological homology was found to be lacking between avian and mammalian sIgA, the similarity was found in the association of chicken IgA with SC in gut secretions (Porter and Parry, 1976).

Therefore, the existence of SC in chicken IgA was proposed for several years (Peppard and Rose, 1972) but cloning of the chicken polymeric immunoglobulin receptor, a key component of secretory IgA, allowed for final proof of its existence (Wieland et al., 2004).
Polymeric Immunoglobulin Receptor Discovered in Chickens

Mucosal immunity and the process by which polymeric immunoglobulin receptor (pIgR) transports polymeric IgA (pIgA) into the lumen on the intestine was studied and understood in mammals some time before it was in birds. Avian research has since allowed for better understanding of avian mucosal immunity, which is necessary to prevent bacterial and viral colonization and infection of mucosal epithelia (Wieland et al., 2004).

The cloning and characterization of the chicken polymeric immunoglobulin receptor (GG-pIgR) gene, the first non-mammalian pIgR orthologue, showed that it is the functional orthologue of mammalian pIgRs. It is associated with polymeric IgA and has high levels of identity with mammalian pIgR sequences. Motifs involved in pIgR functions, including the calmodulin binding motif, the signal for rapid endocytosis, and the initial non-covalent binding site for IgA and the cysteine residues were shown to be conserved in chicken. GG-pIgR was found by Northern-blot analysis to be expressed in intestine, bursa of Fabricius, liver and thymus, but not in heart, caeca, caecal tonsils and spleen. The extracellular, ligand-binding domains of the pIgR are cleaved off on the basolateral surface of epithelia after transcytosis in a vesicle, and the resulting soluble form is SC, mainly found in association with immunoglobulins. The interaction between the chicken secretory component and chicken IgA was studied with co-immunoprecipitation. SC immunoprecipitated with chicken IgA, which confirmed that SC assembles with pIgA into sIgA complexes in chicken (Wieland et al., 2004).

The SC has a protective role of IgA, which is protection from degradation by delaying cleavage in the Fc region of the α-chain (Crottet and Corthesy, 1998; Wieland et al., 2004). It was found that chicken pIgR is mainly expressed in epithelia associated with lymphoid organs. The low level of chicken SC in feces contrasts with its abundance
in bile. This is in agreement with the mRNA expression patterns of intestine and liver tissues, respectively (Wieland et al., 2004).

The Avian Enteric Immune System

Development of the Avian Immune System

The B cell population differentiates in the specialized primary lymphoid organ—the bursa of Fabricius (Davison et al., 2008). The bursa of Fabricius provides a unique microenvironment for proliferation and differentiation of B cells (Ratcliffe, 2006). At hatching, more than 90% of bursa cells are mature B cells. In the embryonic stage, the bursa of Fabricius contains B cell precursor cells, which have already undergone Ig gene rearrangement, generating minimal antibody diversity (Ratcliffe and Jacobsen, 1994). In the bird, IgM and IgA are distributed to the yolk sac and amniotic fluid during embryonic development. The amniotic fluid is imbibed by the embryo, so IgM and IgA are found in the gut at hatching. Further diversification into distinct antibody molecules occurs after hatch (Davison et al., 2008).

The bursa of Fabricius lumen is connected to the gut lumen, and this allows B cell development to occur while in direct contact with molecules derived from the gut. Antigens in the gut are transported to lymphoid follicles and this is initiated several days after hatching, allowing for substantial diversity of B cell secretory Ig receptors. Observations suggest that exposure to gut-derived molecules is critical for normal development of the bursa structure after hatching (Davison et al., 2008).
Gut-Associated Lymphoid Tissues

The mucosal lymphoid tissue of the GI tract is one of the most complex tissues of the immune system as it regulates the development of the IgA response (Fagarasan, 2002). Immunologically mature cells enter the circulation and colonize the gut-associated lymphoid tissues (GALT) along with other secondary, or peripheral, lymphoid tissues. B and T cells occupy different compartments, with B cell compartments being called germinal centers. In lymphoid regions, there are separate areas where non-lymphoid cells present antigen to T cells, and these interact with B cells in order for immunoglobulin production to occur. Avian GALT includes oesophageal tonsils, pyloric tonsils, Peyer’s patches, caecal tonsils and Merkel’s diverticulum (Davison et al., 2008). The mucosae are primary targets for antigens, so chickens have extensive lymphoid tissues in these areas such as GALT. Most secondary lymphoid tissues begin to develop independently of antigen stimulation, but further maturation has been demonstrated to be antigen driven (Hedge et al., 1982).

GALT development begins in the lamina propria of the villus and distinct areas of B and T cells arise. The luminal side of the lamina propria contains a basement membrane with columnar epithelial cells that have mucous-producing goblet cells between them. These epithelial cells synthesize pIgR to which pIgA binds. The receptor-Ig complexes are endocytosed and then transcytosed to the apical surface where sIgA is released into the mucosal lumen by proteolytic cleavage of the receptor ectodomain. Plasma cells are found in the villi, with mainly IgA-producing cells in the lamina propria. During the first week after hatching, intestinal lymphocytes become lamina propria B cells, T-helper cells and epithelial cytotoxic/suppressor cells in function (Davison et al., 2008).

Peyers patches (PP) are lymphoid aggregates with both B and T cells (Davison et al., 2008). Precursor B cells found in PP and caecal tonsils (CT), besides being in the
bursa of Fabricius, were shown to provide IgA-producing plasma cells to the lamina propria (Muir et al., 2000). The ileal Peyer’s patches may serve as an important site for research on the inflammatory and immunologic responses of the host against enteric pathogens since they function as lymphoid inductive sites of the alimentary tract. Macrophages, Dendritic cells, plasma cells, and B and T lymphocytes make up the associated cellular repertoire of this tissue, and antigenic stimulation here can induce effective mucosal and systemic immune responses. Local antigen-specific sIgA production can act as a protective barrier against enteric pathogens in the PP, but the PP may also be manipulated by certain pathogens to serve as a portal of entry. In the ileum, both a distal ileal PP and a proximal ileal PP caudal to Meckel’s diverticulum have been observed in several studies and these most likely serve as integral GALT involved in the chicken enteric immune system (Vaughn et al., 2006).

Cecal tonsils are lymphoid organs which show an increase in lymphocytes the first week after hatching, and germinal center formation in the second week (Davison et al., 2008). Gut flora are essential to the development as shown in germ free chickens (Hedge et al., 1982).

**Mechanism of the Polymeric Immunoglobulin Receptor**

Adaptive humoral immune responses on mucosal surfaces are mediated primarily by secretory IgA (Phalipon et al., 2002). IgA is the primary immunoglobulin isotype produced by the mucosal immune system (Underdown and Schiff, 1986). In the bird, sIgA can be recovered from intestinal fluid, bile, tears, tracheal and lung washings (Tizard, 2002) but the highest concentration is found in the bile fluid (Rose et al., 1981). SlgA is the leading immunoglobulin in mucosal secretions (Chintalacharuvu et al., 1991).
In mammals, sIgA exists primarily as a dimer and possesses secretory component, and this is very similar to what has been found more recently in chickens (Watanabe and Kobayashi, 1974). sIgA primarily functions in immune exclusion, where its binding to an antigen interferes with pathogen attachment and colonization (Muir et al., 2000; Phalipon and Corthesy, 2003). Its role in neutralizing viruses and bacteria through noninflammatory mechanisms both intra and extracellularly promotes intestinal homeostasis (Schneeman et al., 2005).

PlgR, also known as membrane secretory component, is a membrane protein that acts as a specific receptor for IgA and mediates its transport through the epithelial cells lining the intestine to the apical membrane (Chintalacharuvu et al., 1991). The role of plgR in mucosal humoral immunity has received little attention compared to its ligand, sIgA. The maintenance of mucosal homeostasis requires plgR to ensure efficient secretion of non-pathogen and pathogen-specific sIgA (Phalipon and Corthesy, 2003). The affinity of IgA (dimeric or polymeric) for plgR is determined by its J chain and Ig subunit (Brandzaeg and Prydz, 1984). The J chain is a critical component in secretory immunity because it dictates the selective epithelial transport of plgA to form secretory IgA (Brandtzaeg and Johansen, 2001). PlgR transports IgA which can prevent the adherence and invasion of the epithelial layer by pathogens, neutralize viruses and bacteria intracellularly during plgR-mediated transcytosis, and transport immune complexes and pathogens coated in plgA from the basolateral to the apical surface of epithelial cells (Kaetzel, 2001).

The mechanisms of plgR have been studied in vitro using polarized monolayers of transfected MDCK cells. In these cells, an addition of human plgA at the basolateral surface has been shown to stimulate the rate of plgR transcytosis. Ligand-induced transcytosis of rat plgR in the liver has been confirmed in vivo by showing that injection of plgA increased the amount of total SC secreted into the bile. This transcytosis most likely is due to the modification of plgR after its translation, and also due to an
intracellular signal. Protein-tyrosine kinase is activated, and this rapidly phosphorylates phosphatidylinositol-specific phospholipase Cγ1 (PLCγ1). PLCγ1 catalyzes the hydrolysis of phosphatidylinositol-4,5-bis-phosphate into inositol 1,4,5-triphosphate (IP3) and diacylglycerol. Diacylglycerol activates protein kinase C (PKC), stimulating transcytosis. IP3 causes the release of calcium ions into the cytosol from intracellular stores, and an increase of the concentration in the cytosol of Ca ions by thapsigargin also stimulates transcytosis. There seem to be species differences in the sensitivity of pIgR to intracellular calcium, with polymeric IgA binding to human pIgR eliciting intracellular signaling but failing to stimulate transcytosis of pIgR (Giffroy, 2001).

A study generated mice deficient in sIgA by knocking out the pIgR gene in order to determine the ability of the knockout mice to maintain mucosal homeostasis and mucosal and systemic immune functions. Results showed that the knockout mice deficient in sIgA had slightly elevated levels of intestinal IgG, which is not actively transported into the intestinal lumen, and had elevated serum dIgA levels. This may be due to a heightened level of immune induction due to a decreased mucosal barrier function and decreased epithelial integrity due to the antigens associating more with the enterocytes. As seen here, pIgR is important in maintaining mucosal homeostasis (Uren et al., 2003).

Research demonstrated that polarity plays a large role in controlling the release of pIgR in intestinal epithelial cells by regulating its distribution on the basolateral membrane along with the distribution of the SC protease on the apical membrane. The HT-29 human colon carcinoma cell line was used here because it can be induced to differentiate to enterocyte morphology by removal of glucose as a carbon source. Also, it may express pIgR and the cell line has been used to study IgA transport across cultured epithelial cells. To demonstrate the role for cell polarity in controlling the release of pIgR from the cell surface, HT-29.74 cells (selected for induction of pIgR) were cultured in low calcium medium and this disrupted cell-cell contacts and apical/basolateral polarity.
Loss of polarity led to an initial rapid increase followed by significant inhibition of release of pIgR from the cell surface. Reorganization of proteins in the plasma membrane due to loss of polarity is likely to have resulted in contact between basolaterally localized pIgR and apically localized protease. The results demonstrated that the role for cell polarity in pIgR release is consistent with the hypothesis that pIgR is located on the basolateral surface and cleavage of the pIgR-IgA complex to sIgA occurs on the apical surface of cells (Chintalacharuvu et al., 1991).

pIgR, expressed on the basolateral surface of epithelial cells, binds to J-chain-containing pIgA, which is synthesized by plasma cells in the lamina propria (Chintalacharuvu et al., 1991; Giffroy et al., 2001; Hamburger et al., 2004; Pal et al., 2005). pIgR is expressed by most epithelial cells lining the secretory epithelial surfaces and the exocrine glands (Giffroy et al., 2001). Ligand-binding domains of pIgR are cleaved off upon transcytosis to the apical surface of the epithelium. The resulting secretory component is found predominantly associated with immunoglobulin in the secretory IgA complex. Secretory component has a protective role against IgA degradation by delaying cleavage in the Fc region of the α-chain (Wieland et al., 2004; Pal et al., 2005).

The rate of IgA transcytosis may depend on the level of pIgR expression (Bonaz, 2005; Pal et al., 2005; Schneeman et al., 2005), with one molecule of pIgR being synthesized for each molecule of IgA that is transported. Factors that influence pIgR may therefore affect mucosal immunity since it plays a role in mucosal host defense. Its expression in epithelial cells is increased by TNF-α, IL1β, and IL4 (Pal et al., 2005). Microbial byproducts regulate pIgR expression through toll-like receptor (TLR) signaling and hormones in addition to cytokines (Bonaz, 2006), and dsRNA may result in upregulation of the receptor (Schneeman et al., 2005). Receptors may be cleaved at the apical surface even if they are not occupied by IgA, resulting in the same part of pIgR being released as free SC (Brandtzaeg and Johansen, 2001).
When pIgA encounters antigen in the lamina propria of the intestine, the immune complex and excretory pathway are each processed, leading to the release of the antigen-secretory IgA complexes in the intestinal crypts. Antibody binds to antigen and its transcytosis into the lumen by pIgR leads to removal of antigen from infected tissue. The neutralizing mechanism consists of intracellular pIgA transport mediated by pIgR. Free SC may act as a non-specific microbial scavenger. When it associates with specific pIgA, it can clear these pathogens to a greater degree. When SC is free, it is susceptible to proteolytic degradation and this may limit its efficacy (Phalipon and Corthesy, 2003). The efficient transfer of the unoccupied receptors leading to the release of free SC allows for significant levels of it in several secretions, as seen in humans. It possibly may serve other functions (Kaetzel, 2001).

**Secretory Immune System and Vaccines**

Using what has been learned about the secretory immune system in animals, vaccines can be made more efficient. Vaccination against reoviruses in the industry has been aimed towards immunizing breeders with inactivated vaccines to transfer passive immunity to progeny, or to induce active immunity by vaccination of chickens at a young age (one-day old, preferably) with attenuated reovirus strains (Neelima et al., 2003). Oral administration of an antigen induces an effective local intestinal immune response, and this only sometimes occurs with non-replicating antigen. An obstacle for the design of vaccines is that digestive enzymes of the GI tract may degrade some antigens before they reach the immune system. Thus, the small amount that is sampled by GALT (gut-associated lymphoid tissue) tends to result mainly in a suppressor response. In order to have effective vaccines, technologies are needed to avoid the difficulties with non-replicating antigens and induce significant sIgA, and to therefore improve defense at the
intestinal surface. Possible methods include induction of mucosal immune responses by including adjuvants or immunoregulators in vaccines, and manipulation of intestinal microflora. Using a primary intraperitoneal vaccination followed by an oral booster of anti-antigen sIgA overcomes oral unresponsiveness to non-replicating antigens through antigen presentation across the intestinal surface (Muir et al., 2000).

It is important to understand through further research how to identify the tissues where IgA+ B cells reside in order to best design intestinal vaccines (Muir et al., 2000).

**Avian Reoviruses**

**History**

Reoviruses were first isolated from mammals, and later avian reoviruses (ARV) were isolated and characterized serologically. ARV share many morphological and physiochemical features with mammalian reoviruses (MRV). Some avian strains are antigenically related to strains of human reoviruses, and there is similarity between the genomic and capsid structures of avian and human reoviruses (Spandidos and Graham, 1976). However, on a comparative basis ARV have been poorly characterized at the molecular level because they have been considered to be very similar to the well-studied mammalian reoviruses (MRV) (Benavente and Martínez-Costas, 2007).

ARV-induced respiratory disease was first isolated in 1954 by Fahey and Crawley, who isolated the virus from the respiratory tract of chickens with chronic respiratory disease, and named it “Fahey-Crawley virus” (Van der Heide, 2000).
1957, Dr. Norman Olson reported the isolation of an agent, which produced synovitis in broilers, but it was not susceptible to chlortetracycline, furazolidone or streptomycin like the *Mycoplasma synoviae* he was studying (Olson *et al.*, 1966). Its pathogenicity was higher in young chicks. He therefore called this “viral arthritis agent.” It was misdiagnosed first as poxvirus because of its double stranded nucleic acid, but was later identified by electron microscopy as a reovirus (Walker *et al.*, 1972; van der Heide, 2000).

Reovirus-induced tenosynovitis (viral arthritis) in chickens was originally described by Olson and Kerr (Olson and Kerr, 1967; Kerr and Olson, 1969), and numerous studies followed. Several researchers found that the reovirus caused myocarditis and hepatitis in experimentally infected chickens. In 1966, the virus was found to be associated with enteric disease in chickens (Krauss and Uebrenschar, 1966). Isolation of the virus from chickens with arthritis/tenosynovitis and/or enteric lesions occurred in several countries. Different serotypes were reported, and all isolates were indicated to be serologically related, although not all were fully neutralized by the same antiserum (antiserum of S1133) (van der Heide, 2000).

Studies in recent years have revealed important differences between MRV and ARV. Avian reoviruses were found to differ from MRV in their lack of hemagglutinin (Schnitzer *et al.*, 1982; Labrada *et al.*, 2002). Also, the ARV S1 gene was found to be a functional tricistronic gene that possesses three out-of-phase and partially overlapping open reading frames. The initiation of translation of the three S1 cistrons, and the properties and activities displayed by their encoded proteins, are currently important areas for research. The ARV, unlike MRV, are one of the few non-enveloped viruses that cause cell–cell fusion, and their fusogenic phenotype has been associated with a nonstructural 10 kDa transmembrane protein. Finally, ARV are highly resistant to interferon, which makes them useful for studying the mechanisms and strategies that
viruses utilize to counteract the antiviral actions of interferons (Duncan and Sullivan, 1998; Shih et al., 2004; Benavente, and Martínez-Costas, 2007).

Structure and Genomic Organization

Avian reoviruses are members of the Orthoreovirus genus. They are icosahedral, nonenveloped viruses with double-protein capsid shells and genomes consisting of ten double-stranded RNA segments that express structural and nonstructural proteins. The virus encodes at least 10 structural proteins and four non-structural proteins encased within their 2 concentric protein shells (Benavente and Martínez-Costas 2007; Lin et al., 2007, Figure 1.1).

The ten double-stranded RNA genome segments are divided into three size classes based on their electrophoretic mobility (Figure 1.1). These include L (large), M (medium) and S (small). Proteins encoded by the ARV consist of class λ (large), μ (medium) and σ (small). The 10 known structural proteins expressed by ARV include λ A, B, C, μ A, B, BC and BN and σ A, B, and C (Table 1.1). The four known nonstructural proteins are μNS, σ NS, p10 and p17 (Benavente and Martínez-Costas 2007; Table 1.1). Each protein is encoded by an RNA segment consisting of an open reading frame, and the segments are transcribed by core-associated RNA polymerase (Li et al., 1980; Table 1.1).

The ARV strains differ with respect to electropherotype, protein profile and pathogenicity. In all members of the Reoviridae family, co-infection of cells with two virus isolates allows for reassortments, which is the creation of progeny viruses with genome segments from two parental viruses. This is useful for studying reassortments
and characterizing phenotypic properties of the individual genome segments (Ni et al., 1995; Vázquez-Iglesias et al., 2009).

Figure 1.1. Avian Reovirus particle (from Benavente and Martínez-Costas 2007)
<table>
<thead>
<tr>
<th>ARV Protein</th>
<th>Encoded by:</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>λA</td>
<td>L1</td>
<td>Forms the inner core shell that encloses the genome segments and RNA polymerase; Scaffold for core assembly</td>
</tr>
<tr>
<td>λB</td>
<td>L2</td>
<td>Viral core component; possible source or RNA polymerase activity</td>
</tr>
<tr>
<td>λC</td>
<td>L3</td>
<td>Viral capping enzyme by autoguanylation</td>
</tr>
<tr>
<td>μA</td>
<td>M1</td>
<td>Inner capsid component; putative interaction with μNS</td>
</tr>
<tr>
<td>μB, μBN, and μBC</td>
<td>M2</td>
<td>Structure components of the outer capsid, role in release of the virus into the host cell cytoplasm from the lysosomes</td>
</tr>
<tr>
<td>μNS, μNSN, and μNSC</td>
<td>M3</td>
<td>Factory (inclusion) formation in infected cells; recruitment of proteins</td>
</tr>
<tr>
<td>σA</td>
<td>S2</td>
<td>Inner core shell component, dsRNA-binding activity; anti-interferon activity; putative role in ARV morphogenesis</td>
</tr>
<tr>
<td>σB</td>
<td>S3</td>
<td>Component of the outer capsid; function unknown</td>
</tr>
<tr>
<td>σC</td>
<td>S1</td>
<td>Viral cell attachment protein; possible causes apoptosis</td>
</tr>
<tr>
<td>P10</td>
<td>S1</td>
<td>Transmembrane protein; induces cell-cell fusion; destabilizes the membrane (permeabilizing)</td>
</tr>
<tr>
<td>P17</td>
<td>S1</td>
<td>Nucleocytoplasmic shuttling activity; transcriptional activity of the cell; possible DNA-binding activity; possibly regulates cellular proliferation</td>
</tr>
<tr>
<td>σNS</td>
<td>S4</td>
<td>Binds ssRNA; recruited to viral factories of Reo-infected cells; possible roles in RNA packaging and replication</td>
</tr>
</tbody>
</table>
Mode of Avian Reovirus Infection

Natural ARV infections occur via the fecal-oral route, but evidence has been provided for infection via the respiratory tract and vertically via egg transmission. Infection is mainly via oral entry with initial replication in the intestine and bursa of Fabricius (Menendez et al., 1975b; Ni et al., 1995; Benavente and Martínez-Costas, 2007). The small intestine is the most important site for ARV infection, with the spread of infection beginning in the villus and crypt epithelium (Kibenge et al., 1985; Jones et al., 1989). However, infection via the footpad has been demonstrated to be more effective than oral inoculation for the viruses that cause arthritis and tenosynovitis (Ni and Kemp, 1995). Menendez and colleagues demonstrated vertical transmission of ARV by infecting 15 month-old breeders. Hatching eggs at 17, 18 and 19 days post-inoculation, as well as an infertile egg at 16 days post-inoculation, were infected with the virus (Menendez et al., 1975). Egg transmission of ARV plays a large role in maternal antibody protection, as evidenced by the beneficial effects of vaccination (van der Heide, 2000).

Mucosal surfaces, such as the lining of the gut, are important locations for the entry of viruses into the body. Peyers patches (PP) are lymphoid areas of the GI tract with specialized epithelial cells known as microfold (M) cells. A reovirus can enter here after binding to a host cell by way of its outer capsid protein (Bomsel and Alfsen, 2003). Avian reoviruses replicate in the cytoplasm of infected cells and induce fusion of host cells in order to facilitate spread (Vázquez-Iglesias et al., 2009).

After infection in the Peyer’s patches (PP), some enteric reoviruses can invade the central nervous system (CNS) as demonstrated in mice and chickens (van de Zande and Kuhn, 2007) and this has been seen via immunohistochemical detection of viral antigen in areas of the CNS. Birds challenged intramuscularly or orally with the infective strain harbored the virus in the brain. The tropism of the strains for target cells in the CNS is
probably determined by the S1 gene that encodes for viral attachment protein \( \sigma \)C (Forrest and Dermody, 2003; van de Zande and Kuhn, 2007).

**Attachment and Entry**

A virus must cross cell membranes to express its genome within cells. Observation of ARV cells during early infection of chick embryo fibroblast cells suggested that the virus penetrated these cells by receptor-mediated endocytosis (Benavente and Martínez-Costas, 2007). Viruses can interact with epithelial cells and use normal epithelial signaling and pathways of the host cell to facilitate entry and expression of its genome. Viruses use host-cell molecules, also known as co-receptors, and protein receptors to attach to host cells. Reoviruses can also use a junction adhesion molecule (JAM) to penetrate the epithelial-cell cytosol at the epithelial tight junction. This disrupts the tight junction and affects the integrity of the epithelial barrier. Reovirus binding to both sialic acid and JAM is required for efficient apoptosis (cell death) of the host cell (Bomsel and Alfsen, 2003; Forrest and Dermody, 2003; Benavente and Martínez-Costas, 2007; Vázquez-Iglesias *et al.*, 2009).

Reoviruses exploit the transepithelial transport activity of M cells to enter the PP mucosa and initiate infection. After oral ingestion, the outer capsid of native virions is processed by proteases in the lumen of the intestine. This results in infectious subviral particles (ISVPs) that adhere selectively to M-cell surfaces. Once they adhere, they can be transcytosed in vesicles and replicate in the cells of the PP mucosa (Silvey *et al.*, 2001). ISVPs in the lumen of the gut are important for infection by that route (Nibert *et al.*, 1991). ISVPs are important for ARV attachment to or transport across M cells. ISVPs are infectious, like virions, when absorbed to cells. However, they are incapable of transcribing full-length mRNA *in vitro*. The ISVP-to-core conversion is characterized
by loss of infectivity and acquisition of transcriptase activity. Cores but not ISVPs are capable of transcribing full-length mRNA, making it likely that a core is the transcriptionally active form of virus that gains access to the cytoplasm during penetration (Nibert et al., 1991).

**Uncoating**

Uncoating in virus-containing intracellular vacuoles, or endosomes, occurs by acidification. This endosome-mediated proteolytic processing of the major outer capsid protein is necessary for ARV to be ready to replicate. After it is uncoated, the virus core can cross the endosomal membrane and enter into the cytoplasm of the cell for replication (Benavente and Martínez-Costas, 2007; Vázquez-Iglesias, 2009). Purified avian reovirions lost their outer capsid polypeptides upon incubation at 40°C. This suggests that this release of polypeptides in response to this temperature in the host may positively affect virus replication by facilitating penetration of the virus into intestinal cells and by promoting uncoating of the virions within endosomes (Benavente and Martínez-Costas, 2007).

**Replication**

Reovirus cores affect enzymatic activities needed to produce capped mRNA from each dsRNA gene segment and protect the genome from degradation in the cytoplasm. Core proteins may help keep the reovirus genome from generating a high-level interferon (INF)-related antiviral response (Nibert et al., 1991). ARV σA is a dsRNA-binding protein that stabilizes viral core particles and protects the virus against the antiviral action of the INF (Vázquez-Iglesias et al., 2009). Double stranded RNA is a potent inducer of
INF and an essential cofactor for some of the interferon-stimulated antiviral activities in cells. INF interacts with cell-surface receptors and initiates a signal transduction pathway that leads to increased expression of many proteins, some of which possibly play an important role in fighting viral infections. One protein, the dsRNA-activated protein kinase (PKR) plays a key role in regulating intracellular protein synthesis. ARV is highly resistant to antiviral effects of INF. The double-stranded RNA-binding σA protein is capable of reversing the INF-induced antiviral state by down-regulating PKR activity (González-López et al., 2003).

Replication and assembly of ARV takes place in viral factories, or viroplasmas (Touris-Otero et al., 2004), in the cytoplasm of cells. Reoviruses can undergo primary replication in the lymphoid tissue of the PP (Forrest and Dermody, 2003). The gene expression on ARV begins with the synthesis of the ten viral mRNAs. Virus-encoded dsRNA-dependent RNA polymerase starts the process by using the negative strands of the viral genome as templates for mRNA synthesis. RNA polymerase is associated with core components. The viral mRNAs are thought to be made within the inner core. Elongation activity of the dsRNA-dependent RNA polymerase is probably regulated by the physical characteristics of the viral particle (Benavente and Martínez-Costas, 2007; Vázquez-Iglesias et al., 2009).

ARV transcripts, which are identical to the positive strands of their encoding genes, possess a type-1 cap at their 5’ ends, lack a poly-A 3’ tail, and contain short untranslated regions at their 5’ and 3’ ends. These newly made mRNAs program viral protein synthesis at the ribosomes and are templates for dsRNA minus strands (Nibert and Schiff, 2001). ARV morphogenesis takes place with viral cores being assembled first within the first 30 minutes after synthesis of their protein components, and the outer capsid proteins are assembled into these cores over the next 30 minutes, thus completing reovirion morphogenesis (Benavente and Martínez-Costas, 2007; Vázquez-Iglesias et al., 2009).
**Pathogenesis**

Avian reoviruses cause significant economic losses in the poultry industry due to the diseases with which they are associated. They were initially discovered as the disease that induced tenosynovitis in young chickens, and then were found to be ubiquitous among poultry flocks, mostly causing asymptomatic infections. Diseases to which ARV have been associated in poultry include enteric and respiratory diseases, myocarditis, hepatitis, Poult Enteritis and Mortality Syndrome (PEMS) in turkeys and malabsorption syndrome (MAS) in chickens (Heggen-Peay *et al.*, 2002; Benavente and Martinez-Costas, 2007). The ARV infections are important in the poultry industry since mortality, leg weakness and poor feed conversion caused by ARV depresses productivity (Ni *et al.*, 1995). Field outbreaks, mainly among broiler breeders, where the agents have been isolated and identified as reoviruses, have been reported in many parts of the world (Gouvea and Schnitzer, 1982).

Reoviruses isolated from MAS and tenosynovitis can induce lesions in the liver, heart, bursa of Fabricius, bone, proventriculus, intestine and tendons. Reoviruses may be priming agents in early development of intestinal lesions in cases of MAS (Lenz *et al.*, 1998; Songserm *et al.*, 2003). The development of lesions, also known as pathogenicity in the case of ARV, correlated with the presence of replicating virus and its spread in tissues (Ni *et al.*, 1995). Inflammatory lesions in leg tendons were found to occur as a result of ARV infection following oral challenge in chickens (Kibenge *et al.*, 1985). It was demonstrated that challenge of day-old chickens orally and ocularly with ARV causes mild lesions including hyperplasia of lymphocyte aggregates in various organs and mild gizzard erosions. Impaired weight gain, feed conversion and feed passage, along with watery feces, often occur along with lesions in broiler chickens infected with ARV (Lenz *et al.*, 1998). Turkey-origin reoviruses can cause major lesions in the form of
bursal atrophy, which is characterized by lymphoid depletion and fibroplasias (Pantin-Jackwood et al., 2007).

The viremia due to ARV challenge spreads from the villus and crypt epithelium upon initial replication of the virus. It has been re-isolated from plasma, intestines, bursa of Fabricius, pancreas, spleen, liver, kidney, joints and tendons, with the liver becoming a primary site for replication after the virus spreads from the epithelium of the small intestine (Mandelli et al., 1978; Kibenge et al., 1985; Jones et al., 1989). Jones and colleagues (1989) reported that the intestine and bursa of Fabricius serve as portals of entry and sites of replication for ARV, and this initial replication takes place between 12 and 24 hours post-inoculation in the intestine (Jones et al., 1989). In a study describing the distribution of turkey-origin reoviruses in the tissues of infected poult's, only the bursa of Fabricius, intestines and spleens were continually positive for ARV antigen. In both turkey poult's and chickens, the bursa and intestine are the primary sites of entry and replication for the virus, and the spleen is among the first tissues infected after initial replication in the both species. There was no evidence in this study of viral replication in the liver, but this organ has been considered a primary target of ARV (Pantin-Jackwood et al., 2007).

In selenium-deficient ARVCU-98-infected broiler chicken, the duodenal, jejunal, and ileal villi have been found to be thicker, shortened and blunted with deeper crypts of Lieberkuhn. Degeneration of the villus tips occurs, as seen in the sloughing of the villus epithelial cells. The signs of infection were less severe due to the feeding of organic selenium in a high selenium yeast supplement (Sel-Plex) and to a smaller degree due to the feeding of sodium selenite. The live weight gain was greatest in the organic selenium-fed chicks compared to the control (no supplemental selenium) and sodium selenite groups. Also, plasma protein levels were significantly lowered due to the ARV infection, but Sel-Plex maintained plasma protein level at a higher concentration compared with the other dietary treatments at 2 and 3 weeks of age. These findings suggest that nutrients
such as selenium could play a role in the chicken’s ability to resist or regain function due to ARV infection (Edens et al, 2007ab).

Blood plays a role in pathogenesis via influences on tissue tropism and host mechanisms involved in controlling the infection. Kibenge et al. observed ARV viremia in the bone marrow, liver and enteric tissues in chicks challenged orally with ARV. Virus infection was most widely distributed in tissues at 5 days post-inoculation (p.i.) and appeared most persistently in enteric tissues. Maximum titers were found in the duodenum, pancreas and ileum at 3 days p.i. Intermittent titers were detectable in plasma and erythrocyte fractions of blood from 30 hours to 5 days p.i. Titters were only in the mononuclear cell fraction at 10 days p.i. (Kibenge et al., 1985).

Avian reoviruses are ubiquitous in commercial poultry and are frequently isolated from GI and respiratory tracts of chickens with acute or unapparent infections (Gouvea and Schnitzer 1982; Neelima et al., 2003; Benavente and Martínez-Costas, 2007). The viral infections of the gastrointestinal tract in chickens and turkeys result in a broad range of outcomes from unapparent and economically insignificant to severe and economically devastating. Although ARV are known to replicate in intestines of poultry and are frequently isolated from cases of enteritis, malabsorption syndrome in chickens and Poult Enteritis and Mortality Syndrome (PEMS) in turkeys, a causative role for the virus in these diseases is hard to prove. The outcome is due to age and immune status of infected birds, and virulence of the ARV strain. The infections are usually complicated by other infectious agents as well as management, nutrition and environmental factors, which makes the true role of the agents difficult to assess. They do enhance the pathogenicity of many other infectious agents due to immunosuppression, but still are often isolated from clinically healthy chickens. Virus-induced mucosal damage may provide a portal of entry for other potential pathogens. Also, the damage may allow for gastrointestinal attachment of pathogens as a result of loss of normal defense mechanisms. Malabsorption may occur due to mucosal damage, and result in nutritional deficiencies.
Impaired growth and development of lymphoid organs may result (Guy, 1998; Mukiibi-Muka and Jones, 1999). A direct link between the presence of virus and disease, without other viruses and factors involved, has been demonstrated for viral arthritis (tenosynovitis), which is characterized by swelling of the hock joints caused by lesions in the gastrocnemius tendons (Benavente and Martínez-Costas, 2007). Menendez and colleagues demonstrated that an ARV serologically related to the agent that causes viral arthritis can cause widespread infection after inoculation via both the respiratory and alimentary tracts (Menendez, 1975b). In PEMS outbreaks in turkey pouls, ARV (ARV-CU98 was suggested to be a causative agent (Heggen-Peay et al., 2002).

Significant factors that influence the outcome of reovirus infection in chickens are age at infection and route of inoculation. It has been demonstrated that for 1 day old or 2 weeks old broilers inoculated with ARV S1133 via the footpad or oral routes, the greatest pathological effects related to the joints occurred with 1-day-old infection via the footpad. Little disease was seen after infection at 2 weeks old. In chickens infected at one day of age, half of the chicks were depressed and prostrate only 2 days after infection. All returned to normal appearance by the eighth day, but leg swelling was present at 6 weeks of age. In the two youngest groups (1 day old and 2 weeks old) given ARV via oral inoculation, tenosynovitis was first seen as swellings at and below the hock joint from three weeks after infection. The younger the age of the bird at infection, the earlier the lesions developed. Age-linked susceptibility of chickens to reovirus seems to be due to the failure of the young chicks to mount an early and effective immune response (Jones and Georgiou, 1994; Ni et al., 1995; Benavente and Martinez-Costas, 2007). Declining virus titres in the gut have been seen with increasing chick age. When chicks were challenged orally at day of hatch, there was no IgA detected in the intestinal contents. However, those infected at 7 days and 3 weeks had a substantial rise in intestinal IgA (Mukiibi-Muka, 1999).
In another study, three ARV isolates (2177, 2035 and S1733) were used to determine the effect of the age of chickens at inoculation on virulence and persistence of the virus. The highly pathogenic isolate S1733 was re-isolated from several tissues and lesions were observed in a few of these when sampled within a 7-day period following inoculation. The isolate persisted and produced lesions in the gastrocnemius tendons for as long as 22 weeks p.i. The other two isolates were of intermediate pathogenicity (2035) or low pathogenicity (2177) and were isolated less frequently and from fewer tissues than S1733. Isolate 2035 was found in gastrocnemius tendons as long as 7 weeks p.i., while isolate 2177 was never isolated from the tendons and did not produce notable tissue changes. Birds inoculated at the age of one week or older with any of the three ARV pathotypes were more resistant to infection than those inoculated at day 1. There was a decrease in virus re-isolations and a reduction in the severity of lesions in tissues in those inoculated at the older age. Severity of infections were even less in chickens inoculated at 2 weeks of age or older with ARV. The preliminary classification of the ARV pathotypes has been according to their induced severity of mortality, weight reduction, tissue lesions and clinical disease. Isolate 2177 did not differ from controls in mortality, weight gain, tissue lesions or clinical signs. Isolate 2035 produced slightly greater mortality, significant weight depression and intestinal lesions in 1-day-old inoculates. Mortality was as high as 84% in birds infected with isolate S1733 and occurred within 2 weeks following inoculation at 1 day of age. In both 2408 and S1733, signs consistent with those reported in birds suffering from malabsorption syndrome due to ARV (ARV had been isolated) were produced. All in all, highly pathogenic isolates were present more often and at an earlier time p.i., and they persisted longer than less-pathogenic isolates (Rosenberger et al., 1989). A difference in pathogenicity between different strains of turkey origin reoviruses exists with some inducing severe lesions in the bursa of Fabricius, and some inducing mild to moderate lesions (Pantin-Jackwood et al, 2007).
The pathogenicity for 1 day old specific pathogen free chicks infected with seven strains of ARV when inoculated subcutaneously, orally, or via the footpad has been examined. There were no significant differences in the ability of the isolates to infect target tissues, but there was much variance in virulence among the strains. The vaccine strain was the least virulent of all the viruses, having a million fold less LD50 (dose at which 50% of animals die) than its parent strain S1133. With all viruses, the mortality occurred between days 3 and 9 p.i. and the time of death was directly related to the dose of virus received (Gouvea and Schnitzer, 1982).

Malabsorption Syndrome

Malabsorption syndrome was first observed in the late 1970s and was thought to be caused by a reovirus since this pathogen was isolated from broilers with the clinical disease. However, other viruses were often involved, and the complete MAS was found to not be only due to reovirus. It was concluded that the syndrome usually has a multifactorial etiology. Two ARV isolates (S1733 and 2408), however, did individually induce MAS in experimental studies, and these are now present in some inactivated vaccines (van der Heide, 2000). Also known as runting-stunting syndrome, MAS is a global problem for the broiler industry. Birds are susceptible mainly in the first two weeks of age (Songserm et al., 2003). The condition is characterized by enteric disease including diarrhea, inordinate passage of feed, poor feathering, poor feed conversion and stunting (Gouvea and Schnitzer, 1982). Gastrointestinal lesions are often present and cause weight gain depression. The lesions include proventriculitis and enteritis with cellular infiltration, cystic crypts of Lieberkuhn, villus atrophy and villus fusion (Songserm et al., 2003).
Vacuolar degeneration and sloughing of the villus epithelium at 2 days p.i. has been observed in birds infected with reoviruses that are associated with MAS. Reovirus antigen was found mainly in the affected jejunum and ileum in a study where Songserm and colleagues challenged broilers with ARV isolated from cases in The Netherlands and Germany. After day 7, no reovirus antigen was detected in any of the infected groups. Macrophages and monocytes were present in the lamina propria of the jejunum in both groups and increased beginning at 4 days p.i. Although the ARVs caused intestinal lesions in broilers with MAS, none caused weight gain depression. While lesions were mainly at the tip of the villus, antigen was detected at the tip and middle part of the villi. ARV antigen was also detected in the cecal tonsil and bursa. The ARV alone did not cause the same intestinal lesions as those seen in MAS. In commercial breeders, the combination of inoculated reovirus with agents already present in the broiler may be required to cause the effect on weight gain (Songserm et al., 2003).

**PEMS and ARV-CU98**

PEMS is an infectious intestinal disease of turkey poults, and it has been demonstrated that ARV is an agent contributing to its pathogenicity. It is characterized by diarrhea, dehydration, weight loss, anorexia, growth depression and high mortality. Immune dysfunction has been associated with the disease, and humoral and cellular immunity are negatively affected by it. It has been demonstrated that macrophages may have lower phagocytic potential and cytokine profiles may be abnormal. The CD4:CD8 T-cell ratios are altered in the blood and spleen in poults with the illness. It is thought that virus(es) may initiate PEMS, followed by infection by opportunistic pathogens such as *E. coli* strains due to a depressed immune system in the turkey poults (Heggen-Peay et al., 2002). Reynolds has emphasized that multiple viruses are often associated with
enteric diseases, and the detection of a virus does not necessarily mean it is the cause of
disease (Reynolds, 1991).

In a study using a PEMS-associated reovirus, poults were challenged orally with
various filtrates of fecal material from control and PEMS-affected animals. The isolation
of an ARV strain, ARV-CU98, along with characterization of its virulence followed. In
order to ascertain the size of the putative infective agent(s), the challenge filtrates were
from either 100 or 220 nm filtrates of fecal material from PEMS-negative and PEMS-
positive poults. The 100 nm filtrate from the PEMS fecal material was determined to
result in higher mortality and significantly lower body weight and relative weights of the
bursa of Fabricius and thymus, which are clinical signs associated with PEMS. A virus
isolated from the 100 nm filtrate was propagated in liver cells. It was identified as a
reovirus after cross reacting with antisera against another strain, and after electrophoretic
analysis. When inoculated orally into poults, there was a higher incidence of virus-
associated thymic hemorrhaging and gaseous intestines. Relative weights of the bursa of
Fabricius, thymus and liver were significantly lower in birds that received the virus, and
the viral antigen was detected by immunofluorescence in liver sections. In conclusion,
ARV-CU98 may contribute directly to PEMS by affecting the intestine, bursa of
Fabricius, thymus and liver, and may contribute indirectly by increasing susceptibility to
opportunistic pathogens that facilitate the development of clinical PEMS (Heggen-Peay1
et al., 2002). In thesis work performed by Macalintal (2004), bursal atrophy was evident
as early as 4 days post-infection in chickens infected with ARV-CU98 and an astrovirus.
Also, this was seen at 11 days post-infection in birds challenged with both of these
viruses, along with *E. coli*. Relative weights of the bursa of Fabricius and thymus were
significantly lower in birds exposed to combinations of reovirus, astrovirus or *E. coli
(Macalintal, 2004). Thymus filtrate has been used to challenge poults with PEMS, and
resulted in similar disease (diarrhea, growth depression, mortality, immunosuppression)
as poults challenged with intestinal filtrate. Thus, the thymus is an important organ for
the disease pathogenesis (Schultz-Cherry, 2000). Thymic atrophy occurred in pouls given PEMS pathogens including ARV-CU98, astrovirus and \textit{E. coli}, further suggesting that thymus tissues harbor etiologic agents that can cause development of PEMS (Macalintal, 43).

To understand ARV-CU98 as an agent in PEMS, its interaction with various cell types \textit{in vitro} was studied. When macrophages, B cells, T cells and liver cells of chicken or turkey origin were incubated with the virus, only the liver-origin cells demonstrated cytopathic effect, presence of viral antigen, and reduced metabolic activity over time. Distinct pockets of viral particles and viral replication were evident in LMH cells, but not in MQ-NCSU cells. The cytopathic effects seen in liver cells incubated with ARV-CU98 included syncytia formation, sloughing and cell death. The study demonstrated that ARV-CU98 actively infects and replicates in LMH cells, but not in lymphocytes or macrophages. This provides evidence that the liver is a target and site of replication for ARV-CU98 in pouls experiencing PEMS (Heggen-Peay et al., 2002). The altered immune response due to PEMS includes enhancement of interleukin activity (IL-1 and IL-6, specifically) and nitrite production. However, the production of tumor necrosis factor (TNF) by macrophages was lowered. The cytokines that are upregulated contribute to the inflammation in the intestines which leads to diarrhea and mucosal permeability (Heggen \textit{et al.}, 2000). When primary lymphoid organs such as the bursa of Fabricius and thymus are compromised in their growth and development due to agents such as ARV-CU98, immune functions are suppressed. The compromised primary immunocompetent organs result in alterations in B and T cell populations, which then predispose birds to immunosuppression seen in PEMS (Qureshi \textit{et al.}, 1997).

Some ARV isolated from turkeys experiencing enteritis cause illness in turkeys but may replicate poorly and not cause disease in chickens. Molecular characterization of the isolates revealed that turkey and chicken origin reoviruses have identical electropherotype profiles (Spackman \textit{et al.}, 2008). \textit{in ovo} inoculation in broiler chickens
with ARVCU98 (turkey origin) or S1733 (chicken origin) were demonstrated by lower body, liver and lymphoid organ weights, and blood plasma chemistry. Blood plasma glucagon and insulin levels were decreased significantly in both virus-challenged groups, suggesting a causative role in metabolic dysfunction. Hatchability was decreased in both virus groups. Results indicated that pathogenesis due to ARV-CU98 and S1733 can result from in ovo inoculation from day 9 of incubation, and the PEMS-associated ARV-CU98 is able to inhibit growth and development in broiler chickens as seen with turkey pouls (Macalintal, 2004). When ARV-CU98 was orally inoculated into broiler chickens at day of hatch, signs of enteritis were seen at 21 days of age. Infected chicks had heavier and more distended intestines than controls. Histomorphometric analysis of the intestinal villi revealed negative effects due to ARV on integrity of the intestine. Genomic analysis of the responses of chickens to ARV-CU98 demonstrated that the avian reovirus had an effect on the expression of Selenoprotein P, which is a carrier protein for selenium, and polymeric immunoglobulin receptor (pIgR), which transports IgA (Read-Snyder, 2009).

**Secrectory Immune System and ARV**

Different reoviruses can bind at different capacities to intestinal epithelial cells and replicate in the intestine. Those that bind to microfold (M) cells in the Peyer’s patches (PP) promote exposure of the virus to innate and adaptive immune cells in the intestine (Silvey et al., 2001). Reoviruses may bind to the apical surface of cells, which is followed by endocytosis. Reoviruses grow best in the rapidly developing cells of the crypts where they interact with adjacent epithelial cells or are shed in the feces. Infection induces villus shortening and mild mononuclear infiltration in the lamina propria, and even with limited virus replication and shedding, the intestinal epithelial cells are affected by the reovirus. The cellular immune response of the intestines due to
reovirus is dominated by the T helper type 1 cell, which leads to local production of INF-γ. The response in the intestine and the periphery becomes virus specific (Pal et al., 2005).

The polymeric immunoglobulin receptor (pIgR) replication in epithelial cells can clear infection more efficiency when it is up-regulated due to the increase in transport of pIgA in the mucosal secretions. Reovirus up-regulates pIgR expression in the cell line HT-29, and this was suggested to be independent of virus replication but dependent upon binding of the reovirus to its cellular receptors and endosomal acidification leading to uncoating of the virus. Possibly, degradation in the endosomes leads to free viral dsRNA which interacts with TLR-3 to induce signals that lead to up-regulation of pIgR. Intestinal epithelial cells may therefore up-regulate the pIgR expression following exposure to enteric virus by altering cell-signaling pathways that control pIgR expression. UV light-inactivated reovirus increased expression of pIgR to a larger degree than infectious reovirus, which is probably due to the fact that replicating virus usurps or inhibits normal host-cell mRNA and protein synthesis in order to produce infectious virions. Double stranded RNA may trigger cellular responses leading to the activation of NFκB via a calpain-mediated pathway, followed by increased transcription of pIgR. The virus-induced up-regulation of pIgR may be an innate host-defense mechanism against mucosal pathogens (Pal et al., 2005; Schneeman et al., 2005).

Research was conducted to determine if dietary selenium could influence the response of pIgR in broiler chickens infected with avian reovirus, since evidence has shown that organic Se has a positive influence resisting and recovery from double stranded RNA viruses such as this. Birds were challenged orally with ARV-CU98 and tissues were analyzed for expression levels of pIgR using real-time PCR. The eggs used for this study were from Cobb breeders fed Torula yeast diets containing no supplemental selenium, inorganic selenium or organic Se in the form of Sel-Plex and the birds that hatched were placed on diets similar to their parental diets. Results showed that Sel-Plex
supplementation resulted in increased pIgR expression in the liver of infected birds. Expression of pIgR was increased in the bursa of Fabricius and pancreas due to ARV infection. It was therefore suggested that pIgR is involved in the immune response of ARV-infected chickens, and Sel-Plex may allow the infected chickens to maintain more normal villi due to increased pIgR expression (Read-Snyder, 2009).

The role of immunoglobulin A in protection against reovirus entry was shown in a study using infected intestinal mucosa in mice. The reovirus adhered to epithelial M cells and used M cell transport to enter the PP. Orally-inoculated IgA-knockout mice were able to clear the infection as effectively as wild-type mice and could produce higher levels of reovirus specific serum IgG and secretory IgM than wild-type mice. When the knockout mice were re-challenged, the PP became infected. It was therefore shown how sIgA is crucial in protection against reovirus. (Silvey et al., 2001).

A reovirus was obtained from a field outbreak of infectious tenosynovitis, and it was used in a study in which cyclophosphamide was administered to deplete B cells in the chickens. The studies demonstrated that the presence of passively acquired antibodies due to maternal immunity does not preclude the efficacy of early age vaccination with attenuated live reovirus vaccine. Vaccination-induced protection was observed despite only small active antibody formation post-infection. Protection was also present when B cell activity was absent and virus specific antibodies were absent. Therefore, these observations indicated that an antibody-independent means of cell-mediated immunity may protect against ARV (van Loon et al., 2008).

Maternal antibodies are vertically transmitted to the egg. Reovirus-specific antibodies can prohibit attachment of the virus to target cells, facilitate lysis of viral particles or virus-infected cells following complement binding, and can contribute to cytolysis and phagocytosis. Maternal antibodies may interfere with active immunization against infections. This can occur through neutralization of the vaccine before being processed by the immune system, or inhibition of B lymphocytes (Neelima et al., 2003).
Selenium

History

In 1817, Jons Jacob Berzelius, a Swedish scientist, isolated and identified Selenium (Se) when he analyzed a red deposit on a lead chamber used to produce sulfuric acid (Moxon and Rhian, 1943; Spallholz, 1994; Barceloux, 1999; Holben and Smith, 1999). A biological significance of the element was not recognized until its identification as the toxic agent associated with “alkali disease,” now called selenosis. The first written records of Se poisoning occurred in the Dakota and Wyoming territories in the United States in 1856. The cause of that selenosis event in the United States was due to extremely high concentrations of Se in soils and plants in the Dakota and Wyoming territories. Horses affected would loose hair and be unable to move around and search for food due to sore feet. The disease was called alkali disease by farmers that experienced the same problems as these in 1891 because they associated it with alkali seeps and waters of high salt content. In the early 20th century, high doses of selenium dioxide and potassium selenate were advocated briefly in order to treat hematological malignancies. This stopped in 1943 when an animal study reported the occurrence of low-grade tumors in female rats which occurred after 18 months of dosing (Barceloux, 1999). Selenium was therefore considered dangerous until 1957 when Schwarz and Foltz reported that it is an essential trace nutrient (Schwarz and Foltz, 1957; Schwarz et al., 1957; Holben and Smith, 1999; Edens, 2001). Marco Polo was most likely the first to record observations of Se toxicity when he described “hoof rot” disease in horses in Turkestan where Se-rich soils existed (Moxon and Rhian, 1943; Barceloux, 1999, Holben and Smith, 1999).

Selenium was found to be an essential nutrient for rats when Schwarz and Foltz (Schwarz and Foltz, 1957) reported that Se-deficient rats suffered hepatic necrosis. Soon
thereafter, the essentiality of Se as an ultramicronutrient in the nutrition of poultry was discovered. Research indicated that the diets of animals did not need to be supplemented with the element because requirements were low and could easily be met by the diet (Schwarz and Foltz, 1957; Schwarz et al., 1957; Surai, 1992; Spallholz, 1994). In 1957, Patterson et al. made the independent observation that exudative diathesis (dietary liver necrosis) in chickens was prevented when Se was added to the feed (Patterson et al., 1957; Spallholz, 1994). The status of Se as an essential trace element for humans was confirmed by the discovery of Keshan disease in China, which resulted from inadequate levels of Se in the diet. After supplementation of Se in the Chinese diet, incidence of multifocal myocarditis and periacinar pancreatic fibrosis, along with other health issues, declined dramatically (Barceloux, 1999).

The beneficial effects of Se were described at the biochemical level when it was found to be the essential component of the antioxidant enzyme, glutathione peroxidase (GSH-Px). This enzyme also provided a tool for monitoring that status of selenium in animals (Flohé et al., 1973; Rotruck et al., 1973). The essentiality of Se for mammalian species was therefore established in 1973 with the discovery that GSH-Px contained Se (Flohé et al., 1973; Rotruck et al., 1973; Spallholz, 1994).

The United States Food and Drug Administration first approved Se as a feed supplement in 1974. The traditional source of dietary Se for poultry and livestock became sodium selenite, which is inorganic Se (Leeson and Summers, 1991). This was due to cost and the lack of information on selenomethionine. Sodium selenite has a pro-oxidant influence in animals but the use of inorganic Se supplements at the ultramicro levels of 0.1 to 0.3 ppm in feeds has improved the performance of all classes of commercial poultry without signs of toxicity. Currently, the prevailing commercial lines of meat-producing broiler chickens are high-yielding and have higher metabolic rates than older lines, and therefore, they have vastly different nutritional needs that many times have not been met with up-graded diets (Spallholtz, 1997; Terada et al., 1999).
Problems associated with inorganic Se include the extremely low levels in meat proteins and the potential for toxicity if the level of inorganic Se is too high (5 mg/kg and higher) in the diet of chickens (Spallholz, 1997; Terada et al., 1999). Selenium is now added to animal feeds in the United States in levels up to 0.3ppm in order to prevent Se deficiencies. Sodium selenite is still used as the main source in animal feeds despite its documented pro-oxidant influence in all animals tested, including humans. Other limitations for using inorganic Se as a feed supplements include interactions with other minerals, low efficiency of transfer to milk, meat and eggs, and the inability to supply and maintain Se reserves in the body during times of oxidative stress. A high proportion of the inorganic Se consumed is excreted (Hafeman et al., 1974; Csallany and Menken, 1986; Spallholz, 1997; Terada et al., 1999; Surai, 2002).

**Selenium and Oxidative Stress**

A complex interaction between Se and vitamin E was established when the need for adequate levels of both in the diet was demonstrated. Se and vitamin E have compensative effects and the deficiency of both have the potential to cause massive injury (Schwarz, 1951; Schwarz, 1954; Mertz, 1987; Saito et al., 2003). Although the interaction between Se and vitamin E is not direct, there is an enzymatic cascade of events that link antioxidant functions of the two in cellular compartments (Winkler et al., 1994). The two are linked specifically via the selenium-dependent thioredoxin reductase (TrxR), which is a Se-dependent enzyme that maintains disulfide bonds and reduces hydroperoxides, cell proteins, ascorbate and selenite (Tamura and Stadtman, 1996). Since ascorbate (vitamin A) recycles α-tocopheroxyl (oxidized vitamin E, which is inactive) back to α-tocopherol (vitamin E), the lack of the TrxR-facilitated reduction of oxidized ascorbate to reduced ascorbate (vitamin C) would make recycling of vitamin E
less optimal. This lack of ability of inorganic selenium to maintain adequate redox activity at the level of Thioredoxin Reductase (TrxR), which then facilitates redox activity of ascorbate, probably is the basis for a common practice in poultry production to supplement broiler diets with additional vitamin C during stressful times to maintain performance of the birds. Ultimately, the additional vitamin C in the diet will be used for the facilitation of the reduction of oxidized vitamin E back to active form of vitamin E, which then continues to function as one of the most efficient antioxidants associated with cell membranes.

**Selenium and Oncogenesis**

In the last five decades, research interest in Se has increased dramatically as a result of scientific inquiry, which suggested increased risk of cancer with low Se diets (Barceloux, 1999). In 1969, it was proposed that the geographic distribution of Se in the United States was inversely related to cancer mortality rates. Numerous reports followed in which investigations were done to determine whether enhanced Se status reduced the risk of cancer. Biologic mechanisms proposed to explain how Se supplementation could reduce the risk of cancers include antioxidant potential of GSH-Px system, effects on the cellular immune response, carcinogen metabolism and carcinogen-DNA binding, and apoptosis. High rates of cancer related mortality occurred in regions of the United States where there are lower soil Se levels. It was also found, through the Linxian cancer prevention trials, that individuals living in the low soil Se regions of China had less mortality from cancer when treatment contained Se (Clark and Alberts, 1999). Indications from research that Se can have an effect on cancer progression or metastasis include a possible inhibitory effect on tumorigenesis and spread in advanced prostate
cancer, inhibition of growth factors required for progression and metastasis, and the relevancy of glutathione peroxidase to bladder cancer progression (Rayman, 2005).

**Intestinal Absorption/Metabolism**

Different forms of selenium are absorbed in the intestine and metabolized differently. Inorganic Se is found in different inorganic mineral forms as selenite and selenate compounds, and in the nonmetallic selenide form. Selenium is most commonly produced from selenide in many sulfide ores, such as those of copper, silver, or lead. Chemically, Se is related to sulfur and tellurium, and rarely occurs in its elemental state in nature. In organic forms Se has been found in many substances. As a substitute for sulfur in amino acids, it is found in selenomethionine (SeMet) and selenocysteine (SeCys). In nature, animals mainly receive Se in the form of SeMet. Plants absorb Se from the soil in the form of selenite or selenate and synthesize selenoaminoacids, mainly that of SeMet but also Se-methyl-SeMet, SeCys, or Se-methyl-SeCys (Surai, 2002).

Plants can absorb selenite, selenate and SeMet, but selenate is preferred over selenite. The selenium is available in selenite form in acidic, well-aerated and neutral pH soils, while it is available in the readily available soluble selenate form in alkaline and dry/well-aerated soil. In highly acidic soil or those that are very moist, the Se is reduced and forms insoluble adsorption complexes with iron hydroxide. However, soil Se is not a good indicator of plant Se content (Mayland, 1986; Oldfield, 1999; Surai, 2002). Sulfate and selenate compete for common uptake sites in plant roots, and selenate uptake can be inhibited by high sulfate supplies. Also, the toxic effect of Se to some plant species is due to interference with the sulfur metabolism which is necessary for biochemical reactions and cellular enzyme function (Mikkelsen *et al.*, 1989; Marschner, 1995).
In addition to the traditional source for dietary Se for poultry and livestock (sodium selenite), other inorganic sources include sodium selenate and calcium selenate (Echevarria et al., 1988a; Echevarria et al., 1988b), but the commonly used plant and animal-based ingredients in feed contain Se mostly in the organic form, primarily as the selenoaminoacids-SeMet and SeCys (Burk, 1976; Levander, 1986; Cai et al., 1995).

The bioavailability of selenium, including SeMet and SeCys, in plant-derived foods is relatively high while that in animal-derived foods is low to moderate. Se in high Se yeast (such as Sel-Plex™, Alltech Biotechnology Center, Nicholasville, KY) is considered highly available. The bioavailability of selenium as selenite has been demonstrated to be less than that of selenate or SeMet in animal species. The predominant species of Se in drinking water is selenate, and the bioavailability of this is less than that in food. The absorption of Se from amino acids is greater compared with the absorption from inorganic species. The formation of metal complexes with Se reduces bioavailability of Se from fish and water. Differences in bioavailability depend on factors including GSH-Px activity, physiochemical properties (pH), and dietary factors (Combs and Combs, 1986; Barceloux, 1999).

Variable amounts of organic Se (mainly SeMet) and inorganic selenium are in animal diets, depending on what is added to the formulation. Organic Se and inorganic Se are absorbed via different mechanisms. Selenite is absorbed by simple diffusion, whereas selenate is actively absorbed in the ileum by co-transport with sodium ions. Absorption of selenite is not influenced by sulfate, but the absorption of selenate is affected because of a shared absorption route (Wolffram et al., 1986; Schrauzer, 2000). SeMet is absorbed via the sodium dependent neutral amino acid transport system which is shared with and competitively inhibited by methionine. SeCys is thought to be transported in a similar way as cysteine and its transport is inhibited by the transport of cysteine (Wolffram et al., 1989a; Wolffram et al., 1989b). The amount of SeMet available for absorption depends on digestibility of the source, which is specific to the
species of animal, the nature of the ingredient, and the nutritional adequacy of the diet (Combs and Combs, 1986; Barceloux, 1999). While SeMet is not synthesized in animals or humans and must be obtained from feed sources, inorganic Se is absorbed as a mineral and little is retained in tissues. Much of the inorganic Se is excreted with before entering body proteins (Surai, 2002).

In recent years, revisiting organic Se as a feed supplement was necessary. Many years of research led to the approval by the United States Food and Drug Administration of a natural organic selenium that can be used in the poultry industry (Federal Register, 2000; Federal Register, 2002). Sel-Plex™ is a cocktail of Se compounds. SeMet in the Se-enriched yeast cellular protein is the primary form of the selenium in Sel-Plex™. The organic Se profile in the product is similar to that in plants and grains (Kelly and Power, 1995), is readily available, and will be actively absorbed by the animals (Mahan, 1995). This occurs via the Na+-dependent methionine transport system in the intestine (Spencer and Blau, 1962). Increasing levels of dietary inorganic Se are associated with moderate tissue concentrations in a time dependent manner (Echevarria, 1988a,b), but the organic Se from yeast has been demonstrated to be more available to blood and liver proteins than certain inorganic forms and to persist in body proteins for longer periods of time (Moksnes and Norheim, 1986; Vinson, 1987).

Selenium, in general, is more available in diets low in protein, possibly due to decreased total methionine (Cantor and Johnson, 1985). Intestinal absorption varies among species, and Se bioavailability varies among forms (Gabrielsen and Opstevedt, 1980; Douglas et al., 1981; Ringdal et al., 1985; Schen et al., 1997; Wen et al., 1997). When plasma GSH-Px was used as an indicator of the availability of different forms of Se, greater tissue concentrations of Se and GSH-Px were demonstrated in SeMet -fed chickens than in sodium-selenite fed chickens (Cantor and Tarino, 1982; Moksnes and Norheim, 1986).

A major metabolic fate of selenium under physiological conditions is incorporation into selenoproteins (Burk and Hill, 1993). The conversion of dietary Se,
both of organic or inorganic origin, to biologically active selenoproteins occurs once absorption takes place in the small intestine and the selenium is converted to hydrogen selenide. The biologically active selenoproteins, of which about 30 have been identified, are mainly redox enzymes that contain SeCys residues at the active sites (Daniels, 1996; Low et al., 1996). Different Se sources are metabolized differently to SeCys for incorporation into GSH-Px (Surai, 2002) and other selenoproteins, but all must be ultimately metabolized to the selenide form for incorporation into SeCys for incorporation into selenoprotein active sites.

Metabolism of selenium results in reactions including reduction of selenite by cellular glutathione to selenide, incorporation of selenide into selenoproteins via selenocysteine, and methylation of selenide to metabolites that are eliminated. Methylated forms are generally less toxic than the nonmethylated compounds (Barceloux, 1999).

**Selenoaminoacids**

The selenoaminoacids are incorporated into proteins and constitute 50-80 percent of the total selenium in plants and grains (Butler and Peterson, 1967). Selenium functions within animals to perform many different roles and primarily are incorporated into different selenoproteins. SeCys is now recognized as the 21st amino acid (Holben and Smith, 1999) making Se the only trace element to be specified in the genetic code (Rayman, 2005). All presently known selenium enzymes and proteins contain selenium as SeCys. Dietary selenium from inorganic salts and the organic selenium compounds (mainly SeMet from plant and animal foods and SeCys from bacterial processed foods and animal foods) are metabolized into SeCys found in selenoenzymes (Spallholz, 1994).
Because the chemical and physical properties of Se and sulfur are very similar, plants cannot distinguish between the two when synthesizing amino acids. They can make SeMet when Se is available. This was the basis for development of organic Se production from yeast such as Alltech’s product, Sel-Plex™. The medium for yeast growth is designed so that it is deficient in sulfur and supplemented with Se. This allows a strain of *Saccharomyces cerevisiae* yeast to actively synthesize SeMet using Se in lieu of sulfur, and the yeast becomes enriched with the amino acid. This patented *S. cerevisiae* yeast takes up Se salts and forms selenoaminoacids. Some strains can assimilate up to 3000 ppm with 90% of this in the form of SeMet (Kelly and Power, 1996; Surai, 2002). The SeMet in the yeast protein can be fed to animals to supply them with adequate selenoaminoacids.

SeMet is easily converted to SeCys and is required by animals for this purpose. SeCys can be substituted for cysteine in many proteins but it is not incorporated directly into specific selenoproteins. In order for this to occur, organic Se must be converted from its original form to the inorganic form and then back to the organic form. SeMet can be a highly available substrate for many proteins and can substitute for methionine in their structures despite this required conversion (Edens, 2001).

SeMet is incorporated into body proteins in place of methionine and this allows for a reversible storage of Se in organs and tissues. No other selenoaminoacid has this property, which points to a specific physiological function of SeMet. In fact, all needed metabolic forms of Se can be produced from selenomethionine, and this allows SeMet to meet the criteria for an essential amino acid (Schrauzer, 2003). Selenium from selenite is easily released from animal protein subjected to alkaline dialysis, but Se from SeMet is retained as part of the protein (Cummins and Martin, 1967; Latshaw and Osman, 1974). It appears that Se from selenite interacts loosely with the cysteine/thiol group, while Se from SeMet is molecularly integrated into protein. This stored form is in a nonfunctional state and is not used immediately for formation of biologically functional selenoproteins.
(Mahan, 1994; Mahan, 1995). In times of oxidative stress, body protein can be degraded rapidly providing more adequate concentrations of Se that can be used for synthesis of specific selenoproteins (Gowdy and Edens, 2003).

SeCys is found in the body of animals fed inorganic Se. It is necessary for the synthesis of GSH-Px and other selenoproteins in which SeCys is incorporated. Se in Selenocysteine is incorporated using selenide and serine as precursors (Edens, 2001). Inorganic Se from selenite or selenate results in a limited amount of nonspecific insertion of SeCys into protein. This does, however, allow for synthesis of SeCys for insertion into selenoproteins (Schrauzer, 2003).

In a study, selenite labeled with $^{75}\text{Se}$ was given to rats in drinking water, and over 80% of the $^{75}\text{Se}$ was found to be present as SeCys in protein. The selenoproteins with known enzymatic activity are redox enzymes and contain SeCys at their active sites. The effective functions of the enzymes depend on selenium (Burk and Hill, 1993). When Se is incorporated into selenoproteins, it protects tissues and membranes from oxidative stress and controls the redox status of the cells (Rayman, 2005).

Se reserves accumulated in the form of SeMet in muscles are beneficial to an animal when there is overproduction of free radicals due to oxidative stress conditions. The body responds by mobilizing the antioxidant reserves and synthesizing additional selenoproteins. Protein catabolism will release SeMet, and, therefore, supply Se needed for the synthesis of additional selenoproteins. While SeMet and SeCys are the major forms of Se in the body, cells do not contain free pools of these amino acids. They are present as parts of selenoproteins or, in the case of SeMet, in tissue proteins. Supplemented SeMet is non-specifically incorporated into various selenoproteins in place of methionine. More Se is released in the form of SeMet than SeCys by protein catabolism by proteasomes when there are stress conditions resulting in the overproduction of free radicals and lipid peroxidation (Surai, 2002).
Selenoproteins (Enzymes)

In biological systems, Se participates in various physiological functions as integral parts of different selenoproteins. At least 20 eukaryotic proteins and 35 Se-containing proteins or protein subunits are included in the selenoproteins family. The protein expression is dependent upon Se availability, has high tissue specificity, and is sometimes regulated by hormones. Compromising these can contribute to pathological conditions (Surai, 2002).

Important selenoproteins include cellular (or classical) glutathione peroxidase (GSH-Px), plasma (or extracellular) GSH-Px, phospholipid hydroperoxide GSH-Px, gastrointestinal GSH-Px, selenoproteins P, types 1,2,3 iodothyronine deiodinase, selenoprotein W, thioredoxin reductase (TrxR), and selenophosphate synthetase. Cellular and plasma GSH-Px are the parameters used for assessing selenium status (Holben and Smith, 1999). Most contain a single SeCys residue per polypeptide chain, and SeCys insertion is specified by the stop codon, UGA (Surai, 2002).

The optimal expression of some selenoproteins, notably selenoprotein P, requires a higher amount of dietary Se for their efficient synthesis. Individuals differ in their ability to increase selenoprotein activity in response to dietary Se. Requirements for dietary Se for the optimal protection against cancer may be higher in individuals carrying particular functional selenoprotein single nucleotide polymorphisms (SNPs) in the genes for selenoproteins (Rayman, 2005).

It is suggested that the cell’s antioxidant defense is based on the activity of superoxide dismutase, GSH-Px, and catalase. GSH-Px has not received much attention in the poultry industry, but its importance as an antioxidant has become more appreciated in recent years. Since the enzyme depends upon Se, the role of Se in animal nutrition has become more important (Surai, 2002).
GSH-Px was the first selenoprotein described and is the best understood. It exists in four known structurally and genetically different forms (Ursini et al., 1995). The extracellular form has been found in bronchial lavage, is excreted by the placenta into maternal circulation, and is the only form found in human milk (Daniels, 1996). The membrane bound form of GSH-Px, phospholipid hydroperoxide GSH-Px, acts in conjunction with vitamin E as a chain breaking antioxidant that protects the phospholipid membranes. It also may be involved in regulating biosynthesis of leukotriene. The enzyme is expressed mainly in reproductive and endocrine tissues and may explain the role of Se in male reproduction (Beck et al., 1998; Köhrle et al., 2000). It is required for sperm motility and may reduce the risk of miscarriage (Rayman, 2000).

GSH-Px activity, which is dependent upon Se availability, has been used extensively to assess Se nutritional status. Cellular GSH-Px is found in most all cells even though its specific activity varies greatly. In the rat, the enzyme contains more Se than any other selenoproteins (Burk and Hill, 1993). In a study where mice received a coxsackievirus infection in addition to Se deficiency, the mice with a Se deficiency had a fivefold decrease in GSH-Px activity as compared with those fed adequate levels of Se (Beck et al., 2003). Mutations which resulted transformed a normally benign strain of the virus into a strain that induces myocarditis. GSH-Px provides protection against virus-induced damage due to these mutations. Selenium deficiency may lead to the change of an avirulent virus into a virulent one by way of point mutations in its genome (Beck et al., 1998). With knowledge that RNA viruses induce oxidative stress in host cells and cellular redox status plays an important role in regulating viral replication and infectivity, experiments were performed to determine whether GSH blocked influenza infection in cells. It was found that GSH-Px inhibited expression of viral matrix protein, virally induced caspase activation, and Fas up-regulation. Influenza-infected mice given GSH in drinking water had decreased viral titers (Cai et al., 2003).
Glutathione, the pentose phosphate cycle, and glutathione reductase all work together in the GSH-Px system to effectively metabolize hydrogen peroxide and unesterified fatty acid hydroperoxides. Glutathione acts as the reducing agent, the pentose phosphate cycle generates NADPH and hydrogen ions for the reduction of diglutathione and glutathione reductase acts to regenerate glutathione (Wolffram, 1999). Each of the four subunits of active GSH-Px contains one SeCys residue. Hydrogen peroxide and free organic hydrogen peroxides can be reduced by the enzyme in cells. The cellular form regulates the intracellular hydroperoxide concentrations. The enzyme may also be a reservoir for Se, which can be mobilized for other uses. Its reducing agent is present in very low concentrations, implying that there may be a function other than GSH-Px reducing potential. Phospholipid hydroperoxide GSH-Px only contains one subunit and therefore one selenocysteine. Thiol compounds other than glutathione can serve as its reducing agent. It is able to reduce fatty acid hydroperoxides esterified to phospholipids (Burk and Hill, 1993).

Thioredoxin reductase functions to stabilize disulfide bonds, sulfhydryl groups, and to reduce thioredoxin (Tamura and Stadtman, 1996). Thioredoxin is a high capacity electron donor for reductive enzymes and reduces cysteine residues in transcription factors to increase their binding to DNA. This influences gene transcription (Mustacich and Powis, 2000; Powis and Montfort, 2001). Thioredoxin reductase is a Se dependent enzyme that has increased activity after selenium supplementation compared with enzyme activity in animals that are selenium deficient (Hill et al., 1997; Berggre et al., 1999). However, Wu et al. (2003) suggested that TrxR activity is mediated mostly by generation of ROS instead of Se intake. With GSH-Px activity, it appeared to be directly related to Se intake. Observations have suggested that TrxR may be induced as a safety precaution when its SeCys is affected by a severe oxidative stressor, which was in this case due to excess sodium selenite (Berggre et al., 1999). The TrxR system maintains free sulfhydryls, but also controls growth and apoptosis and has co-cytokine and
chemokine activities. The GSH-Px/glutathione system is primarily an antioxidant system. Together, these work to regulate a low intracellular redox potential and maintain free sulfhydryl groups (-SH). Thioredoxin reductases of higher eukaryotes are selenium-dependent proteins with broad substrate specificity that also reduces non-disulfide substrates such as hydrogen peroxides, vitamin C or selenite. (Arner and Holmgren, 2000). Enhanced activity of this enzyme may have beneficial effects on oxidative stress, but can have adverse effects as well. Some in vitro studies have demonstrated inhibitory effects of Se on thioredoxin reductase system correlated with growth inhibition by Se (Ganther, 1999).

There are different effects of organic and inorganic Se supplementation on blood selenoenzyme activities, which may indicate a difference in the metabolic need for Se regulated at the level of Se-dependent cell function. A study demonstrated a more rapid uptake and perhaps turnover of an inorganic source of Se as compared with the organic source. Changes in activity of GSH-Px may reflect subsequent incorporation of Se into selenoproteins in body organs. Selenocysteine incorporation into selenoproteins also involves an inorganic precursor. Most dietary Se is in the organic form of SeCys or SeMet. The in vivo conversion into an inorganic precursor may be an important regulator of Se bioavailability (Brown et al., 2000).

Toxicity

Selenosis, or selenium toxicity, usually occurs when animal ingest plants that have absorbed high quantities of Se from high Se soils (Schrauzer, 2000). It was first confirmed in 1933 when livestock consumed plants that were grown in soils containing selenium up to 1000 ppm (Spallholz, 1994). In the 20th century, the most widespread occurrence of selenium toxicity occurred in the Enshi County of China between 1961 and
1964 when large quantities were ingested due to the intake of corn and vegetables grown in soil containing high Se concentrations. Symptoms included changes in skin, hair and nails, and eventual loss in nervous system function. Also, there were cases of anorexia, abdominal pain, diarrhea, fatigue, irritability, depression, emaciation, pulmonary edema, hemorrhage, liver and kidney necrosis, respiratory distress, and blindness. The United States has had cases of toxicity due to ingestion of hydrogen selenide, selenium oxychloride, selenium dioxide or selenium hexafluoride from industrial accidents and overdoses with Se supplements. The hallmark case in the United States involved an improperly manufactured dietary Se supplement containing 27.3 mg Se per tablet (Holben and Smith, 1999).

The redox catalysis by small inorganic selenium-containing molecules produces superoxide and hydrogen peroxide as a consequence of thiol oxidation. When this thiol oxidation is exceeded by the ability of plants to methylate the excessive metabolites, this leads to development of selenosis in animals consuming the plant (Holben and Smith, 1999).

The toxicity of most forms of Se is low and dependent upon the chemical form of selenium. The United States National Toxicology Program lists selenium sulfide as an animal carcinogen, but there is no evidence that other selenium compounds are carcinogens. Ingestion of selenious acid is almost always fatal. Alkali disease results from the ingestion of grain and forage containing 5-50 ppm Se and is the major disease caused by chronic selenium toxicity (selenosis) in cattle and horses. The variation of selenosis from different selenium species results from differences in physiochemical properties. Generally, organic Se is less toxic than inorganic salts. High levels of Se from a yeast resulted in some gastrointestinal problems. Selenocysteine is the most toxic organic Se compound as seen in animal studies. It is similar to the toxicity of selenite (Barcelou, 1999). In 1978, Ort and Latshaw performed experiments to determine the
level of dietary sodium selenite that is toxic to laying chickens. Five ppm of sodium selenite appeared to be borderline toxic (Ort and Latshaw, 1978).

There have been concerns that SeMet may accumulate and be released from tissues in toxic levels, but a steady state is established which prevents uncontrolled accumulation of Se. Also, selective release of SeMet from body tissues during catabolism does not occur. SeMet does have a lethal dose dependent upon the organism, but the chronic toxicity is lower than that of selenite (Schrauzer, 2000).

Various substances, including arsenic, cysteine, heavy metals, sulfate, and vitamins C and E can alter the toxicity of selenium by reduction (Barceloux, 1999). Dietary silver or copper modify selenium toxicity, possibly by interfering with selenium absorption (Jensen, 1974).

Although sodium selenite is the most commonly used inorganic selenocompound, it is able to produce superoxide radicals and oxidative stress through its reductive reaction with reduced glutathione. Selenite is a pro-oxidant catalyst, producing superoxide, hydrogen peroxide and likely other cascading oxyradicals. This is dependent on oxygen concentration and is ultimately responsible for Se toxicity. Se in the form of selenite, selenate or even free SeMet can generate reactive oxygen species (ROS) in the presence of sulphhydril compounds, resulting in a decrease in cellular protein and enhanced cellular damage as compared with exposure to selenite alone. Selenite has been found to have a much more toxic effect on cells than SeMet, and this was related to superoxide formation, oxidative stress and subsequent induction of apoptosis (Surai, 2002).
Deficiency

In 1957, when Se was reported to be an essential trace element, extensive studies were initiated to discover the metabolic function of the element and the consequences of its deficiency. Animals and humans were affected by deficiency, and this was made manifest in many diseases including liver necrosis, muscular dystrophy, microangiopathy, exudative diathesis, pancreatic fibrosis, poor feathering, retained placenta, mastitis, cystic ovaries, general unthriftiness, Keshan disease, Kashin-Beck disease, cancer, heart diseases, immune deficiencies, reduced fecundity and others. Clinical cases of Se deficiency are clearly recognized and treated today. Yet, Se nutrition today still is less than optimal in both humans and animals in many parts of the world (Edens, 1996; Edens, 2001).

Maas has divided the selenium-responsive diseases into the categories of musculoskeletal, reproductive, gastrointestinal/efficiency, and immunologic (Maas, 1998). An additional category of circulatory/edema disorders, may be important in poultry and other animals such as swine and even humans. A new category centers on the oxidative stability of post-harvest animal food products.

When Se deficiency is combined with low vitamin supply (C and E), there is an increased development of a range of diseases (Surai, 2002). Although Se deficiency is rare, it may exacerbate damage from other disease-causing factors. It appears to drive changes in the viral genome and permit an avirulent virus to acquire virulence (Holben and. Smith, 1999). For example, Keshan disease appeared to result from a Se deficiency along with a coxsackievirus infection. This was later confirmed in mice when the Se-deficient animals developed the myocarditis while those given adequate Se did not. The immune response in the Se deficient mice had been altered such that the proliferative response of T cells was decreased, inflammatory chemokines were expressed to a higher degree, and the expression of interferon gamma was decreased.
Also, the viral pathogen was exposed to a Se-deficient environment which may alter it. Results demonstrated that the genome of the virus that replicated in Se-deficient mice changed by undergoing point mutations and changing from avirulent to virulent. The deficiency in Se led to a decrease in glutathione peroxidase activity, which led to higher susceptibility to the virus and this was demonstrated using glutathione peroxidase-1 knockout mice. The knockout mice had an altered immune response to infection, with greatly decreased neutralizing antibody levels. Therefore, it is clear that Se deficiency can affect host immunity along with changes in the viral pathogen. RNA viruses have a high mutation rate due to a lack of proofreading enzymes during replication, which facilitates reassortment leading to mutant virus appearance. Se deficiency leads to alterations in the immune response of the infected host, which could allow for selection of new viral variants that might be more pathogenic. Increased oxidative stress occurs in Se-deficient mice due to a lack of glutathione peroxidase activity. The increased oxidative stress directly affects the viral RNA, which then promotes mutations that lead to higher pathogenesis (Beck, 2001).

It is known that Se is necessary for cell culture. A study using Jurkat cells demonstrated that deficiency of the element in medium decreased the Se-dependent enzyme activity within cells along with cell viability. Another antioxidant, vitamin E, was able to block Se deficiency-induced cell death. While Se deficiency resulted in an increase of cellular ROS before cell death, sodium selenite was able to inhibit this increase in a dose-dependent manner. ROS, especially lipid hydroperoxides, are involved in the cell death caused by Se deficiency. Se and vitamin E interact in defense against oxidative stress (Saito et al., 2003).

Se may be unique in the manner in which its deficiency is expressed. In mammals, it was likely to be manifest in second generation progeny (Mertz, 1987). Lipid peroxidation is likely a major factor in Se deficiency related diseases (Surai, 2002).
The poor growth efficiency of Se-deficient animals can include ill-thrift, diarrhea and abnormal weight gains (Maas, 1998). The role of Se in thyroid hormone function may explain the low growth rates of animals deficient in the nutrient (Arthur, 1993). Improved growth rate and efficiency has been found in broilers when diets have a higher Se status due to the addition of Sel-Plex™. These studies have suggested the possibility of a higher requirement for selenium in rapidly growing higher yielding modern genetic stock (Naylor et al., 2000; Roch, 2000).

**Selenium and the Host: Virus interaction**

Humans and lower vertebrates have increased susceptibility to infection in association with malnourishment. The increase in severity of an infectious disease in a malnourished host is thought to result from an impaired immune response. Little is known about how the malnourished host may affect the virus’ interaction with the host. Selenium is the ultramicro trace element that seems to play a major role in infections caused by RNA viruses (Beck, 2001; Combs 2001; Field, 2002; Lyons, 2003; Beck, 2004).

Frank Se-deficiency and marginal Se-deficiency are known to decrease the cellular and humoral immune function in humans and laboratory animals (Combs and Combs, 1986; Maas, 1998). Infections with rotavirus, measles, parainfluenza virus and many others are much more severe in malnourished hosts (Beck, 2001).

Many studies have revealed the relationships involving host Se deficiency and virulence of RNA viruses. Beck and colleagues began research on the influence of malnutrition on host:virus interactions upon discovery that new viral variants emerge in a Se-deficient model (Beck 1994, 1995, 1998, 2003). Se-deficient mice were more susceptible to Coxsackievirus B3. The mice infected with a normally harmless form of
the virus developed myocarditis because the avirulent form mutated to a virulent form in Se-deficient mice. The genome mutation was shown to be a result of increased oxidative stress due to the Se deficiency. Beck and colleagues also suggested that influenza virus is influenced by the Se status of the host. Much more severe pathology was seen in Se-deficient mice, and this was due to increased proinflammatory cytokine production in lungs of the Se-deficient mice (Beck 2001; Nelson 2001; Beck 2003; Beck 2004). Similar alterations of virulence and genomic composition of mice given the virus was observed when the animals were fed normal diets but deprived of GSH-Px through knockout of the gene (Beck 2003).
CHAPTER 2: INFLUENCE OF SELENIUM SOURCES ON THE INTEGRITY OF THE INTESTINE IN REOVIRUS-INFECTED BROILER CHICKENS

Abstract

Avian reoviruses cause significant economic losses in the poultry industry due to the diseases with which they are associated, including enteric and respiratory diseases, myocarditis, hepatitis, Poult Enteritis and Mortality Syndrome (PEMS) in turkeys and malabsorption syndrome (MAS) in chickens. Natural avian reovirus infections occur via the fecal-oral route, with infection mainly via oral entry and initial replication being in the intestine and bursa of Fabricius. The small intestine is the most important site for ARV infection regardless of the route of inoculation, with the spread of infection beginning in the villus and crypt epithelium of the small intestine. Different factors may contribute to disease caused by ARV infection. In selenium-deficient ARV-CU98-infected broiler chicken, the duodenal, jejunal, and ileal villi have been found to be thicker, shortened and blunted with deeper crypts of Lieberkuhn. The signs of infection have been demonstrated to be less severe due to the feeding of organic selenium in a high selenium yeast supplement (Sel-Plex) and to a smaller degree due to the feeding of sodium selenite. These findings suggest that nutrients such as selenium could play a role in the chicken’s ability to resist or regain function due to ARV infection. In this study, morphological alteration in the ileal intestinal villi of selenium fed and ARV-challenged broiler chickens was quantified. As chicks began to recover from the ARV infection, there were influences of dietary selenium supplementation demonstrated by villus height: crypt depth ratios. Inorganic Se-fed birds had significantly lower ratios in trial 1 at 16 days p.i. due to shorter villi. Both the potential benefits of selenium supplementation in
the organic form, and the potential disadvantages of sodium selenite during the time of infection and afterwards were important factors that were addressed in this paper.

**Introduction**

Avian reoviruses are members of the *Orthoreovirus* genus. They are icosahedral, nonenveloped viruses with double-protein capsid shells and genomes consisting of ten double-stranded RNA segments that express structural and nonstructural proteins. The virus encodes at least 10 structural proteins and four non-structural proteins encased within their 2 concentric protein shells (Benavente and Martínez-Costas 2007; Lin et al., 2007).

Avian reoviruses cause significant economic losses in the poultry industry due to the diseases with which they are associated. They were initially discovered as the disease that induced tenosynovitis in young chickens, and then were found to be ubiquitous among poultry flocks, mostly causing asymptomatic infections. Diseases to which ARV have been associated in poultry include enteric and respiratory diseases, myocarditis, hepatitis, Poult Enteritis and Mortality Syndrome (PEMS) in turkeys and malabsorption syndrome (MAS) in chickens (Heggen-Peay *et al.*, 2002; Benavente and Martinez-Costas, 2007). The ARV infections are important in the poultry industry since mortality, leg weakness and poor feed conversion caused by ARV depresses productivity (Ni *et al.*, 1995). Field outbreaks, mainly among broiler breeders, where the agents have been isolated and identified as reoviruses, have been reported in many parts of the world (Gouvea and Schnitzer, 1982).

Reoviruses isolated from MAS and tenosynovitis can induce lesions in the liver, heart, bursa of Fabricius, bone, proventriculus, intestine and tendons. Reoviruses may be priming agents in early development of intestinal lesions in cases of MAS (Lenz *et al.*, 2007).
1998; Songserm et al., 2003). The development of lesions, also known as pathogenicity in the case of ARV, correlates with the presence of replicating virus and its spread in tissues (Ni et al., 1995). Impaired weight gain, feed conversion, and feed passage, and watery feces often occur along with lesions in broiler chickens infected with ARV (Lenz et al., 1998). Turkey-origin reoviruses can cause major lesions in the form of bursal atrophy, which is characterized by lymphoid depletion and fibroplasias (Pantin-Jackwood et al., 2007).

Natural avian reovirus infections occur via the fecal-oral route, with infection mainly via oral entry with initial replication in the intestine and bursa of Fabricius. Then, the virus spreads via blood to other tissues and organs (Menendez et al., 1975b; Ni et al., 1995; Benavente and Martínez-Costas, 2007). The small intestine is the most important site for ARV infection regardless of the route of inoculation, with the spread of infection beginning in the villus and crypt epithelium of the small intestine (Kibenge et al., 1985; Jones et al., 1989).

Mucosal surfaces, such as the lining of the gut, are important locations for the entry of viruses into the body. Peyers patches (PP) are lymphoid areas of the GI tract with specialized epithelial cells known as M cells. A reovirus can enter here after binding to a host cell by way of its outer capsid protein (Bomsel and Alfsen, 2003). Avian reoviruses replicate in the cytoplasm of infected cells and induce fusion of host cells in order to facilitate spread (Vázquez-Iglesias et al., 2009).

The viremia due to ARV challenge spreads from the villus and crypt epithelium upon initial replication of the virus. It has been re-isolated from plasma, intestines, bursa of Fabricius, pancreas, spleen, liver, kidney, joints and tendons, with the liver becoming a primary site for replication after the virus spreads from the epithelium of the small intestine (Mandelli et al., 1978; Kibenge et al., 1985; Jones et al., 1989). Jones and colleagues (1989) reported that the intestine and bursa of Fabricius serve as portals of
entry and sites of replication for ARV, and this initial replication takes place between 12 and 24 hours post-inoculation in the intestine (Jones et al., 1989).

Cell proliferation in the chicken intestinal epithelium occurs mainly in the crypt but also along the villus, as seen by immunostaining of the proliferating cell nuclear antigen (Uni et al., 1998). The height of villi can be a direct way to measure intestinal recovery from a stressor, which was confirmed in a feed deprivation study. The rapid recovery of villi height correlated well with intestinal function. Diet-induced changes in cell area and cell mitosis number were similar to changes in villus height. Morphological changes such as villus height and crypt depth can therefore be useful for measuring intestinal function (Shamoto and Yamauchi, 2000). Luminally absorbed nutrients influence villus morphology, and villus morphological recovery from stressors may occur due to enteral nutrient absorption (Tarachai and Yamauchi, 2000). Villus height and crypt depth, along with the ratio of height: crypt depth (H: D), are all direct ways to measure enterocyte turnover (Fan et al., 1997). Villus atrophy or flattening of these cells may be evidenced by reduced H: D ratio (Shah et al., 2000). These parameters are important means for determining intestinal integrity.

In selenium-deficient ARV-CU98-infected broiler chicken, the duodenal, jejunal, and ileal villi have been found to be thicker, shortened and blunted with deeper crypts of Lieberkuhn. Degeneration of the villus tips occurs, as seen in the sloughing of the villus epithelial cells. The signs of infection were less severe due to the feeding of organic selenium in a high selenium yeast supplement (Sel-Plex) and to a smaller degree due to the feeding of sodium selenite. The live weight gain was greatest in the organic selenium-fed chicks compared to the control (no supplemental selenium) and sodium selenite groups in the study. Also, plasma protein levels were significantly lowered due to the ARV infection, but Sel-Plex maintained plasma protein level at a higher concentration compared with the other dietary treatments at 2 and 3 weeks of age. These findings
suggest that nutrients such as selenium could play a role in the chicken’s ability to resist or regain function due to ARV infection (Edens et al., 2007ab).

It has been demonstrated that reoviruses associated with MAS can cause vacuolar degeneration and sloughing of the villus epithelium at 2 days p.i. Reovirus antigen was found mainly in the affected jejunum and ileum in one study where Songserm and colleagues challenged broilers with ARV isolated from cases in The Netherlands and Germany. Macrophages and monocytes were present in the lamina propria of the jejunum in both groups and increased from day 4 p.i. and on. Although the ARVs caused intestinal lesions in broilers with MAS, none caused weight gain depression. While lesions were mainly at the tips of the villi, antigen was detected at the tips and middle parts of the villi. The ARV alone did not cause the same intestinal lesions as those seen in MAS. In commercial breeders, the combination of inoculated reovirus with agents already present in the broiler may be required to cause the effect on weight gain (Songserm et al., 2003).

ARV-CU98 may contribute directly to PEMS by affecting the intestine, bursa of Fabricius, thymus and liver, and may contribute indirectly by increasing susceptibility to opportunistic pathogens that facilitate the development of clinical PEMS (Heggen-Peay et al., 2002a). The effects of in ovo inoculation of broiler chickens with ARV-CU98 from turkey PEMS and S1733 (chicken origin) were demonstrated by changes in body, liver and lymphoid organ weights, and blood plasma chemistry. Hatchability was decreased in both virus groups. Body weights in both groups were also significantly less than controls at 7 and 14 days of age. Also relative weights of the bursa of Fabricius, thymus and liver were decreased significantly. Blood plasma glucagon and insulin levels were decreased significantly in both virus-challenged groups, suggesting a causative role in metabolic dysfunction. Results indicated that both ARV-CU98 and S1733 can be transmitted in ovo from day 9 of incubation, and the PEMS-associated ARV-CU98 is able to inhibit growth and development in broiler chickens as seen with turkey pouls
(Macalintal, 2004). When ARV-CU98 was orally inoculated into broiler chickens at day of hatch, signs of enteritis were seen at 21 days of age. Infected chicks had heavier and more distended intestines than controls. There were diet and reovirus infection effects as seen in the duodenal and ileal measurements. For the duodenal villi, the perimeter and height of the villi were significantly higher in the organic selenium-fed birds within each of the control and infected groups. There was a significant increase in crypt depth due to the virus and a significant increase in the birds not given supplemental selenium or given inorganic Se as compared to those given organic selenium. The H: D ratios for both control and infected groups were increased in birds given organic Se in their diets. In the ileal measurements, villi length was significantly decreased due to ARV. In both control and infected groups, organic selenium-fed birds had the longer villi. Lower crypt depths were seen in organic selenium-fed birds in both control and infected groups. Body weights were decreased as a result of ARV at both 14 and 21 days of age. (Read-Snyder, 2009).

In this study, morphological alteration in the ileal intestinal villi of selenium fed and ARV-challenged broiler chickens was quantified. The dietary treatment groups consisted of diets containing Sel-Plex (Alltech, Inc. Nicholasville, KY) as the source of organic selenium (0.3 ppm), sodium selenite (0.3 ppm) as a source of inorganic selenium or control feed (<.002ppm). Villus height, crypt depth, and the thickness of the submucosalis were measured. H: D ratios were calculated as another parameter for histomorphology.
Materials and Methods

Animal Welfare. This study was conducted following guidelines established by the North Carolina State University Animal Care and Use Committee, which governs all animal-use in experimental procedures.

Animals and husbandry. Feather sexed male Ross 708 broiler chicks were placed at day of hatch in 9 pens in heated metal brood/grow batteries in each of two identical isolation rooms. Each of the isolation rooms was controlled identically for light and temperature. Lighting was provided 24 hours a day through incandescent lamps in the ceiling of the two isolation rooms. The rooms were preheated to 90 ºF before placement of the chicks and held at that temperature for the first week. The room temperature was decreased weekly by 5 ºF.

Twelve birds were placed in each pen. They were identified at placement using plastic neck tags. Three pens of chicks in each of two isolation rooms were fed torula yeast-based diets (Appendix Table A.1) containing either Sel-Plex (Alltech, Inc. Nicholasville, KY) as the source of organic selenium (0.3 ppm), sodium selenite (0.3 ppm), or no supplemental selenium (<0.002ppm). Feed and water were provided on an ab libitum basis in stainless steel pans attached to the frame of the brooder pens.

Tissue sampling and body weight measurements. Tissue samples and body weights from 18 birds at each of 7, 9, 12 and 21 days of age (2, 4, 7, and 16 days post-infection, respectively) were collected. Birds were fasted 12 hours before the beginning of data collection. Body weights were recorded followed by slaughter of the birds by cervical dislocation, bile collection and dissection of the small intestine, pancreas, bursa of Fabricius, and liver. In order to perform an ELISA for secretory IgA (sIgA; chapter 3), intestinal secretions were collected from the duodenal and ileal segments. Small tissue
samples were collected from the pancreas, liver, bursa, ileum, jejunum and duodenum and placed in RNAlater (source of reagent to be given) for analysis using RT-PCR (chapter 4). Small segments of the ileum were collected for histomorphometry, fixed in 10% neutral buffered formalin, dehydrated and embedded in paraffin wax, and 5 micron cross sections were cut, mounted on glass slides and stained with hematoxylin-eosin B (H&E).

**Virus Preparation.** Chicken LMH cells were prepared in high glucose Dulbecco's Modified Eagle Medium (DMEM) (Sigma) containing 1% L-glutamine and 10% fetal bovine serum until the monolayers reached confluency. Ten microliters of ARV-CU98 stock was diluted in 990 microliters of DMEM medium containing 1% L-glutamine, and 2.5% FBS. Three hundred microliters of this was added to each flask of LMH cells. At 48 hours post-infection, the infected cells began to demonstrate cytopathic effects, which included development of loose cells and syncytia. Seventy-two hours after the diluted stock virus was added to drained LMH cells, the cells were collected, frozen, thawed, centrifuged and aliquoted before being frozen in cryovials. A 96-well plate of LMH cells was prepared to perform a 50% Tissue Culture Infective Dose (TCID₅₀) assay by performing 10-fold dilutions on one frozen aliquot of ARV-CU98. It was determined that 2.13 x10⁷ TCID₅₀ units per milliliter were present in each cryovial.

**Avian Reovirus (ARV) Challenge.** At five days post-hatch, 36 chicks in each of the three dietary treatment groups were given an oral gavage containing the virus, ARV-CU98. These birds were in a heated metal-growing battery in a separate isolation room than the birds not given the virus. The oral gavage consisted of 0.1 mL of the reovirus ARV-CU98 (1x10⁵ TCID₅₀ units/bird). Control chicks were given 0.1 mL phosphate buffered saline.
**Feed Efficiency.** Calculations were based on feed consumed and weight gained within each pen and among treatment groups (diet and room) over the three week trial period. Feed-per-gain was calculated as the total feed consumed by the end of the trial divided by the sum of the weights accomplished (gained) by all birds during their life spans. Feed efficiency is the inverse of feed-per-gain.

**Histomorphometry.** A small segment (1cm) of ileum was collected from each of the 18 birds sampled at each of the 4 time points. After collection, the segments were placed in tubes containing 10% neutral buffered formalin. Tissues were processed for histology and stained with hematoxylin and eosinB. A computerized microscope based image analyzer (Southern Micro Instruments, Atlanta, GA) was used to measure the villi heights, crypt depths, and muscularis externa. Villi measured had an intact lamina propria and visible crypts. Up to 50 villi were measured and averaged for each bird, with three birds analyzed in each of the three dietary treatments in both the control and infected groups, and in each of two trials.

**Experimental Design and Statistical Analysis.** All data from this completely randomized experimental design were analyzed using the general linear models procedure of SAS (SAS Institute, 1996). Significant differences were determined by analysis of variance and were separated by Least Significant Difference at $p \leq 0.05$. For the histomorphometric calculations, measurements for each parameter from each bird were analyzed as a one way analysis of variance. Fisher’s Least Significant Difference was used to test differences between means only when the analysis of variance indicated significance at $p \leq 0.05$ (Motulsky, 2005).
Results

Feed Efficiency. There was a significant trial effect ($P \leq 0.05$) for feed efficiency, with birds in trial 1 having an overall feed efficiency of 0.462 and birds in trial 2 having an overall efficiency of 0.575. For trial 1, there was a treatment effect ($P \leq 0.05$) with Selenite-fed chickens having higher feed efficiency than Sel-Plex-fed chickens (Table 2.1). This was independent of reovirus infection. For trial 2, there were no significant differences (Table 2.1). In trial 1, mean feed efficiency followed the trend Selenite$>$No Se$>$Sel-Plex-fed chickens in both the non-infected and virus-infected rooms. Both selenite-fed birds and Sel-Plex-fed birds had higher overall feed efficiency than the control-fed (no Se) birds in both the control and infected groups in trial 2.

Table 2.1. Mean ± SD feed efficiency (FE) of Control and Avian Reovirus-challenged chickens in Trials 1 and 2 at 21 days of age.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Diet</th>
<th>FE</th>
<th>FE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>No Se</td>
<td>0.469$^{a b}$</td>
<td>0.527$^a$</td>
</tr>
<tr>
<td></td>
<td>Selenite</td>
<td>0.482$^a$</td>
<td>0.589$^a$</td>
</tr>
<tr>
<td></td>
<td>Sel-Plex</td>
<td>0.440$^b$</td>
<td>0.565$^a$</td>
</tr>
<tr>
<td></td>
<td>±SD</td>
<td>0.030</td>
<td>0.059</td>
</tr>
<tr>
<td>Infected</td>
<td>No Se</td>
<td>0.444$^b$</td>
<td>0.572$^a$</td>
</tr>
<tr>
<td></td>
<td>Selenite</td>
<td>0.511$^a$</td>
<td>0.594$^a$</td>
</tr>
<tr>
<td></td>
<td>Sel-Plex</td>
<td>0.425$^b$</td>
<td>0.605$^a$</td>
</tr>
<tr>
<td></td>
<td>±SD</td>
<td>0.030</td>
<td>0.065</td>
</tr>
</tbody>
</table>

$^{a, b}$ Within a Trial column, means with unlike superscripts differ significantly ($P \leq 0.05$).
**Body Weights.** All differences in body weights were significant at a level of $p \leq 0.05$. The average body weight for trial 1 chicks at day of hatch was significantly lower than trial 2 chicks, so the hatch weights were used as a covariant in the analysis of the body weights at weeks 1, 2 and 3. At week 1, there was a significant trial effect, after taking into account the weights at day of hatch, with trial 2 birds being heavier. Also, selenite-fed chicks were significantly heavier than Sel-Plex fed chicks. At week 2, the same treatment effect existed, but the trial effect was not seen. At week 3, the treatment effect included a significantly greater body weight for selenite-fed chicks than for both the Sel-Plex and control-fed chicks. A significant trial effect at 3 weeks of age was evident with birds in trial 2 being heavier than birds in trial 1.
Table 2.2. Mean ± SD body weights (grams) of Control and Avian Reovirus-challenged chickens in Trials 1 and 2 at hatch, 7, 14, and 21 days of age, followed by sources of variation.

<table>
<thead>
<tr>
<th>Source of Variation</th>
<th>Significance (p value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trial</td>
<td>0.023 0.0384 NS 0.0063</td>
</tr>
<tr>
<td>Diet</td>
<td>N/A 0.0377 0.0062 0.0008</td>
</tr>
<tr>
<td>Virus</td>
<td>N/A NS NS NS</td>
</tr>
<tr>
<td>Diet*Virus</td>
<td>N/A NS NS NS</td>
</tr>
<tr>
<td>Day of Hatch Weights</td>
<td>N/A 0.0001 NS NS</td>
</tr>
</tbody>
</table>

1NS-Not Significant (p value > 0.05)
Table 2.3. Trial 1 effects of selenium (no supplemental Se, inorganic Se or organic Se) and reovirus challenge on morphology of the ileum in 7 day old chicks (2 days post-infection). Mean ± SEM of villus height (µm), crypt depth (µm), villus height:crypt depth (H:D) ratio, and muscularis thickness (µm).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Control No Se</th>
<th>Control Inorganic Se</th>
<th>Control Organic Se</th>
<th>Infected No Se</th>
<th>Infected Inorganic Se</th>
<th>Infected Organic Se</th>
</tr>
</thead>
<tbody>
<tr>
<td>Villus height</td>
<td>675±25</td>
<td>659±48</td>
<td>704±31</td>
<td>713±46</td>
<td>712±57</td>
<td>599±34</td>
</tr>
<tr>
<td>Crypt depth</td>
<td>99±5.0a</td>
<td>99±3.3a</td>
<td>98±4.5a</td>
<td>132±8.8b</td>
<td>132±19.6b</td>
<td>135±22.2b</td>
</tr>
<tr>
<td>H:D ratio</td>
<td>7.24±0.57a</td>
<td>6.91±0.81a</td>
<td>7.41±0.03a</td>
<td>5.62±0.19b</td>
<td>5.72±0.76b</td>
<td>4.95±1.24b</td>
</tr>
<tr>
<td>Muscularis</td>
<td>175±17.5</td>
<td>170±9.4</td>
<td>184±8.8</td>
<td>172±6.8</td>
<td>154±6.8</td>
<td>156±10.6</td>
</tr>
</tbody>
</table>

abcd In a row, means ± SEM with unlike superscripts differ significantly, p≤0.05.
Table 2.4. Trial 2 effects of selenium (no supplemental Se, inorganic Se or organic Se) and reovirus challenge on morphology of the ileum in 7 day old chicks (2 days post-infection). Mean ± SEM of villus height (μm), crypt depth (μm), villus height:crypt depth (H:D) ratio, and muscularis thickness (μm).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Control No Se</th>
<th>Control Inorganic Se</th>
<th>Control Organic Se</th>
<th>Infected No Se</th>
<th>Infected Inorganic Se</th>
<th>Infected Organic Se</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trial 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Villus height</td>
<td>606±30</td>
<td>549±66</td>
<td>697±70</td>
<td>614±28</td>
<td>692±44</td>
<td>658±49</td>
</tr>
<tr>
<td>Crypt depth</td>
<td>107±11</td>
<td>114±10</td>
<td>141±17</td>
<td>122±13</td>
<td>164±17</td>
<td>143±4</td>
</tr>
<tr>
<td>H:D ratio</td>
<td>6.11±0.59</td>
<td>5.01±0.64</td>
<td>5.17±0.35</td>
<td>5.37±0.64</td>
<td>4.40±0.26</td>
<td>4.72±0.31</td>
</tr>
<tr>
<td>Muscularis</td>
<td>135±6</td>
<td>184±38</td>
<td>171±23</td>
<td>144±11</td>
<td>177±12</td>
<td>147±17</td>
</tr>
</tbody>
</table>

In a row, means ± SEM with no superscripts do not differ significantly, p≥0.05.
Table 2.5. Trial 1 effects of selenium (no supplemental Se, inorganic Se or Organic Se) and reovirus challenge on morphology of the ileum in 9 day old chicks (4 days post-infection). Mean ± SEM of villus height (μm), crypt depth (μm), villus height:crypt depth (H:D) ratio, and muscularis thickness (μm).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Control</th>
<th>Control</th>
<th>Control</th>
<th>Infected</th>
<th>Infected</th>
<th>Infected</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No Se</td>
<td>Inorganic Se</td>
<td>Organic Se</td>
<td>No Se</td>
<td>Inorganic Se</td>
<td>Organic Se</td>
</tr>
<tr>
<td>Trial 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Villus height</td>
<td>674±13</td>
<td>701±51</td>
<td>736±54</td>
<td>678±15</td>
<td>729±7</td>
<td>666±87</td>
</tr>
<tr>
<td>Crypt depth</td>
<td>88±12.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>103±9.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>85±13.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>130±5.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>140±13.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>127±17.1&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>H:D ratio</td>
<td>8.29±1.40&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.17±0.79&lt;sup&gt;a&lt;/sup&gt;</td>
<td>10.18±2.36&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.50±0.26&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.45±0.54&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.46±0.09&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Muscularis</td>
<td>157±13.0</td>
<td>183±9.9</td>
<td>174±7.0</td>
<td>167±12.6</td>
<td>192±6.7</td>
<td>170±26.2</td>
</tr>
</tbody>
</table>

<sup>abcd</sup> In a row, means ± SEM with unlike superscripts are significantly different, p≤0.05.
Table 2.6. Trial 2 effects of selenium (no supplemental Se, inorganic Se or Organic Se) and reovirus challenge on morphology of the ileum in 9 day old chicks (4 days post-infection). Mean ± SEM of villus height (μm), crypt depth (μm), villus height:Crypt depth (H:D) ratio, and muscularis thickness (μm).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Control No Se</th>
<th>Control Inorganic Se</th>
<th>Control Organic Se</th>
<th>Infected No Se</th>
<th>Infected Inorganic Se</th>
<th>Infected Organic Se</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trial 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Villus height</td>
<td>599±48</td>
<td>673±70</td>
<td>637±58</td>
<td>589±21</td>
<td>667±31</td>
<td>630±11</td>
</tr>
<tr>
<td>Crypt depth</td>
<td>133±14.5a</td>
<td>128±6.9a</td>
<td>112±6.8a</td>
<td>160±11.8b</td>
<td>152±7.0b</td>
<td>169±10.7b</td>
</tr>
<tr>
<td>H:D ratio</td>
<td>4.73±0.51a</td>
<td>5.40±0.55a</td>
<td>5.79±0.27a</td>
<td>3.83±0.26b</td>
<td>4.54±0.36b</td>
<td>3.92±0.33b</td>
</tr>
<tr>
<td>Muscularis</td>
<td>158±4.3a</td>
<td>153±11.8a</td>
<td>166±18.4a</td>
<td>196±17.9b</td>
<td>190±4.7b</td>
<td>211±17.8b</td>
</tr>
</tbody>
</table>

abcd In a row, means ± SEM with unlike superscripts are significantly different, p ≤ 0.05.
Table 2.7. Trial 1 effects of selenium (no supplemental Se, inorganic Se or Organic Se) and reovirus challenge on morphology of the ileum in 12 day old chicks (7 days post-infection). Mean ± SEM of villus height (μm), crypt depth (μm), villus height:crypt depth (H:D) ratio, and muscularis thickness (μm).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Control No Se</th>
<th>Control Inorganic Se</th>
<th>Control Organic Se</th>
<th>Infected No Se</th>
<th>Infected Inorganic Se</th>
<th>Infected Organic Se</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Trial 1</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Villus height</td>
<td>745±34</td>
<td>738±17</td>
<td>730±14</td>
<td>795±135</td>
<td>715±52</td>
<td>820±63</td>
</tr>
<tr>
<td>Crypt depth</td>
<td>134±20.6</td>
<td>109±10.0</td>
<td>105±8.9</td>
<td>139±30.9</td>
<td>139±11.3</td>
<td>142±8.0</td>
</tr>
<tr>
<td>H:D ratio</td>
<td>6.01±1.03</td>
<td>7.08±0.62</td>
<td>7.46±0.60</td>
<td>6.01±0.67</td>
<td>5.32±0.12</td>
<td>5.97±0.16</td>
</tr>
<tr>
<td>Muscularis</td>
<td>198±16.7</td>
<td>214±31.5</td>
<td>214±15.5</td>
<td>193±7.9</td>
<td>216±12.7</td>
<td>197±5.3</td>
</tr>
</tbody>
</table>

In a row, means ± SEM with no superscripts do not differ significantly, p≥0.05.
Table 2.8. Trial 2 effects of selenium (no supplemental Se, inorganic Se or Organic Se) and reovirus challenge on morphology of the ileum in 12 day old chicks (7 days post-infection). Mean ± SEM of villus height (μm), crypt depth (μm), villus height: crypt depth (H:D) ratio, and muscularis thickness (μm).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Control No Se</th>
<th>Control Inorganic Se</th>
<th>Control Organic Se</th>
<th>Infected No Se</th>
<th>Infected Inorganic Se</th>
<th>Infected Organic Se</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trial 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Villus height</td>
<td>844±68</td>
<td>858±56</td>
<td>843±74</td>
<td>902±28</td>
<td>851±116</td>
<td>861±22</td>
</tr>
<tr>
<td>Crypt depth</td>
<td>148±15.0</td>
<td>168±13.3</td>
<td>161±19.5</td>
<td>159±13.4</td>
<td>155±25.4</td>
<td>167±1.8</td>
</tr>
<tr>
<td>H:D ratio</td>
<td>6.05±0.86</td>
<td>5.27±0.16</td>
<td>5.42±0.24</td>
<td>5.84±0.35</td>
<td>5.74±0.22</td>
<td>5.24±0.15</td>
</tr>
<tr>
<td>Muscularis</td>
<td>168±13.9</td>
<td>229±4.8</td>
<td>196±36.0</td>
<td>228±10.5</td>
<td>209±22.0</td>
<td>240±7.8</td>
</tr>
</tbody>
</table>

In a row, means ± SEM with no superscripts do not differ significantly, p≥0.05.
Table 2.9. Trial 1 effects of selenium (no supplemental Se, inorganic Se or Organic Se) and reovirus challenge on morphology of the ileum in 21 day old chicks (16 days post-infection). Mean ± SEM of villus height (μm), crypt depth (μm), villus height:crypt depth (H:D) ratio, and muscularis thickness (μm).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Control No Se</th>
<th>Control Inorganic Se</th>
<th>Control Organic Se</th>
<th>Infected No Se</th>
<th>Infected Inorganic Se</th>
<th>Infected Organic Se</th>
</tr>
</thead>
<tbody>
<tr>
<td>Villus height</td>
<td>1045±53</td>
<td>842±41</td>
<td>1093±163</td>
<td>980±74</td>
<td>887±148</td>
<td>970±121</td>
</tr>
<tr>
<td>Crypt depth</td>
<td>151±12.7</td>
<td>157±14.2</td>
<td>160±19.8</td>
<td>151±14.3</td>
<td>159±36.4</td>
<td>121±12.9</td>
</tr>
<tr>
<td>H:D ratio</td>
<td>7.25±0.87b</td>
<td>5.54±0.21a</td>
<td>6.97±0.30b</td>
<td>6.81±0.24b</td>
<td>6.04±0.71a</td>
<td>8.22±0.68b</td>
</tr>
<tr>
<td>Muscularis</td>
<td>236±32.0</td>
<td>265±14.3</td>
<td>239±21.0</td>
<td>224±19.6</td>
<td>241±47.5</td>
<td>226±28.1</td>
</tr>
</tbody>
</table>

abcd In a row, means ± SEM with unlike superscripts are significantly different, p≤0.05.
Table 2.10. Trial 2 effects of selenium (no supplemental Se, inorganic Se or Organic Se) and reovirus challenge on morphology of the ileum in 21 day old chicks (16 days post-infection). Mean ± SEM of villus height (μm), crypt depth (μm), villus height: crypt depth (H:D) ratio, and muscularis thickness (μm).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Control No Se</th>
<th>Control Inorganic Se</th>
<th>Control Organic Se</th>
<th>Infected No Se</th>
<th>Infected Inorganic Se</th>
<th>Infected Organic Se</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trial 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Villus height</td>
<td>997±74</td>
<td>1001±151</td>
<td>994±40</td>
<td>857±130</td>
<td>1000±50</td>
<td>1021±63</td>
</tr>
<tr>
<td>Crypt depth</td>
<td>136±7.1</td>
<td>178±31.2</td>
<td>150±12.1</td>
<td>124±14.5</td>
<td>151±5.9</td>
<td>158±6.7</td>
</tr>
<tr>
<td>H:D ratio</td>
<td>7.46±0.19</td>
<td>5.87±0.75</td>
<td>6.93±0.53</td>
<td>7.08±0.65</td>
<td>6.74±0.14</td>
<td>6.66±0.25</td>
</tr>
<tr>
<td>Muscularis</td>
<td>202±5.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>318±17.7&lt;sup&gt;b&lt;/sup&gt;</td>
<td>239±23.3&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>214±33.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>256±30.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>256±10.8&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a,b</sup> In a row, means ± SEM with unlike superscripts are significantly different, p≤0.05.
**Figure 2.1.** Trial 1 ileal villi mean heights in 7 day old (2 days P.I.), 9 day old (4 days P.I.), 12 day old (7 days P.I.) and 21 day old (16 days P.I.) broiler chickens. Groups include different dietary selenium intake (no supplemental Se, inorganic Se or Organic Se) in control room (no reovirus challenge) and infected room (reovirus challenge).
**Figure 2.2.** Trial 2 ileal villi mean heights in 7 day old (2 days P.I.), 9 day old (4 days P.I.), 12 day old (7 days P.I.) and 21 day old (16 days P.I.) broiler chickens. Groups include different dietary selenium intake (no supplemental Se, inorganic Se or Organic Se) in control room (no reovirus challenge) and infected room (reovirus challenge).
**Figure 2.3.** Trial 1 and 2 crypt depth means in 7 day old (2 days P.I) broiler chickens. Groups include different dietary selenium intake (no supplemental Se, inorganic Se or Organic Se) in control (no reovirus challenge) and infected (reovirus challenge) bird groups.
Figure 2.4. Trial 1 and 2 crypt depth means in 9 day old (4 days P.I) broiler chickens. Groups include different dietary selenium intake (no supplemental Se, inorganic Se or Organic Se) in control (no reovirus challenge) and infected (reovirus challenge) bird groups.
Figure 2.5. Trial 1 and 2 crypt depth means in 12 day old (7 days P.I) broiler chickens. Groups include different dietary selenium intake (no supplemental Se, inorganic Se or Organic Se) in control (no reovirus challenge) and infected (reovirus challenge) bird groups.
Figure 2.6. Trial 1 and 2 crypt depth means in 21 day old (16 days P.I) broiler chickens. Groups include different dietary selenium intake (no supplemental Se, inorganic Se or Organic Se) in control (no reovirus challenge) and infected (reovirus challenge) bird groups.
Figure 2.7. Trial 1 and 2 mean villus height:crypt depth ratios in 7 day old (2 days P.I) broiler chickens. Groups include different dietary selenium intake (no supplemental Se, inorganic Se or Organic Se) in control (no reovirus challenge) and infected (reovirus challenge) bird groups.
Figure 2.8. Trial 1 and 2 mean villus height:crypt depth ratios in 9 day old (4 days P.I) broiler chickens. Groups include different dietary selenium intake (no supplemental Se, inorganic Se or Organic Se) in control (no reovirus challenge) and infected (reovirus challenge) bird groups.
Figure 2.9. Trial 1 and 2 mean villus height:crypt depth ratios in 12 day old (7 days P.I) broiler chickens. Groups include different dietary selenium intake (no supplemental Se, inorganic Se or Organic Se) in control (no reovirus challenge) and infected (reovirus challenge) bird groups.
**Figure 2.10.** Trial 1 and 2 mean villus height: crypt depth ratios in 21 day old (16 days P.I) broiler chickens. Groups include different dietary selenium intake (no supplemental Se, inorganic Se or Organic Se) in control (no reovirus challenge) and infected (reovirus challenge) bird groups.
Figure 2.11. Trial 1 crypt depth means in 7 day old (2 days P.I.), 9 day old (4 days P.I.), 12 day old (7 days P.I.) and 21 day old (16 days P.I.) broiler chickens. Groups include different dietary selenium intake (no supplemental Se, inorganic Se or Organic Se) in control room (no reovirus challenge) and infected room (reovirus challenge).
Figure 2.12. Trial 2 crypt depth means in 7 day old (2 days P.I.), 9 day old (4 days P.I.), 12 day old (7 days P.I.) and 21 day old (16 days P.I.) broiler chickens. Groups include different dietary selenium intake (no supplemental Se, inorganic Se or Organic Se) in control room (no reovirus challenge) and infected room (reovirus challenge).
**Figure 2.13.** Trial 1 height: crypt depth ratio means in 7 day old (2 days P.I.), 9 day old (4 days P.I.), 12 day old (7 days P.I.) and 21 day old (16 days P.I.) broiler chickens. Groups include different dietary selenium intake (no supplemental Se, inorganic Se or Organic Se) in control room (no reovirus challenge) and infected room (reovirus challenge).
Figure 2.14. Trial 2 height: crypt depth ratio means in 7 day old (2 days P.I.), 9 day old (4 days P.I.), 12 day old (7 days P.I.) and 21 day old (16 days P.I.) broiler chickens. Groups include different dietary selenium intake (no supplemental Se, inorganic Se or Organic Se) in control room (no reovirus challenge) and infected room (reovirus challenge).
**Ileum Villus Height.** There was a time dependent increase in ileal villus height noted in this investigation (Figures 2.1 and 2.2), but there were no statistically significant differences associated with dietary treatment or reovirus challenge (Tables 2.3 to 2.10). However, from a purely subjective point of view, in trial 1 control (non-infected) chicks at 7 days of age (2 days p.i.) average villus height was greater in the organic Se-fed chicks than in the control chicks not given supplemental Se (Table 2.3), and in trial 2, at 7 days of age, control chicks given organic Se and infected chicks given either inorganic or organic Se supplement had longer villi than did chicks not given supplemental selenium (Table 2.4). At 4 days p.i., the inorganic Se-fed chicks had longer villi than chicks not given supplemental Se for each trial, within each of the control and infected groups. Also, although not significant, inorganic Se-fed chicks had longer villi than chicks given organic Se in infected groups in both trials at this time point and in the control group at this time point in trial 2 (Tables 2.5 and 2.6). At 7 days p.i., there were longer villi in chicks not given supplemental Se than in chicks given inorganic Se in both control and infected groups in trial 1 and in the infected group in trial 2 (Tables 2.7 and 2.8). Somewhat longer villi in the inorganic Se-fed control birds than in the organic Se-fed control birds were seen at this time point in both trials (Tables 2.7 and 2.8). This was reversed for the infected birds, with organic Se-fed having slightly longer villi than the inorganic Se-fed chicks at 7 days p.i for both trials (Tables 2.7 and 2.8). At 16 days p.i., the three week old birds generally showed longer villi in groups fed organic Se than groups fed inorganic Se, but there were no significant differences among the birds in the different dietary treatments and reovirus challenged groups (Tables 2.9 and 2.10).

Graphical representation of the dynamics of villus growth and responses to dietary treatments and reovirus challenge are shown in Figures 2.1 and 2.2. Ileum villus heights increased from roughly 600 µM at 7 days of age to more than 1000 µM at 21 days of age. Although not significantly different at P≤ 0.05, it can be seen in trial 1 that inorganic Se supplementation was associated with decreased villus height in control and
reovirus-challenged chicks at 21 days of age (Figure 2.1). In trial 2 (Figure 2.2), reovirus-challenged chicks at 21 days of age which were not given supplementary Se had a shorter average villus height than villus heights in all other dietary treatments in both control and reovirus-challenged groups (Figure 2.2).

**Villus Crypt Depth.** In trial 1, the reovirus infected chicks had significantly deeper crypts than did control chicks at both 2 and 4 days p.i. (7 and 9 days of age, respectively; Tables 2.3 and 2.5, respectively), and in trial 2 (Tables 2.4 and 2.6), the difference between control and reovirus infected group crypt averages at day 4 p.i. was significant (p ≤ 0.05). At 2 days p.i. in trial 1, chicks given the three dietary treatments had similar crypt depths within each of the control and infected groups. In trial 2 at 2 days p.i., crypt depth was somewhat deeper in selenium-supplemented birds (Figure 2.3). In this trial, organic Se supplemented feed groups had deeper crypts in control chickens, but inorganic Se feed groups had deeper crypts in the infected chicks. At 4 days p.i., inorganic Se-fed chicks in trial 1 had slightly deeper crypts than the birds fed the other diets within both control and infected groups (Figure 2.4). At 12 days of age (7 days p.i.) crypt depth (Figure 2.5) was not significantly affected by either dietary treatment or reovirus challenge in either trials 1 or 2 (Tables 2.7, 2.8). In trial 1, lack of significance between control and infected crypt depths appeared to be related to a large variation associated with the crypt depths of chicks in the birds given no supplemental selenium compared with the birds given selenium. The trend that developed in trial 1 was to have deeper crypt depths in infected chicks at 7 days p.i. (Table 2.7, Figure 2.5), but this trend was not apparent in trial 2 (Table 2.8). At 21 days of age (16 days p.i.; Tables 2.9, 2.10, Figure 2.6) there were no statistical differences among dietary treatments and reovirus challenge groups.

Examination of crypt depths of chicks in trials 1 and 2 can be seen again in Figures 2.3 to 2.6. At 2 days p.i., it can be seen that reovirus challenge generally was
causing increased crypt depth and that selenium treatments at times appeared to be causing greater crypt depths than found in control chicks (Figure 2.3). At 4 days p.i., it is clear that reovirus challenge had induced greater crypt depths in chicks in both trials 1 and 2 (Figure 2.4), but there was no consistent dietary influence in either control or challenged chicks. By 16 days p.i., reovirus challenged, organic selenium fed chicks in trial 1 had the shallowest crypts, but this was not significant due to variation around means at this age (Table 2.9). Se supplemented birds had slightly, but not significantly, deeper crypts within all other infected and control groups (Figure 2.6).

**H: D Ratio.** Villus height: crypt depth (H: D) ratios are found in tables 2.3 to 2.10. At 2 days p.i. (Figure 2.7), the H: D ratios were decreased (significantly in trial 1 and approached significance in trial 2) in reovirus-challenged chicks (Tables 2.3 and 2.4, respectively), mainly due to the increased crypt depths. At 4 days p.i. (Figure 2.8), in both trials 1 and 2, reovirus challenge caused a significant decrease in the H:D ratios (Tables 2.5 and 2.6). However, at 7 days p.i., there were no significant differences between control and reovirus-challenged H: D ratios in either trials 1 or 2 (Tables 2.7 and 2.8). Nevertheless, the reovirus-challenged chicks tended to smaller ratios than did controls (Figure 2.9). There were no dietary treatment influences on H: D ratios at 7 days p.i. At 16 days p.i., trial 1 differences in H: D were observed (Table 2.9). In trial 1 (Table 2.9, Figure 2.10), H: D ratios in inorganic Se-fed controls and inorganic Se-fed reovirus-challenged chicks were significantly smaller than H: D ratios in other dietary treatment groups. This was mainly the result of decreased average villus height in these groups. The organic Se fed chicks had the highest H: D ratios in trial 1 (Table 2.9) among chicks given supplemental Se, due to the greater villus height. In trial 2, there were no significant differences among H:D ratios for either dietary treatments or for reovirus challenge groups (Table 2.10, Figure 2.10). A graphical representation showing the dynamics of the H: D ratios in chicks given dietary Se treatments and challenged with
reovirus can be examined in Figures 2.7 to 2.10. At 2 and 4 days p.i., it is clear that the reovirus challenge had caused H: D ratios to be depressed (Figures 2.7 and 2.8), and at 7 days p.i., the tendency was for the reovirus to cause depressed H: D ratios, but to a lesser degree than in the younger chicks (Figure 2.9). During the first 7 days after reovirus challenge, there were no indications of a consistent Se form influence on H: D ratios. However, at 16 days p.i., it appeared as if those reovirus-challenged chicks given organic Se were beginning to recover more quickly from their virus challenge than those given the inorganic Se source in trial 1. Mean H: D ratio for organic Se fed, reovirus-challenged chicks were higher than those challenged with ARV-CU98 and given inorganic Se (Figure 2.10). An additional observation for trial 1 at 16 days p.i. was that chicks given the inorganic Se source had shorter ileal villi than the other treatment groups, resulting in significantly lower H:D ratios (Table 2.9).

Ileum Muscularis Width. Ileum muscularis width results for control and infected chicks in trials 1 and 2 are also shown in Tables 2.3 to 2.10. The quantification of this parameter was marked by inconsistent responses, and by the general lack of Se source influences on muscularis width. Only in trial 2 at 4 days p.i. were there significant increases in the muscularis width due to reovirus infection only (Table 2.6). Selenium source effects were only noted in trial 2 at 16 days p.i. There was a significantly higher width in birds that were given inorganic Se in their diets in both control and reovirus challenged chicks (Table 2.10).

Discussion and Conclusions

In this study, an avian reovirus (ARV-CU98) of turkey origin was given to 5 day old broiler chickens fed different selenium sources, and the effects of dietary selenium
sources and ARV challenge on small intestine morphology were quantified using a histomorphometric technique. It had been demonstrated earlier that villi will shorten and become blunted in response to the ARV challenge. In the trials performed in this investigation, there were no statistically significant differences in height associated with reovirus challenge. However, as the chicks began to recover from the ARV infection, there were possible influences of dietary selenium supplementation demonstrated. By 16 days p.i. or at 21 days of age, ARV-challenged chickens given organic selenium, in trial 1 but not trial 2, had greater H: D ratios than did those ARV-challenged chickens not given selenium or given inorganic selenium. This was due to increased villus height along with decreased crypt depth. In trial 2, the reovirus-infected chicks that had not received supplementary Se had lower villus heights than those given inorganic or organic Se. Crypts sometimes showed an ARV challenge effect in their hyperplastic growth followed by increased crypt depth, more so in Se-supplemented birds than control birds. At 16 days p.i. in trial 1, reovirus-challenged chicks given organic Se had shallower crypts than those given the inorganic Se source. Mean height to crypt depth (H: D) ratio for organic Se-fed, reovirus-challenged chicks was the highest ratio in this trial among the 21 day old birds, due to the shallower crypt depth. Inorganic Se-fed birds had significantly lower ratios among control and infected birds in trial 1 at 16 days p.i. due to shorter villi.

It has been known for some time that the small intestine is the most important site for ARV infection (Kibenge et al., 1985; Jones et al., 1989). It now appears that an ultramicro nutrient, such as selenium, also appears to play a role in the chicken’s maintenance of intestinal integrity in the face of a virus challenge and facilitates the chicken’s ability to resist or regain intestinal function due to ARV and other enteric infections (Edens et al., 2007ab). Earlier studies have shown that pathogenesis due to ARV begins in the villus and crypt epithelium in which the initial virus replication occurs (Mandelli et al., 1978; Kibenge et al., 1985; Jones et al., 1989). Jones and colleagues (1989) reported that this initial replication takes place between 12 and 24 hours post-
challenge in the small intestine (Jones et al., 1989) making the small intestine a very important anatomical structure in which histomorphological alterations due to reovirus challenge can be easily examined and quantified.

Spackman et al. (2005) reported that turkey origin reoviruses neither infected nor caused disease in chickens, but in turkeys, they caused mild disease that was characterized by severe damage to some organs of the immune system that resulted in immunodysfunction, which then allowed other agents to cause disease. Nevertheless, it has been shown that the turkey origin reovirus, ARV-CU98, does cause disease in broiler chickens (Macalintal, 2004; Edens et al., 2007ab; Read-Snyder, 2009). In the current report, in selenium-deficient ARV-CU98-infected broiler chicken, the duodenal, jejunal, and ileal villi have been found to be thickened, shortened, and blunted with deeper crypts of Lieberkuhn. The signs of infection were less severe due to the feeding of organic selenium in a high selenium yeast supplement (Sel-Plex) and to a smaller degree due to the feeding of sodium selenite similar to results reported earlier (Edens et al., 2007ab). A later investigation demonstrated that at 21 days of age and post infection, villus height was significantly higher in control and ARV-challenged organic selenium-fed broilers (Edens et al., 2007ab; Read-Snyder, 2009). There was a significant increase in crypt depth due to the virus and a significant increase in the birds given either no supplemental selenium or given inorganic Se as compared to those given organic selenium. The villus H: D ratio for both control and infected groups was increased in birds given organic Se in their diets (Read-Snyder, 2009). Spackman et al. (2010) also characterized enteric reovirus infection in chickens and poults, which displayed shrunken degenerate cells in the villi and crypt epithelium and mild crypt hyperplasia that resulted in increased crypt depth. Mild villus shortening and a increased number of lymphocytes in the lamina propria could be visualized also (Spackman et al., 2010). In this current work, it was noted that the feeding of organic selenium was sometimes associated with greater H: D ratios in ARV-challenged chickens, but this was not seen until the chicks were 21 days
old or at 16 days p.i. This observation was a confirmation of the earlier work with ARV-challenged selenium-fed broilers (Edens et al., 2007ab; Read-Snyder, 2009). Often, the supplementation of inorganic Se in the form of sodium selenite was associated with shortening of villi and/or deepening of the crypts, and therefore lower H: D ratios, by three weeks of age, suggesting its negative effects on intestinal integrity. This observation might even be considered a sign of possible oxidative stress associated with the feeding of inorganic selenium.

These observations are important because the traditional source of dietary Se for poultry and livestock, ever since it was approved initially for inclusion in poultry and livestock diets in 1974, has been inorganic sodium selenite (Leeson and Summers, 1991). Sodium selenite also has a pro-oxidant influence in animals (Spallholz, 1997; Terada et al., 1999), and this pro-oxidant property can be the cause of unrecognized oxidative stress in animals. In fact, Gowdy (2004) observed that as little as 1 ppm of sodium selenite in the diets of high yielding broilers was related to signs of selenium toxicity. The current commercial broiler lines are high-yielding, have higher metabolic rates, and have different nutritional needs, and thereby are subject to increased risk for development of oxidative stress (Spallholz, 1997; Terada et al., 1999). Furthermore, Gowdy (2004) and Mahmoud and Edens (2003) have demonstrated that organic selenium, acting via the glutathione-glutathione peroxidase antioxidant system and also via the thioredoxin-thioredoxin reductase system, maintains a more reduced status in body tissues than inorganic selenium. Therefore, revisiting organic Se as a feed supplement has become necessary especially after organic selenium was approved for incorporation into animal feeds and drinking water (Federal Register, 2000; Federal Register, 2002).

Increasing levels of dietary inorganic Se are associated with greater tissue concentrations, which tend to plateau in a time dependent manner (Norheim and Moksnes, 1985; Moksnes and Norheim, 1986; Echevarria, 1988 a,b), but the organic Se from yeast has been shown to be more available to blood and liver proteins than from
certain inorganic forms (Vinson, 1987) and results in greater tissue concentrations of selenium as compared to inorganic selenium (Norheim and Moksnes, 1985; Moksnes and Norheim, 1986). Selenium in high Se yeast (such as Sel-Plex™, Alltech Biotechnology Center, Nicholasville, KY) is considered highly available. Absorption of organic Se from the intestinal tract is via active transport similar to the absorption of methionine while inorganic Se is absorbed passively (Wolffram et al., 1986; Schrauzer, 2000).

Ostensibly, increased availability of organic selenium should also influence the selenium-dependent antioxidant system. In this current study, antioxidant status was not determined, but work by Mahmoud and Edens (2003), Gowdy (2004), Edens and Gowdy (2004), and Upton et al. (2009) has demonstrated that organic selenium supplementation resulted in a more reduced redox status and higher GSH-px activity in broiler chickens in a variety of experiments in which the selenium-dependent antioxidant system was required to function maximally.

Salim et al. (1990) have noted that the majority of gut viruses infect villus enterocytes, but each virus will have different affinities for enterocytes at different sites on the epithelial cover on the villus. Infection of enterocytes leads to cell death, extrusion into the lumen, and villus atrophy when the rate of cell production in the crypts cannot keep pace with the rate of enterocyte loss and breakdown of tight junctions between epithelia/enterocyts. This results in a reduced surface area as well as impairment of digestive and absorptive functions. This may also result in a net transcellular and paracellular secretory state leading to diarrhea similar to PEMS infection in turkey poultls challenged with ARV-CU98 (Heggen-Peay et al., 2002a). All these changes, along with others, such as reduced enzymatic activity and reduced epithelial integrity, may contribute to the induction of an acute but transient malabsorptive diarrhea which may persist until the digestive/absorptive functions of the enterocyte are restored. In this context, many other morphological alterations occur in the intestine. Certainly, it has been reported that the crypts of Lieberkuhn become significantly deeper as noted in this
investigation and in many others dealing with ARV infections in chickens and in turkeys (Edens et al., 2007ab; Read-Snyder, 2009; Spackmann et al., 2005 and 2010). The hyperplastic response of the epithelial cells in the crypts is due solely to the lost integrity of the villus epithelium, and when the villi shorten and become blunted, the rate at which the surface epithelium is replaced can be increased to regain patency of the epithelial layer. In time, the uninfected epithelial layer is completely replaced and the villi again lengthen to regain normal morphological status. In fact, this series of events in response to ARV-CU98 infection in broiler chickens was seen in this investigation. A recovery period of at least 16 days p.i. was required before the trends became apparent.

Enteric viral infections are inherently associated with oxidative stress and inflammatory activity (Schweizer and Peterhans, 1999). However, Schwarz (1996) has indicated that RNA viruses do not always induce oxidative stress in infected tissues. In the case of enteric avian reovirus infection, which is a double stranded RNA virus, enteric pathology is not always recognized, and ARV can be found even in “healthy flocks” of poultry (Spackman et al., 2010). Likely, the ARV compromises the intestinal barriers and allows secondary bacterial invasion, which then interacts with the virus to cause severe pathology to develop.

In this current study, enteric ARV infection was not associated with decreased body weight or decreased feed efficiencies. The improved intestinal morphological characteristics observed at times in selenium-fed, reovirus-challenged broilers late in the experimental period may be attributed to nutritional influences on other physiological functions. However, this did not always occur and there was much variation. It has long been known that selenium has a role in immunostimulation during times of antigen challenge (Marsh et al., 1986; Leng et al., 2003), and organic selenium feed supplements improve the status of the avian immune system by increasing the ability of immunocompetent cells to respond to an antigen challenge (Leng et al., 2003). Gowdy (2004) showed that organic selenium had a suppressive influence on nitrite production.
from nitric oxide, which can have a stimulatory role in the induction of inflammation. Additionally, Gowdy (2004) demonstrated that humoral antibody was enhanced early in an infection in organic selenium fed broilers and reached higher titers than did selenite fed animals. Tyner et al. (2004) also showed that organic selenium supplementation caused a suppression of nitrite production in *Bordetella avium*-infected turkey poults. Thus, it is possible that the improved morphological profile in organic selenium-fed, ARV-challenged broiler chickens might be related to an improved immunological status as compared with broilers given either no supplemental selenium or sodium selenite. However, this requires additional study for confirmation.

Muscularis width was not generally influenced by either dietary selenium supplementation or by ARV challenge in this investigation. Throughout the time course of this investigation muscularis width was characterized by inconsistencies. However, in trial 2 at 16 days p.i., inorganic Se resulted in thicker muscle walls than the dietary treatment of no supplemental Se. This observation was somewhat in contrast with the reports of Edens et al. (2007ab) who found at 21 days of age and p.i., that both inorganic and organic Se was associated with thicker muscularis in ARV-challenged broilers. The involvement of Se with potentially greater smooth muscle mass in the small intestine suggests that even in the face of an enteric reovirus challenge, growth and development of the intestinal tract can still proceed to meet the nutritional needs of the growing broiler chicken.

In conclusion, selenium is important for chickens as they cope with an ARV challenge. The supplementation of organic selenium and to a lesser extent inorganic selenium in trial 1 may have promoted the protection of the integrity of the ileum during a time when the broilers were combating the ARV-CU98-induced infection. However, on a time course basis, the improved morphological profile of the intestinal villus was not suggested until 16 days p.i. when organic selenium-fed broiler intestines may have been recovering at a faster pace than inorganic selenium-fed broilers. There is a suggestion that
the selenium supplementation in the diets of chickens infected with ARV-CU98 had improved intestinal integrity which was reflected in body weights and feed efficiency. These observations suggest that the presence of inorganic selenium as sodium selenite can induce subtle negative changes in the integrity of the intestinal tract and this is magnified in chickens that become challenged with disease-causing agents such as ARV.
Abstract:

Avian reoviruses cause significant economic losses in the poultry industry due to the diseases with which they are associated, including enteric and respiratory diseases, myocarditis, hepatitis, Poult Enteritis and Mortality Syndrome (PEMS) in turkeys and malabsorption syndrome (MAS) in chickens. Natural avian reovirus infections occur via the fecal-oral route, with infection mainly via oral entry and initial replication being in the intestine and bursa of Fabricius. The small intestine is the most important site for ARV infection regardless of the route of inoculation, with the spread of infection beginning in the villus and crypt epithelium of the small intestine. The signs of infection have been demonstrated to be less severe due to the feeding of organic selenium in a high selenium yeast supplement (Sel-Plex) and to a smaller degree due to the feeding of sodium selenite. The extracellular spaces are protected by the humoral immune response in which antibodies produced by B cells cause the destruction of extracellular microorganisms and prevent the spread of infections within cells. Secretory IgA is the predominant form of antibody in bodily secretions. It is secreted into the gut via bile in the foregut or via the bursal canal in the hindgut. Dimeric IgA, secreted by local plasma cells, is taken up by polymeric immunoglobulin receptor and then transported to the apical membrane, and secreted into the intestinal lumen. Marginal Se-deficiency is known to decrease the cellular and humoral immune function in animals. In this study, the immune response as influenced by the double stranded RNA ARV-CU98 virus and dietary selenium, was studied by determining sIgA levels in intestinal secretions from the
ileum by way of the enzyme-linked immunoglobulin assay (ELISA). IgA levels were also measured in bile. The dietary treatment groups consisted of diets containing Sel-Plex (Alltech, Inc. Nicholasville, KY) as the source of organic selenium (0.3 ppm), sodium selenite (0.3 ppm) as a source of inorganic selenium or control feed (<.002 ppm). Early in the post challenge period, intestinal IgA levels were distributed from highest to lowest levels as follows: inorganic Se > organic selenium > no supplemental selenium. Several days later, organic Se-fed birds were showing an overall higher average ileal IgA level than the inorganic Se-fed and control-fed birds in the challenged groups. By 16 days p.i., there was a significant (p ≤ 0.05) challenge X dietary treatment interaction attributed to infected organic Se-fed birds having significantly higher ileal IgA than the non-challenged, organic Se-fed birds. In both trials, non-challenged organic Se-fed chicks initially had higher bile IgA levels than those not given supplemental selenium, but in the infected groups, chickens not given supplemental Se had the highest sIgA levels, which was greater than inorganic Se-fed chicken sIgA levels, followed by sIgA levels in chickens fed organic Se. In both trials, in both non-challenged and challenged groups at 16 days p.i., the average biliary sIgA was often highest in organic Se-fed birds

Introduction

Avian reoviruses are members of the Orthoreovirus genus. They are icosahedral, nonenveloped viruses with double-protein capsid shells and genomes consisting of ten double-stranded RNA segments that express structural and nonstructural proteins. The virus encodes at least 10 structural proteins and four non-structural proteins encased within their 2 concentric protein shells (Benavente and Martínez-Costas 2007; Lin et al., 2007).

Avian reoviruses cause significant economic losses in the poultry industry due to the diseases with which they are associated. They were initially discovered as the disease
that induced tenosynovitis in young chickens, and then were found to be ubiquitous among poultry flocks, mostly causing asymptomatic infections. Diseases to which ARV have been associated in poultry include enteric and respiratory diseases, myocarditis, hepatitis, Poult Enteritis and Mortality Syndrome (PEMS) in turkeys and malabsorption syndrome (MAS) in chickens (Heggen-Peay et al., 2002; Benavente and Martínez-Costas, 2007). The ARV infections are important in the poultry industry since mortality, leg weakness and poor feed conversion caused by ARV depresses productivity (Ni et al., 1995). Field outbreaks, mainly among broiler breeders, where the agents have been isolated and identified as reoviruses, have been reported in many parts of the world (Gouvea and Schnitzer, 1982).

Natural ARV infections occur mainly via the fecal-oral route with translocation across the mucosal surfaces of the small intestine, which is then followed by initial viral replication in the epithelium of the villi, crypts of Lieberkuhn, and bursa of Fabricius. After initial replication in the small intestine, the virus spreads via blood to other tissues and organs (Menendez et al., 1975b; Kibenge et al., 1985; Jones et al., 1989; Ni et al., 1995; Benavente and Martínez-Costas, 2007). Strategically located on the small intestine are Peyers patches (PP), which are lymphoid areas with specialized epithelial cells known as M cells. The ARV enter these epithelial cells after binding to a host epithelial cell by way of its outer capsid protein, replicating in the cytoplasm of infected cells, and inducing fusion of host cells in order to facilitate viral spread (Bomsel and Alfsen, 2003; Vázquez-Iglesias et al., 2009). Within 12 to 24 hours p.i. in the intestine, the infective ARV have been re-isolated from plasma, intestines, bursa of Fabricius, pancreas, spleen, liver, kidney, joints and tendons, with the liver becoming a primary site for replication after the virus spreads from the small intestine epithelium (Mandelli et al., 1978; Kibenge et al., 1985; Jones et al., 1989).

Protection of the intestinal tract of poultry is achieved by both innate and adaptive immune mechanisms. The primary immune mechanisms used by poultry to protect itself
against viral and bacterial infection in the intestinal tract are via secretory (s) IgA and IgG. Lawrence et al. (1981) have shown that slgG and slgA are produced in the chicken intestine at far greater levels than IgM, and slgA and slgG secretory activity from the intestine follows similar time lines. The extracellular spaces are protected by the humoral immune response in which antibodies produced by B cells cause the destruction of extracellular microorganisms and prevent the spread of infections within cells. Proliferation and differentiation of the B cell into plasma cells that secrete antibody must first occur (Janeway et al., 2005). IgA is the predominant form of antibody in bodily secretions (Underdown and Schiff, 1986; Kerr 1990).

Popiel and Turnbull (1985) have noted that in the chicken hatchling both IgY and IgA participate in the protection of the intestinal tract, but within one week post hatch, the maternally-transferred IgG is no longer effective, and until the maternal antibody is dissipated, host IgG production and secretion is inhibited. Thus, the innately present intestinal slgA then becomes the primary antibody system that protects the intestinal tract against infections of either viral or bacterial origin especially in young chickens. IgG and monomeric IgA are secreted into the gut via bile in the foregut or via the bursal canal in the hindgut (Klipper et al., 2000). Plasma cells secreting IgG or dimeric IgA reside in the intestinal wall as well as in other anatomical sites in the chicken (Bar-Shira and Friedman, 2005), and dimeric IgA is secreted via enterocytes (Mestecky et al., 1999). In this case dimeric IgA, secreted by local plasma cells, is taken up by polymeric immunoglobulin receptor protein (pIgR; Wieland et al., 2004; Read-Snyder, 2009) located in the basal membrane of enterocytes. Secretory IgA is then transported to the apical membrane, and secreted into the intestinal lumen (Mestecky et al., 1999). It is in the lumen that the pre-emptive protection of the intestine is accomplished via the presence of the nonspecific slgA and to specifically directed slgG. The slgA functions in immune exclusion by binding to bacterial and viral antigens, and interfering with attachment and colonization (Muir et al., 2000). Secretory IgA can prevent contact of
pathogens with the mucosal surface by helping to entrap pathogens in the mucus and the lumen by either directly blocking or sterically hindering pathogen attachment proteins that mediate epithelial attachment. IgA also has the capacity to intercept pathogens within cell vesicular compartments (Silvey et al., 2001).

Selenium seems to play a major role in infections caused by RNA viruses (Beck, 2001; Combs 2001; Field, 2002; Lyons, 2003; Beck, 2004). Marginal Se-deficiency is known to decrease the cellular and humoral immune function in humans and laboratory animals, which includes chickens (Combs and Combs, 1986; Maas, 1998). Many studies have revealed the relationships involving host Se deficiency and virulence of RNA viruses. Beck and colleagues reported that in a Se-deficient mouse model new viral variants emerged (Beck 1994, 1995, 1998, 2003) making Se-deficient mice more susceptible to Coxsackievirus B3. The mice infected with a normally harmless form of the virus developed myocarditis because the avirulent form mutated to a virulent form in Se-deficient mice. The genome mutation was believed to be a result of increased oxidative stress due to the Se deficiency, which resulted in reduced activity of glutathione peroxidase (Beck 2001; Nelson 2001; Beck 2003; Beck 2004). Additionally, more severe pathology was seen in Se-deficient mice challenged with an influenza virus, and this was attributed to increased proinflammatory cytokine production in lungs of the Se-deficient mice (Beck 2001; Nelson 2001; Beck 2003; Beck 2004). Little has been reported on the effects of selenium on intestinal immunity of ARV-challenged broiler chickens.

In this study, the immune response as influenced by the double stranded RNA ARV-CU98 virus and dietary selenium, was studied by determining sIgA levels in intestinal secretions from the ileum by way of the enzyme-linked immunoglobulin assay (ELISA). The dietary treatment groups consisted of diets containing Sel-Plex (Alltech, Inc. Nicholasville, KY) as the source of organic selenium (0.3 ppm), sodium selenite (0.3 ppm) as a source of inorganic selenium or control feed (<.002 ppm).
Materials and Methods

Animal welfare. This study was conducted following guidelines established by the North Carolina State University Animal Care and Use Committee, which governs all animal-use in experimental procedures.

Animals and husbandry. Feather sexed male Ross 708 broiler chicks were placed at day of hatch in 9 pens in heated brood/grow batteries in each of two identical isolation rooms. Each of the isolation rooms was controlled identically for light and temperature. Lighting was provided 24 hours a day through incandescent lamps in the ceiling of the two isolation rooms. The rooms were preheated to 90 °F before placement of the chicks and held at that temperature for the first week. The room temperature was decreased weekly by 5 °F.

Twelve birds were placed in each pen. They were identified at placement using plastic neck tags. Three pens of chicks in each of two isolation rooms were fed torula yeast-based diets (Appendix Table A.1) containing either Sel-Plex (Alltech, Inc. Nicholasville, KY) as the source of organic selenium (0.3 ppm), sodium selenite (0.3 ppm), or no supplemental selenium (<0.002ppm). Feed and water were provided on an ab libitum basis in stainless steel pans attached to the frame of the brooder pens.

Body weight determination and tissue collection. Body weights were determined at 1, 2 and 3 weeks of age. Only the birds that were not fasted before sampling or had died prior to weighing were included in the body weight data collections. Birds were fasted 12 hours before tissue samples and weights were collected from 18 birds at 7, 9, 12 and 21 days of age (2, 4, 7, and 16 days post-infection, respectively). After the body weights were recorded, blood samples were collected and this was then followed by slaughter of the birds by cervical dislocation. Bile was collected from each chick for quantification of
secretory IgA (sIgA), and this was followed by dissection of the small intestine, pancreas, bursa of Fabricius, and liver. Intestinal secretions from the duodenum and ileum segments were collected by lavage for determination of sIgA. Small tissue samples were collected from the pancreas, liver, bursa, ileum, jejunum and duodenum and placed in RNAlater (Sigma, St. Louis, MO) for analysis using RT-PCR (chapter 4). Small segments of the duodenum, jejunum, and ileum were collected for histomorphometry (Chapter 2).

**Virus Preparation.** Chicken LMH cells were prepared in high glucose Dulbecco's Modified Eagle Medium (DMEM) (Sigma) containing 1% L-glutamine and 10% fetal bovine serum until the monolayers reached confluency. Ten microliters of ARV-CU98 stock was diluted in 990 microliters of DMEM medium containing 1% L-glutamine, and 2.5% FBS. Three hundred microliters of this was added to each flask of LMH cells. At 48 hours post-infection, the infected cells began to demonstrate cytopathic effects, which included development of loose cells and syncytia. Seventy-two hours after the diluted stock virus was added to drained LMH cells, the cells were collected, frozen, thawed, centrifuged and aliquoted before being frozen in cryovials. A 96-well plate of LMH cells was prepared to perform a 50% Tissue Culture Infective Dose (TCID$_{50}$) assay by performing 10-fold dilutions on one frozen aliquot of ARV-CU98. It was determined that 2.13 $\times 10^7$ TCID$_{50}$ units per milliliter were present in each cryovial.

**Avian Reovirus (ARV) Challenge.** At five days post-hatch, 36 chicks in each of the three dietary treatment groups were given an oral gavage containing the virus, ARV-CU98. These birds were in a heated metal-growing battery in a separate isolation room than the birds not given the virus. The oral gavage consisted of 0.1 mL of the reovirus ARV-CU98 (1$\times 10^5$ TCID$_{50}$ units/bird). Control chicks were given 0.1 mL phosphate buffered saline.
**Feed Efficiency.** Calculations were based on feed consumed and weight gained within each pen and among treatment groups (diet and room) over the three week trial period. Feed-per-gain was calculated as the total feed consumed by the end of the trial divided by the sum of the weights accomplished (gained) by all birds during their life spans. Feed efficiency is the inverse of feed-per-gain.

**Intestinal secretion collection.** A phosphate buffered saline solution containing 1mM phenylmethanesulfonylfluoride (PMSF), 50 mM ethylenediaminetetraacetic acid (EDTA), and 100 mg/L of soybean trypsin inhibitor (all reagents from Sigma, St. Louis, MO) was used to flush out the intestine. The solution was slowly injected and pressure was softly applied to the intestinal wall to allow the solution to flow slowly up the tract. After repeating until a total of about 3.5-4 mL was collected from the intestine, the solution was centrifuged on low speed to separate out cells and debris. Supernatant was collected, and about 10 µL/mL of 0.1M PMSF was added to the solution. After centrifugation at 13000xg, the supernatant was collected in 1 mL aliquots. Fifty microliters of fetal bovine serum (FBS), 10 µL of 0.1 M PMSF, and 10 µL of 1% sodium azide solution were added to each 1 mL aliquoted intestinal secretion.

**Protein concentration assay.** Total protein concentration in all intestinal secretions was determined using the Bicinchoninic acid (BCA) Pierce Protein Assay Reagent Kit (Thermo Scientific, Rockford, IL). Samples were diluted 7-fold in order to prevent the interference of EDTA which was above the concentration compatible with the kit. The absorbance of standards and unknowns was measured at a wavelength of 562 nm. Using SoftMax® Pro Data Analysis Software, protein concentrations were determined and reported with reference to standard dilutions of known concentrations made from bovine serum albumin. A linear-fit standard curve generated by the software consisted of the
average Blank-corrected 562 \text{ nm} measurement for each BSA standard vs. its concentration in \( \mu \text{g/mL} \). The standard curve was used to determine protein concentration of the unknowns.

**ELISA/Chicken IgA Enzyme-Linked Immunosorbent Assay.** All intestinal secretions were diluted in phosphate buffered saline to standardize the total protein concentration in samples to 500 \( \mu \text{g/mL} \) for 7 and 9 day-old birds and 25 \( \mu \text{g/mL} \) for 12 and 21 day-old birds in order to achieve within-range absorption values. The Chicken IgA ELISA Quantification kit (Bethyl Laboratories, Montgomery, TX) was used to quantitatively measure levels of chicken IgA in these intestinal secretions. Microtiter well plates were coated with the capture antibody Goat anti-Chicken IgA. Standards (chicken reference serum) and intestinal secretions were transferred to wells for incubation. Goat anti-chicken IgA-horse radish peroxidase (HRP) conjugate was the HRP Detection antibody for the chicken IgA. Using 3,3',5,5'-tetramethyl benzidine (TMB) peroxidase substrate and solution, HRP was detected. Sulfuric acid (2M) was used to stop the enzyme substrate reaction. The plates were read at a wavelength of 450 \( \text{nm} \). A standard curve (four parameter logistic curve-fit) was generated using SoftMax® Pro Data Analysis Software. O.D. values were used to compare IgA levels in the intestinal secretions.

**Bile IgA quantification.** Bile samples were analyzed for IgA using procedures of Cotter (2000 and 2001). A 5uL sample of each was tested for IgA using an established radial immunodiffusion method in which a lectin (Black Turtle Bean, BTB) replaces antibody. Ring diameters produced by the precipitation of IgA by the lectin depend on IgA quantity (Cotter 2000 and 2001). In 27/141 (19%) of the samples, material precipitated by BTB could not be detected.
Experimental design and statistical analysis. The experiment was a completely randomized design with a 2x3 factorial arrangement of treatments. At day of hatch, 12 chicks were placed in each of the 9 pens in each brooder battery. The two batteries were in separate but identically controlled isolation rooms. One room was for the control group and one for ARV-challenged group. Three dietary treatments (described above) were given to both groups, providing 6 experimental treatments with three replicate pens per treatment. The effects of treatment on IgA levels were determined by the proc-General Linear Models (GLM) procedure in The SAS System for Windows V8 (Citrix Community, 2008). Statements of significance are based on P≤0.05.

Results

Feed Efficiency. There was a significant trial effect (P≤0.05) for feed efficiency, with birds in trial 1 having an overall feed efficiency of 0.462 and birds in trial 2 having an overall efficiency of 0.575. For trial 1, there was a treatment effect (P≤0.05) with Selenite-fed chickens having higher feed efficiency than Sel-Plex-fed chickens (Table 2.1). This was independent of reovirus infection. For trial 2, there were no significant differences (Table 3.1). In trial 1, mean feed efficiency followed the trend Selenite>No Se>Sel-Plex-fed chickens in both the non-infected and virus-infected rooms. Both selenite-fed birds and Sel-Plex-fed birds had higher overall feed efficiency than the control-fed (no Se) birds in both the control and infected groups in trial 2.
Table 3.1. Mean ± SD feed efficiency (FE) of Control and Avian Reovirus-challenged chickens in Trials 1 and 2 at 21 days of age.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Diet</th>
<th>FE</th>
<th>FE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>No Se</td>
<td>0.469&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.527&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Selenite</td>
<td>0.482&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.589&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Sel-Plex</td>
<td>0.440&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.565&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>±SD</td>
<td></td>
<td>0.030</td>
<td>0.059</td>
</tr>
</tbody>
</table>

| Infected  | No Se  | 0.444<sup>b</sup> | 0.572<sup>a</sup> |
|           | Selenite | 0.511<sup>a</sup> | 0.594<sup>a</sup> |
|           | Sel-Plex | 0.425<sup>b</sup> | 0.605<sup>a</sup> |
| ±SD       |        | 0.030 | 0.065 |

<sup>a,b</sup> Within a Trial column, means with unlike superscripts differ significantly (P ≤ 0.05).

**Body Weights.** All differences in body weights were significant at a level of p ≤ 0.05. The average body weight for trial 1 chicks at day of hatch was significantly lower than trial 2 chicks, so the hatch weights were used as a covariant in the analysis of the body weights at weeks 1, 2 and 3. At week 1, there was a significant trial effect, after taking into account the weights at day of hatch, with trial 2 birds being heavier. Also, selenite-fed chicks were significantly heavier than Sel-Plex fed chicks. At week 2, the same treatment effect existed, but the trial effect was not seen. At week 3, the treatment effect included a significantly greater body weight for selenite-fed chicks than for both the Sel-Plex and control-fed chicks. A significant trial effect at 3 weeks of age was evident with birds in trial 2 being heavier than birds in trial 1.
Table 3.2. Mean ± SD body weights (grams) of Control and Avian Reovirus-challenged chickens in Trials 1 and 2 at hatch, 7, 14, and 21 days of age, and sources of variation.

<table>
<thead>
<tr>
<th>Trial 1 Treatment</th>
<th>Diet</th>
<th>Day of hatch</th>
<th>Week 1</th>
<th>Week 2</th>
<th>Week 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>No Se</td>
<td>41±2</td>
<td>123±13</td>
<td>277±33</td>
<td>497±61</td>
</tr>
<tr>
<td>Selenite</td>
<td>40±3</td>
<td>122±12</td>
<td>269±41</td>
<td>497±84</td>
<td></td>
</tr>
<tr>
<td>Sel-Plex</td>
<td>40±3</td>
<td>116±13</td>
<td>251±40</td>
<td>446±83</td>
<td></td>
</tr>
<tr>
<td>Infected</td>
<td>No Se</td>
<td>40±3</td>
<td>117±12</td>
<td>261±34</td>
<td>468±68</td>
</tr>
<tr>
<td>Selenite</td>
<td>41±3</td>
<td>119±13</td>
<td>268±34</td>
<td>491±72</td>
<td></td>
</tr>
<tr>
<td>Sel-Plex</td>
<td>40±3</td>
<td>116±15</td>
<td>254±45</td>
<td>452±93</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Trial 2 Treatment</th>
<th>Diet</th>
<th>Day of hatch</th>
<th>Week 1</th>
<th>Week 2</th>
<th>Week 3</th>
</tr>
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<td>123±17</td>
<td>265±42</td>
<td>496±98</td>
</tr>
<tr>
<td>Selenite</td>
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<td>284±48</td>
<td>533±107</td>
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<tr>
<td>Sel-Plex</td>
<td>41±3</td>
<td>119±12</td>
<td>264±44</td>
<td>491±94</td>
<td></td>
</tr>
<tr>
<td>Infected</td>
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<td>123±16</td>
<td>260±46</td>
<td>491±88</td>
</tr>
<tr>
<td>Selenite</td>
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<td>122±13</td>
<td>274±30</td>
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<tr>
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<td>123±14</td>
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<tr>
<td>Virus</td>
<td>NS</td>
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<td>Diet*Virus</td>
<td>NS</td>
</tr>
<tr>
<td>Day of Hatch Weights</td>
<td>NS</td>
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NS-Not Significant (p value > 0.05)
Figure 3.1. Trial 1 IgA (± SEM) Levels for 7 day old chicks at 2 days post-challenge with ARV-CU98. Diets in the control and infected rooms consisted of either control feed (no supplemented selenium), inorganic selenium or organic selenium.
Figure 3.2. Trial 2 IgA (± SEM) Levels for 7 day old chicks at 2 days post-challenge with ARV-CU98. Diets in the control and infected rooms consisted of either control feed (no supplemented selenium), inorganic selenium, or organic selenium.
Figure 3.3. Trial 1 IgA (± SEM) levels for 9 day old chicks at 4 days post-challenge in the infected room. Diets in the control and infected rooms consisted of either control feed (no supplemented selenium), inorganic selenium, or organic selenium.
Figure 3.4. Trial 2 IgA (± SEM) levels for 9 day old chicks at 4 days post-infection in the infected room. Diets in the control and infected rooms consisted of either control feed (no supplemented selenium), inorganic selenium, or organic selenium.
Figure 3.5. Trial 1 IgA (± SEM) levels for 12 day old chicks at 7 days post-infection in the infected room. Diets in the control and infected rooms consisted of either control feed (no supplemented selenium), inorganic selenium, or organic selenium.
**Figure 3.6.** Trial 2 IgA (± SEM) levels for 12 day old chicks at 7 days post-infection in the infected room. Diets in the control and infected rooms consisted of either control feed (no supplemented selenium), inorganic selenium, or organic selenium.
Figure 3.7. Trial 1 IgA (± SEM) levels for 21 day old chicks at 16 days post-infection in the infected room. Diets in the control and infected rooms consisted of either control feed (no supplemented selenium), inorganic selenium, or organic selenium.
Figure 3.8. Trial 2 IgA (± SEM) levels for 21 day old chicks at 16 days post-infection in the infected room. Diets in the control and infected rooms consisted of either control feed (no supplemented selenium), inorganic selenium, or organic selenium. Data are missing for the control-fed chicks in the infected room.
Two Days Post-Infection. At two days post-infection (7 days of age), trial 1 chicks (Figure 3.1) had slightly higher IgA levels in the infected room, while trial 2 chicks (Figure 3.2) had geometrically higher levels in the control room, which was attributed to higher IgA levels in the control chickens given inorganic-Se supplemented feed. Overall, there was a tendency for IgA levels on average to be higher in the birds fed inorganic Se, followed by those fed organic selenium, and lowest in the birds given no supplemental selenium, but there was much variation in these groups. Additionally, at 2 days p.i., for both trials inorganic Se-supplemented chickens had higher average IgA levels than did those birds given organic Se in the control groups and in the infected group of trial 2. In trial 1, there was no differentiation between inorganic and organic Se supplemented chickens in the infected grouping.

Four Days Post-Infection. At 9 days of age (4 days post-infection), the chicks in trial 1 showed the higher average IgA level in the infected group (Figure 3.3), but in trial 2 the control group had slightly higher average IgA levels than the infected group (Figure 3.4). In both trials, inorganic Se-fed chicks averaged higher IgA levels, followed by organic Se-fed chicks, and the control group averaged the lowest level (same as 2 days prior to this). Birds fed selenium had higher levels than birds not fed supplemental selenium within the control groups of both trials. However, in the infected groups, birds fed no supplemental Se had higher IgA levels than organic Se-fed birds in both trials.

Seven Days Post-Infection. At 7 days p.i., once again trial 1 birds had an overall higher average ileal IgA level in the infected group (Figure 3.5), while in trial 2 the control group had the higher ileal IgA levels (Figure 3.6). When one examines the overall differences among dietary treatments in trials 1 and 2, organic Se-fed birds in the ARV-infected groups averaged higher ileal IgA levels than both the inorganic Se-fed birds and the birds that were not given supplemental Se of either form (Figures 3.5 and 3.6).
**Sixteen Days Post-Infection.** At 16 days p.i., the 21 day old birds had a higher IgA average in the infected group in trial 1 (Figure 3.7), and in the control group in trial 2 (Figure 3.8). However, in trial 2 data are missing for the infected birds fed no supplemental Se, and the average was questionably low for the infected, organic-Se fed group (Figure 3.8). A significant \( p \leq 0.05 \) virus and dietary treatment interaction was seen in trial 1 with the infected, organic Se-fed birds having significantly higher ileal IgA than the organic Se-fed, control birds, but the reverse of this observation was made in trial 2 (Figure 3.8). Although not significant in trial 2, the organic Se-fed control birds had higher ileal IgA than the infected organic Se-fed birds.

**Bile IgA Levels**

![Comparison of Bile IgA Levels at Different Ages-Trial 1](image)

**Figure 3.9:** Trial 1 bile IgA (± SEM) levels for chicks at 7, 9, 12 and 21 days of age. Diets in the control and infected rooms consisted of either control feed (no supplemented selenium), inorganic selenium, or organic selenium.
Comparison of Bile IgA Levels at Different Ages -
Trial 2

Figure 3.10: Trial 2 bile IgA (± SEM) levels for chicks at 7, 9, 12 and 21 days of age. Diets in the control and infected rooms consisted of either control feed (no supplemented selenium), inorganic selenium, or organic selenium.

In trial one, the highest average bile IgA levels for both 7 and 9 day old chicks were in the non-infected, organic Se-fed birds followed by the infected, control-fed birds (Figure 3.9). The highest averages in this trial for 12 and 21 day old chicks were in the infected, organic Se-fed birds (Figure 3.9). At three of the four time points in this trial (7, 12 and 21 days of age), the inorganic Se-fed, non-infected chicks had the lowest average bile IgA. Among the 21 day-old birds, there was a virus effect ($p < 0.05$) with infected birds having overall higher average bile IgA levels than non-infected birds. The correlation between ileal and bile IgA levels in trial 1 were not significant.

In trial 2, the infected, Se-fed chicks had highest average bile IgA levels at both 9 and 12 days of age (Figure 3.10). At 21 days of age, the virus effect was approaching
significance at \( p \leq 0.05 \) with infected birds showing lower average biliary IgA levels than non-infected birds (Figure 3.10), differing from the observations made in trial 1 (Figure 3.9). There was a significant correlation between biliary and ileal IgA at 9 days of age (\( p \leq 0.05 \)) with a Pearson correlation coefficient of -0.58. At 21 days of age, a significant positive correlation (\( p \leq 0.05 \)) between biliary and ileal IgA was seen with a Pearson correlation coefficient of 0.57.

Although bile IgA levels at 2 days post-infection showed no significant diet or virus effects, birds had higher levels of bile IgA in the control group than in the infected group in both trials 1 and 2 (Figures 3.9 and 3.10). In the control groups of both trials, organic Se-fed chicks had higher levels than those not given supplemental selenium. This was opposite in the infected groups, with control-fed (no supplemental Se) chicks having the highest levels, followed by inorganic Se fed chicks and lowest in organic Se-fed chicks. Bile IgA levels at 4 days p.i. were higher in the control birds in trial 1 and higher in the infected birds in trial 2. Organic Se-fed chicks had overall higher biliary IgA than the control-fed chicks in both trials. Within the infected group in both trials at this time point, the chicks fed inorganic-Se had the lowest IgA levels in their bile.

Bile IgA levels at 7 days p.i. were higher in the infected group in both trials, and organic-Se fed birds had higher levels than those not given supplemental selenium in both of the trials (Figures 3.9 and 3.10). In the infected group in each trial, organic-Se fed chicks had the highest average in biliary IgA, followed by chicks not given supplemental Se, and least in those given inorganic selenium. At 21 days of age, a virus effect was noted in trial 1 (Figure 3.9), with higher biliary IgA in the infected birds than in the control birds. However, trial 2, birds had higher bile IgA levels in the control birds than the infected birds (Figure 3.10). In both trials 1 and 2, the average biliary IgA among all birds was highest in the group given organic Se in the diet. Among the control birds in both trials 1 and 2, organic Se-fed birds had higher biliary IgA levels than inorganic Se-fed birds (Figure 3.9 and 3.10). Among the infected birds, organic Se-fed
birds had the highest levels, followed by control fed birds, and lowest in inorganic Se-fed birds.

Discussion and Conclusions:

Surai (2006) reviewed extensively the health effects of dietary selenium supplementation and noted that selenium does not prevent virus-induced disease. However, he did conclude that selenium supplementation appeared to play a pivotal role in reducing the severity of the infection. A similar conclusion, supporting Surai (2006), was made by Edens et al. (2007ab), who reported apparent enhanced recovery of the intestinal integrity of chickens challenged with ARV-CU98.

It is important to recognize the fact that the small intestine is the most important site for development of the initial ARV-induced viremia and that ARV infections spread from the intestine to other body targets regardless of the types of ARV involved (Kibenge et al., 1985; Jones et al., 1989). Mechanism(s) by which selenium improves the chicken’s response to ARV challenge is poorly understood, but it was hypothesized that intestinal sIgA might play a very important role in the chicken’s ability to recover from an enteric ARV infection. In this study, the influence of two forms of selenium, inorganic sodium selenite and organic selenium in selenium yeast, compared to a control diet with no supplemental selenium, were evaluated relative to their role in the output of sIgA in the intestinal tract of chickens subjected to an enteric ARV challenge.

The results from this investigation were difficult to interpret due to trial differences. Yet, certain tendencies in sIgA responses were noted in each of the two trials, but the selenium-related influences on sIgA were equivocal between trials. Baseline differences in IgA levels between control and ARV-challenged groups were likely different in the two trials. The ARV-challenged group had the higher average ileal
IgA as seen in the first samples measured in trial 1, but in trial 2, the control group had the higher average ileal IgA.

Early in the post challenge period (2 and 4 days p.i.) in both trials 1 and 2, intestinal IgA levels were distributed, although with much variation, from highest to lowest levels as follows: inorganic Se > organic selenium > no supplemental selenium. At 4 days p.i., in non-challenged groups, selenium-fed animals showed higher sIgA levels than birds not given supplemental selenium, but in the challenged birds in both trials 1 and 2, birds not given supplemental selenium had higher IgA levels than organic Se-fed birds. The time course changes in intestinal sIgA for both trials 1 and 2 continued to occur as the experiment progressed to 7 days p.i. when organic Se-fed birds had an overall higher average ileal IgA level than the inorganic Se-fed and control-fed birds in the challenged groups. By 16 days p.i., there was a significant (p≤0.05) challenge X dietary treatment interaction attributed to infected organic Se-fed birds having significantly higher ileal IgA than the non-challenged, organic Se-fed birds.

Biliary sIgA levels followed developmental patterns similar to intestinal sIgA. In both trials, non-challenged organic Se-fed chicks initially (2 days p.i.) had higher bile IgA levels than those given no supplemental selenium, but in the infected groups, chickens not given supplemental Se had the highest sIgA levels, which was greater than inorganic Se-fed chicken sIgA levels, followed by sIgA levels in chickens fed organic Se. At 4 days p.i., within the infected groups, the chicks fed inorganic-Se had the lowest levels of biliary sIgA. At 7 days p.i. in both trials, organic-Se fed birds had higher biliary sIgA levels out of the challenged birds, with organic-Se fed > no supplemental Se > inorganic selenium. In both trials, in both non-challenged and challenged groups at 16 days p.i., the average biliary sIgA was often highest in organic Se-fed birds (non-challenged organic Se > inorganic Se), and among challenged birds, organic Se-fed birds had higher sIgA levels > no supplemental Se > inorganic Se.
Rebel et al. (2004) reported that by 14 days post-challenge, chickens exposed to an enteric reovirus began to recover from the initial infection as evidenced by reduced severity of lesions in the villus and crypt regions of the intestine. In chapter 2, it was noted that the height:crypt depth ratios for ARV-CU98-challenged organic selenium-fed chickens were greater than the height:crypt depth ratios for birds given either no supplemental selenium or inorganic selenium. This observation was similar to the observation made by Edens et al. (2007ab) and Read-Snyder (2009). Additionally, in this investigation, by 7 days p.i., sometimes birds fed organic selenium were showing numerically greater levels of ileal and biliary sIgA than did birds fed sodium selenite or no supplemental selenium. Therefore, by extrapolation of the ileal and biliary sIgA responses to ARV-CU98 challenge and to the improved integrity of the intestinal villi and crypt depths in those birds fed organic selenium, one must conclude that dietary organic selenium had imparted some beneficial effects that were not evidenced in ARV-CU98-challenged birds given either no supplemental selenium or sodium selenite in their diets. Organic selenium, therefore, may improve intestinal integrity in reovirus-challenged chickens. It would appear that the improved intestinal integrity in organic selenium-fed, reovirus-challenged chickens was due in large part to a sustained selenium-related induction of sIgA production in both the intestine and the liver.

The beneficial effects of Se were described at the biochemical level when it was found to be the essential component of the antioxidant enzyme, glutathione peroxidase (GSH-Px) (Flohé et al., 1973; Rotruck et al., 1973). It is suggested that the cell’s antioxidant defense is based on the activity of superoxide dismutase, GSH-Px, and catalase. However, there are other selenium-dependent antioxidant enzymes that are involved in maintenance of a reduced status in animals. In poultry, it has been noted that GSH-px and thioredoxin reductase activities are elevated in response to organic selenium supplementation (Mahmoud and Edens, 2003; Edens and Gowdy, 2004; Gowdy, 2004) in
comparison to the activities of these antioxidant enzymes in birds given either no supplemental selenium or sodium selenite (Surai, 2002).

GSH-Px may provide protection against ssRNA virus-induced damage due to viral mutation and reassortments. Selenium deficiency may lead to the change of an avirulent virus into a virulent one by way of point mutations in its genome (Beck et al., 1998). With knowledge that ssRNA viruses induce oxidative stress in host cells and the knowledge that cellular redox status plays an important role in regulating viral replication and infectivity, it has been observed that GSH-Px inhibited expression of viral matrix protein and virally induced caspase activation and Fas up-regulation. (Cai et al., 2003). While specific studies have not ascertained a similar mechanism of action in the dsRNA ARV-CU98 challenged birds, one can speculate that a similar mechanism is in play in ARV-CU98-challenged birds fed organic selenium.

In conclusion, organic selenium appeared to have improved intestinal recovery after an ARV-CU98 enteric reovirus challenge. The apparent recovery was somewhat more visible in organic selenium-fed birds as compared to birds either not given supplemental selenium or given sodium selenite in their diets. These recovery events did not become evident immediately, but did begin to become manifest about 7 days after the infective challenge when the chickens were 12 days old. Improved villus height:crypts of Liberkuhn depths was the hallmark of facilitated intestinal recovery for ARV-CU98 challenge. Associated with this event, there was also the development of a tendency for sIgA from the liver and the intestine of organic selenium-fed chickens to increase in parallel with the the signs of improved villus height:crypt depth ratios from 7 to 16 days p.i. (Chapter 2). Evidence of improved intestinal integrity based on villus height:crypt depth ratios and parallel increases in sIgA from the bile along with intestinal sIgA, suggests that these events might be mediated by organic selenium more efficiently than by inorganic sodium selenite. Overall, selenium, in general, had a tendency to increase sIgA levels in the intestine and in the bile. Whether this increase in biliary and intestinal
sIgA levels was due to a direct or indirect effect of selenium, especially organic selenium, has not yet been ascertained.
CHAPTER 4: INFLUENCE OF SELENIUM AND REOVIRUS CHALLENGE ON THE EXPRESSION OF POLYMERIC IMMUNOGLOBULIN RECEPTOR (PIGR) IN BROILER CHICKENS

Abstract:

Avian reoviruses cause significant economic losses in the poultry industry due to the diseases with which they are associated, including enteric and respiratory diseases, myocarditis, hepatitis, Poult Enteritis and Mortality Syndrome (PEMS) in turkeys and malabsorption syndrome (MAS) in chickens. Natural avian reovirus infections occur via the fecal-oral route, with infection mainly via oral entry and initial replication being in the intestine and bursa of Fabricius. The small intestine is the most important site for ARV infection regardless of the route of inoculation, with the spread of infection beginning in the villus and crypt epithelium of the small intestine. The signs of infection have been demonstrated to be less severe due to the feeding of organic selenium in a high selenium yeast supplement (Sel-Plex) and to a smaller degree due to the feeding of sodium selenite. The extracellular spaces are protected by the humoral immune response in which antibodies produced by B cells cause the destruction of extracellular microorganisms and prevent the spread of infections within cells. Secretory IgA is the predominant form of antibody in organ/gladular secretions. Therefore, the polymeric immunoglobulin receptor (pIgR), which transports sIgA across secretory cells and enterocytes in the intestine, is an important factor involved in the humoral immune response. It has been demonstrated that Sel-Plex supplementation leads to increased pIgR expression in the liver of ARV-CU98 infected birds. This suggests that pIgR is involved in the immune response of ARV-infected chickens and Sel-Plex may allow the infected chickens to maintain more normal intestinal villi due to maintenance of an
immune response in which pIgR is transporting sufficient antibodies for controlling the infection. By quantifying the expression of the in the duodenum, jejunum, ileum and liver tissues, it was suggested that selenium was having an influence. Throughout the infection period, inorganic Se-supplemented birds showed overall higher expression than organic Se-supplemented birds, and this might be due to a stronger immune system (fighting the virus more efficiently) or a weaker immune system (needing to compensate for a weaker overall response). By 16 days post-infection, once the virus had ran its course, those infected birds that had been given organic Se were often showing decreased levels of expression. Possibly, the response was low for this group due to sufficient recovery from the virus challenge. It was difficult to make conclusions about the functioning of the immune system at the level of pIgR in this study due to a lack of consistent trends. However, it appears as though selenium supplementation is having an influence on the expression of this receptor, therefore affecting the response to ARV at the level of sIgA.

**Introduction:**

Avian reoviruses are members of the *Orthoreovirus* genus. They are icosahedral, nonenveloped viruses with double-protein capsid shells and genomes consisting of ten double-stranded RNA segments that express structural and nonstructural proteins. The virus encodes at least 10 structural proteins and four non-structural proteins encased within their 2 concentric protein shells (Benavente and Martínez-Costas 2007; Lin *et al.*, 2007).

Avian reoviruses cause significant economic losses in the poultry industry due to the diseases with which they are associated. They were initially discovered as the disease that induced tenosynovitis in young chickens, and then were found to be ubiquitous among poultry flocks, mostly causing asymptomatic infections. Diseases to which ARV
have been associated in poultry include enteric and respiratory diseases, myocarditis, hepatitis, Poult Enteritis and Mortality Syndrome (PEMS) in turkeys and malabsorption syndrome (MAS) in chickens (Heggen-Peay et al., 2002; Benavente and Martínez-Costas, 2007). The ARV infections are important in the poultry industry since mortality, leg weakness and poor feed conversion caused by ARV depress productivity (Ni et al., 1995).

Natural avian reovirus infections occur via the fecal-oral route, with infection mainly via oral entry and initial replication occurring in the intestine and bursa of Fabricius. Then, the virus spreads via blood to other tissues and organs (Menendez et al., 1975b; Ni et al., 1995; Benavente and Martínez-Costas, 2007). Mucosal surfaces, such as the lining of the gut, are important locations for the entry of viruses into the body. Peyers patches (PP) are lymphoid areas of the GI tract with specialized epithelial cells known as M cells. A reovirus can enter here after binding to a host cell by way of its outer capsid protein (Bomsel and Alfsen, 2003). Avian reoviruses replicate in the cytoplasm of infected cells and induce fusion of host cells in order to facilitate further replication and spread (Vázquez-Iglesias et al., 2009).

The viremia due to ARV challenge spreads from the villus and crypt epithelium upon initial replication of the virus. It has been re-isolated from plasma, intestines, bursa of Fabricius, pancreas, spleen, liver, kidney, joints and tendons, with the liver becoming a primary site for replication after the virus spreads from the epithelium of the small intestine (Mandelli et al., 1978; Kibenge et al., 1985; Jones et al., 1989).

In selenium-deficient ARV-CU98-infected broiler chickens, the villi have been found to be thicker, shortened and blunted with deeper crypts of Lieberkuhn. The signs of infection were less severe due to the feeding of organic selenium in a high selenium yeast supplement (Sel-Plex) and to a smaller degree due to the feeding of sodium selenite. The live weight gain was greatest in the organic selenium-fed chicks compared to the control (no supplemental selenium) and sodium selenite groups. Also, plasma
protein levels were significantly lowered due to the ARV infection, but Sel-Plex maintained plasma protein level at a higher concentration compared with the other dietary treatments at 2 and 3 weeks of age. These findings suggest that nutrients such as selenium could play a role in the chicken’s ability to resist or regain function due to ARV infection (Edens et al., 2007ab).

ARV-CU98 may contribute directly to PEMS in turkey poults by affecting the intestine, bursa of Fabricius, thymus and liver, and may contribute indirectly by increasing susceptibility to opportunistic pathogens that facilitate the development of clinical PEMS (Heggen-Peay et al., 2002). *in ovo* inoculation of broiler chickens with ARV-CU98 (turkey origin) and S1733 (chicken origin) resulted in changes in body, liver and lymphoid organ weights, and blood plasma chemistry. Results indicated that both ARV-CU98 and S1733 can be transmitted *in ovo* from day 9 of incubation, and the PEMS-associated ARV-CU98 is able to inhibit growth and development in broiler chickens as seen with turkey poults (Macalintal, 2004). When ARV-CU98 was orally inoculated into broiler chickens at day of hatch, signs of enteritis were seen at 21 days of age as demonstrated via histomorphometric analysis of the intestinal villi. Genomic analysis of the responses of chickens to ARV-CU98 demonstrated that the avian reovirus had an effect on the expression of Selenoprotein P, which is a carrier protein for selenium, and polymeric immunoglobulin receptor (pIgR), which transports IgA (Read-Snyder, 2009).

The chicken polymeric immunoglobulin receptor gene, GG-pIgR, was first found to be expressed in intestine, bursa of Fabricius, liver and thymus cells. The extracellular, ligand-binding domains of the plgR are cleaved off at the apical surface of epithelial and endocrine cells after transcytosis, and the resulting soluble form is secretory component (SC), which is mainly found in association with immunoglobulins. SC assembles with plgA into sIgA complexes in chicken (Wieland et al., 2004). The SC protects IgA from degradation (Crottet and Corthesy, 1998; Wieland et al., 2004). It was demonstrated that
chicken pIgR is mainly expressed in epithelia associated with lymphoid organs. It appears to be abundant in bile (Wieland et al., 2004). It is hypothesized that the rate of IgA transcytosis across epithelial and endocrine cells depends on the level of pIgR expression (Bonaz, 2005; Pal et al., 2005; Schneeman et al., 2005) with one molecule of pIgR needing to be synthesized for each molecule of IgA that is transported.

Research was conducted to determine if dietary selenium could influence the response of pIgR in broiler chickens infected with avian reovirus, since evidence has shown that organic Se has a positive influence on resisting and recovery from double stranded RNA viruses such as this. The eggs used for the study were from Cobb breeders fed Torula yeast diets not containing supplemental selenium, containing inorganic selenium or containing organic Se in the form of Sel-Plex. The birds that hatched were placed on diets similar to their parental diets. Results showed that Sel-Plex supplementation resulted in increased pIgR expression in the liver of infected birds. Expression of pIgR was increased in the bursa of Fabricius and pancreas due to ARV-CU98 infection. It was therefore suggested that pIgR is involved in the immune response of ARV-infected chickens and Sel-Plex may allow the infected chickens to maintain more normal villi (Read-Snyder, 2009), since this was seen using histomorphometric analysis.

SIgA is the primary antibody involved in antigen-antibody interactions in the mucosal immune system (Harriman et al., 1999). When pathogens establish a contact with a host at a mucosal surface, the adaptive immune defense at the site is initiated by lymphocytes and secretion of secretory sIgA (Reese et al., 2006). The sIgA functions in immune exclusion by binding to bacterial and viral antigens, and interfering with attachment and colonization (Muir et al., 2000). Secretory IgA may prevent contact of pathogens with the mucosal surface by helping to entrap pathogens in mucus followed by peristaltic or ciliary clearance. IgA may directly block or sterically hinder the attachment proteins that mediate epithelial attachment. It may intercept pathogens within cell
vesicular compartments (Silvey et al., 2001). Therefore, the receptor which transports sIgA through cells, pIgR, is an important part of the response to antigen in the intracellular and extracellular spaces. The rate of sIgA transcytosis may depend on the level of pIgR expression (Bonaz, 2005; Pal et al., 2005; Schneeman et al., 2005), with one molecule of pIgR being synthesized for each molecule of IgA that is transported. Factors that influence pIgR would therefore affect mucosal immunity since it plays a role in mucosal host defense (Pal et al., 2005).

Animals have increased susceptibility to infection in association with malnourishment. The increase in severity of an infectious disease in a malnourished host is thought to result from an impaired immune response. Little is known about how the malnourished host may affect the virus’ interaction with the host. Selenium is the ultramicro trace element that seems to play a major role in infections caused by RNA viruses (Beck, 2001; Combs 2001; Field, 2002; Lyons, 2003; Beck, 2004). Se-deficiency and marginal Se-deficiency are known to decrease the cellular and humoral immune function in humans and laboratory animals (Combs and Combs, 1986; Maas, 1998). Many studies have revealed the relationships involving host Se deficiency and virulence of RNA viruses. Beck and colleagues began research on the influence of malnutrition on host and virus interactions upon the discovery that new viral variants emerge in a Se-deficient model, which made the mice more susceptible to Coxsackievirus (Beck 1994, 1995, 1998, 2003). The genome mutation responsible for this was suggested to be a result of increased oxidative stress due to the Se deficiency (Beck 2001; Nelson 2001; Beck 2003; Beck 2004). Beck and colleagues also suggested that influenza virus is influenced by the Se status of the host. Much more severe pathology was seen in Se-deficient mice, and this was due to increased proinflammatory cytokine production in lungs of the Se-deficient mice (Beck 2001; Nelson 2001; Beck 2003; Beck 2004).

In this study, the immune response, specifically involving pIgR, as affected by ARV-CU98 and dietary selenium, was studied by quantifying the expression of pIgR
using quantitative real-time PCR (qRT-PCR). The dietary treatment groups consisted of diets containing Sel-Plex (Alltech, Inc. Nicholasville, KY) as the source of organic selenium (0.3 ppm), sodium selenite (0.3 ppm) as a source of inorganic selenium or control feed with no supplemental selenium(<.002ppm). One half of each treatment group was challenged with ARV-CU98 at 5 days of age, and the other half was not challenged.

Materials and Methods:

Animal Welfare. This study was conducted following guidelines established by the North Carolina State University Animal Care and Use Committee, which governs all animal-use in experimental procedures.

Animals and husbandry. Feather sexed male Ross 708 broiler chicks were placed at day of hatch in 9 pens in heated brooder batteries in each of two identical isolation rooms. Each of the isolation rooms was controlled identically for light and temperature. Lighting was provided 24 hours a day through incandescent lamps in the ceiling of the two isolation rooms. The rooms were preheated to 90 °F before placement of the chicks and held at that temperature for the first week. The room temperature was decreased weekly by 5 °F.

Twelve birds were placed in each pen. They were identified at placement using plastic neck tags. Three pens of chicks in each of two isolation rooms were fed torula yeast-based diets (Appendix Table A.1) containing either Sel-Plex (Alltech, Inc. Nicholasville, KY) as the source of organic selenium (0.3 ppm), sodium selenite (0.3 ppm), or no supplemental selenium (<0.002ppm). Feed and water were provided on an ab libitum basis in stainless steel pans attached to the frame of the brooder pens.
**Tissue sampling and body weight measurements.** Tissue samples and body weights from 18 birds at each of 7, 9, 12 and 21 days of age (2, 4, 7, and 16 days post-infection, respectively) were collected. Birds were fasted 12 hours before the beginning of data collection. Body weights were recorded followed by slaughter of the birds by cervical dislocation, bile collection and dissection of the small intestine, pancreas, bursa of Fabricius, and liver. In order to perform an ELISA for secretory IgA (sIgA), intestinal secretions were collected from the duodenal and ileal segments. Small tissue samples were collected from the pancreas, liver, bursa, ileum, jejunum and duodenum and placed in Rnalater (source of reagent to be given) for analysis using RT-PCR. Small segments of the ileum were collected for histomorphometry, fixed in 10% neutral buffered formalin, dehydrated and embedded in paraffin wax, and 5 micron cross sections were cut, mounted on glass slides and stained with hematoxylin-eosin B (H&E).

**Virus Preparation.** Chicken LMH cells were prepared in high glucose Dulbecco's Modified Eagle Medium (DMEM) (Sigma) containing 1% L-glutamine and 10% fetal bovine serum until the monolayers reached confluency. Ten microliters of ARV-CU98 stock was diluted in 990 microliters of DMEM medium containing 1% L-glutamine, and 2.5% FBS. Three hundred microliters of this was added to each flask of LMH cells. At 48 hours post-infection, the infected cells began to demonstrate cytopathic effects, which included development of loose cells and syncytia. Seventy-two hours after the diluted stock virus was added to drained LMH cells, the cells were collected, frozen, thawed, centrifuged and aliquoted before being frozen in cryovials. A 96-well plate of LMH cells was prepared to perform a 50% Tissue Culture Infective Dose (TCID<sub>50</sub>) assay by performing 10-fold dilutions on one frozen aliquot of ARV-CU98. It was determined that 2.13 x10<sup>7</sup> TCID<sub>50</sub> units per milliliter were present in each cryovial.
**Avian Reovirus (ARV) Challenge.** At five days post-hatch, 36 chicks in each of the three dietary treatment groups were given an oral gavage containing the virus, ARV-CU98. These birds were in a heated metal brood/grow battery in an isolation room separate from the birds not given the virus. The oral gavage consisted of 0.1 mL of the reovirus ARV-CU98 (1x10⁵ TCID₅₀ units/bird). Control chicks were given 0.1 mL phosphate buffered saline.

**Feed Efficiency.** Calculations were based on feed consumed and weight gained within each pen and among treatment groups (diet and room) over the three week trial period. Feed-per-gain was calculated as the total feed consumed by the end of the trial divided by the sum of the weights accomplished (gained) by all birds during their life spans. Feed efficiency is the inverse of feed-per-gain.

**RNA Extraction and Qualitative Assessment.** Tubes containing small samples of duodenum, jejunum, ileum and liver in RNAse later were thawed. All tubes used for RNA extraction were RNase free, and RNase Away® (Molecular Bioproducts, Inc. San Diego, CA) was used to wipe down tools and laboratory benches that would possibly come into contact with the tissue samples. One milliliter of Trizol® (Invitrogen) was pipetted into each 2 ml conical, screw top microcentrifuge tube. A tiny piece of tissue was placed in each of tube. Using a mini-beadbeater-96 (Biospec, Inc. Bartlesville, OK), the tissues were homogenized. Three hundred microliters of chloroform was then added to each tube, followed by vortexing. After incubating at room temperature for approximately 5 minutes, the tubes were centrifuged for at least 5 minutes at 12,000x g and 4°C. The clear, top aqueous layer was then removed and placed in new tubes which contained 600 µl of 100% ethanol. The tubes were mixed and cooled in the freezer for at least 30 minutes. They were then centrifuged at 12,000xg for 15 minutes and 4°C, and supernatant was discarded, leaving a pellet. After adding 600 µl of 70% ethanol to the
tube in order to wash the RNA pellet, the tubes were centrifuged at 12,000xg for 5 minutes and 4°C and dried for 10-15 minutes. Remaining ethanol was pipetted from the pellet. The pellets were re-suspended in nuclease free water (50-100 μl) and stored at -80°C.

Frozen tubes of extracted RNA were thawed on ice and the RNA was quantified using a Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE). Tubes with a concentration above 850 ng/μl RNA were doubled in volume and re-measured. RNA was re-extracted from tissue samples containing less than 100 ng/μl RNA. The purity of RNA was considered acceptable if the 260/280 ratio was greater than 1.5. RNA was re-extracted from tissue samples with a ratio below 1.5. The quality of the RNA was visualized by running an agarose gel with ethidium bromide for about 150 of the 432 samples in order to determine whether 2 distinct bands for ribosomal RNA were present. These two bands were not present when the RNA concentration was too low and the RNA sample needed to be re-extracted (see Figure 4.1)
Figure 4.1. Gel image of various RNA extracts from duodenum, jejunum, ileum and liver samples. The far left lane is the 1kb DNA ladder.

cDNA Synthesis. The High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) was used to synthesize DNA from 1000 ng of each RNA sample that was extracted. For each 10 μl sample (diluted appropriately so 1000 ng RNA was present), 10 μl of 2X Reverse Transcription (RT) Master Mix was used. This consisted of 2 μl 10x RT buffer, 0.8 μl 25x dNTP mix, 2 μl 10x RT random primers, 1 μl MultiScribe™ reverse transcriptase, and 4.2 μl nuclease free water. The samples and mastermix were added to a 96 well plate, and this was centrifuged briefly followed by running in a thermocycler with conditions optimized for use with the High Capacity cDNA Reverse Transcription Kit.

Real-Time PCR. The cDNA from each of three birds for each trial, age, treatment and tissue group were pooled together and diluted 1:20 before amplification by qRT-PCR. The expression level of pIgR was analyzed using the BioRad iCycler iQ Real Time PCR
system (BioRad, Hercules, CA) and the iQ-SYBR Green Supermix kit (Applied Biosystems, Foster City, CA) following the manufacturer’s protocols. The real time reaction consisted of 1 μl of pooled cDNA, 0.4 μl of both the forward and reverse primers (Table 4.1), 0.2μl of fluorescein, 10μl SYBR green and 8μl of water.

Thermocycling parameters programmed for the BioRad iycler iQ Real Time PCR system consisted of the following: 94°C for 7 minutes; 50 cycles at 95°C for 30 seconds, appropriate Tm (Table 4.1) for 30 seconds, 72°C for 30 seconds; 72°C for 5 minutes; 95°C for 1 minute; 55°C for 1 minute, 80 cycles at 55°C for 30 minutes. Fluorescence measurements were collected at every cycle during the extension step (72°C).

Gene expression was normalized using 18S ribosomal RNA and a standard curve was run to determine PCR efficiency (Table 4.1). The dilution intervals for the standard curve consisted of a pool of all undiluted cDNA samples diluted 1:5, 1:25, 1:125 and 1:625. Cycle Threshold (Ct) values were automatically calculated by the iQ software and corresponded to the cycle in which the amplification rate was maximal.

**Table 4.1.** Primers used for qRT-PCR analysis (from Read-Snyder, J., 2009)

<table>
<thead>
<tr>
<th>Target Gene</th>
<th>Accession Number</th>
<th>5’ Primer</th>
<th>3’ Primer</th>
<th>Primer Efficiency</th>
<th>Tm (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>18 S</td>
<td>M59389</td>
<td>CGGAGAGGGAGCCTGAGAA</td>
<td>GCCAGCTCAGTCCCAAGA</td>
<td>93.3%</td>
<td>58.6</td>
</tr>
<tr>
<td>pIgR</td>
<td>AF303371</td>
<td>CAGGAGAGGAGGAGAATC</td>
<td>TTAAGTCCACCGTGACC</td>
<td>106.6%</td>
<td>56</td>
</tr>
</tbody>
</table>

**Experimental Design and Statistical Analysis.** The experiment was a completely randomized design with a 2x3 factorial arrangement of treatments. At the day of hatch,
12 chicks were placed in each of the 9 pens in each brooder battery. The two batteries were in separate but identically controlled isolation rooms. One room was for the control group and one for ARV-challenged group. Three dietary treatments (described above) were given to both groups, providing 6 experimental treatments with three replicate pens per treatment. The effects of treatment on pIgR expression were determined by the proc-General Linear Models (GLM) procedure in The SAS System for Windows V8 (Citrix Community, 2008). Statements of significance are based on $P \leq 0.05$.

The $C_t$ values obtained from the iQ software were converted to reflect the normalized gene expression, based upon the $C_t$ for the 18S ribosomal RNA for the sample. The inverse of the difference was used so the higher the ratio is, the higher the normalized gene expression level. The equation for this conversion was as follows:

$$1/ (pIgR \ C_t - 18S \ C_t)$$

The values obtained using the above equation were used in order to determine statistical significance (Table 4.2) before further manipulating the data in order to reflect fold changes, which were acquired by using the Pfaffl equation (Pfaffl, 2001) below:

$$\text{Ratio} = (E_{\text{target}}^{(\Delta C_t \text{Target})}) / (E_{\text{reference}}^{(\Delta C_t \text{Reference})})$$

where $E$ is the efficiency of the target (pIgR) and reference (18S) genes (Table 4.1), and $\Delta C_t$ for these genes is the $C_t$ for the control treatment (no virus, no supplemental Se) minus the $C_t$ for the treatment within that trial and age group.
Table 4.2. Analysis of variance table for expression of the polymeric immunoglobulin receptor (pIgR) gene in chicken duodenum, jejunum, ileum and liver at 2, 7 and 16 days p.i. in trials 1 and 2.

<table>
<thead>
<tr>
<th>Source</th>
<th>Duodenum Prob&gt;F</th>
<th>Jejunum Prob&gt;F</th>
<th>Ileum Prob&gt;F</th>
<th>Liver Prob&gt;F</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>TRIAL 1</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>2 Days P.I.</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Virus</td>
<td>0.8286</td>
<td>0.4886</td>
<td>0.0002</td>
<td>0.0037</td>
</tr>
<tr>
<td>Diet</td>
<td>0.0017</td>
<td>0.5623</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Virus*Diet</td>
<td>&lt;.0001</td>
<td>0.1939</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td><strong>7 Days P.I.</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Virus</td>
<td>0.3188</td>
<td>0.0138</td>
<td>0.6296</td>
<td>0.0036</td>
</tr>
<tr>
<td>Diet</td>
<td>0.3371</td>
<td>0.0099</td>
<td>0.0001</td>
<td>0.087</td>
</tr>
<tr>
<td>Virus*Diet</td>
<td>0.1083</td>
<td>0.0007</td>
<td>0.0075</td>
<td>0.0534</td>
</tr>
<tr>
<td><strong>16 Days P.I.</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Virus</td>
<td>&lt;.0001</td>
<td>0.8166</td>
<td>0.0003</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Diet</td>
<td>&lt;.0001</td>
<td>0.0031</td>
<td>0.0549</td>
<td>0.1305</td>
</tr>
<tr>
<td>Virus*Diet</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
<td>0.0026</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td><strong>TRIAL 2</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>2 Days P.I.</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Virus</td>
<td>0.0017</td>
<td>&lt;.0001</td>
<td>0.9459</td>
<td>0.9787</td>
</tr>
<tr>
<td>Diet</td>
<td>&lt;.0001</td>
<td>0.0299</td>
<td>&lt;.0001</td>
<td>0.0031</td>
</tr>
<tr>
<td>Virus*Diet</td>
<td>0.032</td>
<td>0.0435</td>
<td>0.0186</td>
<td>0.0002</td>
</tr>
<tr>
<td><strong>7 Days P.I.</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Virus</td>
<td>0.0032</td>
<td>0.0362</td>
<td>0.0098</td>
<td>0.1055</td>
</tr>
<tr>
<td>Diet</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
<td>0.0788</td>
<td>0.0053</td>
</tr>
<tr>
<td>Virus*Diet</td>
<td>0.0007</td>
<td>0.0146</td>
<td>0.5538</td>
<td>0.0086</td>
</tr>
<tr>
<td><strong>16 Days P.I.</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Virus</td>
<td>0.6936</td>
<td>0.6187</td>
<td>0.5769</td>
<td>0.5616</td>
</tr>
<tr>
<td>Diet</td>
<td>0.2309</td>
<td>0.0003</td>
<td>0.4206</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Virus*Diet</td>
<td>0.0001</td>
<td>0.0008</td>
<td>&lt;.0001</td>
<td>0.0013</td>
</tr>
</tbody>
</table>
Results:

**Feed Efficiency.** There was a significant trial effect ($P \leq 0.05$) for feed efficiency, with birds in trial 1 having an overall feed efficiency of 0.462 and birds in trial 2 having an overall efficiency of 0.575. For trial 1, there was a treatment effect ($P \leq 0.05$) with Selenite-fed chickens having higher feed efficiency than Sel-Plex-fed chickens (Table 2.1). This was independent of reovirus infection. For trial 2, there were no significant differences (Table 2.1). In trial 1, mean feed efficiency followed the trend Selenite>No Se>Sel-Plex-fed chickens in both the non-infected and virus-infected rooms. Both selenite-fed birds and Sel-Plex-fed birds had higher overall feed efficiency than the control-fed (no Se) birds in both the control and infected groups in trial 2.

**Table 4.3.** Mean ± SD feed efficiency (FE) of Control and ARV-challenged chickens in Trials 1 and 2 at 21 days of age.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Diet</th>
<th>Trial 1 FE</th>
<th>Trial 1 FE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>No Se</td>
<td>0.469 $^a$</td>
<td>0.527 $^a$</td>
</tr>
<tr>
<td></td>
<td>Selenite</td>
<td>0.482 $^a$</td>
<td>0.589 $^a$</td>
</tr>
<tr>
<td></td>
<td>Sel-Plex</td>
<td>0.440 $^b$</td>
<td>0.565 $^a$</td>
</tr>
<tr>
<td></td>
<td>±SD</td>
<td>0.030</td>
<td>0.059</td>
</tr>
<tr>
<td>Infected</td>
<td>No Se</td>
<td>0.444 $^b$</td>
<td>0.572 $^a$</td>
</tr>
<tr>
<td></td>
<td>Selenite</td>
<td>0.511 $^a$</td>
<td>0.594 $^a$</td>
</tr>
<tr>
<td></td>
<td>Sel-Plex</td>
<td>0.425 $^b$</td>
<td>0.605 $^a$</td>
</tr>
<tr>
<td></td>
<td>±SD</td>
<td>0.030</td>
<td>0.065</td>
</tr>
</tbody>
</table>

$^a,b$ Within a Trial column, means with unlike superscripts differ significantly ($P \leq 0.05$).

**Body Weights.** All differences in body weights were significant at a level of $p \leq 0.05$. The average body weight for trial 1 chicks at day of hatch was significantly lower than...
trial 2 chicks, so the hatch weights were used as a covariant in the analysis of the body weights at weeks 1, 2 and 3. At week 1, there was a significant trial effect, after taking into account the weights at day of hatch, with trial 2 birds being heavier. Also, selenite-fed chicks were significantly heavier than Sel-Plex fed chicks. At week 2, the same treatment effect existed, but the trial effect was not seen. At week 3, the treatment effect included a significantly greater body weight for selenite-fed chicks than for both the Sel-Plex and control-fed chicks. A significant trial effect at 3 weeks of age was evident with birds in trial 2 being heavier than birds in trial 1.
Table 4.4. Mean ± SD body weights (grams) of Control and Avian Reovirus-challenged chickens in Trials 1 and 2 at hatch, 7, 14, and 21 days of age, and sources of variation.

<table>
<thead>
<tr>
<th>Trial 1</th>
<th>Treatment</th>
<th>Diet</th>
<th>Day of hatch</th>
<th>Week 1</th>
<th>Week 2</th>
<th>Week 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>No Se</td>
<td>41±2</td>
<td>123±13</td>
<td>277±33</td>
<td>497±61</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Selenite</td>
<td>40±3</td>
<td>122±12</td>
<td>269±41</td>
<td>497±84</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sel-Plex</td>
<td>40±3</td>
<td>116±13</td>
<td>251±40</td>
<td>446±83</td>
<td></td>
</tr>
<tr>
<td>Infected</td>
<td>No Se</td>
<td>40±3</td>
<td>117±12</td>
<td>261±34</td>
<td>468±68</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Selenite</td>
<td>41±3</td>
<td>119±13</td>
<td>268±34</td>
<td>491±72</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sel-Plex</td>
<td>40±3</td>
<td>116±15</td>
<td>254±45</td>
<td>452±93</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Trial 2</th>
<th>Treatment</th>
<th>Diet</th>
<th>Day of hatch</th>
<th>Week 1</th>
<th>Week 2</th>
<th>Week 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>No Se</td>
<td>41±3</td>
<td>123±17</td>
<td>265±42</td>
<td>496±98</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Selenite</td>
<td>41±3</td>
<td>127±13</td>
<td>284±48</td>
<td>533±107</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sel-Plex</td>
<td>41±3</td>
<td>119±12</td>
<td>264±44</td>
<td>491±94</td>
<td></td>
</tr>
<tr>
<td>Infected</td>
<td>No Se</td>
<td>41±3</td>
<td>123±16</td>
<td>260±46</td>
<td>491±88</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Selenite</td>
<td>41±2</td>
<td>122±13</td>
<td>274±30</td>
<td>530±59</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sel-Plex</td>
<td>41±3</td>
<td>123±14</td>
<td>257±36</td>
<td>480±81</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Source of Variation</th>
<th>Significance (p value)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Trial</td>
<td>0.023</td>
<td>0.0384</td>
</tr>
<tr>
<td>Diet</td>
<td>N/A</td>
<td>0.0377</td>
</tr>
<tr>
<td>Virus</td>
<td>N/A</td>
<td>NS</td>
</tr>
<tr>
<td>Diet*Virus</td>
<td>N/A</td>
<td>NS</td>
</tr>
<tr>
<td>Day of Hatch Weights</td>
<td>N/A</td>
<td>0.0001</td>
</tr>
</tbody>
</table>

*NS-Not Significant (p value > 0.05)
Duodenum

Figure 4.2. Trial 1 influence of diet and reovirus challenge on gene expression of plgR in the duodenum of chicks at 2 days post-infection. a,b,c Columns in the histogram containing unlike lower case letters differ significantly, p<0.05.
Figure 4.3. Trial 2 influence of diet and reovirus challenge on gene expression of pIgR in the duodenum of chicks at 2 days post-infection. Columns in the histogram containing unlike lower case letters differ significantly, $p \leq 0.05$. 
Figure 4.4. Trial 1 influence of diet and reovirus challenge on gene expression of pIgR in the duodenum of chicks at 7 days post-infection.
Figure 4.5. Trial 2 influence of diet and reovirus challenge on gene expression of pIgR in the duodenum of chicks at 7 days post-infection. a,b,c,d Columns in the histogram containing unlike lower case letters differ significantly, p<0.05.
Figure 4.6. Trial 1 influence of diet and reovirus challenge on gene expression of pIgR in the duodenum of chicks at 16 days post-infection. a,b,c Columns in the histogram containing unlike lower case letters differ significantly, $p \leq 0.05$. 

![Trial 1-Duodenal PIGR Expression at 16 Days P.I.](image)
**Figure 4.7.** Trial 2 influence of diet and reovirus challenge on gene expression of pIgR in the duodenum of chicks at 16 days post-infection. Columns in the histogram containing unlike lower case letters differ significantly, $p \leq 0.05$. 
Figure 4.8. Trial 1 fold changes for duodenal pIgR relative to control room, control feed at each of 2, 7 and 16 days post-infection.
Figure 4.9. Trial 2 fold changes for duodenal plgR relative to control room, control feed at each of 2, 7 and 16 days post-infection.
Duodenum

Two days post-infection. At 2 days p.i., an interaction effect between diet and reovirus challenge existed in both trials 1 and 2 (Figures 4.2 and 4.3, respectively) on the expression of pIgR in the duodenum. In trial 1, these 7 day old birds showed the highest levels of expression in the control group that was not given supplemental Se and in the infected group that was given inorganic Se (Figure 4.2). All other treatment groups had the lower levels and they were not significantly different from one another. In trial 2 (Figure 4.3), this age group contained the highest levels of expression in the organic Se-fed groups, regardless of reovirus infection. The control group birds that were not given supplemental Se actually showed the lowest pIgR expression in this trial.

Seven days post-infection. There were no effects due to diet or reovirus challenge on the expression of pIgR in the duodenum of these 12 day old birds in trial 1 (Figure 4.4). However, out of the infected chicks, it was obvious that expression was slightly increased in birds that had been on the inorganic Se-supplemented diet. In trial 2 (Figure 4.5), the control birds on the inorganic Se-supplemented diet had significantly higher pIgR expression than all other treatment groups at this time. Infected birds on this diet also had increased expression. Both control and challenged chicks, which were given the control diet, showed the lowest levels of pIgR expression. Overall, bird groups that received either form of Se supplementation appeared to have had the higher levels of pIgR expression, regardless of the ARV-CU98 challenge status.

Sixteen days post-infection. Among the 21 day old birds, expression of pIgR in the duodenum showed both similar and different diet and reovirus challenge interaction effects between trials 1 and 2 (Figures 4.6 and 4.7, respectively). In trial 1(Figure 4.6),
the birds that had been infected 16 days earlier showed highest pIgR expression in the
group which was not given supplemental Se, while this group showed one of the lowest
levels of expression in trial 2 (Figure 4.7). The infected birds given inorganic Se,
however, showed some of the highest levels of expression among treatment groups in
both trials at this time. Also, in both trials, the birds that had been infected and were on
the organic Se-supplemented feed showed some of the lowest levels of pIgR expression.

Duodenal Fold Changes. In trial one, the highest fold changes were seen at 16 days p.i
(Figure 4.8). The one data point that stood out from the rest was over 40 times that of the
control birds on the control diet, and it occurred in the infected group on the control diet.
There were high fold changes relative to the control also seen in the infected group on the
inorganic Se-supplemented diet, followed by both Se-supplemented groups in the control
room. The greatest fold change in trial 2 (Figure 4.9) occurred at 7 days p.i. Here, the
inorganic Se-fed control group was over 80 times that of the control birds given the
control diet. This was followed by the control, organic and infected, inorganic groups
which were both over 20 times that of the control birds on the control diet. Due to
different values from the Pfaffl equation for the control room, control feed groups, the
dynamics of the fold change graphs were very different between the two trials. In trial
1(Figure 4.8), the fold changes relative to the control increased between 7 and 16 days
p.i., while in trial 2 (Figure 4.9) they decreased.
Figure 4.10. Trial 1 influence of diet and reovirus challenge on gene expression of pIgR in the jejunum of chicks at 2 days post-infection.
Figure 4.11. Trial 2 influence of diet and reovirus challenge on gene expression of pIgR in the jejunum of chicks at 2 days post-infection. Columns in the histogram containing unlike lower case letters differ significantly, p<0.05.
Figure 4.12. Trial 1 influence of diet and reovirus challenge on gene expression of pIgR in the jejunum of chicks at 7 days post-infection. Columns in the histogram containing unlike lower case letters differ significantly, $p \leq 0.05$. 
Figure 4.13. Trial 2 influence of diet and reovirus challenge on gene expression of pIgR in the jejunum of chicks at 7 days post-infection. Columns in the histogram containing unlike lower case letters differ significantly, p<0.05.
Figure 4.14. Trial 1 influence of diet and reovirus challenge on gene expression of pIgR in the jejunum of chicks at 16 days post-infection. Columns in the histogram containing unlike lower case letters differ significantly, $p \leq 0.05$. 
**Figure 4.15.** Trial 2 influence of diet and reovirus challenge on gene expression of pIgR in the jejunum of chicks at 16 days post-infection. \(^{a,b,c}\)Columns in the histogram containing unlike lower case letters differ significantly, \(p \leq 0.05\).
Figure 4.16. Trial 1 fold changes for jejunal plgR relative to control room, control feed at each of 2, 7 and 16 days post-infection.
Figure 4.17. Trial 2 fold changes for jejunal pIgR relative to control room, control feed at each of 2, 7 and 16 days post-infection.

Jejunum

Two days post-infection. While there were no significant differences in jejunal pIgR expression among treatment groups in trial 1 at 2 days post-infection (Figure 4.10), the birds that had been challenged with ARV-CU98 and given organic Se had increased expression, which was also seen in trial 2 (Figure 4.11). In trial 2, the challenged birds which were given inorganic Se had increased expression.
Seven days post-infection. These 12 day old birds showed the highest pIgR expression in the control group that was given inorganic Se in both trials 1 and 2 (Figures 4.12 and 4.13, respectively). Some of the lowest levels of expression were found to be in the organic Se-fed control and challenged groups in both trials 1 and 2, along with the inorganic Se-fed challenge group in trial 1 (Figure 4.12). Birds that were not given supplemental Se showed low expression levels in comparison to other groups in trial 2 (Figure 4.13).

Sixteen days post-infection. Within the infected group of birds in trials 1 (Figure 4.14) jejunal pIgR was increased in birds not given supplemental Se, while in trial 2 (Figure 4.15), expression of jejunal pIgR at 16 days p.i. was decreased in birds not fed supplemental Se compared to those fed inorganic or organic Se forms. Within the control groups of both trials, the birds given inorganic Se showed the lowest expression. The control group in trial 2 (Figure 4.15) showed the highest expression in birds given the organic Sel-Plex supplementation.

Jejunal fold changes. The highest fold change for the expression of pIgR in the cells of the jejunum for trial 1(Figure 4.16) occurred at only 2 days p.i. and was only just over 2 times that of the control birds on control feed. It occurred in the control group on the inorganic Se supplemented diet. The fold changes for this treatment group dropped throughout the time course of the experiment so that it was the lowest of all fold changes relative to the control at 16 days p.i. All fold changes were low relative to the control by this age. In trial 2 (Figure 4.17), the same group as in trial 1 (Figure 4.16; control group given the inorganic Se supplemented diet) had the highest fold change relative to the control, and this occurred at 7 days p.i. Once again, this group had the lowest fold change relative to the control at 16 days p.i. The control group given organic Se in feed had the highest fold change relative to the control.
Figure 4.18. Trial 1 influence of diet and reovirus challenge on gene expression of pIgR in the ileum of chicks at 2 days post-infection. Columns in the histogram containing unlike lower case letters differ significantly, p≤0.05.
Figure 4.19. Trial 2 influence of diet and reovirus challenge on gene expression of pIgR in the ileum of chicks at 2 days post-infection. Columns in the histogram containing unlike lower case letters differ significantly, p<0.05.
Figure 4.20. Trial 1 influence of diet and reovirus challenge on gene expression of pIgR in the ileum of chicks at 7 days post-infection. Columns in the histogram containing unlike lower case letters differ significantly, $p \leq 0.05$. 

Trial 1-Ileal PlgR Expression at 7 Days P.I.
**Figure 4.21.** Trial 2 influence of diet and reovirus challenge on gene expression of pIgR in the ileum of chicks at 7 days post-infection. a,b Columns in the histogram containing unlike lower case letters differ significantly, p≤0.05.
Figure 4.22. Trial 1 influence of diet and reovirus challenge on gene expression of pIgR in the ileum of chicks at 16 days post-infection. $a,b,c,d$ Columns in the histogram containing unlike lower case letters differ significantly, $p \leq 0.05$. 

\[ \frac{1}{(Ct \text{ pIgR} - Ct \text{ 18S})} \]
**Figure 4.23.** Trial 2 influence of diet and reovirus challenge on gene expression of pIgR in the ileum of chicks at 16 days post-infection. a,b,c,d Columns in the histogram containing unlike lower case letters differ significantly, p<0.05.
Figure 4.24. Trial 1 fold changes for ileal pIgR relative to control room, control feed at each of 2, 7 and 16 days post-infection.
Figure 4.25. Trial 2 fold changes for ileal pIgR relative to control room, control feed at each of 2, 7 and 16 days post-infection.

Ileum:

Two days post-infection. The infected birds fed the control feed in trials 1 and 2 (Figures 4.18 and 4.19, respectively) had some of the lowest expressions of pIgR in the ileum at 2 days p.i. Significantly increased expression occurred in birds fed selenium among infected birds in trial 1(Figure 4.18) and among both control and infected birds in
trial 2 (Figure 4.19). Often, the birds fed inorganic Se showed higher levels than those fed organic Se within the same group.

**Seven days post-infection.** In trial 1 at 7 days p.i. (Figure 4.20), there was a diet X reovirus interaction for ileal pIgR expression, but only a reovirus effect (higher expression in challenged group) in trial 2 (Figure 4.21). In trial 1, birds given selenium had increased pIgR expression compared to birds on the control diet. The trend in both control and challenged groups here follows: no selenium < inorganic Se < organic Se.

**Sixteen days post-infection.** While there were diet and reovirus interaction effects in the ileal pIgR expression of three week old birds in both trials (Figures 4.22 and 4.23), trends were not found here. Sometimes Se supplementation appeared to have resulted in increased levels, and sometimes the control diet appeared to have done this.

**Ileal fold changes.** The dynamics of the fold change graphs for the ileal pIgR are quite different. For the trial 1 (Figure 4.24) fold changes relative to the control room, control feed group, treatment groups increased between 2 and 7 days p.i. and decreased from 7 to 16 days p.i. This was opposite in trial 2 (Figure 4.25). Highest relative fold change in trial 1 occurred with the control group that was given organic Se. This group was highest at 7 days p.i., being more than 80 times that of the control. In trial 2, the control group that was given inorganic Se had the highest relative fold change both at 2 and 16 days p.i. The infected group that was not given supplemental Se had the lowest relative fold change at 2 days p.i.
Figure 4.26. Trial 1 influence of diet and reovirus challenge on gene expression of pIgR in the liver of chicks at 2 days post-infection. a,b,c,d Columns in the histogram containing unlike lower case letters differ significantly, p<0.05.
Figure 4.27. Trial 2 influence of diet and reovirus challenge on gene expression of pIgR in the liver of chicks at 2 days post-infection. $^{a,b,c}$ Columns in the histogram containing unlike lower case letters differ significantly, $p<0.05$. 
Figure 4.28. Trial 1 influence of diet and reovirus challenge on gene expression of pIgR in the liver of chicks at 7 days post-infection. a,b Columns in the histogram containing unlike lower case letters differ significantly, $p \leq 0.05$. 
Figure 4.29. Trial 2 influence of diet and reovirus challenge on gene expression of pIgR in the liver of chicks at 7 days post-infection. Columns in the histogram containing unlike lower case letters differ significantly, p≤0.05.
Figure 4.30. Trial 1 influence of diet and reovirus challenge on gene expression of pIgR in the liver of chicks at 16 days post-infection. a,b,c,d Columns in the histogram containing unlike lower case letters differ significantly, p≤0.05.
Figure 4.31. Trial 2 influence of diet and reovirus challenge on gene expression of pIgR in the liver of chicks at 16 days post-infection. Columns in the histogram containing unlike lower case letters differ significantly, p≤0.05.
Figure 4.32. Trial 1 fold changes for liver plgR relative to control room, control feed at each of 2, 7 and 16 days post-infection.
Figure 4.33. Trial 2 fold changes for liver pIgR relative to control room, control feed at each of 2, 7 and 16 days post-infection.

Liver

Two days post-infection. Among the 7 day old chicks, there was no significant difference between control and infected group expression levels for pIgR in either trial 1 or trial 2 (Figures 4.26 and 4.27, respectively). In the liver cells, pIgR expression appeared elevated in the ARV challenged, Se-supplemented groups in trial 1 (Figure
4.26), but lower in these groups compared to the control group in trial 2 (Figure 4.27). Both times, the birds fed inorganic Se had higher levels than those fed organic Se. The organic Se-supplemented group in trial one which did not receive the virus challenge had significantly higher pIgR expression than the other two dietary treatment groups but similar expression to the challenged, organic Se-supplemented group in this trial.

**Seven days post-infection.** At 7 days p.i., the now 12 day old birds sometimes were showing increased expression in the ARV-CU98 challenged group (trial 1; Figure 4.28). While there were no significant treatment differences within infected and control groups in this trial, the birds given organic Se supplementation had increased levels of pIgR expression. In trial 2 (Figure 4.29), the highest expression of pIgR was seen in control, inorganic Se-fed birds, and infected birds either not fed a Se supplement or given organic Se.

**Sixteen days post-infection.** In trial 1 (Figure 4.30), birds that were now 21 days old and had not been challenged with ARV showed increased expression of pIgR in the group given a Se supplement in the organic form, and showed decreased expression of pIgR in the group given a Se supplement in the inorganic form. Among infected birds, the groups given the control diet or inorganic Se supplementation showed significantly higher expression. In trial 2 (Figure 4.31), there were no significant dietary treatment differences in the control group. Among the treatment groups both in trial 1 and 2 (Figures 4.30 and 4.31, respectively) that had been challenged with the virus, there was a significantly lower level of pIgR expression found in cells of birds that had been fed diets containing the organic Se supplementation. In trial 2 (Figure 4.31), the infected group that was not given a Se supplement showed increased expression of pIgR.

**Liver fold changes.** Relative fold changes were very different between trials and among treatment groups. In trial 1 (Figure 4.32), the only time that these increased was from 2
to 7 days p.i. follow by a drop between 7 and 16 days p.i. in the infected group that was given Sel-Plex. This group had the highest relative fold change at 7 days p.i., with it being over 4 times that of the control. At day 16, the infected, inorganic Se-fed group had the highest relative fold change, followed by the control, organic Se-fed group and then the infected, control-fed group. In trial 2 (Figure 4.33), once again, the dynamics of the infected, organic-fed group was that it increased from 2 to 7 days p.i. followed by a drop between 7 and 16 days p.i. The control, organic Se-fed group also followed this response pattern, but to a greater magnitude of change. The highest relative fold change at 2 days p.i. was in the infected, control-fed group, being 5 times that of the control group. At 16 days p.i. it was also highest, being 3 times that of the control.

Discussion and Conclusions

Since the small intestine is the most important site for ARV infection (Kibenge et al., 1985; Jones et al., 1989), and nutrients such as selenium could play a role in the chicken’s ability to resist or regain function due to ARV infection (Edens et al., 2007ab), the effects of reovirus challenge and different dietary selenium sources on the immune response, characterized by sIgA (chapter 3), by way of the polymeric immunoglobulin receptor were studied here.

The polymeric immunoglobulin receptor (pIgR) transports IgA which can prevent the adherence and invasion of the epithelial layer by pathogens, neutralize viruses and bacteria intracellularly during pIgR-mediated transcytosis, and transport immune complexes and pathogens coated in pIgA from the basolateral to the apical surface of epithelial cells (Kaetzel, 2001). This transcytosis is likely due to the modification of pIgR after its translation, and also due to an intracellular signal such as protein-tyrosine kinase (Giffroy, 2001). pIgR is expressed by most epithelial cells lining the secretory
epithelial surfaces and the exocrine glands (Giffroy et al., 2001). Factors that influence pIgR may therefore affect mucosal immunity since it plays a role in mucosal host defense. Its expression in epithelial cells is increased by TNF-α, IL1β, and IL4 (Pal et al., 2005). Double stranded RNA, such as reoviruses, may result in up-regulation of the receptor (Schneeman et al., 2005). Receptors may be cleaved at the apical surface even if they are not occupied by IgA, resulting in the same part of pIgR being released as free SC (Brandtzaeg and Johansen, 2001). Free SC may act as a non-specific microbial scavenger. When it associates with specific pIgA, it can clear these pathogens more efficiently. When SC is free, it is susceptible to proteolytic degradation and this may limit its efficacy (Phalipon and Corthesy, 2003).

Reovirus alone may up regulate pIgR. A study demonstrated that reovirus up-regulates pIgR expression in the cell line HT-29, and this was suggested to be independent of virus replication but dependent upon binding of the reovirus to its cellular receptors and endosomal acidification leading to uncoating of the virus. Possibly, degradation in the endosomes leads to free viral dsRNA which interacts with TLR-3 to induce signals that lead to up-regulation of pIgR. Intestinal epithelial cells and liver endocrine cells, therefore, might up-regulate the pIgR expression following exposure to enteric virus by altering cell-signaling pathways that control pIgR expression. Double stranded RNA may trigger cellular responses leading to the activation of NFκB via a calpain-mediated pathway, followed by increased transcription of pIgR. The virus-induced up-regulation of pIgR may be an innate host-defense mechanism against mucosal pathogens (Pal et al., 2005; Schneeman et al., 2005).

In this investigation, when 4 tissues were analyzed for pIgR expression, several trends among the dietary and virus treatment groups were found. In the duodenal cells, increased expression occurred at 2 and 16 days p.i. in the infected birds which had been given an inorganic selenium supplement. In the jejunal cells at 2 days p.i., the birds that had been challenged with ARV-CU98 and given organic Se had increased expression of
pIgR in both trials. Five days later, highest pIgR expression in jejunal cells occurred in the control group that was given inorganic Se in both trials. Some of the lowest levels of expression were found to be in the organic Se-fed control and challenged groups in both trials. Ileal pIgR expression was increased overall in birds that received a form of Se supplementation by 2 days p.i. Among the infected groups in both trials, the groups that did not receive supplemental Se had the lowest expression. Inorganic Se-supplementation usually resulted in higher levels of expression than organic Se supplementation. In the liver cells, the birds fed inorganic Se had higher levels than those fed organic Se. At 7 days p.i., increased expression occurred in organic Se-fed groups, but by 16 days p.i., among previously infected birds, the groups given the control diet or inorganic Se supplementation showed significantly higher expression. Those that had been challenged with the virus had a significantly lower amount of pIgR expression among birds that had been fed diets containing the organic Se supplementation. Fold changes reflect the trend in the infected, organic Se-fed group pIgR expression. In both trials, the expression relative to the control increases between 2 and 7 days p.i. and decreases between 7 and 16 days p.i.

The expression of pIgR in intestinal and liver cells among the 6 treatment groups at each of the three post-infection times was quite variable, but one can see some similarities. At 2 days p.i., increased expression was often found in birds that were in infected rooms and in the Se-supplemented dietary treatment groups. Inorganic Se resulted in higher levels of pIgR expression than did organic Se in most of these infected groups, with an exception being found for jejunal cells. At 7 days p.i., once again increased expression was often found in the infected, Se-supplemented groups, and this also was seen several times in the control, inorganic Se-supplemented groups. By 16 days p.i., there were several instances where the birds that had been infected and had been given inorganic Se were showing greater expression of pIgR. Those infected birds
that had been given organic Se were often showing decreased levels of expression at comparable times p.i.

Selenium appeared to result in improved intestinal recovery after an ARV-CU98 enteric reovirus challenge (chapter 2). The apparent recovery was somewhat more visible in organic selenium-fed birds as compared to birds either not given supplemental selenium or given sodium selenite in their diets. These recovery events did not become evident immediately, but did begin to become manifest about 7 days after the infective challenge when the chickens were 12 days old. Improved villus height:crypts of Liberkuhn depths was the hallmark of facilitated intestinal recovery for ARV-CU98 challenge. Associated with this event, there was also the development of a tendency for sIgA from the liver and the intestine of organic selenium-fed chickens to increase (chapter 3). Evidence of improved intestinal integrity based on villus height:crypt depth ratios along with increases in sIgA from the bile along with intestinal sIgA suggests that these events might be mediated by organic selenium more efficiently than by inorganic sodium selenite. Overall, selenium, in general, had a tendency to increase sIgA levels in the intestine and in the bile (chapter 3). Whether this increase in biliary and intestinal sIgA levels was due to a direct or indirect effect of selenium, especially organic selenium, has not yet been ascertained. By quantifying the expression of the receptor (pIgR), which transports sIgA, in the duodenum, jejunum, ileum and liver tissues, it was suggested that selenium was having an influence. Other factors were involved in the the host response to ARV-CU98, and therefore these measurements were not sufficient to evaluate the overall immune status of the bird. Possibly, pIgR expression is elevated when other components of the immune response are lacking. Throughout the infection period, inorganic Se-supplemented birds showed overall higher expression than organic Se-supplemented birds, and this might be due to a stronger immune system (fighting the virus more efficiently) or a weaker immune system (needing to compensate for a weaker
overall response). By 16 days p.i., those infected birds that had been given organic Se were often showing decreased levels of expression.

Not much is known about how the malnourished host may affect the virus’ interaction with the host. Selenium is the ultramicro trace element that seems to play a major role in infections caused by RNA viruses. Deficiency in the nutrient may decrease the cellular and humoral immune function. In this study, it was difficult to make conclusions about the functioning of the immune system at the level of pIgR due to a lack of consistent trends. However, it appears as though selenium supplementation is having an influence on the expression of this receptor, and therefore affecting the response to ARV at the level of sIgA.
REFERENCES


Echevarria, M.G., P.R. Henry, C.B. Ammerman, P.V. Roa and R.D. Miles. 1988a. Estimation of the relative bioavailability of inorganic selenium sources
for poultry. 1. Effect of time and high dietary selenium on tissue selenium uptake. *Poult. Sci.*, 67:1295-1301


Touris-Otero, F. Cortez, S., San Martin, M., Martínez-Costas, J. and Benavente, J. 2004. Avian reovirus morphogenesis occurs within viral factories and
begins with the selective recruitment of \( \sigma_{\text{NS}} \) and \( \lambda_{\text{A}} \) to \( \mu_{\text{NS}} \) inclusions. 


APPENDIX

Table A.1. Diets consisted of either control feed (no supplemented selenium), selenite (inorganic selenium), or Sel-Plex (organic selenium).

<table>
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<th>Control Diet</th>
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<th>Organic Se</th>
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<tr>
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