THE ROLES OF MULTIPLE INFECTIOUS AGENTS IN THE PREDISPOSITION OF TURKEYS TO COLIBACILLOSIS

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(ABSTRACT)

Colibacillosis is considered one of the more costly diseases encountered in the production of market turkeys. It is responsible for a significant amount of mortality in birds between the ages of 6-12 weeks.

Research conducted over the past 5 years has shown that within the Shenandoah Valley production area, multiple primary infectious agents are responsible for the predisposition of turkeys to colibacillosis. These agents were first identified as potential contributors through field case studies. They include hemorrhagic enteritis *(HE)* virus, Newcastle disease virus, and *Bordetella avium*. Further retrospective serologic studies affirmed the role of these primary agents and uncovered the potential involvement of *Mycoplasma meleagridis*.

Trials were conducted to determine the reproducibility of some multiple agent interactions under laboratory conditions. It was found that Newcastle disease virus or *B. avium* infection followed by *HE* virus and *Escherichia coli* challenge produced

clinical colibacillosis.

It is believed that hemorrhagic enteritis virus is the pivotal agent in this process of predisposition. Almost all turkeys are vaccinated for hemorrhagic enteritis in the field. The virulent strains of the virus are known to be immunosuppressive. It is suspected that the vaccine strains are mildly so. Infection with *HE* vaccine virus was shown to cause an increase in CT8+ cells in peripheral blood. These cells are believed to be suppressor T-cells and may account for the reputed immunosuppressive effects of the virus.

Thus, interactions of multiple infectious agents including Newcastle disease virus, *B. avium*, *M. meleagridis*, and *HE* virus appear to be involved in the predisposition of turkeys to secondary *E. coli* infections.

✠ Gloria Deo ✠

Gratias Adamus Domino Deo Nostro
Dignum Et Justum Est

To: Mary Ellen, Rachael, Sarah, Dad,

and Mom.

Your patience and love will always astound me.

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1.1 Introduction

> "If fowls are kept clean, and well sheltered from the wind and wet; are not overfed, and have a due proportion of both soft and green food, with a neverfailing supply of clean water, they will remain free from disease, unless infected by strangers. And when a fowl becomes ill, the best cure in nearly every case is to kill it before it is to bad to be eaten. Only in the case of valuable birds, which people are naturally unwilling to sacrifice, do we recommend much attempt at a cure, and even then only where the disease is so defined and evident that the treatment is sure. To prescribe for a fowl in the dark is one of the most hopeless speculations that can well be." (111)

There are few who deal with poultry today who would not agree with the wisdom expressed in this quotation taken from The Practical Poultry Keeper by Lewis Wright. Although over 100 years old, this passage seems to accurately describe the dilemma facing today's technologically advanced poultry industry.

Since the turn of the century, we have made considerable progress in the areas of poultry genetics, management, nutrition, and health. The explosion of scientific information occurring over the last half century, particularly in the area of disease research, has facilitated exponential growth in the poultry industry. Much of this advancement in understanding has been based on the rudimentary hypothesis of "one disease, one agent" as interpreted from Koch's postulates. For years, this approach has been predictably rewarding, so much so that we are now left with only

a few diseases of unknown etiology. Those that do remain however, have proven to be refractory to standard methods of investigation. This is likely due to the myriad of factors, i.e. infectious, nutritional, environmental, and genetic which potentially influence the health of birds raised in today's large confinement poultry operations. Instead of getting to the "bottom of the disease barrel" it seems that we have begun to enter a new level of disease complexity born out of our advances in production technology. Thus we find ourselves once again faced with the "hopeless speculation" of "prescribing for a fowl in the dark".

Colibacillosis appears to be one of those "dark" diseases which defy simple explanation. To date, it's occurrence in the field and reproduction in the laboratory have been dependably unpredictable, which not only emphasizes its complexity but galvanizes it against dissolution via traditional research methods.

1.2 Colibacillosis - The Terminal Event in a Cascade of Pathology.

As described by Gross (48), *Escherichia coli* infections in poultry may take a variety of forms depending on the predominant locus of infection. These include colibacillosis, coliseptisemia, Hjarre's disease, coligranuloma, peritonitis, salpingitis, synovitis, omphalitis, air sac disease and any other disease condition for which *E. coli* is the primary infectious isolate. The most common and most costly form of the disease observed in commercial chicken and turkey production is the respiratory / septicemic form referred to as colibacillosis (25). In the mid 1980's, annual losses

resulting from colibacillosis in the United States were estimated to be in excess of $100 million for broiler chickens (94) and $40 million for turkeys (78). It would be inappropriate to say that *E. coli* is the only, distinct etiological agent associated with colibacillosis, since it appears that a variety of agents and factors can lead to its development. However, it would be appropriate to say that *E. coli* infection is the terminal event in a cascade of pathological insults and therefore the direct reason for mortality.

E. coli is a gram negative, non-sporeforming, flagellated, bacillus (48). Based on the Ewing classification system, there are over 154 O antigen, 84 K antigen, and 49 H antigen serotypes of *E. coli* listed (48). In the case of poultry, a large majority of isolates obtained from clinical cases of colibacillosis are from serogroups O1, O2, O35, and O78 (38, 89, 102). It is estimated that close to 15.0% of normal, healthy broiler chickens harbor virulent serotypes of *E. coli* in their intestines (62). Therefore fecal contamination from carrier birds is most likely the reason for the high numbers of *E. coli* found in poultry litter, poultry house dust (61), and contaminated water supplies (77).

Virulent and non-virulent isolates have similar biochemical characteristics and drug sensitivity patterns (26, 89). However, there appears to be a distinct physical difference between pathotypes in that virulent forms are piliated (75, 79). Pathogenic serotypes express pili when cultured *in vitro* whereas, avirulent or mildly virulent serotypes are sparsely or non-piliated (11). This is of importance *in vivo*

since pili seem to enhance adherence to tissues like tracheal epithelium (30) and thus enable the establishment of an infection which may progress to lower respiratory disease and septicemia. Virulent strains are better able to resist clearance from the blood by hepatic macrophages (9) but are indeed susceptible to antibody dependant phagocytosis (7, 8). An additional characteristic thought to correlate with virulence is the ability to bind Congo Red dye *in vitro* (19).

In the 1950's, research began to emerge which indicated that respiratory colibacillosis was not solely due to infection with *E. coli* but was actually the result of an interaction of multiple infectious agents (35, 110). It was reported that chickens infected with *Mycoplasma gallisepticum* and infectious bronchitis virus or Newcastle disease virus and subsequently exposed to aerosols containing pathogenic strains of *E. coli* developed lesions consistent with "air sac disease" or colibacillosis (43). In fact, it was determined that Newcastle disease virus or infectious bronchitis virus alone were capable of predisposing birds to *E. coli* infection, with vaccine strains being almost as effective as field strains (42, 44, 101). A window of susceptibility was noted to occur 2-44 days after primary mycoplasmal/viral exposure but only 2-5 days were required for respiratory disease to develop after secondary challenge with *E. coli* (42, 44). Fowl adenoviruses (29), infectious bursal disease virus (112) and coccidia (76) have also been implicated as agents capable of predisposing chickens to respiratory and septicemic *E. coli* infections.

In turkeys, field cases of colibacillosis have been associated with hemorrhagic

enteritis virus infection (104). No overt evidence of hemorrhagic enteritis virus infection was detected other than enlarged spleens. The levels of virus in splenic tissue were too low to be detected on agar gel precipitin test but there were sufficient amounts present to produce infection in poults orally inoculated with homogenized splenic material. The involvement of hemorrhagic enteritis virus in the predisposition of turkeys to colibacillosis was corroborated by a series of laboratory experiments using an avirulent strain of hemorrhagic enteritis virus, and a known pathogenic strain of *E. coli* (O1:K1) (67).

In addition to hemorrhagic enteritis virus, other infectious agents have been implicated in colibacillosis of turkeys. An increased incidence of respiratory *E. coli* infection has been reported in the field subsequent to aerosol vaccination with the La Sota strain of Newcastle disease virus (1). This appears to be associated with viral damage to respiratory epithelium resulting in a decrease in tracheal mucus transport and bacterial clearance from the respiratory tract (37). Infection with *Bordetella avium*, the etiologic agent of turkey coryza, has likewise been shown to increase susceptibility to *E. coli* infection (36) presumably by virtue of the damage it causes to tracheal epithelium (90). Paramyxovirus-2 and *Chlamydia psittaci* have also been isolated from field cases of respiratory disease complicated by superinfection with *E. coli* (6).

It has been suggested that a variety of non-infectious agents might also contribute to the development of respiratory colibacillosis. High concentrations of

gaseous ammonia prevalent in confinement poultry operations have been noted to decrease bacterial clearance from the respiratory tract and thus increase susceptibility to secondary infections (4, 5, 74). Dust in poultry houses may likewise cause respiratory irritation as well as aid in the transport of *E. coli* into the respiratory tract (24). Physiologic stress induced by adverse environmental or social conditions i.e. chilling, overheating, inclement weather, feed changes, food or water deprivation, crowding and disruption of social order (24, 42, 43, 46, 50), in addition to inherent immunological deficiencies imposed by genetic selection for high performance, may all work to reduce resistance to secondary bacterial infections (56).

Subsequent to these predisposing events and challenge with virulent *E. coli*, the pathologic progression of disease can be rapid. The first microscopic lesions visible may actually be remnants of responses to primary agents, i.e. *Mycoplasma gallisepticum*, Newcastle disease virus, etc. These includes hyperplasia of respiratory epithelium with multifocal lymphocytic aggregations and heterophil infiltration. Changes in response to *E. coli* challenge may be seen microscopically as early as 12 hrs post challenge and consist of edema, with heterophil and phagocytic monocyte infiltration. As the pathology evolves, monocyte numbers increase and giant cell formation occurs around areas of necrosis (48). Sepsis is a common sequela. Virulent strains of *E. coli* will readily cause a bacteremia in birds exposed by aerosol (10) or air sac inoculation (86). The development of lesions in a variety of other

organs and tissues follows a logical progression. Post-mortem findings consistent with septicemia often develop (Figure 1), i.e. congestion of parenchymatous organs, hepatomegaly, splenomegaly, thickening and vascular injection of the pericardial sac, fibrinous pericarditis, perihepatitis, peritonitis and airsacculitis (48).

Prevention and control of colibacillosis appears to rest predominantly with the abrogation of primary causes. Although several inactivated vaccines have been developed for use against *E. coli* in poultry (58, 59, 63, 83), none have received widespread acceptance. Vaccination against infectious agents like Newcastle disease virus, infectious bronchitis virus, infectious bursal disease virus, *Bordetella avium* and hemorrhagic enteritis virus (12, 87), when birds are in good health and otherwise unstressed, should and can be helpful. As previously noted however, under adverse conditions some of the same vaccines may result in predisposition to secondary infections. Finally, obtaining birds from mycoplasma free breeding stock, good biosecurity, management, and sanitation are of the utmost importance as they relate to stress and the reduction of infectious agent challenge (48, 87).

Treatment of secondary *E. coli* infections can be accomplished through the use of antimicrobials, but in many cases, isolates obtained from the field are resistant to the more common, economical antibiotics (26).

The key to overcoming colibacillosis as a major disease of poultry resides in an increased understanding of how and why secondary bacterial infections develop. An awareness of mechanisms of immunocompromise and the roles of various

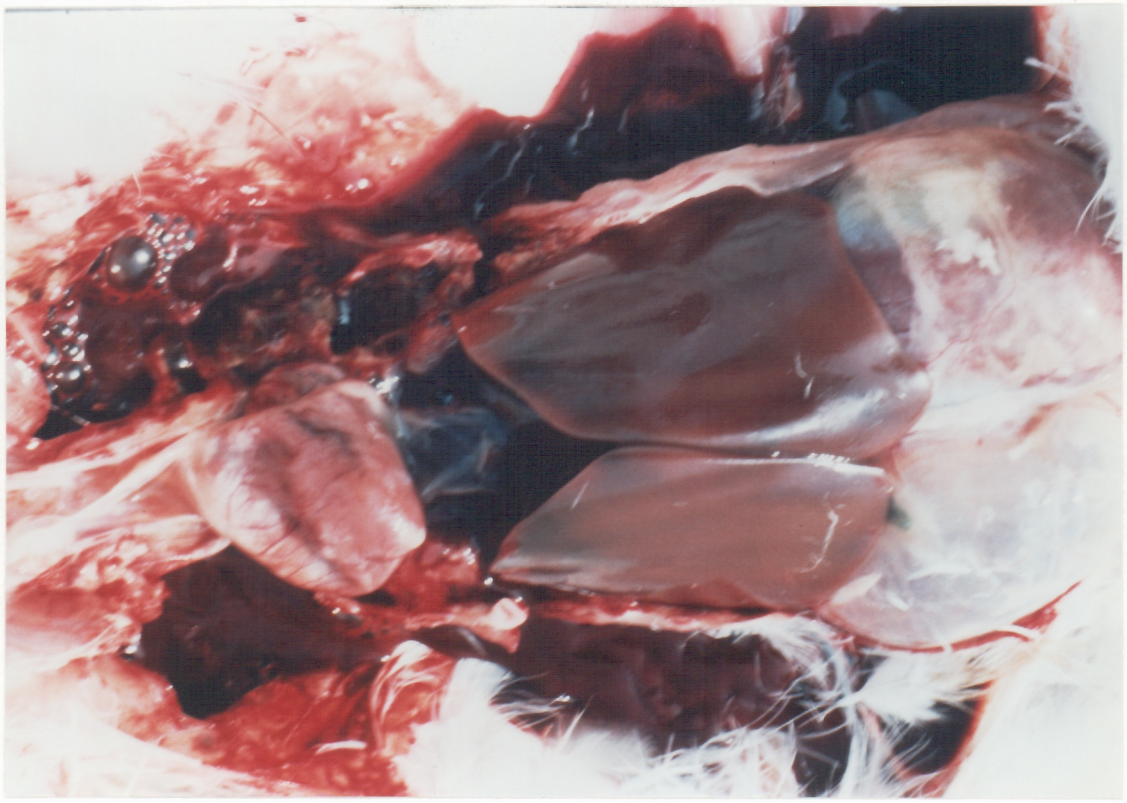

Figure 1. Pericarditis, perihepatitis, and airsaculitis associated with colibacillosis in a turkey.

infectious and noninfectious agents in this process is essential to solving the problem.

1.3 Mechanisms of Immunocompromise

When one attempts to define or describe the mechanisms by which an animal maintains good health, a somewhat nebulous and complicated interface of humoral and cellular, physiologic, and anatomic components is encountered. Similarly, the ways by which these components, as a result of negative influences, fail in their efforts to maintain good health are equally complex.

The most common term used to describe these ill effects is "immunosuppression" which has been defined as "a state of temporary or permanent dysfunction of the immune response resulting from damage to the immune system and leading to increased susceptibility to disease" (32). This definition is rather narrow and relates only to a direct impairment of the immune system and its ability to adaptively respond to disease challenge. It does not consider cases in which there is an increased susceptibility to disease due to failure of primary defense mechanisms, i.e. the respiratory mucociliary apparatus or respiratory macrophage system.

A term which seems to more adequately describe both effects might be "immunocompromise". In other words, damage to a primary anatomical/cellular line of defense may compromise an animals ability to resist infection but may or may not result in the development of disease depending on the capacity of the second line of

defense e.g., the immune system, to respond. Damage to the first line of defense may substantially increase the impact of an infectious agent on the second. Damage to both lines of defense would almost insure increased susceptibility to infection and disease. Immunocompromise should be considered inclusive of immunosuppression. Thus by definition, immunocompromise would be a state of temporary or permanent dysfunction of the immune response resulting from damage to either primary anatomical/cellular defenses or the immune system and leading to increased susceptibility to disease.

When one considers the pressures and circumstances under which we raise poultry commercially, a variety of potential immunocompromising agents present themselves. These may be loosely categorized as: infectious, toxic, nutritional, physical or environmental stressors, and genetic.

1.3.1 Immunocompromise Mediated by Infectious Agents

The effect of infection by a primary agent on the defense mechanisms of a host are obviously related to the site of predilection for that agent. An agent like Newcastle disease virus which replicates in a wide variety of tissues including respiratory epithelium (3) can cause pathological changes that will impair the normal function of these tissues. Destruction of the respiratory epithelium associated with Newcastle infection has been reported to cause a decrease in tracheal mucus transport rate and quantitative clearance of bacteria from the respiratory tract (36).

Another example of damage to primary defenses resulting in secondary disease has been noted in the case of coccidiosis in chickens. The destruction of intestinal mucosa that takes place during the replication of *Eimeria* species is believed to permit local bacterial invasion and subsequent sepsis (76).

As recorded by Specter et.al., the concept that one infectious agent could influence the immune response to another was first alluded to by von Pirquet in 1908 when he noted that human patients infected with measles virus had a reduced delayed hypersensitivity response to tubercle bacillus antigens (103). Since then, extensive advances in the areas of immunology and infectious disease research have permitted us to develop a greater understanding of the mechanisms of superinfection. The role played by viruses in immunosuppression has received particular attention (Figure 2). One of the most obvious ways in which an infectious agent like a virus can cause immunosuppression is by infection of immune cells resulting in their destruction or impairment. Extensive infection and lysis of lymphocytes or macrophage cell types can reduce the population of functional cells to a point at which immunoresponsiveness is diminished. The potential then exists for an imbalance in the immune system due to the destruction of a specific cell population critical to immune function. Examples of this would include human immunodeficiency virus (HIV) which is known to infect a specific population of T-helper lymphocytes (88), and in avian species, Marek's disease virus, a herpes virus infecting both T- and B-lymphocytes (84, 97). Other avian infectious agents

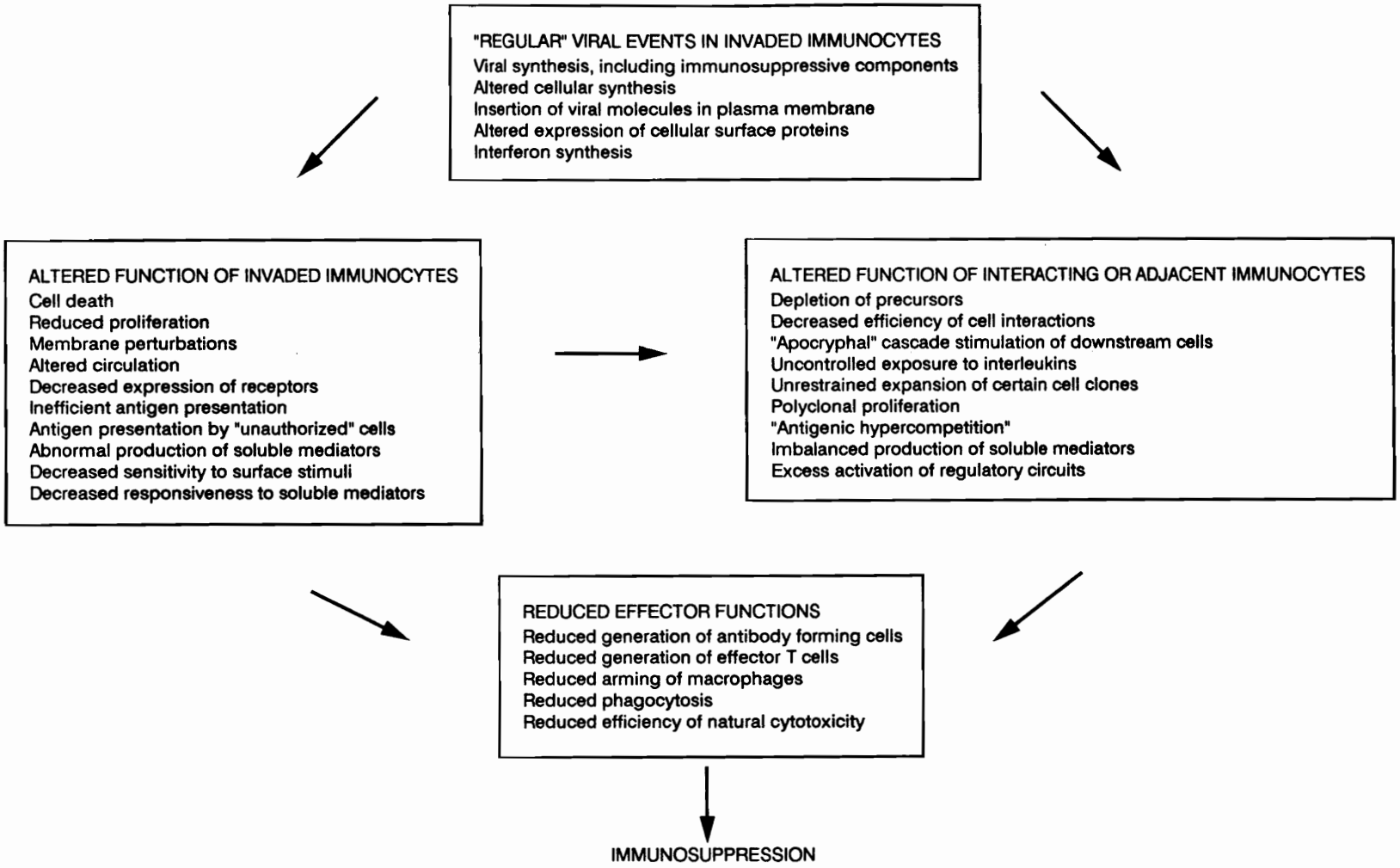

Figure 2. How viral invasion of immunocytes might lead to immunosuppression (103).

known or thought to cause immunosuppression through destruction of immunocytes are infectious bursal disease virus (96), chicken infectious anemia agent (40), and hemorrhagic enteritis virus of turkeys (95). Short of a fully developed cytolytic infection, there exists the possibility of interim impairment of target cell function or long term dysfunction as a result of persistent or latent infections (97).

Additional mechanisms by which viruses can produce immunosuppression include the enhancement of suppressor T-cell activity and the generation of transformed cells which themselves produce excessive levels of suppressor substances, i.e. chicken fetal antigens. Both are demonstrable with Marek's disease virus infection (68, 80). In a similar fashion, viral structural proteins which are produced in abundance in many infections may also have a direct suppressive effect on cells of the immune system. Examples of this would include the penton proteins of human adenoviruses and unidentified structural components of the avian leukosis and sarcoma viruses (21, 34, 109).

Immunosuppression as a direct result of bacterial and parasitic infection is also documented. The production of surface antigens by such organisms, i.e. the glycolipid lepromin, in the case of *Mycobacterium leprae,* facilitates survival of the organism and maintenance of infection through direct suppression of the host immune response (20).

1.3.2 Immunocompromise Mediated by Toxic Substances

Of the various toxic substances capable of suppressing the immune response, none have received more attention than the fungal toxins, collectively known as mycotoxins. Those considered to be of the most importance are aflatoxin, ochratoxin-A, cyclopiazonic acid, and the tricothecenes. These compounds, depending on dosage, are capable of producing pathological effects ranging from acute mortality to reduced performance (85). The immunosuppressive effects of mycotoxins have been recently reviewed with particular emphasis on avian species (27). These may take the form of destruction or impaired function of T- and B-cells, macrophages and effector cells, or reduced antibody production and complement activity. The molecular basis for these effects is believed to be related to the ability of mycotoxins to impair DNA, RNA and/or protein synthesis. Injury at such an elemental level of cellular activity has the potential of undermining the regulation and overall function of the entire immune system.

Other agents such as gaseous ammonia and dust which are present in the atmosphere of poultry houses could also be considered as agents of toxic immunocompromise by virtue of their ability to disrupt the normal function of primary defense mechanisms, i.e. the respiratory mucociliary apparatus (4, 5, 24, 74).

1.3.3 Nutritional Immunocompromise

The quantity and quality of nutrients in feed as well as the pattern of feeding

can affect the immunocompetence of an animal. This is because the immune system is not an autonomous entity but is influenced by other physiological systems (17). The maintenance of metabolic equilibrium in times of physiologic stress requires the redirection of nutrients into compensatory mechanisms. The immune system may be considered one of those mechanisms. Therefore, it is logical to assume that the generation of an immune response places a drain on the nutrient pool. The obverse should also be true.

Total removal of feed and water for 48 hours will cause potentially detrimental alterations in the immune function. In chickens this is seen as a decrease in the relative weights of lymphoid organs (18) and an increase in the ratio of circulating heterophils to lymphocytes (55). However, such changes may represent the indirect effect of perceived stress and a subsequent elevation in serum corticosterone, not a distinct nutritional deficiency. Long term starvation with exhaustion of body reserves undoubtedly results in immunocompromise.

Infection causes distinct changes in protein metabolism (17). New proteins and energy are required to mount a successful immune response. Thus, one might conclude that diets deficient in protein or essential amino acids could produce immunocompromise. Available data on this topic indicate a variable effect based on the type of infectious agent involved. Low levels of dietary protein seem to reduce mortality in birds with coccidiosis (23) and higher levels appear to cause increased mortality with *Salmonella gallinarum* (22) and Newcastle disease virus infections

(64). However, in the case of *E. coli* infection, higher levels of dietary protein seemed to reduce mortality (22).

Vitamins and minerals, when deficient, may depress immune function due to their critical roles in metabolism. Documented examples include deficiencies of vitamins A and E, pantothenic acid, and the minerals sodium, chloride, and selenium. A deficiency in vitamin A may compromise immunity by causing a degeneration of epithelial cells which serve as the primary defense against invasion by infectious agents (93). It can also reduce the antibody response to infection with agents like *Salmonella pullorum* (82) and Newcastle disease virus (28). Diets low in vitamin E and selenium can likewise result in epithelial degeneration (71) and a decrease in humoral response (72). With vitamin E and selenium, the effect seems to be the result of a decrease in free radical scavenging and subsequent cell destruction (92). Deficiencies in pantothenic acid (82), sodium and/or chloride (26) have also been shown to reduce humoral responses.

Interestingly, certain vitamins may actually be immunostimulatory. High levels of vitamins A and E appear to promote a more rapid clearance of *E. coli* from the blood (106). Vitamin C may also be protective but the response appears to require optimal dosing, since the addition of vitamin C to the diet in levels which are too high or low actually potentiates mortality due to colibacillosis (47).

1.3.4 Immunocompromise Mediated by Physical or Environmental Stressors

Changes in the environment, adverse conditions, noxious stimuli, and any other real or perceived threat to homeostasis may be considered stressors. In such cases, an animal must mount an adaptive physiological response to restore balance. What may be considered a stressor in one situation may be of no consequence in another due to the presence of modifying factors (Figure 3), i.e. the severity of stimuli, duration, novelty, and genetic variability among respondees (31, 41). Also important is the perception of stress, that is to say that the amount of change detected between current circumstances and a new set of circumstances will determine how stressful the new circumstances seem (46). Thus, a stressor may have a variable effect on host response and subsequent well-being.

The general adaptation syndrome (GAS) proposed by Hans Seyle as reviewed by Moberg (73), describes a nonspecific response pathway driven by catecholamines, in which the initial perception of stress results in the mounting of a "fight or flight" response. If the stressor persists, the animal begins a "conservation withdrawal reaction" which enables it to resist and cope with the increased physiological demands necessary to maintain homeostasis. Long term stress leads to eventual exhaustion and the development of pathology. The physiologic mechanism behind the GAS involves the hypothalamic-pituitary-adrenal axis which is essentially a cascade of events resulting in the release of glucocorticoids which stimulate gluconeogenesis and thus boost the levels of glucose available for vital

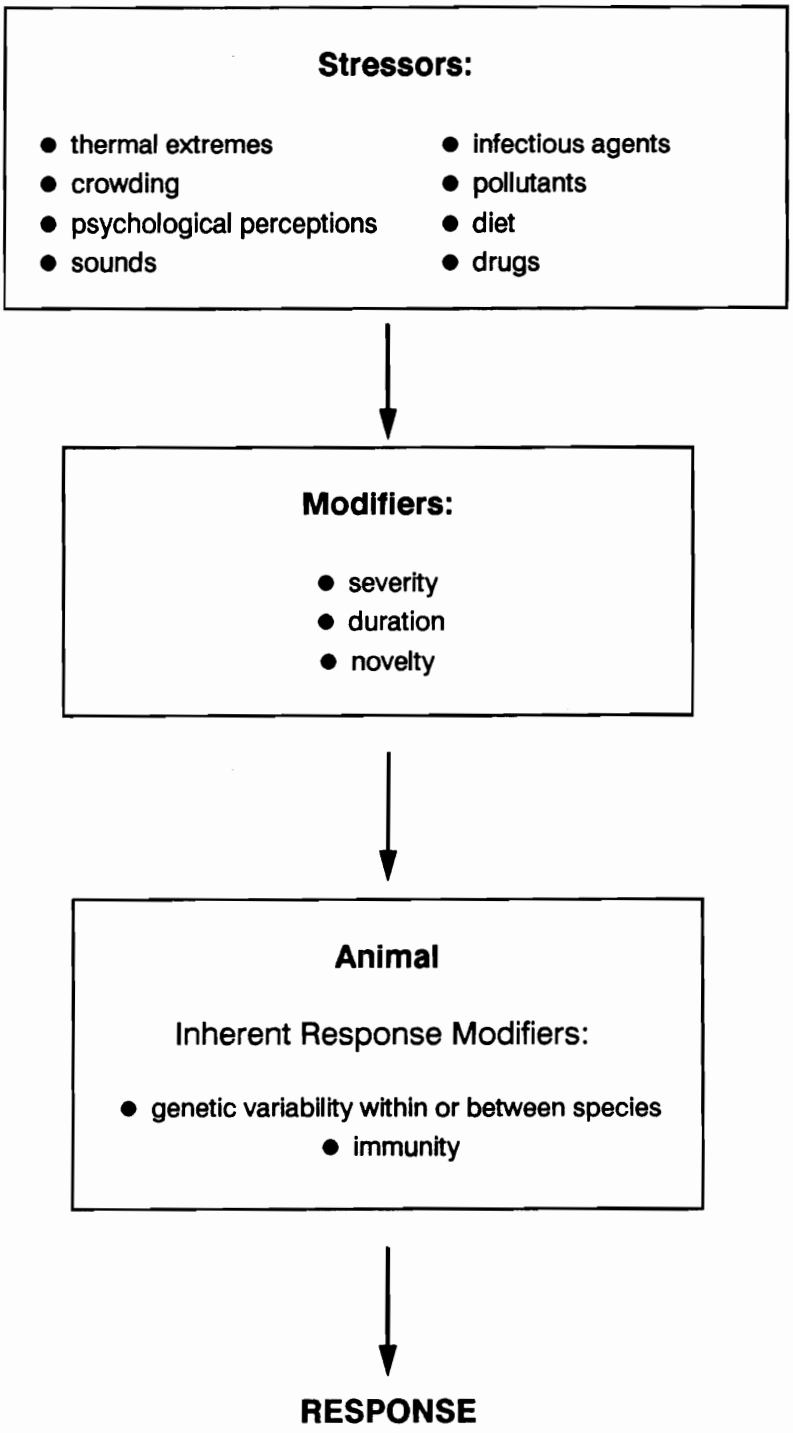

Figure 3. The impact of varied stressors on an animal is influenced by stress modifiers (31). Adapted from Dohms and Metz, 1991 (31).

functions.

As already mentioned, the short term effects of this response are of benefit to the immediate survival of the animal, however there are side effects which have the potential of being detrimental to immune function. Susceptibility to viral and mycoplasmal infections is increased as the level of environmental stress increases (51). It is also documented that exogenous glucocorticoids have the ability to cause peripheral lymphopenia (54) and a reduction in lymphoid tissue mass (39) and humoral immune response (52). Elevated levels of endogenous corticosterone produced through genetic selection have also been shown to correlate with a decrease in lymphocyte blastogenic response and mitogen induced cellular cytotoxicity in Marek's disease virus infected chickens (107).

Despite the fact that stress can be detrimental to immune function, it has been proposed that a certain amount of stress is necessary (53) and can be potentially beneficial with regard to defense against certain disease challenges. It has been demonstrated that resistance to *E. coli* (51), coccidia (45), and northern fowl mites (60) is greater when birds are subjected to high levels of social stress. Thus it can be generally stated that stress may enhance resistance to certain bacterial and parasitic diseases and decrease resistance to viral and mycoplasmal diseases.

1.3.5 Genetic Immunocompromise

The rapid growth of the poultry industry has been due in part to production advances made through genetic selection. Yet genetic improvements in performance have probably had their cost with regard to other factors, in particular immunocompetence. Chickens genetically selected for a low level of antibody response to sheep red blood cells (SRBC) had higher body weights as juveniles, lower weights as adults, better feed efficiency, matured earlier, laid more eggs, and maintained fertility longer than those selected for high antibody response (70, 100). Commercial broilers have been found to respond to SRBC with antibody response similar to those seen in birds deliberately selected for low antibody titers to SRBC (98). Therefore, it is not surprising that birds specifically bred for high body weight have lower antibody titers to SRBC than those bred for low body weight (56). A genetic constitution favoring performance over antibody production appears to result in increased susceptibility to mycoplasmal, viral, coccidial, and ectoparasitic diseases and resistance to certain bacterial diseases (57). This characteristic is believed to be under polygenic control (99).

Genetic control of the avian cellular immune response has been recently reviewed (14). Cells of the avian immune system, not unlike those of mammals have a variety of accessory and cell adhesion molecules on their surfaces. They possess cell surface receptors, i.e. T-cell receptors (TcR) and major histocompatibility complex (MHC) proteins, that are integral components of cellular interactions

necessary for humoral and cell mediated (CMI) immune responses. All of which are under genetic control. Cytokines including the interleukins, growth factors, toxic factors, and interferons which influence cell proliferation and activation as well as the receptors for these molecules are also regulated gene products.

The avian MHC and its relationship to disease resistance has also been recently reviewed (13, 65). In the chicken, the MHC genes and their products are divided into three classes: class I, II, and IV. These are also designated as B-F, B-L, and B-G because of their linkage to the B blood group. The Class III MHC genes and their products as identified in mammals have not been found in chickens. Class I (B-F) gene products are found on the surface of all cells. Class II (B-L) products are found on the surfaces of monocytes, macrophages, B-cells and some T-cells and class IV MHC products, found only in chickens, are expressed on the surface of erythrocytes and a few other cells. The MHC antigens are of importance in the cell to cell interactions which take place during an immune response (66). In the case of helper T and B-cell interactions, the cells must have at least one haplotype of the class II MHC in common for interaction to take place (108). Similar restricted interactions may also be important with regard to the function of cytotoxic T-cells in the destruction of virus infected and transformed cells (69, 91). A wide variety of production and performance traits have been correlated with MHC haplotype (13). Likewise, certain MHC haplotypes have been shown to be correlated with susceptibility to Marek's disease, Rous sarcoma, avian lymphoid leukosis, and

spontaneous autoimmune thyroiditis (2, 15, 16, 91). There are however aspects of the immune response that are not under MHC control as demonstrated by the fact that birds which are genetically divergent with the exception of MHC haplotype have been shown to differ in their susceptibility to disease (81, 105).

1.3.6 Immunocompromise as a Function of Resource Allocation

Experiments involving the genetic selection of birds based on their antibody response to SRBC have led to an understanding of how the various mechanisms of immunocompromise are inter-related. As already indicated, there appears to be a negative correlation between performance and immunocompetence at least with regard to humoral response (56, 57, 100). It has been suggested that the basis for this effect may be a deliberate reallocation of resources towards production and away from immune function (70). Infectious agents, toxins, nutrition, stress and genetics all have the potential to produce immunocompromise. To the degree that any of these place a demand on the resources of the bird, there may be a compensatory redirection of resources away from the immune system. For example, if a specific viral defense is called for, and the drain on resources prolonged, a decrease in resistance to bacterial disease or rate of weight gain might be detected. Similarly, a severe environmental stress placed on an individual which has been selected for improved performance may necessitate the maintenance of homeostasis at the expense of immunoresponsiveness. If there is also a heavy bacterial challenge

in the environment, the eventual overpowering of what is now a multiply compromised immune system could result in the production of disease (49).

1.4 Causation and Disease

Most of today's large poultry companies are regionally based. In fact, a typical production area in the United States may be home to several companies, each with hundreds of houses, all in close proximity to one another. Each individual house contains thousands of chickens or turkeys, all potentially subject to some inherent genetic deficiencies as well as occasional nutritional and environmental insults. Add one or more infectious agents to this pressurized situation and it is easy to understand the devastation and complexity of disease, not to mention the difficulty of establishing a distinct cause.

In his paper entitled "Causation and Disease: The Henle-Koch Postulates Revisited", A.S. Evans has detailed the evolution of causal theory over the last century (33). The original criteria outlined by Jacob Henle and further developed by his pupil Robert Koch, were formally presented in 1890 before an international congress held in Berlin. The postulates are summarized in Table 1. Koch was of the noted opinion that if these criteria could be met then the relationship between "parasite and disease could not be considered accidental". Koch felt that several pathogens and their respective diseases fully met the criteria i.e. anthrax, tuberculosis, erysipelas, tetanus and "almost all diseases which are infectious for

Table 1. The Henle-Koch postulates[1]

1. The parasite occurs in every case of the disease in question and under circumstances which can account for the pathological changes and clinical course of the disease.
2. It occurs in no other disease as a fortuitous and nonpathogenic parasite.
3. After being fully isolated from the body and repeatedly grown in pure culture, it can induce the disease anew.

[1] Based on Rivers' translation as cited by Evans, 1976 (93).

animals". There were other situations however i.e. cholera, where causality was highly suspected but in which there was failure to fulfill the third postulate. Thus, almost at the time of their advent and acceptance, Koch was becoming convinced that rigid adherence to all three postulates was unnecessary, especially in cases where causality was substantially supported by fulfillment of the first two.

In as much as it is human nature to desire clear cut guidelines and neatly packaged explanations, it is the dynamic nature of nature itself to thwart such desires. The exponential increase of our knowledge base in the medical sciences alone has made the desimplification of these criteria inevitable. Thus, the pursuit of a reliable systematic approach to the establishment of causality will inherently be fraught with limitations. A consciousness of these limitations permits us to realistically interpret the information obtained through a criterion based method. Some of the more obvious limitations to the establishment of causality in cases of acute infectious and chronic disease have been described by Evans and are listed in Table 2. Regard for these limitations and reference to the work of predecessors like Rivers, Huebner, W. Henle, Yerushalmy and Palmer permitted Evans to develop a more highly evolved "Unified Concept for Consideration of Causality" (Table 3).

The limitations and criteria outlined by Evans should be helpful in the interpretation of information currently available on colibacillosis. As guidelines they should also serve as a reasonable framework for the construction of experiments

Table 2. Limitations in the pursuit of causality[1]

1. The same pathologic or clinical condition can be produced by different etiologic agents.
2. Causative agents may vary in different geographic areas, in different age groups, or with different patterns of host susceptability.
3. Some diseases require the presence of two or more agents or cofactors acting together to produce the disease.
4. A single agent may produce different clinical and pathological responses in different settings.
5. Any cause or set of causes usually produces a biologic gradient of response which may vary from no observable or even detectable reaction to mild clinical or pathologic changes, to classic and recognized disease.
6. The nature and severity of the host response following exposure to an infectious or non-infectious agent varies with certain host characteristics, of which behavioral patterns, social position, genetics, age, immunologic status, and exposure to other cofactors is important.

[1] Adapted from Evans (32).

Table 3. Criteria for causation: a unified concept[1]

1. Prevalence for the disease should be significantly higher in those exposed to the putative cause than in case controls not so exposed.[2]

2. Exposure to the putative cause should be present more commonly in those with the disease than in controls without the disease when all risk factors are held constant.

3. Incidence of the disease should be significantly higher in those exposed to the putative cause than in those not so exposed as shown in prospective studies.

4. Temporally, disease should follow exposure to the putative agent with a distribution of incubation periods on a bell shaped curve.

5. A spectrum of host responses should follow exposure to the putative agent along a logical biological gradient from mild to severe.

6. A measurable host response following exposure to the putative cause should regularly appear in those lacking this before exposure or should increase in magnitude if present before exposure; this pattern should not occur in animals so exposed.

7. Experimental reproduction of the disease should occur in higher incidence in animals or man appropriately exposed to the putative cause than those not so exposed; this exposure may be experimentally induced in the laboratory, or demonstrated in controlled regulation of natural exposure.

8. Elimination or modification of the putative cause or of the vector carrying it should decrease the incidence of the disease.

9. Prevention or modification of the host's response on exposure to the putative cause should decrease or eliminate the disease.

10. The whole thing should make biological and epidemiological sense.

[1] Adapted from Evans (32).
[2] The putative cause may exist in the external environment or in a defect in host response.

and studies aimed at clarifying our understanding of the disease.

1.5 Conclusion

By all accounts, colibacillosis is an enigmatic and complicated disease which has cost the poultry industry millions of dollars. It will continue to do so unless a full understanding of its pathogenesis is aquired. Accomplishing this will reqiure a thorough epidemiological dissection of the various components which collaboratively predispose poultry to secondary *E. coli* infections. Most notably, the potential interactions of a variety of infectious agents must be investigated. These agents, by virtue of their ability to impair primary anatomical defenses or by their direct suppression of the immune system, are capable of opening the door to infection. The impact of other factors, including nutrition, genetics, and environment, must also be comsidered. With the matrix of interactions thus disclosed, the elimination or control of each individual component can be effected in such a way so as to diffuse colibacillosis before it ever gets started.

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Chapter 2

Preliminary Assessment of the Causes of Colibacillosis in Turkeys Raised in the Shenandoah Valley of Virginia.

2.1 Summary

The purpose of this study was to assess whether certain primary infectious agents could be considered as contributors to the problem of respiratory *Escherichia coli* infections in turkeys raised in the Shenandoah Valley of Virginia. Turkey flocks experiencing clinical signs and mortality consistent with colibacillosis were sampled to ascertain whether in addition to *Escherichia coli*, evidence of previous or concurrent infection with hemorrhagic enteritis virus, Newcastle disease virus, and *Bordetella avium* was present. Results suggested that all three agents were individually or collectively important in the predisposition of turkeys to colibacillosis.

2.2 Introduction

Colibacillosis is believed to be the culmination of a disease process resulting from the interaction of a variety of infectious, toxic, nutritional, environmental, and/or genetic factors (11). Each of these factors may influence immunity (4, 7) and to the degree that the influence is negative, opportunists such as *Escherichia coli* are able to establish themselves and produce disease. Empirically, the specific factors and combinations thereof may vary from flock to flock, and locality to locality. It is therefore important to methodically assess field cases of colibacillosis to determine

causality.

It was decided, because of the potential multiplicity of interactions, that investigations should be limited to infectious agents with a high probability of occurrence in turkeys. The role of hemorrhagic enteritis virus in the predisposition of turkeys to secondary *E. coli* infections has been established (12, 19). Newcastle disease virus (1, 9) and *Bordetella avium*, the organism responsible for turkey coryza, (17, 20) have been similarly implicated. The purpose of this investigation was to determine whether any one or combination of these agents could be incriminated with regard to cases of colibacillosis in turkeys raised in the Shenandoah Valley of Virginia.

2.3 Materials and Methods

Twenty turkey flocks experiencing clinical signs and mortality associated with what appeared to be colibacillosis were identified by company personnel. Each flock was subsequently observed by the investigators and information pertaining to flock age, sex, vaccination status, and mortality was obtained from written records or through personal interviews with farm and company personnel. Ten moribund birds from each affected flock were bled and killed by cervical dislocation. Necropsies were performed on site and samples for diagnostic microbiology obtained. Blood samples were taken from ten additional birds chosen at random in the flock.

E. coli (EC) infection was diagnosed by visual observation of lesions and

confirmed through direct culture of air sacs, pericardial sacs and/or livers. Material was plated on Congo Red medium and incubated as prescribed (2, 5). Isolate identities were confirmed using the API 20E system (API Analab Products, Sherwood Medical, Plainview, NY).

To determine the presence of *B. avium (BA)*, tracheal swabs were plated on Peptone agar (18) and incubated as prescribed. Formal isolate identification was performed using the API Rapid NFT system (API Analab Products, Sherwood Medical, Plainview, NY) and pathogenicity was determined by a modified guinea pig erythrocyte agglutination assay (3, Appendix 1).

Concomitant infection with hemorrhagic enteritis *(HE)* virus was determined based on the presence of viral antigen in spleen using an agar gel immunodiffusion assay (AGID) (8). Seroconversion as a result of *HE* virus infection was also determined by AGID.

Seroconversion resulting from infection with Newcastle disease *(ND)* virus was detected using an enzyme-linked immunosorbent assay (ELISA) (IDEXX Corporation, Portland, ME).

2.4 Results

A summary of historical information is presented in Table 1. Of the 20 turkey flocks sampled, 60.0% were female and 40.0% were male. Thirty-five percent (35%) of the flocks sampled received no vaccinations. Of the 65.0% that were vaccinated,

all received hemorrhagic enteritis vaccine (splenic) at approximately 4 to 5 weeks-of-age, 15.0% received Newcastle disease vaccine (B1-LaSota combination) via the drinking water at 2 weeks-of-age and 15.0% were vaccinated against bordetellosis at 1 day of age except in one case where a second vaccination was given at 3 weeks-of-age (Art-Vax®, American Scientific Laboratories, Omaha, NB). Only one flock was vaccinated against all three diseases. Flocks were approximately 6.8 weeks-of-age when weekly mortality climbed above 0.5%.

Table 2 summarizes the bacteriological information obtained. Approximately 95.0% of the flocks identified by company personnel as exhibiting clinical signs and mortality consistent with colibacillosis were cultured positive for pathogenic *EC* according to the criteria established by Berghoff and Vinal (5). These isolates were mostly obtained from the livers of euthanized, moribund birds and occasionally from air sacs or pericardial sacs. *BA* was isolated from a total of 30.0% of the flocks, ten percent (10.0%) of which represented flocks that had been previously vaccinated with Art-Vax® at the hatchery. The remaining 20.0% were not vaccinated with Art-Vax® and therefore presumably represented actual field infections.

Virological information is summarized in Table 3. Evidence of exposure to *HE* virus was present in 90.0% of the flocks sampled. Of these, 10.0% were vaccinated and in the process of active infection as indicated by the presence of *HE* viral antigen in their spleens. Fifty percent (50.0%) had been vaccinated and were seropositive. Thirty percent (30.0%) of the flocks had not been vaccinated for *HE*

but were seropositive indicating field infection. Serologic evidence of *ND* virus exposure was detected in 85.0% of the flocks sampled. Fifteen percent (15.0%) was presumably attributable to vaccination and 70.0% to field infection.

Table 4 shows the potential agent interactions that were encountered and their frequency of occurrence. Flocks demonstrating *HE* viral antigen in the spleen or serum antibody were considered collectively as *HE* positive. Fifteen percent (15.0%) of the flocks sampled were positive for *HE* alone. Five percent (5.0%) were positive for *ND* alone. Fifty percent (50.0%) of the flocks were positive for both *HE* and *ND*. Five percent (5.0%) were positive for both *ND* and *BA*. And 25.0% were positive for *HE, ND,* and *BA*.

2.5 Discussion

Although information from healthy flocks is necessary for a thorough comparison, the data obtained in this study suggests that *HE, ND,* and *BA* are likely contributors to the problem of secondary *EC* infections in 6 to 12-week-old turkeys.

It appears that hens had a higher probability than toms of experiencing *EC* related mortality between 6 to 12 weeks-of-age, especially since there were fewer hen flocks than tom flocks at risk in the population (46.3% vs 53.7% respectively) during the time of this study. This is consistent with mortality figures more recently obtained from the industry, which show that 62.0% of hen and 57.0% of tom flocks placed during 1991-92 had at least one week between 6 to 12 weeks-of-age where

mortality was 0.5% or greater. No reasonable explanation for the observed sex predilection is available at this time.

The fact that 90.0% of the flocks sampled were positive for *HE*, either serologically or by antigen detection, suggests an important, perhaps pivotal role for this agent. The short period of time between *HE* vaccination at 5 weeks-of-age and the onset of mortality at 6.8 weeks-of-age is also suggestive of an *HE* role. It is well accepted that *HE* vaccination can help reduce losses due to colibacillosis (16) by providing protection against infection with the highly immunosuppressive, virulent strains of *HE* virus (13, 14, 15). Yet, the vaccine strain of *HE* virus, would seem to be mildly immunosuppressive in its own right by virtue of its ability to increase susceptibility to colibacillosis under laboratory conditions (12). The fact that 30.0% of the flocks were seropositive but had no history of vaccination or clinical *HE* implies field exposure to the vaccine strain or some other mild pathotype of the virus.

In this investigation, a large number (85.0%) of the flocks experiencing mortality due to colibacillosis were seropositive for *ND*. Most had not been actively vaccinated. The Shenandoah Valley is densely populated with broiler chickens as well as turkeys. It is currently standard practice to vaccinate all broilers against *ND*. This means that there is potentially a large amount live Newcastle virus present environmentally and the risk of cross exposure for turkeys is great. Exposure to the La Sota strain of *ND* virus which is present in much of the vaccine used, has been

shown experimentally to increase the susceptibility of turkeys to secondary *EC* infections (10).

Concomitant infection with *BA* was detected in approximately 30% of the flocks tested. In 2 of the 6 cases, there was a history of vaccination at the hatchery with Art-Vax®, a temperature sensitive mutant which produces mild, subclinical infections (6). Thus, isolates obtained from these flocks could represent residual vaccine, pathogenic field isolates that have overcome the protection afforded by vaccination or both. Four of the 6 cases were most likely due to field challenge since no history of vaccination existed. *BA* has been shown to increase the susceptibility of turkeys to colibacillosis in the field and under laboratory conditions (9, 20).

In the cases of colibacillosis observed in this survey, it is highly likely that the primary agents identified may have worked in concert to produce mortality. Although each of the agents has the potential to predispose birds to colibacillosis, their combined effect should intuitively be even more devastating. Circumstantially, the observed interactions appear legitimate. Whether they are coincidental and can occur in healthy flocks with minimal impact on mortality is yet to be determined.

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Table 1. Summary of historical data obtained from turkey flocks experiencing mortality due to colibacillosis.

	Number of flocks	**Percent**
Sex		
Male	8/20	40.0%
Female	12/20	60.0%
Vaccination Status		
No vaccinations	7/20	35.0%
Hemorrhagic enteritis	13/20	65.0%
Newcastle disease	3/20	15.0%
Bordetellosis	3/20	15.0%
Mean age at mortality onset	6.8 weeks	

Table 2. Summary of bacteriological data obtained from turkey flocks experiencing mortality due to colibacillosis.

	Number of flocks	**Percent**
Escherichia coli (EC) - total flocks from which Congo Red positive *EC* strains isolated	18/19[1]	94.7%
Bordetella avium (BA)		
Flocks vaccinated with Art-Vax® from which *BA* isolated	2/20	10.0%
Flocks not vaccinated with Art-Vax® from which *BA* isolated	4/20	20.0%
Total flocks from which *BA* isolated	6/20	30.0%

[1] Samples not obtained from 1 flock.

Table 3. Summary of virological data obtained from turkey flocks experiencing mortality due to colibacillosis.

	Number of flocks	**Percent**
Hemorrhagic Enteritis Virus		
Flocks vaccinated for *HE* and positive for *HE* viral antigen	2/20	10.0%
Flocks vaccinated for *HE* and seropositive for *HE*	10/20	50.0%
Flocks not vaccinated for *HE* but positive for *HE* viral antigen	0/20	0.0%
Flocks not vaccinated for *HE* but seropositive for *HE*	6/20	30.0%
Total flocks positive for *HE*	18/20	90.0%
Newcastle Disease Virus		
Flocks vaccinated for *ND* and seropositive	3/20	15.0%
Flocks not vaccinated for *ND* but seropositive	14/20	70.0%
Total flocks positive for ND	17/20	85.0%

Table 4. Various infectious agent combinations found in turkey flocks experiencing mortality due to colibacillosis.

Agent(s)	Number of flocks	Percent
HE	3/20	15.0%
ND	1/20	5.0%
BA	0/20	0.0%
HE x ND	10/20	50.0%
HE x BA	0/20	0.0%
ND x BA	1/20	5.0%
HE x ND x BA	5/20	25.0%

Chapter 3

Immunoexposure Profiles of Turkey Flocks Experiencing Mortality Due To Colibacillosis

3.1 Summary

Turkey flocks which experienced a peak in mortality between 6 and 12 weeks-of-age were compared with healthy flocks using serological and performance data. Statistical analysis of serological data, revealed that primary infection with hemorrhagic enteritis virus, Newcastle disease virus, *Bordetella avium*, and *Mycoplasma meleagridis* in various combinations was probably responsible for the predisposition of turkeys to colibacillosis between 6 to 12 weeks-of-age. An explanation of these disease interactions, their effect on performance and production data are discussed.

3.2 Introduction

In the mid 1980's, annual losses due to *Escherichia coli* infections in turkeys were estimated at $40 million dollars nationwide (9). Much of this problem was believed to be related to infection with hemorrhagic enteritis virus (11). Vaccination of turkeys with the "Virginia Avirulent 1" strain of the virus has been shown to significantly reduce such losses (1, 2). However, despite the widespread use of hemorrhagic enteritis vaccine, colibacillosis is still considered one of the more costly disease problems of 6 to 12-week-old turkeys. Industry mortality figures from

1991-92 have shown that as high as 62% of hen flocks and 57% of tom flocks in this age group have at least 1 week of 0.5% mortality or greater (10). Based on data previously generated by our laboratory, it is believed that this mortality is due to the interactions of several primary infectious agents (Chapter 2). These include hemorrhagic enteritis virus, Newcastle disease virus , and *Bordetella avium*. Any one of these agents has the potential to increase the susceptibility of turkeys to secondary *Escherichia coli* infections (3, 7, 14). Other possible contributors to the problem may include *Mycoplasma gallisepticum* (4), *Mycoplasma synoviae* (12), and *Mycoplasma meleagridis* (13). The purpose of this study was to document, by means of serology, the history of infectious agent exposure, e.g. the immunoexposure profile, of sick versus healthy flocks, and then by statistical comparison incriminate or absolve the aforementioned agents with regard to their roles in the predisposition of turkeys to colibacillosis.

3.3 Materials and Methods

A total of 20 turkey flocks were incorporated in the study. Ten turkey flocks between 6 and 12 weeks-of-age experiencing mortality due to colibacillosis were identified by company personnel. Diagnosis was affirmed by the investigators based on clinical signs and gross necropsy findings. Serum was obtained from 20 randomly selected birds in each flock at the onset of mortality and again 2 weeks later. Ten apparently healthy flocks having a similar distribution of age and sex were also

sampled.

Sera were assayed for the presence of antibodies against *Mycoplasma gallisepticum (MG), Mycoplasma synoviae (MS), and Mycoplasma meleagridis (MM)* by hemagglutination inhibition (HI) test (6) with antigens supplied by the National Veterinary Services Laboratory, U.S.D.A., Ames, IA. Hemorrhagic enteritis virus *(HE)*, Newcastle disease virus *(ND)*, and *Bordetella avium (BA)* serologies were determined by enzyme-linked immunosorbent assay (ELISA). Sera were also tested by ELISA for the presence of antibodies against *Pasteurella multocida (PM)* because of the clinical similarities between fowl cholera and colibacillosis in turkeys. The *HE* ELISA technique developed by van den Hurk (15) was employed. Nunc-immuno Maxisorb 4® (Nunc-Intermed, Denmark) plates were coated with affinity purified *HE* virus hexon supplied by Dr. J.V. van den Hurk, V.I.D.O., Saskatoon, Saskatchewan, Canada. Antigen for the *BA* ELISA was prepared with some modification, according to the method of Marshall et.al. (8). Briefly, field isolate 84-105, provided by Dr. J.K. Skeeles, University of Arkansas, Fayetteville, AR, was grown for 48 hr at 37°C on brain heart infusion agar. Bacteria were harvested in 0.05 M carbonate-bicarbonate buffer (pH 9.6), disrupted by sonication using a Sonic Dismembrator Model 300® (Fisher Scientific Co., Norcross, GA) and diluted to obtain a final absorbance of approximately 1.0 at 540 nm. Nunc-immuno Maxisorb 4® plates were incubated with the sonicate for 16-18 hrs at 4°C and then frozen at -20°C until use. The remainder of the assay was developed *de novo* in our

laboratory. Plates were thawed and washed 3 times with phosphate buffered saline containing 0.05% Tween 20 (PBST). Control sera provided by Dr. J.K. Skeeles, University of Arkansas, Fayetteville, AR, and unknown field samples were diluted 1:500 in PBST with 1.0% fetal bovine serum (Sigma Chemical Co., St. Louis, MO) (PBST+FBS). Sera were added to appropriate wells and incubated for 1.0 hr at room temperature on a rotating shaker (Thermolyne Roto-Mix Model 50800, Thermolyne Corp., Dubuque, IA). Plates were washed and conjugate consisting of peroxidase labeled, goat anti-turkey IgG heavy and light chain (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, MD) diluted 1:1000 with PBST+FBS was added to each well. After 1.0 hr incubation at room temperature, plates were washed and substrate containing 5-aminosalicylic acid (0.08%, w/v) and H_2O_2 (0.005%, v/v), pH 6.0 was added. Plates were incubated for a final 30 min at room temperature and the absorbance at 570nm was read on a Dynatech Model MR650 Autoreader® (Dynatech Corp., Chantilly, VA). Newcastle disease virus and *Pasteurella multocida* ELISAs were performed using commercially available kits (IDEXX Corporation, Portland, ME). Seropositivity was established based on comparisons with predetermined sample positive ratios previously determined for individuals of known exposure.

Flocks with 2 or more sera positive out of 20 on either the first or second bleeding were considered exposed to a given agent. A one-tailed Fisher's exact test for 2 x 2 contingency data was used for statistical evaluation of flock

immunoexposure profiles when data cells contained less than 5 observations. When data cells contained more than 5 observations an uncorrected chi square analysis was used. A chi square analysis for linear trends was performed to determine the cumulative effect of multiple agent exposure. Statistical significance was assigned at $p \leq .05$ (16).

Routine information regarding the performance of each flock was obtained after slaughter. A statistical comparison between sick and healthy flocks was made using a two sample t-test with unpooled standard deviations. Significance was again assigned at $p \leq .05$ (16).

3.4 Results

Flocks were chosen for comparison in this study based on the presence or absence of a peak in mortality between 6 and 12 weeks-of-age. Based on a conservative interpretation of previous data (Chapter 2), those having a weekly mortality above 1.0% were considered sick, those below 1.0% were considered healthy. As previously indicated, mortality was directly attributed to colibacillosis but indirectly assumed to be the result of predisposition by one or several primary agents. The mean percent weekly mortalities for sick vs healthy flocks are plotted over time in Figure 1. The peak mean mortality for sick flocks was 3.63% at approximately 8.0 weeks-of-age. The average onset of mortality appears to have been between 6 and 7 weeks-of-age. The highest mean weekly mortality for healthy

flocks during the 6-12 week period was 0.24% at 6 weeks-of-age. A scattergram of the individual weekly mortalities for each flock is shown in Figure 2. A wide range of mortality figures for sick flocks was especially evident between 6 and 10 weeks-of-age. The highest recorded weekly mortality for a sick flock was 16.6%. The lowest was 1.12%. The highest value recorded for a healthy flock was 0.58% and the lowest was 0.02%.

Sick and healthy flocks were compared on the basis of serologic evidence of exposure to *MG, MS, MM, HE, ND, BA,* and *PM.* An "immunoexposure profile" for each flock was generated. Since none of the flocks examined were positive for *MG* or *MS*, the profiles were inspected for 1, 2, 3, 4, and 5-way agent interactions.

Table 1 shows the relationship between mortality pattern and evidence of exclusive exposure to a single infectious agent. The term "exclusive" is used to describe an effect attributable only to the infectious agent in question. For there to be an exclusive effect, no other agent tested for could be present in the immunoexposure profile. Only 3 flocks out of the 20 examined had profiles positive for one agent alone. These 3 were healthy, had been vaccinated for *HE*, and were seropositive for the same.

There were many instances in which a specific agent was present in a profile in addition to other agents. Thus, the data was retabulated to ascertain whether a single agent could be important in determining mortality pattern regardless of whether other agents were present. This was termed the "inclusive effect" (Table 2).

It appears that *BA* exposure may have contributed in a significant manner ($p \leq .01$) to the observed mortality in sick flocks. A contribution by other agents in the profile to this effect may not have taken place. However, since there were no sick flocks positive for *BA* only, a synergistic effect with other agents is likely to have occurred. Notably, all healthy and sick flocks were positive for *HE*. This meant that in later comparisons, it was statistically unnecessary to include *HE*.

Table 3 begins the evaluation of multiple agent combinations. It should be understood that these are in addition to the *HE* exposure present in all flocks. Thus the only significant 2 agent (plus *HE*) interaction observed was that of *ND x BA*. The inclusive effects of 3 agent combinations, e.g. those flocks which had a specific 3 agent combinations as well as *HE* in their profiles are shown in Table 4. Flocks having *MM x ND x BA* (plus *HE*) present in their profile had significantly higher probability of being sick. There were no flocks which had serologic evidence of exposure to 4 agents plus *HE*, e.g. a 5 agent profile.

The cumulative effect of infectious agent exposure on flock mortality is shown in Table 5. Statistical analysis revealed that as the number of agents in the exposure profile increased so did the likelihood of mortality due to colibacillosis. This trend was highly significant ($p \leq .01$).

The effect of multiple agent exposure on flock performance were unsurprising. Table 6 shows that flock livability, e.g. the percentage of original birds placed that survived until market, was significantly lower in sick flocks and

medication charges were significantly higher when compared with healthy flocks. There were observed numerical differences in feed conversion and production costs which likewise, although not statistically significant, do equate with dollars lost or gained by the individual grower and company.

3.5 Discussion

The data presented in this study clearly suggest the involvement of multiple infectious agents in the predisposition of turkeys to colibacillosis. Sick flocks characteristically had evidence of exposure to *HE, ND, BA,* and/or *MM* in various combinations. The more agents involved, the greater the likelihood of mortality. In all the cases, *HE* exposure was attributable to vaccination. It is suggested that the benefit derived from *HE* vaccination is abrogated by prior or concomitant exposure to the other three agents. The appearance of these agents in the profiles of some healthy flocks may be a reflection of the relative timing of agent exposure in relation to the pivotal agent, *HE.* In other words, *BA, ND,* and/or *MM* exposure if separated from *HE* vaccination (exposure) by a sufficient period of time could conceivably cause seroconversion without provoking a serious interaction with *HE.* The role of multiple agents and critical nature of timing in the course of developing secondary *E. coli* infection has been previously addressed in the study of respiratory disease complex of chickens (4, 5). Whether the sequence of events described for chickens is applicable to turkeys and how a non-respiratory agent such as HE fits into this

pathogenic mechanism is not completely understood. This in addition to the demonstrable economic impact of multiple agent exposures is justification for further research which may more fully define the sequence of events responsible for colibacillosis.

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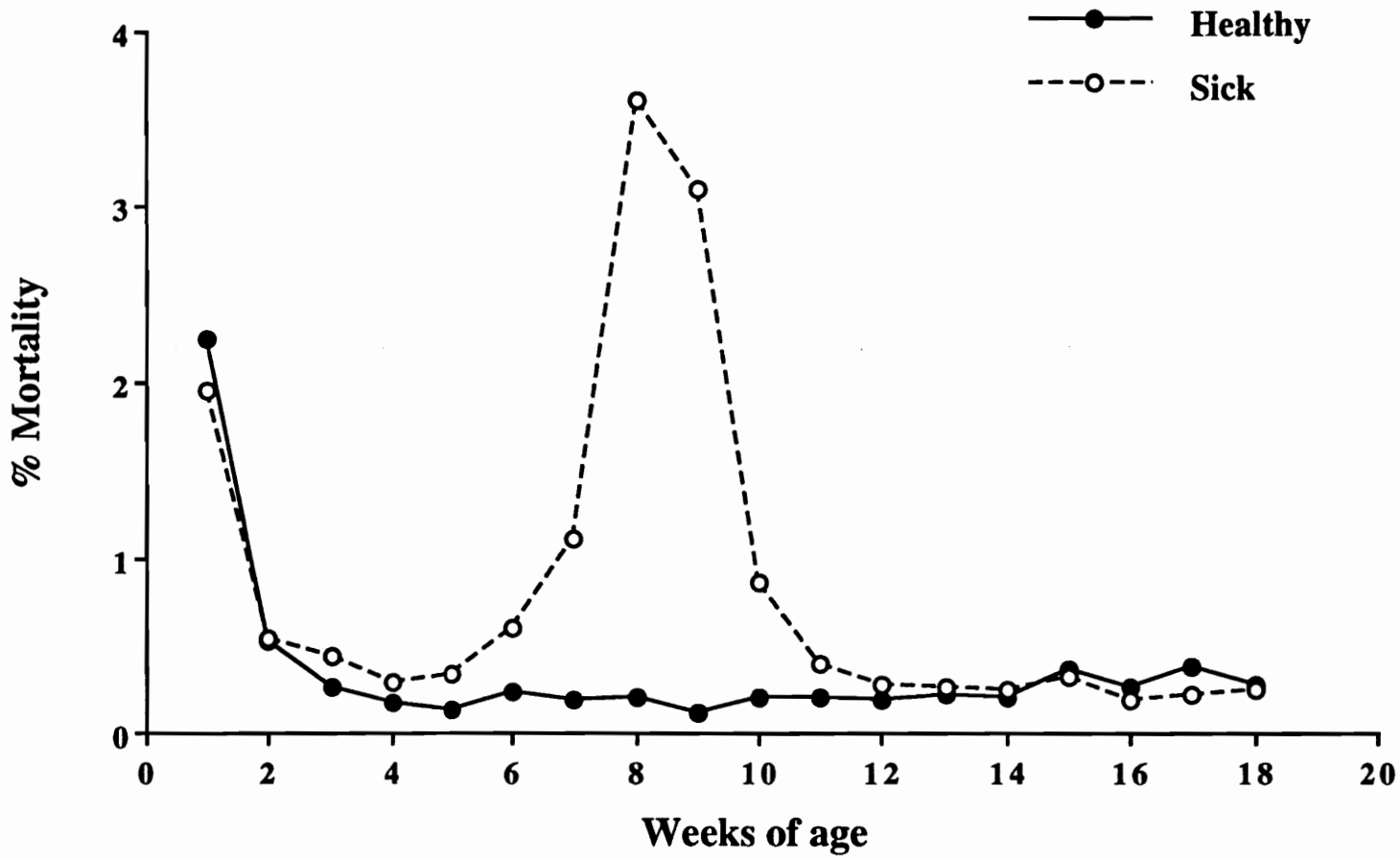

Figure 1. Mean weekly mortality of healthy vs sick flocks.

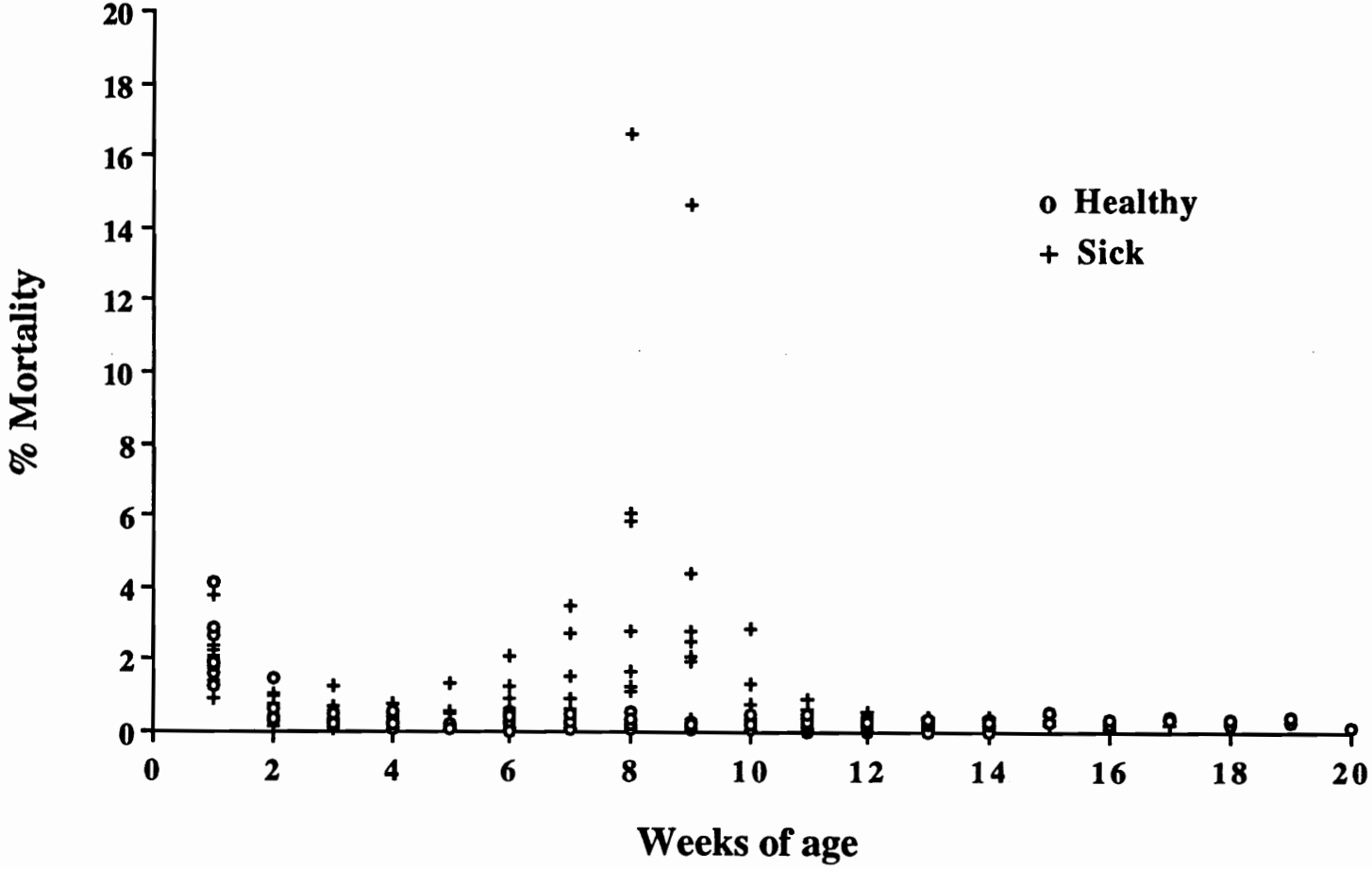

Figure 2. Scattergram of individual weekly mortalities for healthy vs sick flocks.

Table 1. The exclusive effect of various infectious agents on flock mortality pattern.[1]

Agent	Number positive		p-value
	Healthy Flocks	Sick Flocks	
MG	0/10	0/10	--[2]
MS	0/10	0/10	--
MM	0/10	0/10	--
HE	3/10	0/10	.1053
ND	0/10	0/10	--
BA	0/10	0/10	--
PM	0/10	0/10	--

[1] The term exclusive is used to describe an effect attributable only to the infectious agent in question. Therefore, no other agents could be present in the immunoexposure profile.
[2] Unable to analyze because all flocks were negative; -- indicates no effect.

Table 2. The inclusive effect of various infectious agents on flock mortality pattern.[1]

Agent	Number positive		p-value
	Healthy flocks	Sick flocks	
MG	0/10	0/10	--[2]
MS	0/10	0/10	--
MM	3/10	6/10	.1849
HE	10/10	10/10	--[3]
ND	5/10	9/10	.0704
BA	2/10	8/10	.0073
PM	1/10	1/10	.7632

[1] The term inclusive is used to describe an effect potentially attributable to the agent in question. Other agents may or may not have been been present in the total immunoesxposure profile.
[2] Unable to analyze because all flocks were negative; -- indicates no effect.
[3] Unable to analyze because all flocks were positive; -- indicates no effect.

Table 3. The inclusive effect of various two-agent combinations on flock mortality pattern.[1]

Agents	Number positive		p-value
	Healthy flocks	Sick flocks	
MM x ND	2/10	6/10	.0849
MM x BA	1/10	4/10	.1518
MM x PM	1/10	0/10	.5000
ND x BA	0/10	7/10	.0015
ND x PM	1/10	1/10	.7632
BA x PM	0/10	1/10	.5000

[1] The term inclusive is used to describe an effect potentially attributable to the agent combination in question. Other agents may or may not have been present in the immunoexposure profile.

Table 4. The inclusive effect of various three-agent combinations on flock mortality pattern.[1]

Agents	Number positive		p-value
	Healthy flocks	Sick flocks	
MM x ND x BA	0/10	4/10	.0433
MM x ND x PM	1/10	0/10	.5000
ND x BA x PM	0/10	1/10	.5000

[1] The term inclusive is used to describe an effect potentially attributable to the agent combination in question. Other agents may or may not have been present in the immunoexposure profile.

Table 5. The cumulative effect of infectious agent exposure on flock mortality pattern.

Health Status	Number of agents in profile			
	1	2	3	4
Healthy	3/10	4/10	2/10	1/10
Sick	0/10	1/10	4/10	5/10

Chi Square for linear trend = 7.382, p value = .0066

Table 6. A comparison of healthy vs sick flocks with regard to performance data and production costs.

	Mean ± SE	
	Healthy flocks	Sick flocks
Livability[1]	94.37 ± 0.59%	84.90 ± 3.20%
Feed conv. at farm[2]	2.367 ± 0.042	2.475 ± 0.059
Feed conv. after condem.[3]	2.392 ± 0.044	2.503 ± 0.062
Poult cost/lb[4]	$0.0638 ± 0.0025	0.0715 ± 0.0042
Feed cost/lb[5]	0.2459 ± 0.0044	0.2598 ± 0.0057
Medication cost/lb[6]	0.0016 ± 0.0004	0.0042 ± 0.0007
Litter cost/lb[7]	0.0044 ± 0.0004	0.0032 ± 0.0004
Fuel cost/lb[8]	0.0067 ± 0.0014	0.0074 ± 0.0018
Total cost/lb[9]	0.3821 ± 0.0052	0.3965 ± 0.0090

[1] Livability is the percent of birds originally placed that survived until marketing. A Significant difference was observed between healthy and sick flocks at $p < .05$.
[2] Feed conversion at farm is expressed as lbs of feed / lb of body weight sent to market.
[3] Feed conversion after condemnation is expressed as lbs of feed / lb of body weight after subtraction of lbs condemned at processing.
[4] Dollars spent per poult to produce 1 lb at processing.
[5] Dollars spent for feed to produce 1 lb at processing.
[6] Dollars spent for medication to produce 1 lb at processing. A highly significant difference was observed between healthy and sick flocks at $p \leq .01$.
[7] Dollars spent for litter to produce 1 lb at processing.
[8] Dollars spent for fuel to produce 1 lb at processing.
[9] Total dollars spent to produce 1 lb at processing.

Chapter 4

The Production of Colibacillosis in Turkeys by Sequential Exposure to Newcastle Disease Virus or *Bordetella avium*, Hemorrhagic Enteritis Virus, and *Escherichia coli*.

4.1 Summary

Female large white turkeys were intranasaly inoculated with either Newcastle disease virus *(ND)* or *Bordetella avium (BA)* at 4 weeks-of-age. This was followed by oral inoculation with hemorrhagic enteritis virus *(HE)* at 5 weeks and intravenous inoculation with *Escherichia coli (EC)* at 6 weeks. Controls received no treatment, *ND, BA, EC alone* or *HE* followed by *EC*. Turkeys receiving a single agent prior to *EC* challenge did not experience a significant increase in mortality or pericarditis. Those exposed to *ND* or *BA* followed by *HE* and *EC* experienced a significant elevation in mortality and pericarditis. A highly significant, positive correlation between the number of infectious agents encountered during primary exposure and the incidence of colibacillosis after *EC* challenge was demonstrated.

4.2 Introduction

Field data collected over the past 4 years has suggested that mortality associated with colibacillosis in 6 to 12-week-old turkeys is the result of interactions between multiple primary infectious agents. These have been identified as hemorrhagic enteritis virus, Newcastle disease virus, *Bordetella avium* and

Mycoplasma meleagridis (8, Chapters 2 and 3). The ability of each of these agents to increase susceptibility to secondary infection with *Escherichia coli* has been demonstrated under laboratory conditions (5, 7, 10). However, assessments made in the field typically fail to reveal evidence of only one agent. Laboratory replications of combined Newcastle disease virus, *B. avium*, and hemorrhagic enteritis infections have not been previously described. Thus, the purpose of this study was to attempt to duplicate experimentally some of these apparent field interactions.

4.3 Materials and Methods

In each of 2 replicates, 112 4-week-old, commercial Large White female turkeys were assigned to 7 treatment groups each containing 16 birds. All were determined to be seronegative for Newcastle disease *(ND)* virus, *B. avium (BA)*, and hemorrhagic enteritis *(HE)* virus prior to the study by enzyme-linked immunosorbent assay (ELISA). They were housed in Horsfahl-Bauer isolators, 4 per unit, and provided with feed and water *ad libitum*.

ND challenge inoculum was prepared by diluting commercial B1-LaSota vaccine (Clonevac-30-T®, Intervet America Inc., Millsboro, DE) in phosphate buffered saline (PBS) to a final concentration of 10 label doses / ml. This was then aliquoted and frozen at -40°C until use. *B. avium* (ATCC strain # 35086) inoculum was prepared fresh on the day of challenge according to the technique of Arp and

Brooks (1) and was estimated by standard dilution plate method to contain 10^6 colony forming units (cfu) / ml. *HE* challenge inoculum was prepared from splenic material obtained from turkeys infected with the "Virginia Avirulent 1" strain of *HE* virus (3). Spleens were homogenized and diluted in PBS to produce a final inoculum estimated by live bird titration to contain 20 turkey infectious $dose_{50}$ (TID_{50}) / ml. This was also frozen at -40°C until use. *E. coli (EC)* challenge inoculum was prepared by diluting a 6 hour, log phase, brain heart infusion broth culture of strain O1:K1 in PBS. The final inoculum was estimated to contain 10^5 cfu/ml by standard dilution plate methodology.

The protocol sequence is shown in Table 1. On day 0 (4 weeks-of-age), 32 birds comprising treatment groups 4 and 6 were inoculated intranasally with 0.05 ml (0.5 label doses) of the *ND* preparation and 32 birds comprising groups 5 and 7 were inoculated intranasally with 0.05 ml ($5.0x10^4$ cfu) of the *BA* preparation. Seven days later (day 7), birds in groups 3, 6, and 7 were dosed orally with 0.5 ml (10 TID_{50}) of *HE* inoculum. Also at this time, four birds from groups 1, 4 and 5 were euthanized by cervical dislocation and nasal turbinates cultured (2, 9) to determine the presence or absence of *BA* infection. On day 12, 5 days after inoculation with *HE*, 4 birds from groups 2, 3, 6, and 7 were euthanized to determine the presence or absence of *HE* infection. Spleens were removed and tested for *HE* viral antigen by agar gel immunodiffusion (4). On day 14, the birds in groups 2-7 were inoculated via the brachial vein with 0.2 ml ($2x10^4$ cfu) of *E. coli* inoculum. Birds were inspected

every 12 hrs and dead were removed. After 48 hours, the remaining birds were bled, euthanized, and necropsied along with earlier mortalities to determine the incidence of pericarditis. Birds were considered to have developed pericarditis if the pericardium was thickened, opaque, vascularized, and filled with a light yellow or white, fibrinous exudate (6, Figure 1).

The effect of treatment was determined based on the occurrence of mortality or pericarditis. Treatment groups 3-7 were compared with negative controls in group 1 and the *EC* controls in group 2. Data from both trials were combined for the statistical analysis. A one-tailed Fisher's exact test was employed when data cells contained 5 or less observations and an uncorrected chi square was used when cells contained more than 5 observations. A chi square analysis for linear trends was performed on grouped data. Significance was assigned at $p \leq .05$ (11).

4.4 Results

As shown in Table 1, birds in groups 1, 4, and 5, were euthanized 7 days after inoculation with *BA* and *ND*. All birds in the *BA* inoculated group were positive for *BA* on culture; those in the *ND* inoculated and control groups were not. Five days after inoculation with *HE* (day 12), birds were sacrificed in groups 2, 3, 6, and 7. The spleens obtained from birds in the *HE* inoculated groups were positive on AGID for *HE* virus antigen, those from the uninoculated group were negative. Sera obtained on day 16 from all groups (trial 1) were tested for antibodies against *ND*

and *BA*. Birds in groups 4 and 6 were seropositive for *ND* and seronegative for *BA*. Birds in groups 5 and 7 were seropositive for *BA* and seronegative for *ND*. Groups 1, 2, and 3 were seronegative for *ND* and *BA*.

The effect of multiple agent combinations on the incidence of mortality or pericarditis is shown in Table 2. When compared with the negative and *E. coli* controls, there were no significant increases in mortality or pericarditis observed in those groups which received only one primary agent prior to *EC* challenge, e.g. groups 3, 4, and 5. However those that received *ND* or *BA* in addition to *HE* prior to *EC* challenge, e.g. groups 6 and 7, had significantly higher incidences of mortality and pericarditis when compared to both control groups. The chi square analysis for linear trends was applied to data grouped on the basis of the number of agents involved in an interaction. This revealed a highly significant, ($p \leq .01$) positive correlation between the number of infectious agents that birds were exposed to and the occurrence of mortality or pericarditis.

4.5 Discussion

Laboratory replication of the field scenario described in previous studies was successful. Of interest was the fact that primary exposure to *HE, ND*, or *BA* alone did not significantly increase susceptibility to *EC* challenge. The only significant effect seen was with sequential infections of *ND* and *HE* or *BA* and *HE* followed by *EC* challenge. This substantiates data obtained from the field (Chapters 2 and 3),

which suggests that *ND, BA* or both prior to, or concomitant with *HE* vaccination is responsible for elevations in mortality in 6 to 12-week-old turkeys. The *BA x HE* combination appears to be particularly effective in predisposing turkeys to secondary infection with *EC*. The time interval between *ND* or *BA* exposure and *HE* vaccination is probably critical with regard to the development of colibacillosis. A period of 1 week between exposures seems to result in additive or synergistic immunocompromise. However, field observations suggest that the administration of vaccines at least 2 weeks apart may prevent the overlap of effects. Therefore, one could speculate that effective control of colibacillosis might be achieved with more precise timing of *ND, BA,* and *HE* vaccinations, taking into account the risk of field exposure, the waning of passive maternal immunity, and the potential for agent interaction. With this in mind, the determination of optimal vaccination schedules for market turkeys should command a high research priority.

4.6 References

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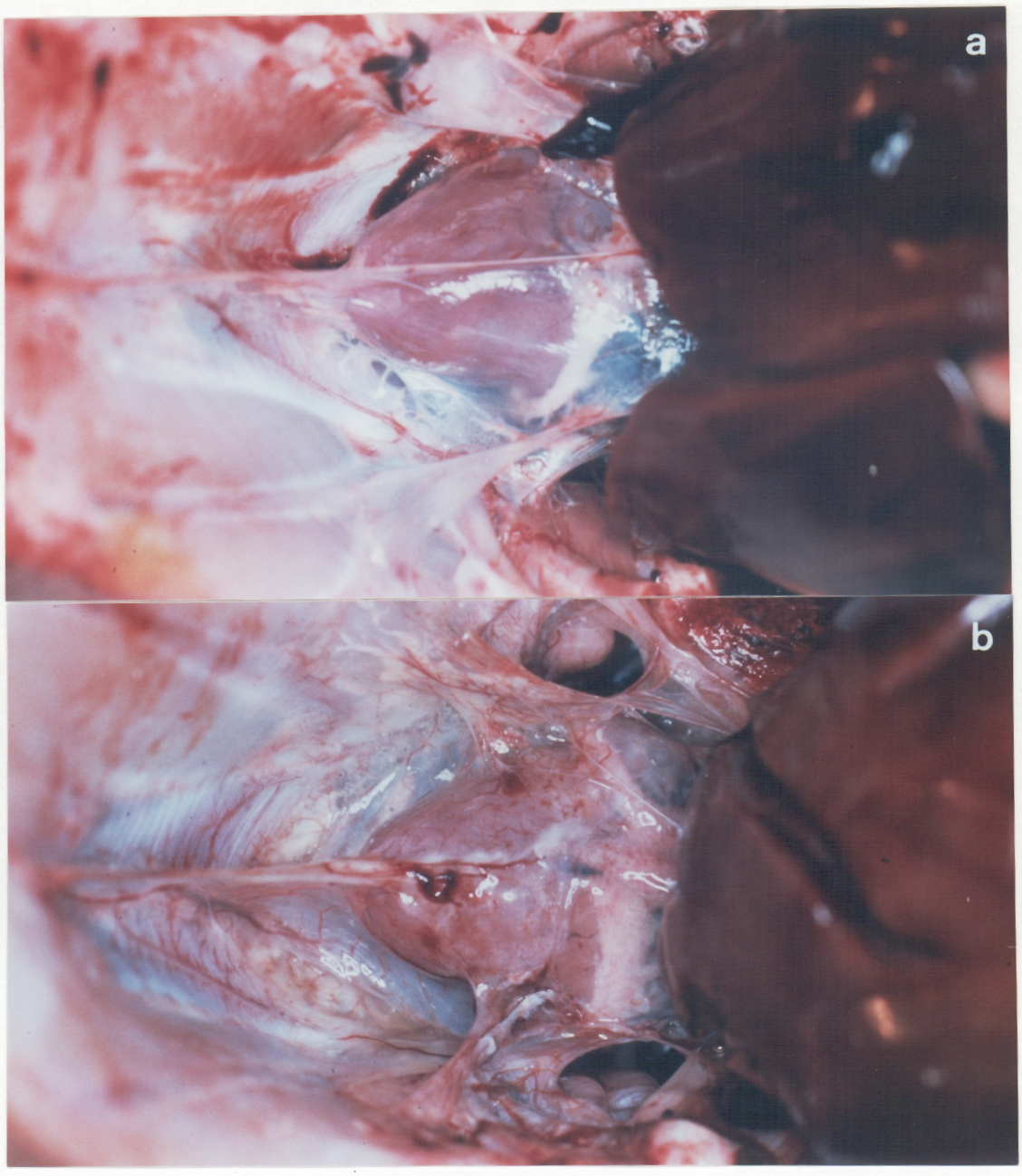

Figure 1. a) Normal (control) turkey heart and pericardium. b) Pericarditis associated with secondary *E. coli* infection. Note edema, opacity, and increased vascularity.

Table 1. Schedule of inoculation, euthanasia, and sample collection.

Treatment group	Day				
	0	7	12	14	16
1. Negative Control	-	(4)[1]	-	-	(12)
2. *EC*	-	-	(4)	*EC*	(12)
3. *HE x EC*	-	*HE*	(4)	*EC*	(12)
4. *ND x EC*	*ND*[2]	(4)	-	*EC*	(12)
5. *BA x EC*	*BA*	(4)	-	*EC*	(12)
6. *ND x HE x EC*	*ND*	*HE*	(4)	*EC*	(12)
7. *BA x HE x EC*	*BA*	*HE*	(4)	*EC*	(12)

[1] ( ), number of birds euthanized, necropsied, and sampled.
[2] *ND, BA, HE,* and *EC* represents inocula administered.

Table 2. The effect of multiple agent combinations on the incidence of mortality and pericarditis.

Treatment	Number affected/Number challenged	
	total[1]	grouped[2]
1. Negative Control	0/24	0/24
2. *EC*	1/24	1/24
3. *HE x EC*	4/24	8/72
4. *ND x EC*	2/24	
5. *BA x EC*	2/24	
6. *ND x HE x EC*	$7/24^{Ab}$	17/48
7. *BA x HE x EC*	$10/24^{AB}$	

[1] Trials 1 and 2 combined. Superscript A denotes a significant difference between treatment groups and the Negative Control group at $p \leq .01$. Superscript b and B denote significant differences between treatment groups and the *E. coli* control group at $p \leq .05$ and $p \leq .01$ respectively.

[2] Treatments grouped according to number of agents in interaction; 0, 1, 2, and 3. Chi square for linear trend = 17.253, p-value = 0.00003, Significance assigned at $p \leq .05$.

Chapter 5

The Effect of Hemorrhagic Enteritis Virus Infection on CT4 and CT8 Antigen Bearing Cells in the Peripheral Blood of Turkeys.

5.1 Summary

Six-week-old turkeys were orally inoculated with a vaccine strain of hemorrhagic enteritis *(HE)* virus. Beginning on the day of inoculation and continuing every other day for the next 20 days, infected birds and an equal number of uninfected controls were bled. Peripheral blood leukocytes were isolated and stained with primary monoclonal antibodies directed against chicken CT4 and CT8 lymphocyte surface antigens followed by a secondary fluorescein isothiocyanate antibody conjugate. Cells were enumerated by flow cytometry and the percentage of CT4+ and CT8+ cells determined. On day 6, Cells were also sorted on the basis of surface antigen and visualized by transmission electron microscopy (TEM).

Two-way analysis of variance revealed a statistically significant elevation in the percentage of bright staining CT8+ cells at 8-10 days post inoculation in infected vs control birds. No statistical differences were detected with regard to CT4+ cells. TEM revealed the cells to be mononuclear, presumably lymphocytic, and devoid of visible intracellular viral particles.

The observed effect of *HE* virus infection on the percentage of bright CT8+ cells in peripheral blood may represent a normal cytolytic T-cell response to viral infection. It may also constitute a suppressor T-cell response which could provide

an explanation for *HE* virus associated immunosuppression.

5.2 Introduction

Hemorrhagic enteritis is an acute disease of turkeys 4 weeks-of-age and older characterized by depression, bloody droppings, and sudden death. Morbidity approaches 100.0% whereas mortality may vary between 1% and 60% with an average of about 10-15% (8). It is caused by a group II avian adenovirus which appears to replicate in reticuloendothelial cells (2, 12, 13).

Due to the extensive use of vaccines, the disease is now only occasionally seen in its classic form. Most if not all of the vaccines currently available utilize a low virulence strain of the virus originally isolated by Domermuth et. al. (9). This strain does not produce overt clinical disease but can apparently have a slight adverse effect on immune function as demonstrated by its ability, under laboratory conditions, to predispose birds to secondary infection with *E. coli* (14, Chapter 4). Field observations have likewise indicated that the vaccine strain may act synergistically with other agents such as Newcastle disease virus, *Bordetella avium*, and *Mycoplasma meleagridis* to predispose turkeys to colibacillosis (Chapters 2 and 3). The exact mechanism of *HE* immunosuppression is unclear. Research has indicated that infection with highly virulent *HE* virus causes a reduction in the T-cell mitogenic response (17). Transient inhibitions of antibody responses to sheep erythrocytes (15) and Newcastle disease virus (16) have also been observed.

In humans, monoclonal antibodies have been used to help more clearly define the immunopathogenesis of several viral infections including cytomegalovirus (CMV), Epstein-Barr virus, (EBV), and human immunodeficiency virus (HIV) (3, 6, 7, 21). Particularly useful have been monoclonal antibodies prepared against the CD4 and CD8 surface antigens of helper and cytotoxic/suppressor T-cells respectively. The avian homologues of these antigens, CT4 and CT8, are believed to be associated with functionally similar lymphocyte subpopulations (4). This study was designed to evaluate the effect of *HE* virus infection on CT4 and CT8 antigen bearing cells in the peripheral blood of turkeys.

5.3 Materials and Methods

Six-week-old, Large White, female turkeys, seronegative for hemorrhagic enteritis were used in 2 experimental trials. The first consisted of 3 groups of 4 birds each. The second consisted of 3 groups of 6 birds each. Groups were given the designation of negative-control, infected, and infected-control. Infected and infected-control groups were housed in battery units at the same location. Negative control groups were housed in batteries at a separate location to prevent cross contamination. All groups were given feed and water *ad libitum*.

On day 0 of the experiment the birds in the infected and infected-control groups were orally inoculated with 10 turkey infectious $dose_{50}$ (TID_{50}) of hemorrhagic enteritis virus (Virginia Avirulent 1 strain) (9). Birds in the negative-control groups

were not inoculated. Five ml of heparinized blood were obtained from the brachial vein of each bird in the infected and negative-control groups. It was then processed as prescribed by Chan et.al. (4) with only a few modifications to the procedure. Briefly, blood was centrifuged at 55 x g for 8 minutes. Buffy coats containing peripheral blood leukocytes (PBLs) were collected and washed in Hanks Balanced Salt Solution (HBSS). 2×10^6 viable PBLs were incubated with mouse monoclonal antibodies prepared against chicken CT4 and CT8 lymphocyte surface antigens obtained from Dr. C.H. Chen, Birmingham, AL. These monoclonals are known to cross react with turkey lymphocytes (5, Appendix 2). Heat inactivated, nonspecific mouse serum was used as a control. After washing with Phosphate Buffered Saline (PBS) containing 1.0% Bovine Serum Albumin and 0.1% sodium azide, cells were incubated with fluorescein isothiocyanate (FITC) conjugated rabbit-anti-mouse IgG (Sigma Chemical Co., St. Louis, MO). Cells were washed again and then fixed in PBS containing 3.0% paraformaldehyde (w/v), pelleted and resuspended in PBS. The entire protocol was repeated on 2, 4, 6, 8, 10, 12, 14, 16, 18, and 20 days post inoculation. Cells were analyzed on the basis of fluorescence intensity using a Coulter EPICS 752 Flow Cytometer.

Birds in the infected-control group were euthanized at 5 days post-inoculation. To confirm infection, the presence of *HE* viral antigen in the spleen was determined by agar gel immunodiffusion (10).

Earlier work has indicated that the highest number of infected cells in

peripheral blood would most likely be found 6 days after oral inoculation (11). Therefore, CT4 and CT8 positive cells obtained on day 6 from the infected and negative-control birds (trial 2) were sorted, collected, and pooled by group. They were then post-fixed in 1.0% osmium tetroxide, dehydrated in ethanol, pelleted and embedded in Polybed 812 (Polysciences, Inc., Warrington, PA). Five micron sections were stained with uranyl acetate followed by lead citrate and examined with a JOEL JEM-100CX II electron microscope to determine cell type. They were also screened for the presence of viral particles (20).

Sera collected on day 20 of the study were assayed for the presence of antibodies against *HE* virus using enzyme-linked immunosorbent assay (ELISA) (23).

Flow cytometric data obtained from both trials was pooled and statistically analyzed using a two-way analysis of variance to determine the effect of *HE* virus infection over time on the percentages of CT4+ and CT8+ cells in peripheral blood (24). The assignment of significance was based on a $p \leq .05$.

5.4 Results

Flow cytometric analysis of turkey PBLs revealed the presence of distinct populations of cells positively stained with the anti-chicken CT4 and CT8 monoclonals (Figures 1a and 1b). After subtraction of the control peak, it was apparent that the CT4+ and CT8+ peaks were bimodal in nature. Thus the

analyses included whole cell populations as well as bright and dim subpopulations.

The percentage of total PBLs staining positively for CT4 antigen over time in negative-control vs infected turkeys is shown in Figure 2. Although numerical differences between infected and negative-control birds were observed 8 days post-inoculation (Figures 1c and 1e), statistical analysis revealed no significance. Likewise, when bright (Figure 3) and dim (Figure 4) subpopulations were analyzed separately, no significant differences were found.

The effect of *HE* virus infection on the percentage of CT8+ PBLs in peripheral blood over time is shown in Figure 5. No significant differences in total CT8+ cells were observed between the infected and negative-control groups. However, when the bright CT8+ subpopulation was analyzed separately, a distinct effect was noted (Figures 1d, 1f, and 6). Infected birds had a higher mean percentage of bright CT8+ cells in peripheral blood on days 8 and 10 when compared to negative-controls. There were no observed differences between negative-control and infected birds noted with regard to the dim CT8+ subpopulation (Figure 7).

PBLs obtained on day 6 of the experiment were sorted on the basis of immunofluorescence, collected and prepared for transmission electron microscopy (TEM) as described. TEM grids were scanned to determine the types of cells present. The majority of the cells appeared to be mononuclear, presumably lymphocytic (Figure 8). An occasional red cell or heterophil was also seen. No

distinct morphological differences were noted between CT4+ and CT8+ cells. One-hundred cells from each of the pooled samples were scrutinized for the presence of viral particles. None were found.

The viability of the *HE* virus inoculum was confirmed by the presence of viral antigen in 10/10 (trials 1 and 2 combined) of the spleens taken from infected-control birds 5 days post-inoculation. Ten out of 10 birds (trials 1 and 2 combined) in the infected groups were found to be seropositive at 20 days post-inoculation. The negative-controls were all seronegative for *HE*.

5.5 Discussion

In general, the response of the avian immune system to viral infection is similar to that of mammalians. Helper T-cells first recognize foreign antigen, in this case viral, on the surface of an antigen presenting cell by means of a T-cell receptor (19, 22). Antigen is presented in association with class II MHC molecules in a restricted fashion, after which the helper T-cells undergo blast transformation and clonal proliferation. Cytokines secreted by helper T-cells further enhance T- and B-cell proliferation as well as stimulate a cell mediated immune response (3). At this point antigen specific, MHC restricted T- and B-cell cooperation takes place which initiates the production of antibodies directed against the foreign antigen (22).

The predominant mechanism of immunity against viral infections is cell mediated. This involves natural killer (NK) cells early in the course of infection and

cytotoxic T-lymphocytes (CTLs) after an infection becomes established. Mammalian CTLs are known to bear CD8 antigen on their surfaces. Similar cells bearing the avian homologue, CT8 have been identified (4). CTLs are capable of recognizing viral antigen in association with class I MHC molecules on the surface an infected cell. This facilitates the lysis of infected cells and the stimulation of intracellular enzymes which degrade viral nucleic acids (1). The cytolytic capabilities of avian CT8+ cells have been demonstrated *in vitro* (3). Interestingly, CT8+ cells are also capable of suppressor activity as evidenced by the fact that compliment mediated elimination of CT8 reactive cells results in an increased *in vitro* response to T-cell growth factor (3). Suppressor T-cells may represent a subpopulation of CT8+ lymphocytes involved in the cell mediated, antiviral immune response which are distinctly different from CTLs.

In the current study, the most notable effect of *HE* virus infection appears to have been an elevation in the percentages of bright CT8+ cells 8-10 days post-inoculation. Similar responses have been noted with human lymphocytopathic viruses such as cytomegalovirus (CMV) (3), Epstein-Barr virus (EBV) (6), and human immunodeficiency virus (HIV) (7). Maximal *HE* virus replication appears to take place in the spleen about 5 days after oral inoculation with *HE* virus (9). The peak cellular response in the spleen which is lymphoid in nature, occurs 6-7 days after inoculation. However, by 8 days post-inoculation, spleen size has returned to normal (9), presumably because cells have entered the peripheral circulation. If

CT8+ lymphocytes were the predominant cell type in the splenic response, this might explain the increase in their numbers observed in peripheral blood 8-10 days post-inoculation.

As previously stated, *HE* virus is believed to be immunosuppressive and probably lymphocytopathic. However, no evidence of viral replication in CT4+ or CT8+ cells was detected. Although only a proportionately small number of cells was visualized, immunosuppression due to massive cytolytic infection of these cells seems unlikely. Thus, other mechanisms may be responsible for the *in vitro* depression of T-cell blastogenesis and antibody production reported in the literature (15, 16, 17).

CD8 (CT8) antigen appears to be present on the surfaces of T-cells with suppressor activity as well as those that have cytolytic capabilities (3, 18). In humans, an increase in the number of CD8+ T-cells with suppressor activity has been reported in peripheral blood during the acute phases of CMV (3) and EBV (6) infection. A temporal association between elevations in absolute numbers of CD8+ cells in peripheral blood and a decrease in the level of HIV antigen has also been observed (7). It has been suggested in the case of HIV infection, that these CD8+ cellular responses may reflect attempts to suppress the viral antigen expression (7). Yet, with EBV (21) and CMV (3), the increase in CD8+ cells has been associated with a decrease in lymphocyte proliferative responses to mitogens and antigens. The combination of these effects seems counterproductive but perhaps the latter is actually a negative side effect of the former peculiar to lymphocytopathic viral

infections. If *HE* virus is indeed lymphocytopathic, then the immunosuppression observed with infection may be the result of an elevation of CT8+ cells intended for the purpose of repressing viral replication but adversely accompanied by a transient depression in general immunoresponsiveness.

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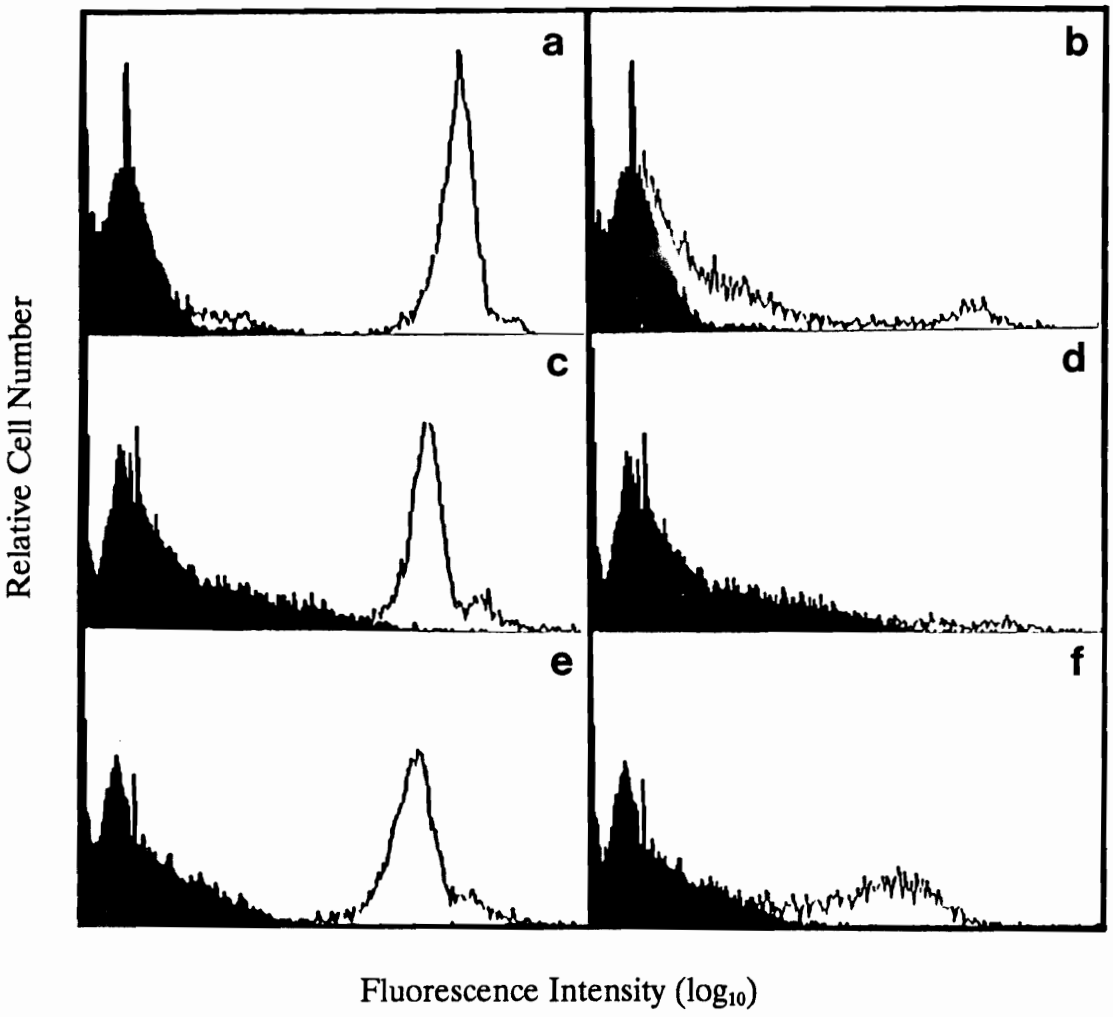

Figure 1. Relative cell number vs fluorescence intensity (log_{10}) of PBLs stained for CT4 and CT8 surface antigen. a) Representative analysis of PBLs obtained from either the negative-control or infected group on day 0. Cells were stained with mouse-anti-CT4 followed by FITC-conjugated rabbit-anti-mouse IgG (CT4+). Dark peaks represent non-specific mouse sera controls. b) Representative analysis of PBLs obtained from either the negative-control or infected group on day 0. Cells were stained with mouse-anti-CT8 followed by FITC conjugated rabbit-anti-mouse IgG (CT8+). c) Representative analysis of CT4+ PBLs from the negative-control group 8 days post-inoculation. d) Representative analysis of CT8+ PBLs from the control group 8 days post-inoculation. e) Representative analysis of CT4+ PBLs from the infected group 8 days post-inoculation. f) Representative analysis of CT8+ PBLs from the infected group 8 days post-inoculation.

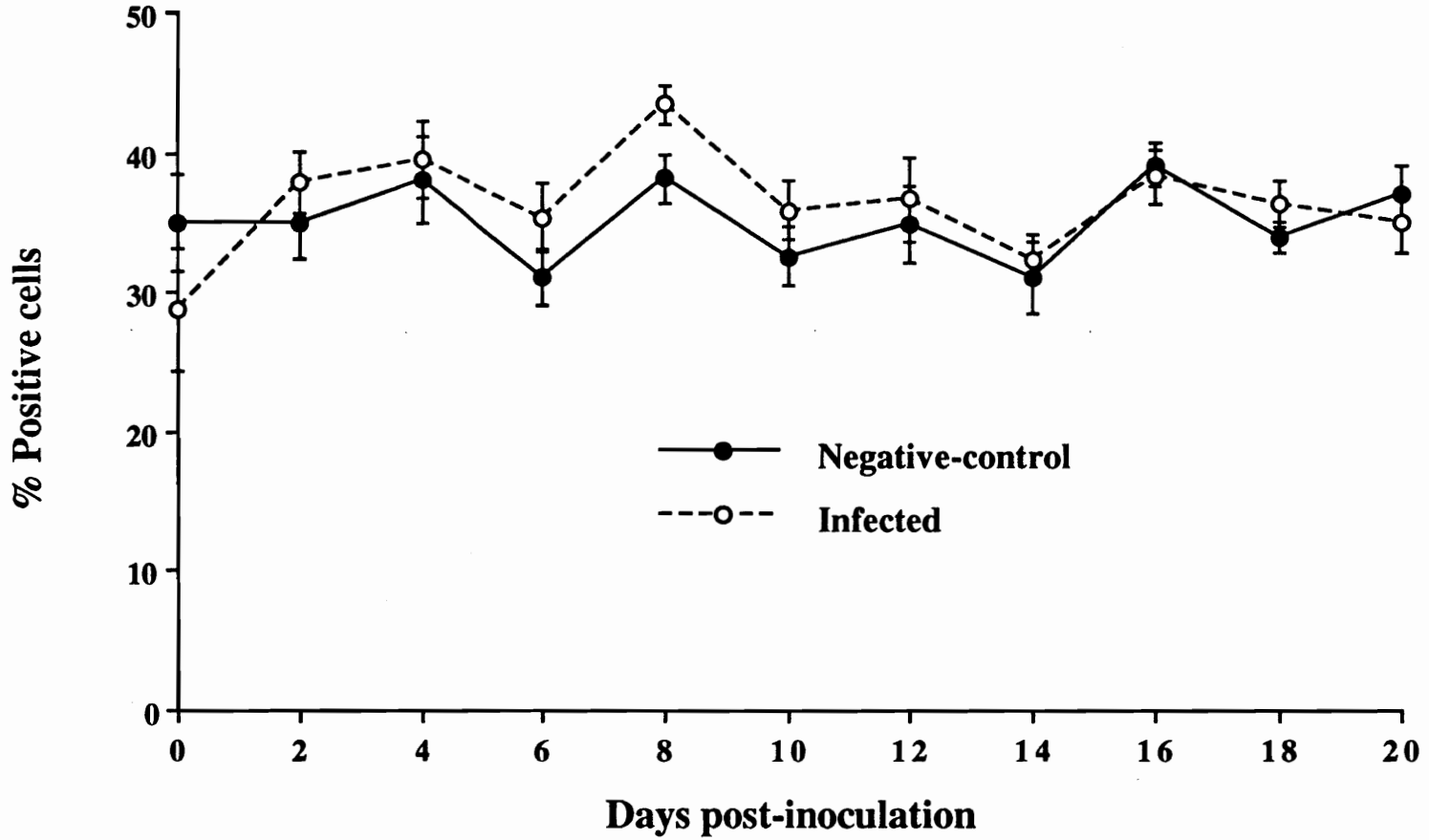

Figure 2. The effect of *HE* virus infection over time on the percentage of peripheral blood leukocytes expressing CT4 surface antigen.

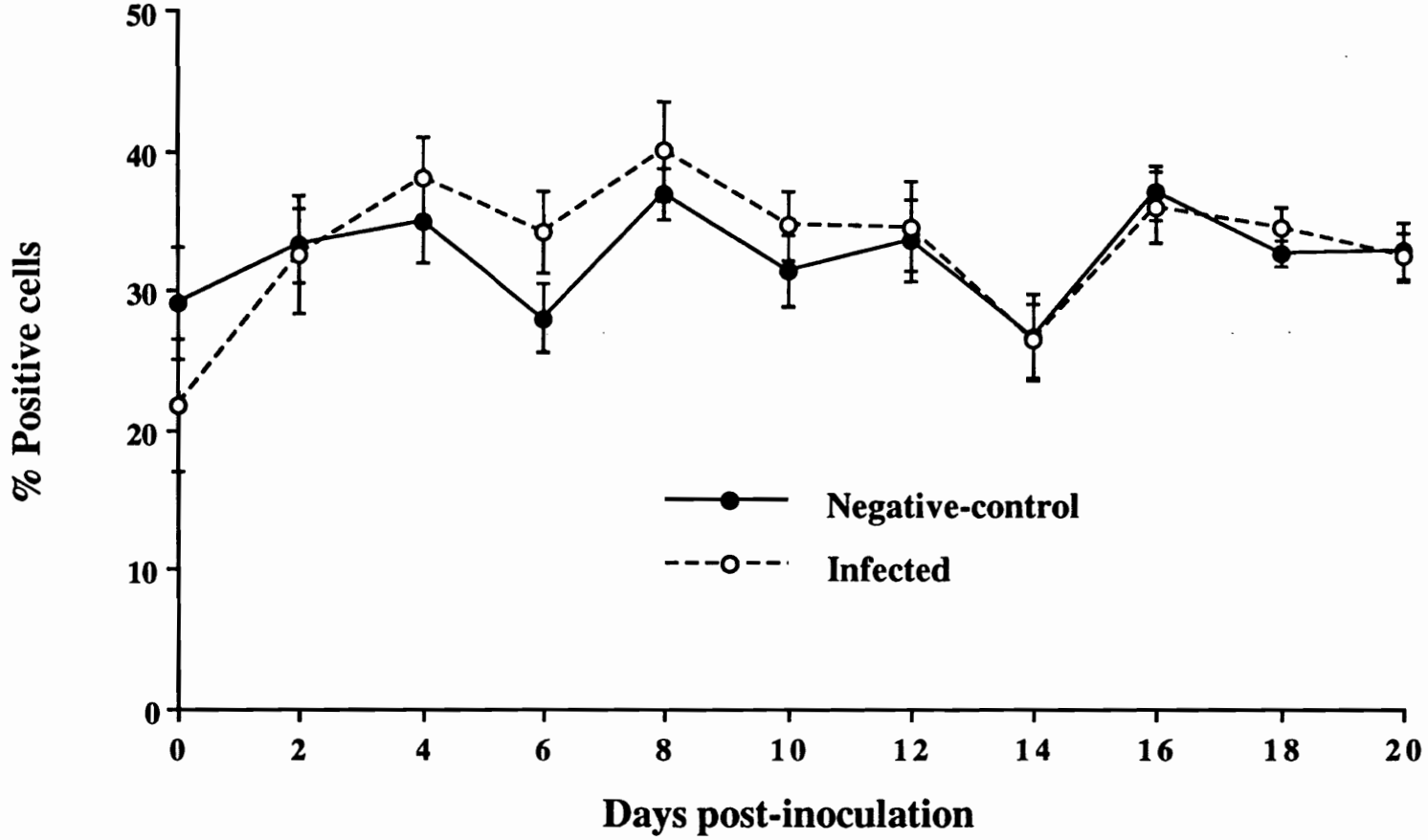

Figure 3. The effect of *HE* virus infection over time on the percentage of peripheral blood leukocytes exhibiting bright immunofluorescense associated with CT4 surface antigen.

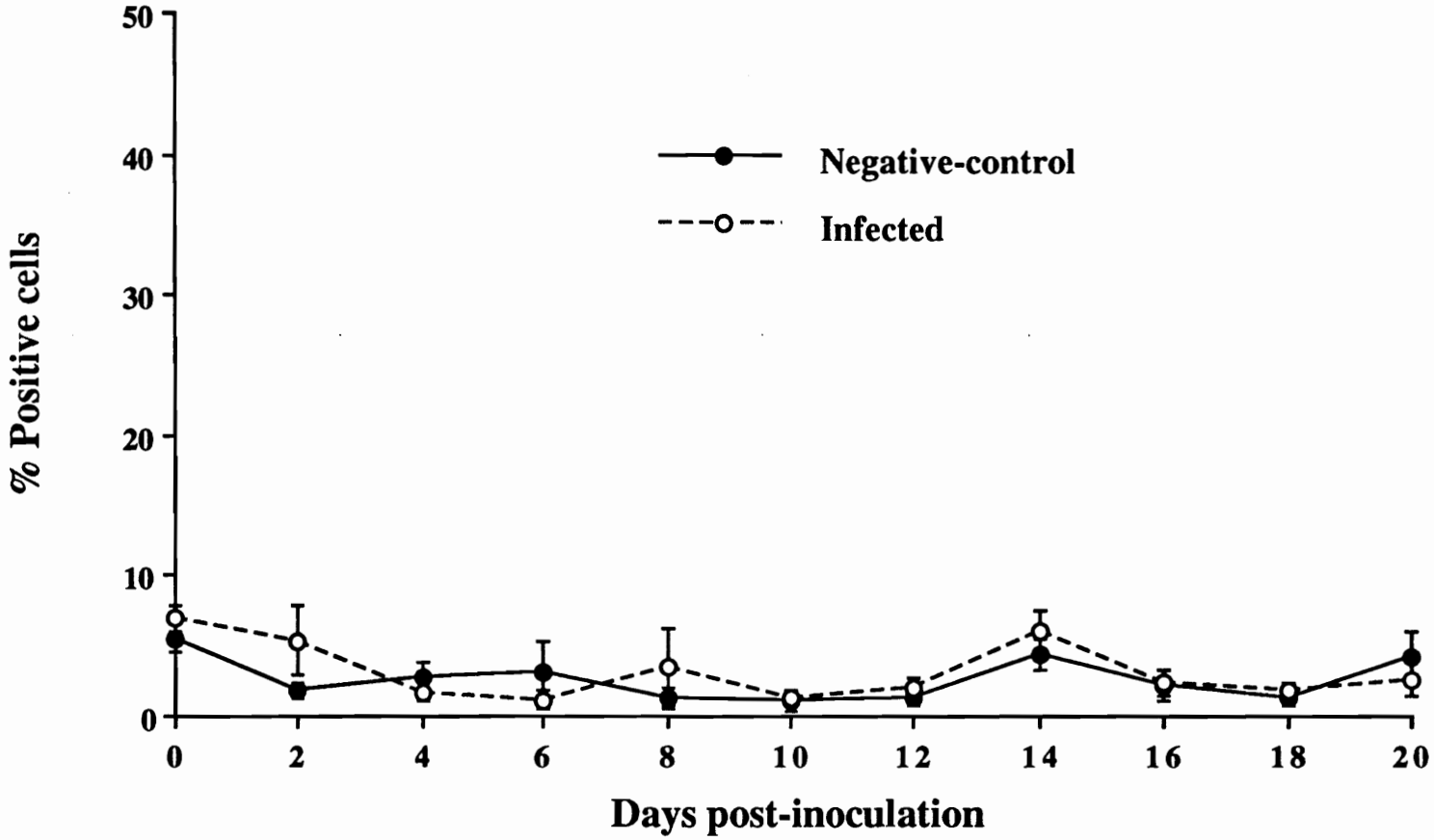

Figure 4. The effect of *HE* virus infection over time on the percentage of peripheral blood leukocytes exhibiting dim immunofluorescense associated with CT4 surface antigen.

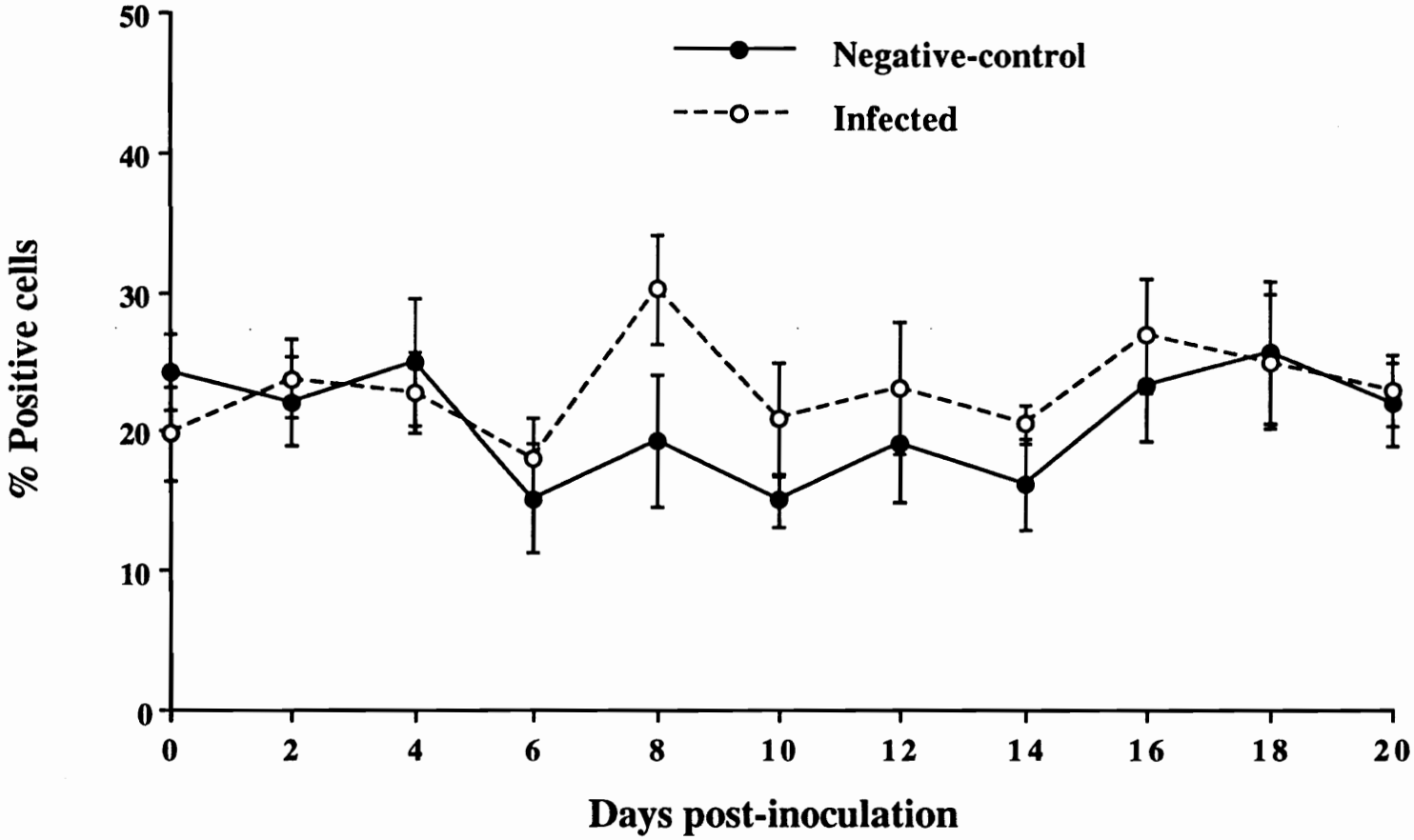

Figure 5. The effect of *HE* virus infection over time on the percentage of peripheral blood leukocytes expressing CT8 surface antigen.

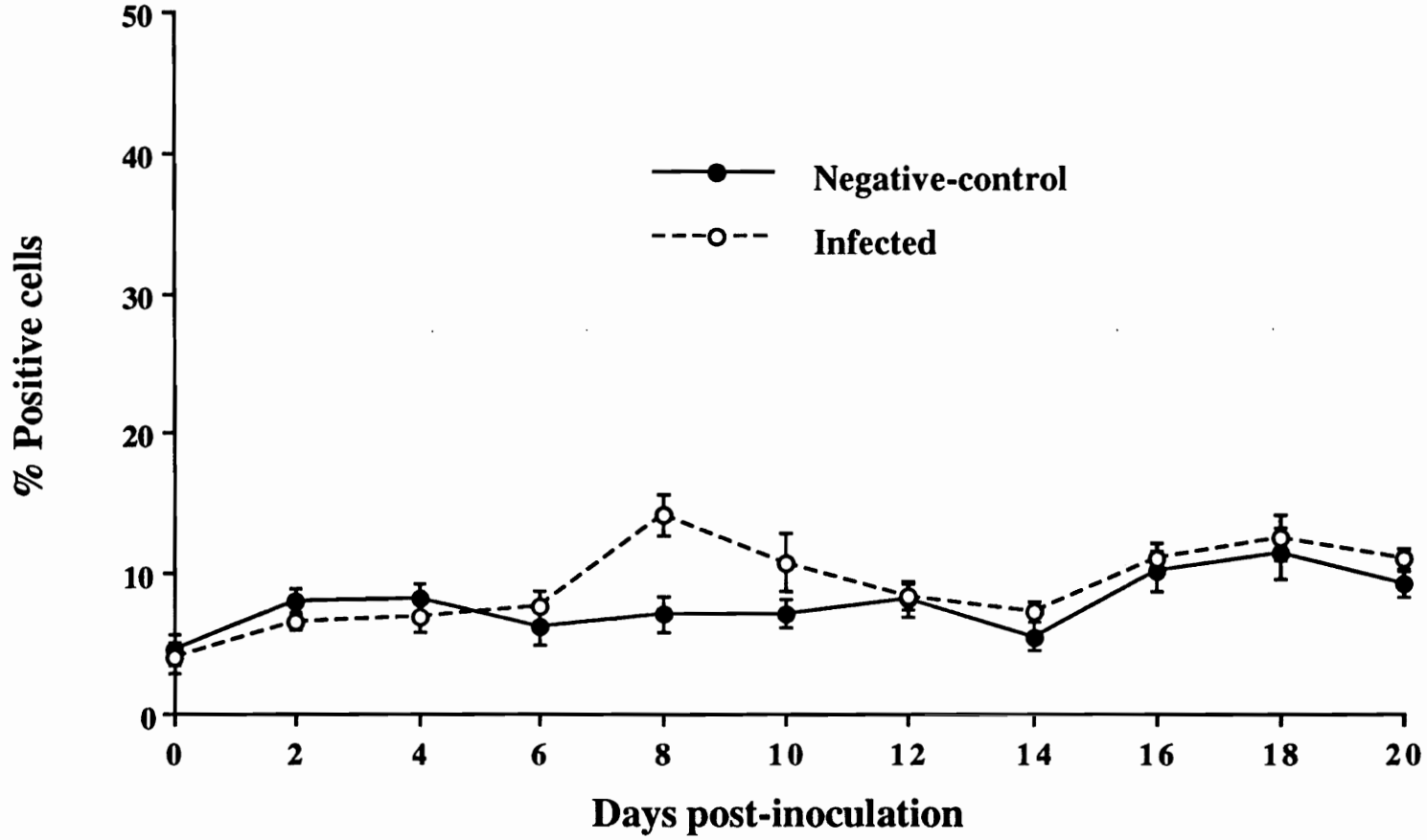

Figure 6. The effect of *HE* virus infection over time on the percentage of peripheral blood leukocytes exhibiting bright immunofluorescense associated with CT8 surface antigen.

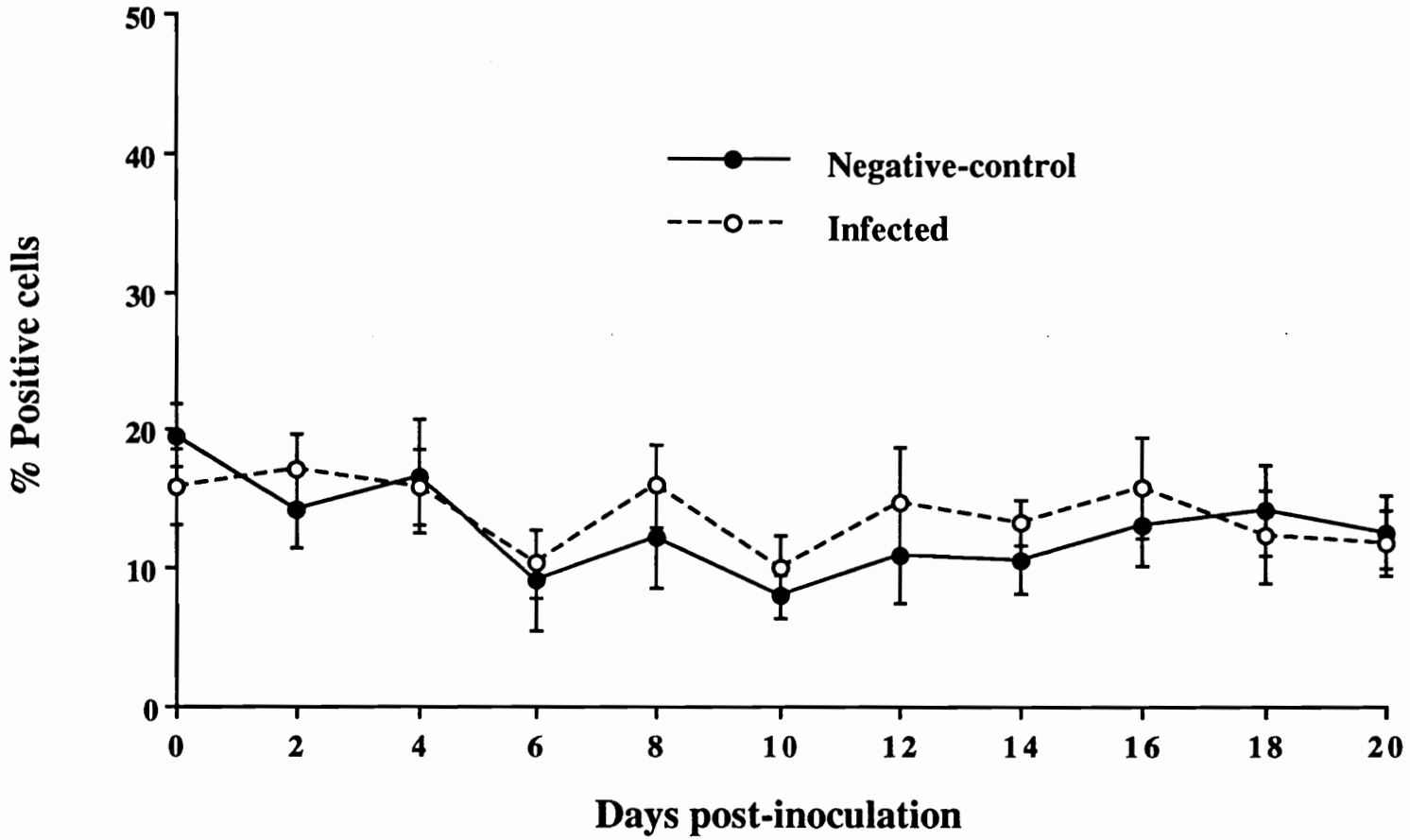

Figure 7. The effect of *HE* virus infection over time on the percentage of peripheral blood leukocytes exhibiting dim immunofluorescense associated with CT8 surface antigen.

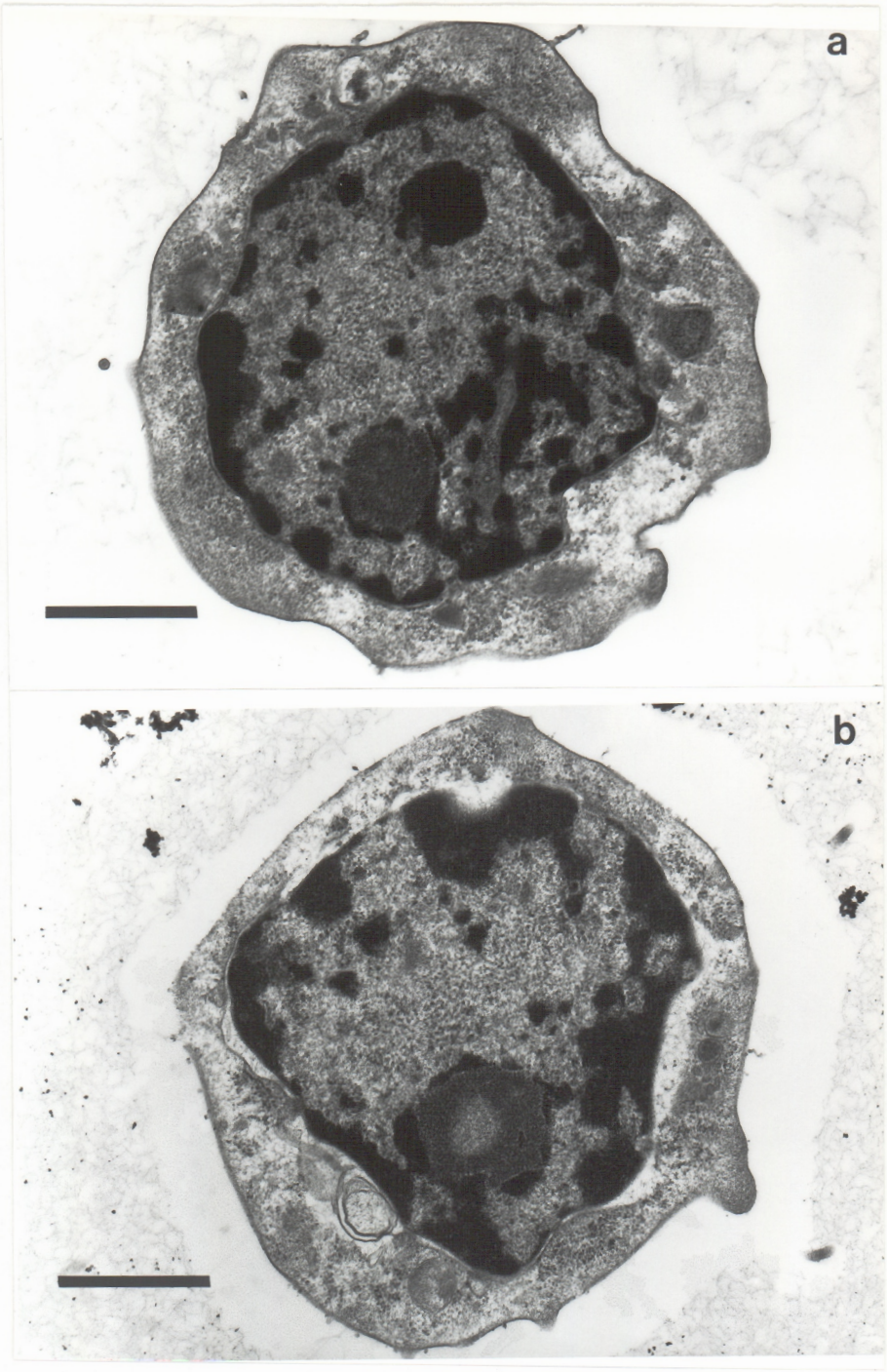

Figure 8. Transmission electron micrographs of a) CT4+ and b) CT8+ cells. Note high nuclear to cytoplasmic ratio characteristic of lymphocytes. CT4+ and CT8+ cells are morphologically indistinguishable. Bar represents 1 μm.

Chapter 6

General Discussion and Conclusion

6.1 The roles of multiple infectious and non-infectious agents in the predisposition of 6 to 12-week-old turkeys to colibacillosis.

The abilities of certain infectious and non-infectious agents to compromise the immune system have already been discussed. Relatively little, however, is understood about the synergistic interactions of such agents which apparently take place under complicated field conditions.

Work pioneered by Gross (3, 4, 6) has shed some light on the intricate pathogenic mechanisms involved in the production of colibacillosis. It has been suggested that the avian respiratory tract, uninfected by viral or mycoplasmal agents is quite able to defend itself against such agents or *Escherichia coli (EC)*. However, the key word is "or". Apparently, the mounting of an effective response to a viral and/or mycoplasmal challenge may be at the expense of bacterial defenses. The immune response to primary viral or mycoplasmal challenge is lymphoid in nature and highly specialized (6). It is an appropriate response with regard to the primary challenge, but is functionally inappropriate and inadequate to prevent a coincidental *EC* challenge from progressing to infection. In fact, existing immunologic obstacles to secondary bacterial infection may be actively reduced or be allowed to passively decay as a result of the re-allocation of resources to the primary response (6). Essentially, the bird attempts to cope with what is perceived as the most imminent,

life threatening situation. In this case, the establishment of an immunologic priority appears to be self defeating. One must therefore consider an infectious agent not only with regard to the direct pathology it produces but also the degree of host response it elicits. It seems that in some cases, an unbalanced, overspecialized immune response can be as detrimental to the ultimate survival of the host as the primary agent against which it is mounted.

To this already complex interaction add non-infectious factors such as genetic background, nutrition, ammonia, dust, weather extremes, stressful social interactions, etc. Some of these are inherent and fairly unalterable facts of modern poultry production. Others can be controlled by good management but often are not. And still others are unpredictable and uncontrollable. All have potential to either cause direct physical impairment of defense mechanisms or induce a physiologic stress response which can increase susceptibility to primary infection.

Now, if one specifically looks at the variables influencing the well-being of market turkeys, the complexity of colibacillosis becomes apparent. In particular, around the time of hemorrhagic enteritis *(HE)* vaccination at 4 to 5 weeks-of-age, numerous things are happening. As noted in previous chapters, exposure to Newcastle disease virus *(ND)*, *Bordetella avium (BA)* and *Mycoplasma meleagridis (MM)* is probably taking place. These agents have the ability to produce clinical disease and predispose turkeys to colibacillosis individually. They may however occur as subclinical infections. Thus the average flock owner or company

service-person may overlook the occurrence and follow through with *HE* vaccination as prescribed. This is where the threshold of susceptibility to *EC* may be crossed. *HE* vaccination appears to produce a certain amount of immunosuppression which under normal circumstances would be tolerable if no other agents were present. If however, an interaction of multiple primary agents takes place at this time, the synergistic effect could result in an increased susceptibility to colibacillosis.

Also around the time of *HE* vaccination, certain management related changes are occurring which may be perceived as stressful. Just prior to vaccination, alterations in the form and content of feed are taking place. Poults have to adjust to a pelleted feed containing lower levels of protein and higher levels of fat. After vaccination, in the "two-stage" grow-out operations that characterize the Shenandoah Valley, poults are physically being moved to a new environment. This move is often only as far as the other end of a partitioned house, yet the moving process and the resultant destruction of established pecking orders can be extremely stressful.

Based on the information obtained during the course of this research, the following sequence of events leading to the development of colibacillosis in 6 to 12-week-old turkeys is proposed (Figure 1). Between 3 and 5 weeks-of-age, exposure to *ND, BA,* and/or *MM* appears to be occurring. This results in either a subclinical or clinical infection depending on the size of the challenge or virulence of the offending agent(s). A cellular response commensurate with the challenge is elicited. At this point the immune response begins to become specialized, yet existing

defenses against an ever-present *EC* challenge probably remain adequate. At 5 weeks-of-age *HE* vaccine is administered. Approximately 8 days post-vaccination an elevation in CT8+ suppressor T-cells occurs. This response is mounted in an attempt to suppress viral replication in infected lymphocytic target cells. The increase in suppressor T-cells also produces a transient immunosuppression which in conjunction with the already existing overspecialization of the immune response, results in a fatal reduction in *EC* defenses. At approximately 6.8 weeks, 2-5 days after *EC* resistance is overcome, mortality due to colibacillosis begins (3, 5). Peak mortality occurs around 8 weeks-of-age and then returns to normal between 9 and 10 weeks-of-age.

It is standard practice in many turkey operations to vaccinate for *HE* at approximately 4 weeks-of-age. Thus, with all other aspects equal, one would expect the onset, peak, and decline in mortality to also be shifted by approximately 1 week.

6.2 Based on the data presented, does the concept of synergistic interaction adequately explain the occurrence of colibacillosis?

To establish whether or not this explanation of the events leading to colibacillosis is adequate, we must first determine the confines under which assessment is to be made. For this, let us return to the "Limitations in the pursuit of causality" as proposed by Evans (1). The applicability of these limitations to the occurrence of colibacillosis in 6 to 12-week-old turkeys is delineated in Table 1. In

essence, all of the described limitations seem appropriate for application to this scenario.

To substantiate the claim that colibacillosis is the result of synergistic interaction, compliance with Evans' "criteria for causation" (1) must be assessed. Table 2. provides an evaluation of conformity with these criteria. Evans' "Unified Concept of Causality" is perhaps the most evolved of the epidemiological criterion models, yet it still falls short of clearly addressing the interaction of multiple agents. Therefore, it is necessary to consider the multiple agents involved in the production of colibacillosis as a collective cause. Having made this assumption, it is possible with the proposed explanation to meet 7/10 of Evans' criteria. This would appear to substantiate the concept that synergistic interactions of multiple agents are responsible for the predisposition to colibacillosis.

6.3 In light of the explanation proposed for the development of colibacillosis in turkeys, what control measures might be implemented?

Given the perceived role of primary infectious agents in the development of colibacillosis, strategic vaccination needs to be employed. As the exposure to various agents may differ from farm to farm, immunoexposure profiling of problem flocks should become a routine part of health management. The availability of this type of information is critical to the development of effective, customized vaccination programs. Intuitively, the tests necessary for inclusion in serologic panels will differ

between geographical regions. Thus some type of preliminary assessment should always be made to determine which agents pose the greatest risk in any area.

The data presented in this dissertation implicates *HE* virus, *ND* virus, *BA*, and *MM* in the production of colibacillosis in the Shenandoah Valley. Thus vaccination against these agents should be performed when justified by immunoexposure profiling. A suggested protocol for *BA, ND,* and *HE* vaccination is presented in Table 3. This takes into account the potential for overlapping effects by providing at least 2 weeks between *ND* and *HE* vaccinations. Since the La Sota strain of *ND* virus was successfully used to produce colibacillosis under laboratory conditions, it is suggested that vaccines containing only the milder, B1 strain be used for early exposures. This should help reduce the interaction between *HE* and *ND* viruses. As *MM* vaccines are not available for turkeys, serologic monitoring of breeders for *MM* and the dipping of eggs from seropositive flocks should be continued in an attempt to reduce the vertical infection of progeny.

Apparently *ND, BA, HE,* and *MM*, whether vaccine in origin or wild, are endemic in the Shenandoah Valley and thus the need for improved biosecurity on all farms should be emphasized.

Finally, management related problems, especially ventilation, and moving procedures for two-stage grow-out operations should be addressed. Principles and techniques for proper ventilation should be an ongoing part of company training programs for service personnel and growers. Likewise, the importance of allowing

turkeys enough time to move from brooding to growing facilities should be emphasized. Given minimal prodding and adequate time, turkeys should be able to move from one end of a house to the other without major disruptions in social structure. This would help to minimize what are believed to be a major stressors.

6.4 The modification of HE vaccine and its use.

One possible avenue for the reduction of colibacillosis is the modification of *HE* vaccine and its use. The alteration of *HE* vaccine to decrease or eliminate its immunosuppressive features should receive a high research priority. Currently, alternative approaches to the delivery of immunoreactive, non-suppressive, *HE* virus proteins are being investigated. Methods which utilize live delivery vehicles such as genetically engineered bacteria may help to meet the requirements of high antigen mass, low cost, and reduced labor intensity necessary for vaccination of market turkeys.

Another approach to reducing the immunosuppressive effects of *HE* virus infection would be to optimize the scheduling of vaccination to take advantage of maternal antibody. This would require the standard vaccination of all breeder flocks and periodic assessment of breeder and progeny antibody titers. A determination of how early progeny could be vaccinated so as to minimize infection while maximizing immunity would also have to be made. It has been reported that turkeys can be infected as early as 2.5 weeks-of-age without the development of intestinal

lesions or splenic enlargement (7). However, other reports indicate that maternal antibody interferes with vaccination up to 5 weeks post-hatch (2). If one considers that the titers of maternal antibody present in progeny from vaccinated breeders are distributed normally in a bell shaped curve, then it follows that some individuals will become susceptible to infections earlier than others. Vaccinating at the earliest possible time would permit susceptible birds to become infected and begin shedding virus. Thus, a continuous viral challenge would be present which should facilitate the infection of remaining birds as soon as maternal antibody drops below threshold level. Theoretically, each bird would become infected in a titrated fashion whereby enough maternal antibody would be present to mitigate an overt cellular (CT8+) reaction and yet permit the development of active immunity. Solid protection might therefore be afforded by vaccination at 3 to 4 weeks-of-age and again at 5 weeks to be sure all birds are adequately challenged.

6.5 References

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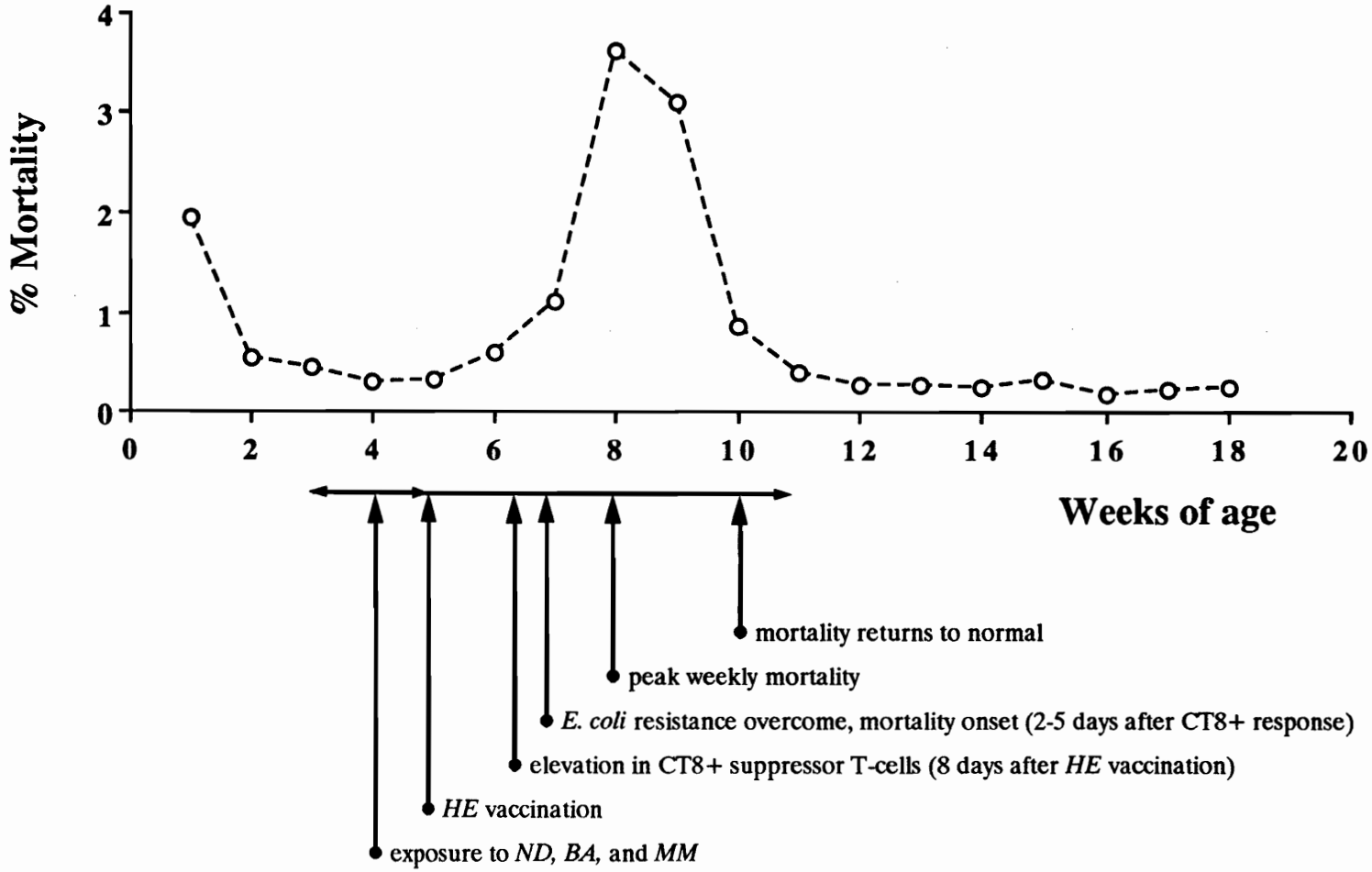

Figure 1. A proposed sequence of events leading to the development of colibacillosis in 6 to 12 week old turkeys.

Table 1. Evans' "limitations in the pursuit of causality" as applied to the scenario of colibacillosis in 6 to 12 week old turkeys.[1]

Does the limitation apply?	Limitation
Yes	1. The same pathologic or clinical condition can be produced by different etiologic agents.
Yes	2. Causative agents may vary in different geographic areas, in different age groups, or with different patterns of host susceptibility
Yes	3. Some diseases require the presence of two or more agents or cofactors acting together to produce the disease.
Yes	4. A single agent may produce different clinical and pathological responses in different settings.
Yes	5. Any cause or set of causes usually produces a biologic gradient of responses which may vary from no observable or even detectable reaction, to mild clinical or pathologic changes, to classic and recognized disease.
Yes	6. The nature and severity of the host response following exposure to an infectious or non-infectious agent varies with certain host characteristics, of which behavioral patterns, social position, genetics, age, immunologic status, and exposure to other cofactors is important.

[1]Adapted from Evans, (1)

Table 2. The conformity of the concept of synergistic interaction with Evans' "criteria for causation".[1]

Does the conformity exist?	**Criteria and points of conformity**
Yes	1. Prevalence for the disease should be significantly higher in those exposed to the putative cause then in case controls not so exposed.[2] * Birds exposed to *HE, ND, BA,* and *MM* were more likely to have elevated mortality due to colibacillosis.
Yes	2. Exposure to the putative cause should be present more commonly in those with the disease than in controls without the disease when all risk factors are held constant. * For the purpose of field studies the risk of exposure was assumed to be constant. However, as previously stated, there are a variety of other factors and agents which may influence immunocompetance. These are often uncontrolable and difficult to measure.
Not Determined	3. Incidence of the disease should be significantly higher in those exposed to the putative cause then in those not so exposed as shown in prospective studies. * A prospective field study has not yet been performed.
Partially Determined	4. Temporally, disease should follow exposure to the putative agent with a distribution of incubation periods on a bell shaped curve. * The precise timing of events, and distribution of incubation periods has yet to be determined, although a partial understanding of the sequence of exposure exists.

Table 2. continued

Yes	5. A spectrum of host responses should follow exposure to the putative agent along a logical biological gradient from mild to severe. * This was apparent in the laboratory trials where a gradient in the severity of clinical mainfestations occurred. Birds experienced everything from no detectable response to death. In the field this may also take place and is probably tempered by the pathotype and number of primary agents involved. Gradation in the percent of mortality appears to be related to these factors.
Yes	6. A measurable host response following exposure to the putative cause should regularly appear in those lacking this before exposure or should increase in magnitude if present before exposure; this pattern should not occur in animals so exposed. * If one considers mortality as the measurable host response, it is apparent that low levels exist prior to exposure. After exposure to the appropriate combinations of agents, percent mortality climbs above 0.5%. Undoubtedly the presence of antibody as a measurable host response also enables this criterion to be met.
Yes	7. Experimental reproduction of the disease should occur in higher incidence in animals appropriately exposed to the putative cause than those not so exposed; this exposure may deliberate in subjects, experimentally induced in the laboratory, or demonstrated in controlled regulation of natural exposure. * The laboratory replication of colibacillosis using combinations of *ND, BA, HE,* and *EC* reveals this to be so.

Table 2. continued

Yes	8. Elimination or modification of the putative cause or of the vector carrying it should decrease the incidence of the disease. * If one considers the combination of agents collectively as the putative cause then modification by removing individual agents would appear to reduce the incidence of mortality due to colibacillosis as revealed by laboratory studies.
Probable	9. Prevention or modification of the host's response on exposure to the putative cause should decrease or eliminate the disease. * Based on field observations, lessening of the host response with appropriate, well timed vaccinations appears to reduce the incidence of colibacillosis.
Yes	10. The whole thing should make biological and epidemiological sense.

[1] Adapted from Evans (1).
[2] The putative cause may exist in the external environment or in a defect in host response.

Table 3. A proposed vaccination schedule for market turkeys raised in the Shenandoah Valley of Virginia.

Age	Vaccine and method of administration
1 day	Art-Vax®[1], spray cabinet
1 week	B1 Newcastle, water
2 weeks	Art-Vax®, water
3 weeks	B1 Newcastle, water
4 weeks	--
5 weeks	Hemorrhagic enteritis, water

[1] Art-Vax®, attenuated *Bordetella avium* vaccine, American Scientific Laboratories, Omaha, NB.

Appendix 1

Fixation of Guinea Pig Red Blood Cells for Use with Hemagglutination Assays

1) Obtain 5.0 ml of packed pig red blood cells using approximately 1.0 ml of citrate dextrose per 5.0 ml of whole blood.

2) Centrifuge at 600 g for 10 min. Add packed RBC's to fixing solution containing 40.0 ml of phosphate buffered saline (PBS) and 10.0 ml of 40.0% formaldehyde. Final pH of solution should be approximately 7.4.

3) Mix gently on rotary shaker at room temperature overnight.

4) Centrifuge at 600 g for 10.0 min and wash pellet ten times with PBS.

5) Resuspend RBC's in PBS at a final concentration of 1.0% and store at 4.0°C (cells should be stable for at least 6 mos).

Bordetella avium Hemagglutination Assay

1) Pick several suspect colonies off of a peptone agar plate or blood agar plate (5.0% sheep blood). G20G medium which contains antibiotics has caused some variability in the expression of hemagglutination so this medium should preferably be avoided for subculturing prior to HA determinations.

2) Suspend bacterial cells in sterile saline at a density approximately equal to that of a 5.0 McFarland standard.

3) Combine 50.0 μl of bacterial suspension with 50.0 μl of fixed guinea pig RBC's in a V bottom microtiter well. Mix the contents of the well thoroughly with the dispensing pipette.

4) Run concurrently with positive and negative controls. The positive control we are currently using is the 85-105-1a strain of *B. avium* from Dr. J. Kurt Skeeles, Department of Animal Sciences, University of Arkansas. Our negative controls are *B. avium*-like Iso 128 T5 from Dr. Y. Mo Saif, O.A.R.D.C., Wooster, Ohio; the ATCC 8750 *Alcaligenes faecalis*. Using all of these isolates is probably not necessary for diagnostic purposes. A well with only 50.0 μl of RBC's is also useful as an additional control.

5) Allow to stand at room temperature for at least 1.0 hr before reading.

Appendix 2

The cross-reactivity of the anti-chicken CT4 and CT8 monoclonal antibodies (courtesy of Dr. C.H. Chen, Birmingham, AL) with anitgens on the surface of turkey peripheral blood leukocytes (PBLs) was confirmed in the following manner.

Isolation of PBLs, and the processing cells was identical to that described in Chapter 2 up to the point of incubation with the fluorescein isothiocyanate conjugate. At this step, goat-anti-mouse IgG conjugated to crystalline tetramethylrhodamine isothiocyanate (TRITC) was substituted. After incubation with the conjugate, the cells were washed and resuspended in PBS. 10μl drops were placed on glass microscope slides, air dried, fixed in methanol, and mounted with PBS/glycerin (1:1 v/v). Slides were viewed by epi-illumination using an Olympus BH-2 fluorescent microscope with photomicrographic capabilities and equipped with a filter suitable for TRITC excitation.

Photomicrographs of turkey PBLs stained for either CT4 or CT8 are shown in Figures 1 and 2. In each case, it was apparent that specific populations of cells were being stained. Functional differences between populations were not verified.

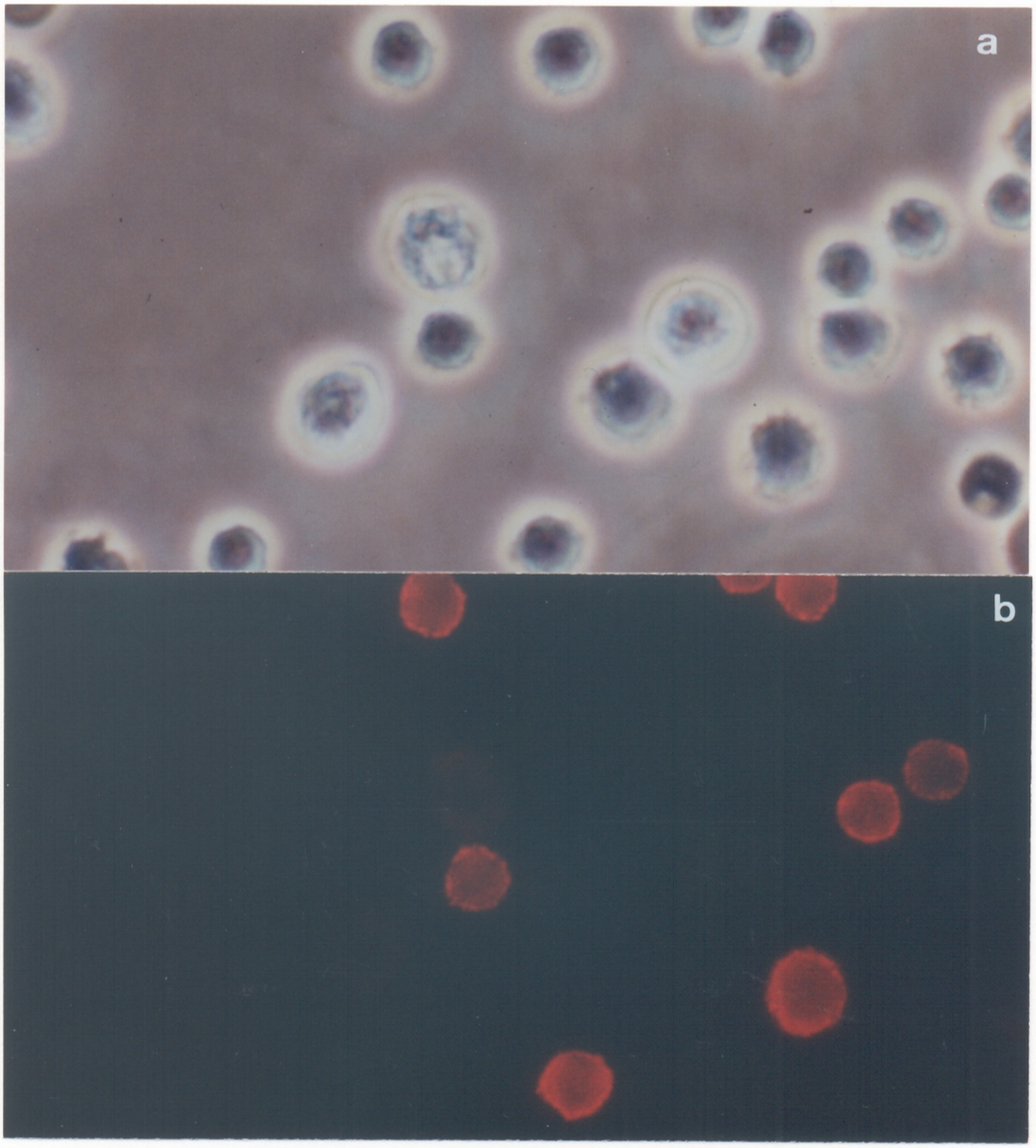

Figure 1. Photomicrographs of peripheral blood leukocytes stained for the presence of CT4 surface antigen. a) Cells visualized by phase contrast and b) epifluorescence.

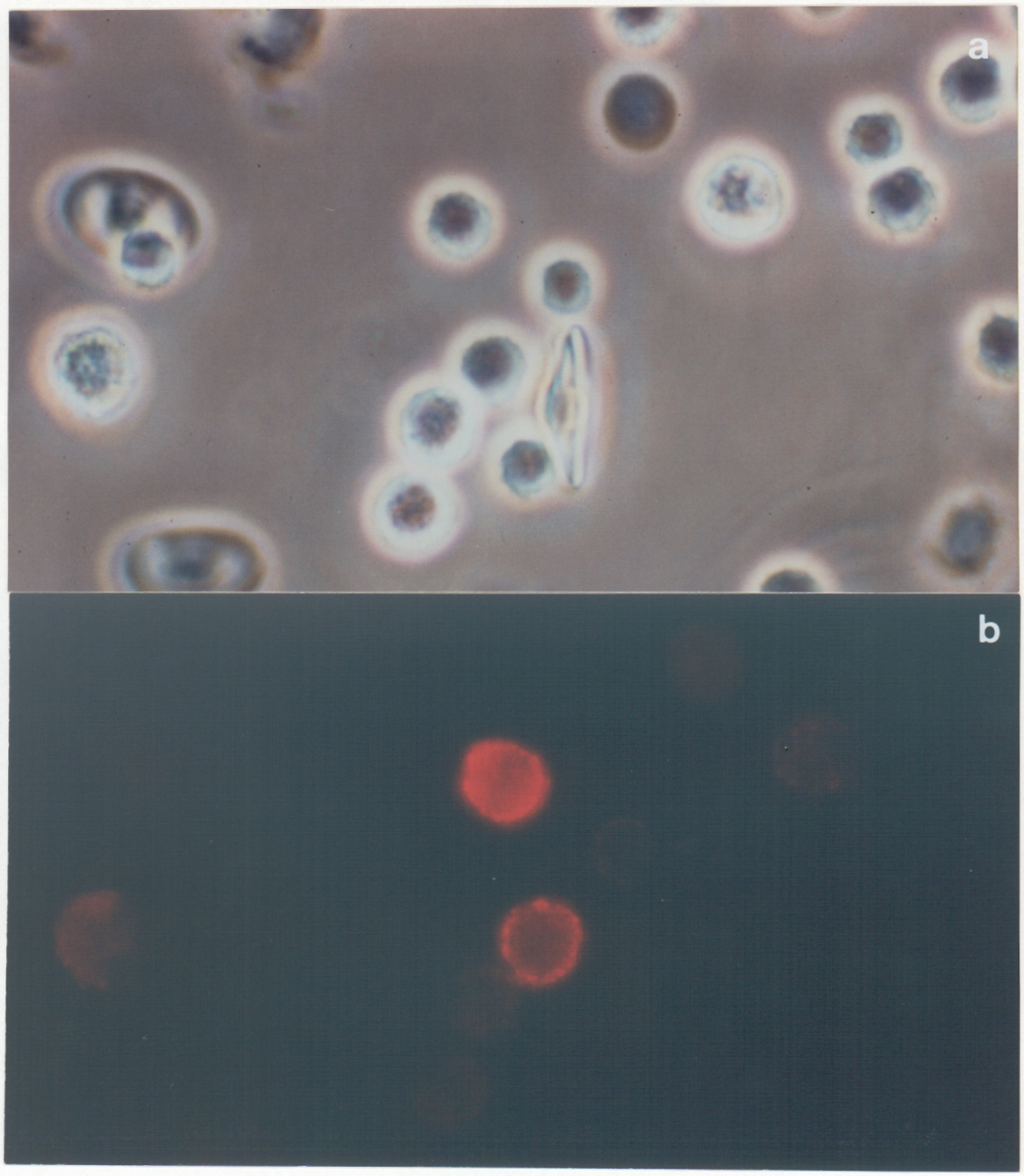

Figure 2. Photomicrographs of peripheral blood leukocytes stained for the presence of CT8 surface antigen. a) Cells visualized by phase contrast and b) epifluorescence.

Vita

Frank William Pierson, son of Frank and Mildred Pierson, was born on May 3, 1956 in Baltimore, MD. In 1974, he graduated from Towson Senior High School in Towson, Maryland. He earned a B.S. degree in Agriculture from the University of Delaware in 1978 and a M.S. degree in Animal Science from Purdue University in 1980. He received his D.V.M. from the Virginia-Maryland Regional College of Veterinary Medicine in 1984 and entered private veterinary practice. In 1987, he returned to Virginia Polytechnic Institute and State University to pursue doctoral studies in Avian Medicine and completed his Ph.D. in February 1993.

During his academic career, Dr. Pierson has received numerous awards including the Poultry Science Association's Certificate of Excellence in Graduate Research in 1979, First Place in the V.M.R.C.V.M. Graduate Research Competition in 1988, a V.M.R.C.V.M. College Teaching Award in 1992, and a Virginia Tech Certificate of Teaching Excellence in 1992. He has also be elected to membership in several national honor societies, including Phi Kappa Phi, Gamma Sigma Delta, Phi Zeta, and Sigma Xi.

He is an active member of the American Veterinary Medical Association, the Association of Avian Pathologists, and the Association of Avian Veterinarians.