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The role of iron in the pathogenesis of Escherichia coli septicemia of turkeys (Meleagris gallopavo)

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THE ROLE OF IRON IN THE PATHOGENESIS OF ESCHERICHIA COLI SEPTICEMIA OF TURKEYS (MELEAGRIS GALLOPAVO)

Iowa State University

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The role of iron in the pathogenesis of *Escherichia coli*
septicemia of turkeys (*Meleagris gallopavo*)

by

Carole Ann Bolin

A Dissertation Submitted to the
Graduate Faculty in Partial Fulfillment of the
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GENERAL INTRODUCTION

*Escherichia coli* is a common cause of respiratory disease and septicemia in poultry. Colisepticemia develops most often in young birds and causes lesions in the respiratory tract, heart, liver, and spleen. Subacute fibrinopurulent polyserositis may develop in birds surviving the septicemic phase of infection. The respiratory tract is the primary route of infection, followed by bacteremia and septicemia when *E. coli* invade pulmonary vasculature.

Tendency to produce bacteremia is correlated with virulence of strains of *E. coli* for poultry. Virulent strains, in contrast to avirulent strains, contain factors which permit passage into or survival in the blood. It is likely that bacterial or host factors which influence the development of bacteremia and septicemia are important in the pathogenesis of this economically important disease.

Host restriction of iron is an important non-specific host defense mechanism against bacterial infection. Administration of iron to animals increases the incidence and severity of a number of bacterial infections. The role of host iron-restriction in the pathogenesis of *E. coli* septicemia of turkeys has not been determined.

*Escherichia coli* growing in an iron-restricted environment, in vitro or in vivo, produce new outer membrane proteins involved in iron acquisition. These proteins are antigenic but their role as protective antigens against *E. coli* infection is not known.

The objectives of this study were to i) determine if administration of exogenous iron potentiates systemic *E. coli* infection in turkey
poults and ii) determine if antibody against *E. coli* outer membrane proteins produced in iron-restricted medium protects turkeys from colisepticemia.

This dissertation is presented in the alternate format. The chapters are prepared in the style of the American Journal of Veterinary Research. The first manuscript has been published in the American Journal of Veterinary Research and the second manuscript has been submitted to Infection and Immunity. A review of the literature precedes the first chapter. All literature cited appears at the end of the dissertation. The candidate was the principal author for each manuscript.
Avian colibacillosis

*Escherichia coli* is a common cause of respiratory disease and septicemia in chickens and turkeys. Three forms of colibacillosis are seen clinically. First, acute colisepticaemla occurs most often in young birds and produces lesions characteristic of gram-negative septicemia. Lesions include swollen and hyperemic viscera, hemorrhage in the subcutis and joints, pulmonary edema, pericardial effusion, and focal necrosis of liver, spleen, and lymphoid tissues. Secondly, subacute fibrinopurulent polyserositis occurs as a sequela to septicemia, in birds which survive the acute phase of disease. Lesions typically include fibrinopurulent pericarditis, pleuritis, peritonitis, and airsacculitis. Synovitis, panophthalmitis, and salpingitis may also occur. Lastly, a low incidence of chronic granulomatus pneumonitis, enteritis, and hepatitis occurs.

Natural infection occurs primarily following inhalation of poultry house dust and litter contaminated with pathogenic *E. coli*. The most common serotypes of *E. coli* isolated from birds with septicemia and airsacculitis are 01:K1, 02:K1, and 078:K80. *Escherichia coli* septicemia and respiratory disease have been produced experimentally following exposure to virulent *E. coli* by aerosol, intratracheal, air sac, intravenous, and intramuscular routes. Oral inoculation failed to produce respiratory lesions but produced a severe pyogranulomatus bursitis.
Natural infections often develop as a complication of viral, mycoplasmal, or other bacterial infections. However, under conditions of confinement housing and overcrowding, primary colibacillosis may occur. Colibacillosis often complicates infections of chickens with Mycoplasma gallisepticum and turkeys with Mycoplasma meleagridis. Infection with Newcastle disease virus or infectious bronchitis virus also predisposes birds to colibacillosis.

Development of bacteremia is important in the pathogenesis of colibacillosis in poultry. Escherichia coli are thought to gain access to the bloodstream by invasion through pulmonary microvasculature or lymphatics. Arp et al. demonstrated that after aerosol exposure of turkey pouls to an avirulent strain of E. coli, bacteremia was not produced, although the organism persisted in the lung for long periods. In contrast, a virulent isolate of E. coli readily produced bacteremia and lesions typical of colisepticemia. These results indicate that virulent strains contain certain factors, which permit passage into or survival in the blood, but are absent in avirulent strains.

Prevention of colibacillosis is difficult because of multiple serotypes of E. coli causing disease, confinement management systems, and persistence of pathogenic serotypes in the environment. Effective experimental bacterins have been developed to protect poultry from disease caused by serotypes 078 and 02 of E. coli. Commercially available bacterins are killed, whole cell products containing
E. coli of serotypes 078, 02, and 01. In general, E. coli bacterins fail to provide cross-protection for a serotype not contained in the bacterin. 41,42

Iron and infection

Introduction The factors involved in bacterial infection are numerous and complex; however, a common factor in all infections is the ability of the invading pathogen to multiply in the host. 58 In septicemic conditions, the bacterium must gain access to and survive in the bloodstream. Non-specific host factors which influence the ability of bacteria to survive and replicate in blood include bactericidal and static effects of serum, temperature, pH, oxygen tension, and availability of nutrients. 58 One non-specific host defense mechanism which is thought to limit bacterial growth in host tissue and fluids is the limited availability of iron. 58,106

Iron is an essential nutrient for most life forms, including bacteria. 105,106 The presence of cytochromes and non-heme iron in respiratory chains of aerobic and facultative anaerobic organisms, dictates a central role for iron in the energy metabolism of microorganisms. 105,106 In addition, iron is required by enzymes involved in hydrogen peroxide metabolism, DNA synthesis, and nitrogen fixation. 30,105,106

Limitation of iron in animal tissues and fluids Although animals have an abundance of iron in the body, availability of free iron for microorganisms is limited. 49,149 The majority of iron in the body is found as intracellular stores of ferritin, hemosiderin,
hemoglobin, and myoglobin. Extracellular iron is complexed with high-affinity iron-binding glycoproteins. Complexes of iron are present as transferrin, lactoferrin, ovotransferrin, ferritin, ceruloplasmin, and haptoglobin-heme. In normal tissues, the concentration of free ionic iron is $10^{-18}$ M, which is far below the 0.4 to 4.0 μM required for bacterial growth and metabolism.

Inflammation in host tissues results in a further reduction in available iron. Depressed serum iron values are recognized as a biochemical manifestation of most infectious diseases. Generally, serum iron levels decrease rapidly during the incubation period, remain depressed during the active phase, and return to normal during the convalescent phase of an infectious disease.

Hypoferremia can be produced experimentally by injection of endotoxin or by creating a site of sterile inflammation, and can be prevented by blockade of the mononuclear phagocyte system by injection of carbon particles. Depression in serum iron levels is accompanied by an accumulation of iron in tissue storage depots. Iron accumulates (as ferritin or hemosiderin) at sites of inflammation, in the liver, and in cells of the mononuclear phagocyte system.

Hypoferremia, as a result of inflammation, could be produced by accelerated removal of iron from the plasma pool, or by impaired return of intracellular iron to the plasma pool, or both. Van Snick et al. provide evidence that accelerated removal of plasma iron occurs during infection or inflammation. This process is believed to be mediated, in part, by interleukin-1 released by phagocytes at sites of
Interleukin-1 causes release of lactoferrin from neutrophil granules. Lactoferrin combines with iron in blood and tissues and the complexes are rapidly sequestered in cells of the mononuclear phagocyte system. While it is clear that lactoferrin can compete successfully with transferrin for iron at sites of inflammation, it is doubtful that such a mechanism would operate efficiently in the plasma. Indeed, Letendre and Holbein, using radiolabeled iron-transferrin, found no evidence of accelerated removal of iron from the plasma transferrin pool during the hypoferreemic response to Neisseria meningitidis infection of mice.

Letendre and Holbein showed that hypoferremia following infection or inflammation is primarily the result of increased incorporation of plasma and heme-derived iron into the insoluble, low-turnover, ferritin iron pool of cells of the mononuclear phagocyte system. This resulted in a decrease in return of iron to the plasma transferrin pool and, therefore, lowered serum iron concentrations. The cause of these shifts in intracellular iron metabolism during infection and inflammation is unknown.

Ceruloplasmin is an acute phase protein and a plasma ferroxidase. Concentrations of ceruloplasmin rise sharply during an inflammatory process, and increased plasma ferroxidase activity promotes release of iron from intracellular storage, thus returning serum iron levels to normal.

In addition to the factors mentioned above, there is a decrease in gastrointestinal iron absorption and an increase in iron
excretion during inflammation which may contribute to hypoferremia seen during infectious diseases.

**Iron level and susceptibility to infection** Alteration of serum iron values, changes the susceptibility of the host to infection. Parenteral administration of exogenous iron has been shown to enhance infections in a number of experimental systems. Over a hundred years ago, Trousseau observed recrudescence of quiescent tuberculosis in patients given iron supplements. More recently, many reports have shown that added iron enhances the ability of many microbes to grow in body fluids, cells, or tissues. The disease enhancing effect of added iron has been demonstrated with fungi, protozoa, and gram-positive, gram-negative and acid-fast bacteria.

Generally, an amount of added iron sufficient to double the saturation of the iron-binding protein used in the experimental system has a marked effect on the growth rate of responsive strains of bacteria. A variety of sources of iron, e.g., inorganic salts, organic compounds, heme, ferritin, and hemoglobin are effective in enhancing infections. Activity of iron in these systems is not dependent on processing of the iron compounds by the cells of the mononuclear phagocyte system and does not function by neutralizing complement. The most important mechanism whereby excess iron enhances disease is by its growth enhancing effect for the invading microbe. In addition, iron may also impair cellular defense mechanisms of the host as will be discussed.
Although most research on iron-stressed hosts has involved mammals, some information is available on the effects of iron in avian species. Virulence and replication of gram-negative bacteria increase in iron-treated chicken embryos.\(^{14,111}\) In contrast, studies show that oral or parenteral administration of some forms of iron increases the survival of chickens inoculated with *E. coli*, *Salmonella gallinarum*, or *Mycobacterium avium*.\(^{73,127,128,130}\) It is not clear why administration of iron lessens the severity of these bacterial infections in birds. With the exception of laying hens,\(^9\),\(^{94,115}\) iron metabolism\(^{141}\) and the hypoferremic response as a result of inflammation\(^{29,81,129,133}\) differ little between mammals and birds.

Clinical observations in humans with iron overload confirm the results obtained in experimental studies. Iron overload of human tissue or fluids occurs from 1) excess ingestion, injection or inhalation of iron; 2) destruction of iron storage cells (for example, during hepatitis); 3) hemolysis; or 4) decreased synthesis of transferrin.\(^{81,99}\) Case reports indicate that persons with these conditions are at increased risk of developing systemic infections.\(^{137,149}\) For example, infants given intramuscular injections of iron dextran during the first week of life were found to have a 20-fold increase in the incidence of *E. coli* septicemia and meningitis, compared to similar infants not given iron.\(^9,11\) Other examples of the effects of iron overload and susceptibility to infection are reviewed elsewhere.\(^{51,99,148,149}\)
Enhancement of host iron withholding mechanisms decreases the incidence and intensity of bacterial infections.\textsuperscript{77,149} Rats fed low-iron diets prior to inoculation with \textit{Salmonella typhimurium}, for example, had lower serum iron values at the time of inoculation and a significantly increased survival rate as compared to rats fed a normal diet.\textsuperscript{149} Mice fed a copper deficient diet become hypoferremic because of decreased ceruloplasmin levels. These mice are significantly more resistant to \textit{N. meningitidis} infection than mice fed normal diets, or those fed copper deficient diets which were given either iron or ceruloplasmin.\textsuperscript{93}

Hypoferremia produced by injection of endotoxin,\textsuperscript{29,84,129,139} bacterial cell walls,\textsuperscript{148} turpentine,\textsuperscript{10,35} or interleukin-\textsuperscript{143,148} has been associated with a decrease in incidence and intensity of infection.\textsuperscript{10,148,149} Mice given an intramuscular injection of turpentine become hypoferremic and are protected from \textit{N. meningitidis} infection.\textsuperscript{10} The protective effect is reversed by administration of iron.

Circumstantial evidence indicates that naturally occurring hypoferremia decreases the incidence of infectious diseases in humans. Hypoferremia is the normal condition for Masai tribesmen of Africa, whose diet is composed primarily of milk. Despite poor sanitation practices, the incidence of amebiasis in this population is less than 9%. When some individuals were supplemented with iron, sufficient to return serum iron values to normal, the attack rate of amebiasis rose to 83%. The incidence of other infectious diseases, including malaria, also increased markedly in the iron-supplemented group.\textsuperscript{149} Presumably
the hypoferremic condition provides some protection from these infectious diseases.

Mammals which are severely iron deficient from prolonged or severe iron deprivation become more susceptible to disease. This is thought to occur because of a depression in humoral and cellular immune function in these animals. Therefore, attempts to decrease susceptibility to infectious diseases by iron-restriction must be carefully controlled.

Effect of iron-restriction on bacterial structure and function

Bacteria which grow in iron-restricted environments have developed highly efficient mechanisms to obtain iron. These mechanisms include low affinity and high affinity iron assimilation systems.

Low affinity iron-acquisition systems allow most microorganisms to grow in conditions of relatively high iron concentration (10 μM), as are usually present in enriched culture medium. Bacteria are able to use highly insoluble, polymeric forms of iron when these compounds are present in high concentration. Molecular details of the low affinity systems are not known. Presumably some of the surface iron atoms in the polymeric molecules are more loosely bound and hence available to the bacteria. Alternatively, low affinity iron-binding sites may be incorporated in the bacterial envelope.

High affinity iron-acquisition systems allow microorganisms to grow in extremely low iron concentrations, as are present in animal tissues and fluids. High affinity systems consist of two
components, the siderophore and the corresponding bacterial membrane transport system. Siderophores are low molecular weight (500-1000 daltons) ligands with high association constants for ferric iron. Production of siderophores is tightly regulated by the availability of iron in the extracellular milieu. Many microbial siderophores have been identified and characterized, but most can be classed chemically as either catechols or hydroxamates.

Production of outer membrane protein receptors and enzymes involved in the uptake and release of iron from siderophores are the second components of the high affinity system of bacterial iron-acquisition. Production of outer membrane protein siderophore-receptors is regulated by iron concentration in the growth medium. In gram-negative bacteria, the outer membrane constitutes a permeability barrier to water-soluble molecules larger than 500 daltons. Therefore, ferric-siderophores require outer membrane receptors and transport mechanisms to pass into the cell. Siderophore-receptors also act as receptors for bacteriophages and colicins.

Escherichia coli produce the catechol siderophore, enterochelin, a trimer of 2,3-dihydroxybenzoylserine. Enterochelin is a product of the enzymatic conversion of chorismate, a compound produced during aromatic amino acid biosynthesis. The chemistry of enterochelin has been characterized in detail and it has the highest iron binding constant of any chelator known. Ability to produce
Enterochelin is widespread among isolates of *E. coli* and genes responsible for its production are located on the chromosome. Enterochelin is produced in a variety of iron-restricted media *in vitro*. In addition, enterochelin was recovered from the peritoneal washings of guinea pigs infected with *E. coli*, indicating that *E. coli* excrete enterochelin *in vivo*. In a number of bacterial species, loss of the ability to produce enterochelin, or other catechol siderophores, has been associated with a decrease in virulence and loss of the ability to survive and replicate in serum. Production of enterochelin by *E. coli* is associated with ability to replicate in body fluids, but has not been linked to ability to cause invasive disease.

Many invasive strains of *E. coli* causing septicemia in poultry, and other species, contain the Col V plasmid. This plasmid codes for the production of the hydroxamate siderophore aerobactin, the associated outer membrane receptor, and colicin V. Ability of strains of *E. coli* to cause septicemia in chickens and mice is increased when Col V plasmids are introduced, and decreased when the plasmids are cured. Smith reported that when chickens or calves were inoculated with a mixture of *E. coli* containing Col V plasmids and those not containing Col V, nearly 100% of the isolates subsequently recovered from the blood and liver of these animals contained the Col V plasmid. Colicin V activity is not responsible for the increased virulence of *E. coli* carrying Col V. Therefore, production of the
aerobactin iron-transport system is thought to be responsible for virulence enhancement of those E. coli which contain Col V,2,151,154. Recently, genes which code for the production of aerobactin have been located in the chromosome of some strains of E. coli.66,142

Another hydroxamate siderophore, ferrichrome, produced by other microorganisms (primarily fungi), can be used by E. coli but the significance of this system during infection is unknown.8,106,108

Escherichia coli produce several new outer membrane proteins in iron-limiting conditions in vitro37,65 and in vivo.64 Some of these proteins have been identified as siderophore receptors.107 These include the ferric-enterochelin receptor (81,000 daltons), the ferric-ferrichrome receptor (78,000 daltons), and the ferric-aerobactin receptor (74,000 daltons).36,39,57,107 The number and molecular weight of various iron-regulated outer membrane proteins of E. coli vary somewhat, depending on the particular iron-restricted medium used and on the strain of E. coli.37,65 Iron-regulated outer membrane proteins are major components of the outer membrane of E. coli grown in vivo.64

Some aspects of the interaction of ferric-enterochelin with its outer membrane receptor have been determined. The metal-catechol portion of the ferric-enterochelin molecule binds to the receptor.47,98 The ferric-enterochelin complex is transported into the periplasmic space by an energy-dependent process, where it accumulates, until needed inside the cell.47,98 Iron is released from the ligand by the activity of a cytoplasmic esterase, which may destroy the enterochelin
molecule in the process. Similar details of the aerobactin system are not known.

There are several reasons why production of aerobactin confers a selective advantage for in vivo growth upon *E. coli* which can also produce enterochelin. Thermodynamically, enterochelin is clearly superior to aerobactin as an iron-chelator, but aerobactin functions more efficiently in vivo. Enterochelin binds to plasma proteins, is less effective at low chelator concentrations than aerobactin, and is not recyclable. Aerobactin does not bind to plasma proteins and can be recycled. In addition, genetic determinants of aerobactin biosynthesis and uptake are more readily expressed than those of enterochelin as iron concentrations become limited.

In addition to synthesis of siderophores and new outer membrane proteins, other changes occur in *E. coli* grown in iron-limited conditions. Iron-restricted *E. coli* produce populations of undermodified tRNA molecules in vitro and in vivo. These iron-related changes may be associated with cellular regulation. Amino acid synthesis and aromatic amino acid transport are affected by the presence of undermodified tRNA molecules.

Production and excretion of some bacterial toxins are also enhanced in low iron concentrations. The β-phage cytotoxin of *Corynebacterium diphtheriae* is produced under iron-restricted conditions. Production of a Shigella-like toxin by some adhering and effacing strains of *E. coli* is markedly enhanced in low-iron medium.
Effect of iron on phagocytes and the immune response

Although the primary effect of iron levels on bacteria is a difference in growth rate, other factors in host defense may also be affected by iron levels. Excess iron inhibits the chemotactic and bactericidal action of neutrophils. Iron interferes with intracellular killing mechanisms of neutrophils by inhibition of oxidative killing and cationic protein activity. Excess iron may also be cytotoxic for neutrophils. Iron overload decreases monocyte phagocytic activity and destabilizes macrophage lysosomes. Severe iron deficiency may also decrease intracellular bacterial killing.

There is evidence that both iron overload and iron deficiency can impair immune responsiveness. Patients with iron overload secrete abnormally low levels of immunoglobulin. Serum from iron deficient patients fails to provide sufficient iron for optimal T or B lymphocyte proliferation in response to mitogens, in vitro.

Iron, antibodies, and host resistance

The ability of transferrin, lactoferrin, or other iron-binding proteins to bind iron is usually essential for the bacteriostatic effect of host body fluids. In 1940, Schade and Caroline showed that the bacteriostatic effect of egg white could be reversed by addition of iron. Similarly, the bacteriostatic effect of serum for E. coli or N. gonorrhoea, for example, is completely abolished when iron is added to the serum. Loss of serum bacteriostatic effect is not because of interference with complement or lysozyme.
Milk has a direct bacteriostatic or bactericidal effect for *E. coli*.\(^3,15,28\) Purified, iron-free lactoferrin or specific antibody against *E. coli* each have a mild bacteriostatic effect.\(^3,28\) However, the combination of unsaturated lactoferrin and specific antibody has a powerful bacteriostatic effect, which is reversible upon the addition of iron.\(^3,15,20,28,97\) The milk bacteriostatic system requires bicarbonate and does not involve complement.\(^15,28,61\) The reason why antibody and lactoferrin act synergistically to prevent bacterial growth remains to be shown. Antibodies specific for the O antigen of *E. coli* prevent the secretion of enterochelin.\(^50,118,119\) Therefore, specific antibody in milk may interfere with the ability of *E. coli* to obtain iron in the iron-restricted conditions created by the presence of unsaturated lactoferrin.\(^118,119\)

Experiments with vaccinated mice show that iron administration has a deleterious effect on acquired immunity.\(^87\) Mice vaccinated with killed *S. typhimurium* were protected from subsequent challenge with virulent *S. typhimurium*. Administration of iron to vaccinated animals increased mortality rates, in a dose dependent manner, when the animals were challenged.\(^89\) Therefore, iron-restriction in vivo appears to be important in defense against bacterial infections, even in the presence of a specific acquired immune response.\(^87,89\)

Antibodies against enterochelin have been found in serum from healthy humans.\(^102,103\) Enterochelin-specific antibody interferes with ferric-enterochelin uptake by *E. coli* and prevents enterochelin-mediated growth stimulation of an *E. coli* strain deficient in
enterochelin synthesis. This antibody is of the IgA class and is thought to result from antigenic stimulation of B lymphocytes in the intestinal tract with enterochelin secreted by bacteria in the intestinal lumen.

Iron-regulated outer membrane proteins are antigenic, and serum from healthy humans, mice, rabbits, and guinea pigs contain antibodies against the iron-regulated outer membrane proteins of \textit{E. coli}. In wild type, smooth strains of \textit{E. coli}, iron-regulated outer membrane proteins are exposed at the surface of the bacteria, and are able to interact with antibody molecules. Antibodies against the ferric-ferrichrome receptor of \textit{E. coli} and against a siderophore receptor (for ferric-pyochelin) of \textit{Pseudomonas aeruginosa} inhibit siderophore-mediated iron uptake in the respective organisms \textit{in vitro}. Antibodies against the ferric-pyochelin receptor are also opsonophagocytic and passively protect mice from experimental \textit{P. aeruginosa} burn infections. The role of iron-regulated outer membrane proteins of \textit{E. coli} as possible protective antigens has not been determined.
EFFECTS OF EXOGENOUS IRON ON *ESCHERICHIA COLI* SEPTICEMIA

OF TURKEYS
EFFECTS OF EXOGENOUS IRON ON *ESCHERICHIA COLI* SEPTICEMIA
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SUMMARY

The effect of inoculation with *Escherichia coli* on serum iron concentrations of turkeys and the effect of exogenous iron, as ferric ammonium citrate, on *E. coli* septicemia in turkeys were determined. Inoculation of air sacs with *E. coli* produced hypoferremia in 18-day-old turkeys. Administration of iron with *E. coli* significantly (p<0.01) increased mortality, frequency and degree of bacteremia, and severity of lesions in inoculated turkeys, compared with those in turkeys given *E. coli* but not given iron. Similar results were seen whether iron was inoculated at the same location as *E. coli* or at a different location.


INTRODUCTION

Iron, an essential nutrient for bacterial growth, is largely unavailable in animal tissues. Most iron in the body is found as intracellular stores, and extracellular iron is combined with high affinity iron-binding glycoproteins, e.g., transferrin, lactoferrin, and ovotransferrin. In healthy tissues, the concentration of available iron is approximately $10^{-18}$ M, which is far below that required for bacterial growth. Inflammation in host tissues results in a further reduction in available iron by transfer of serum iron to iron storage pools and by decreased gastrointestinal iron absorption. These processes are believed to be mediated by interleukin-1 released by phagocytes at sites of inflammation. Hypoferremia, as a result of inflammation, may be an important nonspecific host defense mechanism against bacterial infection.

Interference with the hypoferremic response of mammals by administration of iron increases the occurrence and severity of a variety of bacterial infections. Virulence and replication of gram-negative bacteria also increase in iron-treated chicken embryos. Paradoxically, studies revealed that oral or parenteral administration of some forms of iron increases survival of chickens inoculated with *Escherichia coli*, *Salmonella gallinarum*, or *Mycobacterium avium*. It is not clearly understood why administration of iron lessens the severity of these bacterial infections in birds. With the exception of laying hens, iron
metabolism\textsuperscript{141} and the hypoferremic response, as a result of inflammation, \textsuperscript{81,112,128} differ little between mammals and birds.

The purpose of the present report was to determine the effect of iron on \textit{E. coli} infection of turkeys. \textit{Escherichia coli} septicemia and associated respiratory tract diseases are an important cause of death and production losses in the poultry industry. \textit{Escherichia coli} infection of turkeys is also a convenient and economical model for study of the pathogenesis of gram-negative bacterial septicemias.
MATERIALS AND METHODS

**Turkeys**

One-day old Nicholas Broad-Breasted White turkeys were raised to 18 days of age in heated brooders. Food and water were provided *ad libitum*. Samples of blood, liver, lungs, and air sacs were collected from 2 birds of each lot, and samples were cultured on blood agar to detect contamination with *E. coli*.

**Bacterial inoculation**

The *E. coli* strain (078:K80:H9) was isolated originally from the liver of a turkey that died of colisepticemia. Cells from an 18-hour trypticase soy broth culture of *E. coli* were suspended in 0.01 M phosphate-buffered saline solution at a concentration of 7.5 to 8.4 x 10^4 colony-forming units/ml. Turkeys, at 18 days of age, were exposed by injection with 0.1 ml of *E. coli* suspension into the caudal portion of the left lesser abdominal air sac. Noninfected controls were similarly injected with 0.1 ml sterile saline solution.

**Injection of iron**

Abdominal air sacs of turkeys were injected with 0.1 ml of saline solution containing 1.5 mg (6 mg/kg of body weight) of iron as

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*a* Rochester Hatchery, Rochester, MN.

*b* BBL Microbiology Systems, Cockeysville, MD.
ferric ammonium citrate. In preliminary studies, this dose doubled the saturation of serum iron-binding capacity in inoculated turkeys for at least 6 hours after inoculation (Appendix A). Control turkeys were given 0.1 ml of saline solution containing 7.5 mg (30 mg/kg of body weight) of ammonium citrate in the air sac.

**Experimental design**

To determine whether the dose of *E. coli* and inoculation procedure used would cause hypoferremia in turkeys, 12 birds were inoculated with *E. coli*. Serum was obtained at intervals after inoculation, and serum iron values were determined using a colorimetric ferrozine assay kit. This assay was found to be valid and reliable for use with turkey serum.

To determine the effect of iron on *E. coli* infection, turkeys were given *E. coli* alone, *E. coli* and ferric ammonium citrate in the left air sac, or *E. coli* and ammonium citrate. To determine whether effects of iron were local or systemic, a group of turkeys was given *E. coli* in the left air sac and ferric ammonium citrate in the right air sac. Noninfected control groups were given saline solution and ferric ammonium citrate or saline solution and ammonium citrate.

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*a* Em Diagnostic Systems, Gibbstown, NJ.

To determine whether ferric ammonium citrate might enhance disease by damaging the air sac membrane, samples of left lesser abdominal air sac were collected from noninfected turkeys given ferric ammonium citrate or ammonium citrate. Samples were fixed, embedded, sectioned, and stained for histologic and electron microscopic examination using routine methods. 78, 96

Assessment of disease severity

At 6, 12, 24, and 48 hours after inoculation, turkeys from each group were examined for bacteremia and severity of lesions of E. coli infection. Number of birds examined at each time was 3 for groups given saline solution and ferric ammonium citrate, 2 for those given saline solution and ammonium citrate, 3 for those given E. coli only, 6 for those given E. coli and ammonium citrate, 6 for those given E. coli and iron in the same location, and 3 for those given E. coli and iron in opposite air sacs. A 1-ml blood sample was collected from each turkey with 0.05 ml of 5% polyanetholsulfonic acid as anticoagulant. The number of bacteria in blood was determined, using a serial dilution and blood agar plate count method. Turkeys were killed, carcasses opened aseptically, and a swab of the inner surface of the left lesser abdominal air sac obtained for culture. Randomly selected colonies from blood and air sac cultures were tested for 078 antigen using a slide agglutination test with anti-078 antiserum. Gross lesions were

aSigma Chemicals, St. Louis, MO.
recorded and a lesion score was determined for each bird. A Student's t-test was used to compare results between groups of turkeys.
RESULTS

Inoculation of air sacs with *E. coli* produced hypoferremia (Figure 1). Turkeys given *E. coli* only or *E. coli* and ammonium citrate remained clinically normal; whereas, turkeys given *E. coli* and iron had ruffled, feces-stained feathers and rapid, shallow respirations. Deaths (12 of 40 inoculated turkeys) occurred only in groups given *E. coli* and iron. All turkeys dying before the time of blood sampling and necropsy were examined and had severe bilateral airsacculitis and pericarditis, with hemorrhage and edema in the subcutis.

Administration of iron with *E. coli* significantly ($p<0.01$) increased the frequency and degree of bacteremia and the severity of lesions in inoculated turkeys, compared with those in turkeys not given iron (Table 1; Figures 2 and 3). Because differences between turkeys given *E. coli* only and those given *E. coli* and ammonium citrate were not significant, results were grouped for statistical analysis (Table 1). The average frequency of *E. coli* isolation from air sacs of birds given *E. coli* was not significantly different between treatments (92% vs 100%), but significantly ($p<0.01$) more *E. coli* organisms were isolated from air sacs of iron-treated birds than from those not given iron. The effect of iron on disease was similar whether the iron and *E. coli* were given in the same or different sites, although a time lag in disease-enhancing effect occurred when iron and *E. coli* were given in contralateral air sacs (Figures 2 and 3). All colonies tested were of serotype 078, as determined by slide agglutination.
Figure 1. Serum iron values in control turkeys (dashed line) and turkeys given *Escherichia coli* in the air sac (solid line). Control values represent the mean from 10 birds and each other point is the mean from 3 birds given *E. coli*.
Table 1. Culture and necropsy results for turkeys exposed to *Escherichia coli*

<table>
<thead>
<tr>
<th>Treatment(^a) (No. inoculated)</th>
<th>Bacteremic (%)</th>
<th>(\log_{10}) CFU(^b) of E. <em>coli</em>/ml of blood</th>
<th>Gross lesion score(^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. <em>coli</em> (12)</td>
<td>50</td>
<td>0.86</td>
<td>1.3</td>
</tr>
<tr>
<td>E. <em>coli</em> +AC (24)</td>
<td>46</td>
<td>0.72</td>
<td>0.8</td>
</tr>
<tr>
<td>E. <em>coli</em> +FeL (28)</td>
<td>94</td>
<td>2.94</td>
<td>2.6</td>
</tr>
<tr>
<td>E. <em>coli</em> +FeR (12)</td>
<td>80</td>
<td>2.35</td>
<td>2.3</td>
</tr>
<tr>
<td>E. <em>coli</em> without Fe(^d) (36)</td>
<td>48 + 9</td>
<td>0.79 ± 0.11</td>
<td>1.0 ± 0.18</td>
</tr>
<tr>
<td>E. <em>coli</em> with Fe (40)</td>
<td>87 + 7**</td>
<td>2.65 ± 0.34**</td>
<td>2.5 ± 0.27**</td>
</tr>
</tbody>
</table>

\(^a\) Turkeys were given E. *coli*, E. *coli* and ammonium citrate (E. *coli* + AC), E. *coli* and ferric ammonium citrate at the same site (E. *coli* + FeL) or E. *coli* and ferric ammonium citrate in contralateral air sacs (E. *coli* + FeR).

\(^b\) CFU = Colony-forming unit.

\(^c\) 0 = no gross lesions; 1 = mild to moderate left airsacculitis; 2 = moderate left airsacculitis with mild right airsacculitis and pericarditis; 3 = severe left airsacculitis with moderate pericarditis and right airsacculitis; 4 = severe bilateral airsacculitis, pericarditis, pneumonia, and subcutaneous hemorrhage.

\(^d\) Groups given E. *coli* but not treated with iron (E. *coli* and E. *coli* + AC) and those given E. *coli* and treated with iron (E. *coli* + FeL and E. *coli* + FeR) were combined for statistical analysis. Data are expressed as mean ± SEM.

** Values are significantly different (p<0.01) than those for birds given E. *coli* without iron.
Figure 2. Mean colony-forming units (CFU) of *Escherichia coli* isolated per milliliter of blood from bacteremic turkeys given *E. coli* (☉), *E. coli* and ammonium citrate (□), *E. coli* and ferric ammonium citrate in the same air sac (▼) or *E. coli* and ferric ammonium citrate in contralateral air sacs (◆). *Bacteremia was not detected in *E. coli* or *E. coli* and ammonium citrate inoculated groups at 6 hours after inoculation. †All turkeys given *E. coli* and ferric ammonium citrate in the same air sac died within 48 hours after inoculation.
LOG₁₀ CFU OF E. coli/ml OF BLOOD FROM BACTEREMIC BIRDS

HOURS AFTER INOCULATION

48  24  12  6
Figure 3. Average gross lesion scores for turkeys given *Escherichia coli* (\(\square\)), *E. coli* and ammonium citrate (\(\boxdot\)), *E. coli* and ferric ammonium citrate in the same site (\(\blackbox\)), or *E. coli* and ferric ammonium citrate in contralateral air sacs (\(\triangledown\)). Gross lesion scores were determined using the following scale: 0 = no gross lesions; 1 = mild to moderate left airsacculitis; 2 = moderate left airsacculitis with mild right airsacculitis and pericarditis; 3 = severe left airsacculitis and moderate right airsacculitis and pericarditis; 4 = severe bilateral airsacculitis, pericarditis, pneumonia, and hemorrhage in the subcutis.

*All turkeys given *E. coli* and ferric ammonium citrate in the same site died within 48 hours after inoculation.*
*Escherichia coli* was not isolated from, and gross lesions were not seen in, turkeys treated with ferric ammonium citrate or ammonium citrate alone.

Small, multifocal areas of epithelial cell loss and fibrin accumulation were seen in air sacs treated with ferric ammonium citrate or ammonium citrate (Appendix B). Differences in number or extent of these lesions could not be detected among groups. The remaining air-sac membrane was normal.
DISCUSSION

Administration of iron potentiates systemic *E. coli* infection after air-sac inoculation of young turkeys. The effect of iron is systemic and is not caused by an irritant effect on the air sac membrane. Although hyperferremia probably occurs rarely in healthy, immature poultry, effects of iron administration on *E. coli* infection indicate a role for host iron withholding mechanisms in resistance to this disease. Virulence of *E. coli* isolates for poultry is strongly associated with a superior ability of these isolates to grow in iron-deficient conditions. Production of the siderophore aerobactin is responsible for increased iron acquisition by these isolates. This is also evidence that relative unavailability of iron in the host may contribute to natural resistance of birds to *E. coli* septicemia.

Isolation of significantly more *E. coli* from the blood and air sacs of turkeys given iron indicates an enhanced ability of *E. coli* to replicate in the tissues of those birds as compared with that in birds not given iron. Administration of iron promotes bacterial growth in tissues by increasing the availability of iron to bacteria and may interfere with the bactericidal activity of heterophils. Iron interferes with intracellular killing mechanisms of neutrophils by inhibition of oxidative killing and cationic protein activity. Cationic proteins are thought to be important in bactericidal activity of avian heterophils, that lack myeloperoxidase. Therefore, excess
iron may decrease bacterial killing by heterophils and allow increased bacterial growth in tissues by this mechanism.

A delay was present in the development of bacteremia and lesions in turkeys given E. coli and iron in different sites as compared with that in birds given both in the same location. The delay probably represents the time required for E. coli to gain access to the blood, and therefore, the increased amounts of iron. Alternatively, the delay might represent the time required for iron to be absorbed into the blood from the air sac. This is unlikely to be a major part of the delay because increases in serum iron values are present within 30 minutes of an air-sac inoculation with ferric ammonium citrate.a

The present results differ from reports73,127,128,130 of the effect of iron on bacterial infections in birds. Differences in experimental protocol including dose, route of administration, and form of iron used, make direct comparison between those reports and this one difficult. Smith et al.128 and Harry73 report increases in survival from S. gallinarum or E. coli infection in chickens fed a diet containing high concentrations of iron. Increases in serum iron concentrations and saturation of serum iron-binding capacity of birds fed diets high in iron were small, compared with those in the current study in which the dose of iron given was sufficient to double the saturation of serum iron-binding capacity in treated turkeys. Smith

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et al.⁰¹²³,⁰¹²⁷,⁰¹³⁰ report a beneficial effect of parenteral administration of iron dextran, but not ferric ammonium citrate, for chickens inoculated with *S. gallinarum* or *M. avium*. Doses of iron used in those studies were 8 to 33 times the dose used in the current study. Because the present results are similar to those⁰⁴⁹,⁰⁵⁸,⁰¹⁴⁹ obtained with other gram-negative bacteria in mammalian species, *E. coli* infection of turkeys would be an appropriate model for studies of the role of iron in the pathogenesis of bacterial septicemias.
PASSIVE IMMUNIZATION WITH ANTIBODIES AGAINST IRON-REGULATED
OUTER MEMBRANE PROTEINS PROTECTS TURKEYS FROM
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PASSIVE IMMUNIZATION WITH ANTIBODIES AGAINST IRON-REGULATED OUTER MEMBRANE PROTEINS PROTECTS TURKEYS FROM ESCHERICHIA COLI SEPTICEMIA

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SUMMARY

*Escherichia coli* septicemia is a common disease of young poultry, as well as in several species of mammals. Rabbit antiserum was prepared against iron-regulated outer membrane proteins of *E. coli*. Eighteen-day-old turkeys were passively immunized with antiserum and challenged by air-sac inoculation with 1 to $2 \times 10^6$ colony-forming units of *E. coli* (078:K80:H9). Turkeys injected with normal rabbit serum or saline solution prior to challenge served as controls. Fatalities (8 of 51 inoculated) occurred only in groups given saline solution or normal rabbit serum. Remaining turkeys were necropsied 96 hours after challenge. Passive immunization with antiserum significantly (p<0.05) reduced the frequency of bacteremia at 96 hours after challenge, the frequency of recovery of *E. coli* from air sacs, and the severity of gross lesions in inoculated birds as compared to birds given normal rabbit serum or saline solution.
INTRODUCTION

Iron is an essential nutrient for bacterial growth but is largely unavailable in animal tissues. The majority of iron found in the body is in intracellular stores. Extracellular iron is complexed with high affinity iron-binding glycoproteins, e.g., transferrin, lactoferrin, and ovotransferrin. In normal tissues, the concentration of available iron is approximately $10^{-18}$ M which is far below that required for bacterial growth. This iron-restricted environment induces changes in the metabolism and outer membrane composition of bacteria growing in vivo.

Escherichia coli growing in an iron-restricted environment, in vitro or in vivo, synthesize low molecular weight iron chelators (siderophores) and outer membrane proteins (OMP) not found when adequate concentrations of iron are available. Some of the iron-regulated OMP are membrane receptors for ferric-siderophore complexes, as part of the high affinity iron acquisition system of E. coli. Iron-regulated OMP are antigenic and sera from healthy humans, mice, rabbits, and guinea pigs contain antibodies against iron-regulated OMP of E. coli. In wild type, smooth strains of E. coli, iron-regulated OMP are exposed at the surface of bacterial cells. Consequently, they are able to interact with large protein molecules, and presumably antibody. The role of iron-regulated OMP of E. coli as protective antigens has not been determined.

The purpose of this study was to evaluate the effect of passive immunization of turkeys with antibody against E. coli iron-regulated
OMP on the severity of disease produced following challenge with virulent E. coli. Escherichia coli septicemia of turkeys is a significant cause of economic loss in the poultry industry and is a convenient, economical model of E. coli septicemia in mammals.
MATERIALS AND METHODS

E. coli and growth conditions

The E. coli (078;K80:H9) used was isolated originally from the liver of a turkey that died from colisepticaemia. This strain produces enterochelin and aerobactin as determined by bioassay and colony hybridization. For isolation of iron-regulated OMP, E. coli were grown in iron-restricted medium composed of tryptcase soy broth containing 300 μM of the iron chelator α,α'-dipyridyl. For isolation of OMP from iron-replete bacteria and for challenge exposure of turkeys, E. coli were grown in tryptcase soy broth that did not contain chelator. Bacteria were incubated at 37°C for 18 hours on a rotary shaker.

OMP preparation

Outer membrane protein-enriched fractions were prepared from E. coli grown in iron-restricted and iron-replete media. Cells were harvested by centrifugation, suspended in distilled water, and disrupted by sonication using a Branson model S75 Sonifier at maximum power output. Whole cells and debris were removed by centrifugation at

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a William P.H. University of Leicester, Leicester UK: Personal communication, 1986.

b BBL Microbiology Systems, Cockeysville, MD.

c Sigma Chemicals, St. Louis, MO.

d Branson Scientific, Danbury, CT.
4000 xg for 20 minutes at 5°C and cell membranes were collected by centrifugation at 105,000 xg for 45 minutes at 5°C. Membranes were suspended in 20 mM tris(hydroxymethyl)aminomethane\(^a\) (Tris) solution (pH 7.2) containing 1% sodium n-lauroylsarcosine\(^a\) and incubated for 30 minutes at 22°C. The detergent-insoluble OMP-enriched fraction was collected by centrifugation at 105,000 xg for 45 minutes at 5°C, suspended in 20 mM Tris solution (pH 7.2), and stored at -70°C. Protein concentrations were determined using a dye-binding assay.\(^b\)

**Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE)**

Proteins were separated by SDS PAGE using a 3.75% stacking gel, an 8% resolving gel, and the two buffer system of Laemmli.\(^91\) Outer membrane protein-enriched fractions were boiled for 5 minutes in a sample buffer solution (2% sodium lauryl sulfate,\(^a\) 10% glycerol,\(^a\) 5% 2-mercaptoethanol\(^b\) and 0.003% bromphenol blue\(^b\) in 0.0625 M Tris (pH 6.8). For analytical gels, samples were loaded at 10 \(\mu\)g of protein per well and for preparative gels at 200 to 400 \(\mu\)g of protein per gel. A constant current of 40 mA per gel was used. A mixture of high molecular weight standards\(^b\) was used for molecular weight determinations. Gels were stained with Coomassie blue\(^b\) and then destained in 50% methanol, 10% acetic acid solution.

\(^a\)Sigma Chemicals, St. Louis, MO.

\(^b\)Biorad, Richmond, CA.
Antiserum against iron-regulated OMP

Outer membrane protein-enriched fractions from E. coli grown in iron-restricted conditions were separated by SDS PAGE, stained, and destained. Iron-regulated OMP were identified as a group of proteins in the molecular weight range of 73,000 to 85,000 daltons which were present in the OMP fraction of E. coli grown in iron-restricted medium. These proteins were not present, or were present in reduced quantities, in OMP preparations of iron-replete bacteria. Iron-regulated OMP were excised from gels, rinsed briefly in a solution of 10 mM sodium phosphate, 0.9% sodium chloride, pH 7.2 (PBSS), and emulsified in PBSS solution with a tissue grinder. An adult New Zealand White rabbit was given a subcutaneous injection of 125 μg of iron-regulated OMP antigen. The rabbit was given a similar amount of antigen subcutaneously, 2 weeks later, and after an additional week, was given 250 μg of antigen intramuscularly. The rabbit was exsanguinated and antiserum collected 7 days after the third injection. Serum was collected from a healthy adult rabbit and used, without further treatment, as a control.

Adsorption of rabbit antiserum

Twenty ml of rabbit antiserum, prepared as described above, was adsorbed with lipopolysaccharide-sensitized rabbit red blood cells to remove antibodies to lipopolysaccharide. Lipopolysaccharide was extracted from E. coli 078:K80:H9 by the procedure of Westphal and

aSmall Stock Industries, Pea Ridge, AR.
Jann and sensitized red cells were prepared as described. Rabbit serum was mixed with 6 ml of sensitized red blood cells, incubated for 30 minutes at 37°C, the red cells removed by centrifugation, and the procedure repeated. Rabbit antiserum was further adsorbed to remove antibodies against OMP other than the iron-regulated OMP. Serum was mixed with 20 mg of OMP-enriched fraction isolated from E. coli 078:K80:H9 grown in iron-replete medium and incubated at 37°C for 2 hours. Outer membrane material was removed from the serum by centrifugation at 45,000 xg for 1 hour at 5°C. The adsorbed antiserum was precipitated by addition of ammonium sulfate to 35% saturation and the precipitated material was dissolved in 10 ml of PBSS for passive immunization of turkeys.

Passive immunization

One-day-old Nicholas Broad-Breasted White turkeys were obtained commercially and raised in isolation. At 18 days of age, groups of ten birds were given intravenous injections of 0.5 ml of undiluted antiserum, antiserum diluted 1:5, undiluted normal rabbit serum, normal rabbit serum diluted 1:5, or sterile PBSS. In a replicate experiment, eighteen turkeys were given 0.5 ml of undiluted antiserum intravenously and eighteen birds were given similar injections of sterile PBSS. Serum samples were collected from 5 birds in each group prior to passive immunization and two hours after immunization.

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aMidwest Hatchery, Dike, IA., or Willmar Turkey Co., Willmar, MN.
**Challenge**

Two hours after immunization, all turkeys received 0.2 ml of PBSS containing 1 to $2 \times 10^6$ colony-forming units of *E. coli* in the left lesser abdominal air sac. Ninety-six hours after challenge, turkeys were necropsied, gross lesions recorded, and a lesion score determined using criteria described previously. One ml of blood was collected from each bird prior to necropsy and incubated in brain heart infusion broth and on blood agar to detect bacteremia. A swab of the inner surface of the left lesser abdominal air sac was incubated in brain heart infusion broth to detect *E. coli*. Turkeys that were moribund before necropsy were scored as fatalities. Randomly selected colonies from blood and air sac cultures were tested for 078 antigen using a slide agglutination test with anti-078 antiserum.

**Detection of antibodies**

Antibodies to *E. coli* OMP antigens were detected by immunoblotting using the method of Towbin et al. After separation on SDS PAGE gels, proteins were transferred electrophoretically to nitrocellulose membrane (.45 μM pore size) using a Biorad transblot cell. Transfer was done at 0.11 A for 16 hours followed by 2 hours at 0.22 A in a

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[a] Dimed, St. Paul, MN.
[b] Schleicher and Schuell, Keene, NH.
[c] Biorad, Richmond, CA.
buffer solution containing 25 mM Tris, 192 mM glycine\textsuperscript{a} and 20% methanol. Blots were blocked by immersion in PBSS containing 0.05% tween 20.\textsuperscript{a} Blots were then immersed for 1 hour in tween-PBSS containing dilutions (1:5 to 1:1,000) of the serum to be tested. Blots were washed in tween-PBSS for thirty minutes, and then incubated with an appropriate dilution of biotinylated anti-turkey immunoglobulin\textsuperscript{b} or biotinylated anti-rabbit immunoglobulin\textsuperscript{c} for 30 minutes. After washing as before, blots were incubated for 30 minutes in Vectastain ABC reagent,\textsuperscript{c} prepared according to the manufacturer's directions. Blots were washed in PBSS and immersed for 5 to 10 minutes in a peroxidase substrate solution containing 6 mg 4-chloro-1-napthol\textsuperscript{d} dissolved in 2 ml methanol, 10 ml PBSS, and 5 µL of 30% hydrogen peroxide. Antibodies to 078 lipopolysaccharide were detected using an indirect hemagglutination assay as previously described.\textsuperscript{41} Titers were reported as the reciprocal of the highest dilution of serum to cause agglutination of sensitized red cells.

\textbf{Statistical analysis}

The effect of passive immunization with antiserum on each variable was assessed using a Student's $t$-test or Chi Square analysis.

\textsuperscript{a}Sigma Chemicals, St. Louis, MO.

\textsuperscript{b}Zymed Laboratories, San Francisco, CA.

\textsuperscript{c}Vector Laboratories, Burlingame, CA.

\textsuperscript{d}Biorad, Richmond, CA.
RESULTS

Iron-regulated OMP were detected in OMP-enriched fractions from *E. coli* grown in medium containing α,α'-dipyridyl (Figure 1).

Antibodies to the major OMP (porins) and to the iron-regulated OMP of *E. coli* were detected in a 1:10 dilution of rabbit serum collected prior to injection of OMP antigen (Figure 2). Rabbit antiserum harvested after 3 injections of antigen contained antibodies against a similar array of *E. coli* OMP, but antibodies to iron-regulated OMP were detectable at a serum dilution of at least 1:1,000 (Figure 2). After adsorption of antiserum, antibodies against iron-regulated OMP of *E. coli* remained (Figure 2). Antibodies to the major OMP of *E. coli* were only detectable in adsorbed rabbit antiserum using low serum dilutions. The titer of antibodies to 078 lipopolysaccharide in the rabbit serum was 40 before, and 80 after injection of antigen, and not detectable after adsorption of the rabbit antiserum.

Antibodies reactive with major OMP and with the iron-regulated OMP of *E. coli* were detected in a 1:10 dilution of the control normal rabbit serum. The reaction with the iron-regulated OMP was faint. Antibodies to 078 lipopolysaccharide were not detected in the normal rabbit serum.

Serum collected from turkeys prior to immunization had low to undetectable concentrations of antibody against 078 lipopolysaccharide. Antibodies against *E. coli* OMP, including iron-regulated OMP in some cases, were detected in preimmunization turkey serum at a dilution of
Figure 1. SDS PAGE preparation of outer membrane protein-enriched fractions isolated from *Escherichia coli* grown in iron-replete medium (lane 2) and in iron-restricted medium (lane 3). The location of the iron-regulated outer membrane proteins (FEOMP) and position of molecular weight standards (lane 1) are indicated.
Figure 2. Immunoblots of separated outer membrane proteins of iron-restricted *Escherichia coli* reacted with serum from the rabbit used for antiserum production (lanes 1-3) or a passively immunized turkey (lanes 4-6). Approximate positions of the iron-regulated outer membrane proteins (A) and the major outer membrane proteins (B) are indicated. Lanes:

1 = rabbit preimmunization serum (1:10 dilution); 2 = rabbit antiserum (1:1000 dilution); 3 = adsorbed rabbit antiserum (1:1000 dilution); 4 = preimmunization turkey serum (1:10 dilution) with biotinylated anti-turkey secondary antibody; 5 = preimmunization turkey serum (1:10 dilution) with biotinylated anti-rabbit secondary antibody; and 6 = turkey serum collected 2 hours postimmunization (1:50) with biotinylated anti-rabbit secondary antibody.
1:10 (Figure 2). Two hours after passive immunization, and immediately before challenge with *E. coli*, serum from turkeys given rabbit antiserum, diluted or undiluted, contained rabbit antibody against iron-regulated OMP of *E. coli* but rabbit antibodies against the major OMP were not detected (Figure 2). Serum from turkeys given diluted or undiluted normal rabbit serum contained rabbit antibodies which reacted faintly with the major OMP of *E. coli* but rabbit antibodies against the iron-regulated OMP were not detected. Rabbit antibodies were not detected in the serum of turkeys given PBSS.

Turkeys passively immunized with antiserum remained clinically normal after challenge with *E. coli*. Turkeys given normal rabbit serum or PBSS had ruffled, feces-stained feathers and rapid, shallow respirations 48 hours after inoculation with *E. coli*. Two birds given PBSS were dead 24 hours after inoculation with *E. coli* and 4 birds given PBSS and 2 birds given diluted normal rabbit serum were moribund 96 hours after challenge.

Passive immunization with rabbit antiserum significantly reduced the incidence of bacteremia (*p*<0.05), the frequency of air sac cultures yielding growth of *E. coli* (*p*<0.05), and the severity of gross lesions (*p*<0.01) in turkeys 96 hours after challenge with *E. coli* as compared to turkeys given PBSS or normal rabbit serum (Table 1). Because differences between groups given undiluted or diluted antiserum and those between groups given undiluted or diluted normal rabbit serum were not significant, results were combined for statistical analysis (Table 1). Turkeys given undiluted normal rabbit serum had a lower
Table 1. Gross lesions, bacteremia, and air sac culture results from turkeys given *Escherichia coli*

<table>
<thead>
<tr>
<th>Treatment(^a)</th>
<th>Mean gross lesion score(^b) (SEM)</th>
<th>Air sac culture(^c)</th>
<th>Bacteremia(^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Experiment 1:</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Antiserum (UD)</td>
<td>0.8 (.29)**</td>
<td>5/10(^*)</td>
<td>2/10(^*)</td>
</tr>
<tr>
<td>Antiserum (1:5)</td>
<td>0.9 (.30)**</td>
<td>6/10(^*)</td>
<td>3/10</td>
</tr>
<tr>
<td>Normal serum (UD)</td>
<td>1.9 (.36)</td>
<td>9/10</td>
<td>5/10</td>
</tr>
<tr>
<td>Normal serum (1:5)</td>
<td>2.6 (.38)</td>
<td>8/10</td>
<td>6/10</td>
</tr>
<tr>
<td>PBSS(^d)</td>
<td>2.8 (.32)</td>
<td>11/13</td>
<td>7/11</td>
</tr>
<tr>
<td><strong>Experiment 2:</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Antiserum (UD)</td>
<td>1.1 (.24)**</td>
<td>11/18(^**)</td>
<td>7/18(^**)</td>
</tr>
<tr>
<td>PBSS</td>
<td>3.0 (.22)</td>
<td>18/18</td>
<td>14/18</td>
</tr>
</tbody>
</table>

\(^a\)Turkeys were given 0.5 ml of the indicated solution 2 hours before challenge with *E. coli*. UD = undiluted; 1:5 = diluted 1:5; PBSS = phosphate buffered saline solution.

\(^b\)0 = no gross lesions; 1 = mild to moderate left airsacculitis; 2 = moderate left airsacculitis with mild right airsacculitis and pericarditis; 3 = severe left airsacculitis with moderate pericarditis and right airsacculitis; 4 = severe bilateral airsacculitis, pericarditis, pneumonia, and subcutaneous hemorrhage. Data are expressed as mean (standard error of the mean).

\(^c\)Number of samples with growth of *E. coli*/total samples.

\(^d\)Thirteen birds were used in this group to ensure sufficient data even if birds died prior to necropsy.

*Values are significantly (p<0.05) different than those for turkeys given normal rabbit serum or PBSS. In experiment 1 antiserum undiluted and diluted groups were combined and normal rabbit serum undiluted and diluted groups were combined for statistical analysis.

**Values are significantly (p<0.01) different than those for turkeys given normal rabbit serum or PBSS.
mean gross lesion score than birds given PBSS, although the
difference was not significant. This partially protective effect was
not seen when the normal rabbit serum was diluted. In contrast,
antiserum was protective whether given diluted or undiluted. Because
normal rabbit serum provided no significant protective effect in
experiment 1, the control group in experiment 2 was given PBSS.

Gross lesions in turkeys given antiserum were usually restricted
to mild airsacculitis at the site of E. coli injection. Gross lesions
in birds given normal serum or PBSS were extensive and consisted of
severe bilateral airsacculitis and pericarditis. Turkeys which died or
were moribund prior to necropsy had severe bilateral airsacculitis,
pericarditis, perihepatitis, and hemorrhage and edema in the subcutis.
All E. coli colonies tested were of serotype 078 by slide agglutination
and other pathogenic bacteria were not detected.
DISCUSSION

Turkeys passively immunized with antibody against iron-regulated OMP of *E. coli* were protected from experimental colisepticemia. Although birds given antiserum developed mild to moderate airsacculitis at the site of *E. coli* injection, they did not develop generalized lesions characteristic of colisepticemia.\(^{38}\) It is likely that the antibodies enhanced clearance of the bacteria by host defense mechanisms and therefore may have limited the development of disseminated disease.

The mechanism whereby antibody against iron-regulated OMP of *E. coli* provided protection to immunized poults is not clear. Antibody may have bound to the bacteria and acted as an opsonin, either directly or as a result of complement activation.\(^{21}\) Antibody-mediated complement activation may also have caused bacterial membrane injury.\(^{21}\) Antibodies, prepared in a manner similar to that used here, against an iron-regulated OMP of *E. coli* (the ferric-ferrichrome receptor) interfere with iron uptake ability of *E. coli in vitro*.\(^{39}\) Antibodies against an iron-regulated OMP of *Pseudomonas aeruginosa*, inhibit siderophore-mediated iron uptake *in vitro* and passively protect mice from *P. aeruginosa* septicemia using a burn wound infection model.\(^{131}\) It is not known whether antibodies against iron-regulated OMP interfere with iron uptake by *E. coli in vivo*, or if such an effect would be beneficial in inhibiting bacterial growth in animal tissues.

Antiserum used to immunize turkeys was not monospecific. Its predominant reactivity was with the iron-regulated OMP, but antibodies
reactive with the major OMP of *E. coli* were present. Although antibodies to 078 lipopolysaccharide were not detected in the antiserum using a sensitive assay, their presence cannot be excluded. It is possible, therefore, that a portion of the protective effect of the antiserum was because of antibodies of other specificities. However, the lack of protection by normal rabbit serum, which also contained antibodies against major OMP of *E. coli*, indicates that the primary protective effect of the antiserum was likely because of antibodies to iron-regulated OMP.

In general, *E. coli* bacterins provide little protection from disease caused by serotypes not present in the bacterin. Because at least one of the iron-regulated OMP of *E. coli* is antigenically conserved across a variety of serotypes, it is possible that antibodies reactive with iron-regulated OMP may provide some cross-serotype protection. Bacterins currently available for protection of poultry from colisepticemia are prepared in enriched culture medium containing fairly high levels of iron. Therefore, it is unlikely that these bacterins contain antigenic quantities of the iron-regulated OMP of *E. coli*. The results of this study indicate that inclusion of iron-regulated OMP of *E. coli* in a bacterin preparation may be beneficial in protecting immunized animals from colisepticemia.

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GENERAL DISCUSSION AND SUMMARY

Administration of iron potentiates systemic *E. coli* infection after air-sac inoculation of young turkeys. Isolation of significantly more *E. coli* from blood and air sacs of turkeys given iron indicates an enhanced ability of *E. coli* to replicate in the tissues of those birds, compared with that in birds not given iron. Effects of iron administration on *E. coli* infection indicate a role for host iron withholding mechanisms in resistance of poultry to systemic colibacillosis.

Results of this study are in contrast to other reports of the effect of iron on bacterial infections in birds. Differences in experimental protocol including dose, route of administration, and form of iron used, make comparison between those reports and this one difficult. Because the present results are similar to those obtained with other gram-negative bacteria, in mammalian species and chicken embryos, *E. coli* infection of turkeys would be an appropriate model for studies of the role of iron in the pathogenesis of bacterial septicemias.

Passive immunization with antibodies against iron-regulated outer membrane proteins protects turkeys from *E. coli* septicemia. Passively immunized birds developed mild airsacculitis after challenge with virulent *E. coli*, but did not develop generalized lesions characteristic of colibacillosis. Although the mechanism of protection was not determined, it is likely that the antibody enhanced clearance of *E. coli* by host defense mechanisms. Antibody may have acted as an opsonin, either directly, or by activation of complement.
mediated complement activation may also have caused bacterial membrane injury.21

Antibodies against siderophore receptors of *E. coli* and *P. aeruginosa* interfere with siderophore-mediated iron uptake by the respective organisms *in vitro*.39,131,132 A similar effect may occur *in vivo* and be an additional mechanism, independent of complement and phagocytes, whereby antibody against iron-regulated outer membrane proteins of *E. coli* protects turkeys.

Currently available bacterins for protection of poultry from colibacillosis are unlikely to contain antigenic quantities of *E. coli* iron-regulated outer membrane proteins. The results of this study indicate that inclusion of these proteins in the bacterins may be of benefit in protecting immunized birds from colibacillosis. Because siderophore-receptor outer membrane proteins are antigenically conserved between serotypes of *E. coli*,36 antibodies against these proteins may also provide some cross-serotype protection from *E. coli*. 
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My family, my sons, and especially my husband, Steven, provided the incentive and love necessary to the realization of this goal.
APPENDIX A: SERUM IRON VALUES
Table 1. Effect of dose of iron on serum iron parameters of 18-day-old turkeys

<table>
<thead>
<tr>
<th>Dose(^a) (no. inoculated)</th>
<th>0 HPI(^b)</th>
<th>6 HPI</th>
<th>12 HPI</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SI(^c)</td>
<td>%S(^d)</td>
<td>SI</td>
</tr>
<tr>
<td>0</td>
<td>18.4</td>
<td>35</td>
<td>-</td>
</tr>
<tr>
<td>3.0</td>
<td>-</td>
<td>-</td>
<td>35.1</td>
</tr>
<tr>
<td>6.0</td>
<td>-</td>
<td>-</td>
<td>60.1</td>
</tr>
<tr>
<td>12.0</td>
<td>-</td>
<td>-</td>
<td>75.2</td>
</tr>
</tbody>
</table>

\(^a\)Dose of iron in mg/kg of body weight as ferric ammonium citrate given to each turkey by air sac inoculation.

\(^b\)Hours post-inoculation.

\(^c\)Mean serum iron value in \(\mu\)M/L.

\(^d\)Mean percent saturation of serum iron-binding capacity.

\(^e\)Not done.

\(^f\)All birds given 12.0 mg/kg of iron died before 12 HPI.
APPENDIX B: ELECTRON MICROSCOPY OF AIR SACS
Figure 1. Epithelial cell loss and fibrin accumulation in an air sac of a turkey given an air-sac inoculation with ferric ammonium citrate. Similar changes were seen in air sacs treated with ammonium citrate.

Figure 2. Air sac epithelium of a turkey treated with ferric ammonium citrate. The majority of air sac samples examined from birds given ferric ammonium citrate or ammonium citrate appeared as in this micrograph.