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Characterization of turkey complement and its role in defense against *Escherichia coli* infection

by

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ABSTRACT

Two distinct pathways of complement activation were identified in turkey serum. The first pathway, indicated by the lysis of sheep red blood cells sensitized with antibody (SSRBC) was inhibited by ethylenediamine tetraacetic acid (EDTA), ethylene glycol-bis-βaminoethyl ether-N,N,N',N'-tetraacetic acid (EGTA) and carrageenan. In contrast, lysis of horse red blood cells (HRBC) was antibody-independent and was inhibited only by EDTA. Hence, the hemolytic activity against SSRBC and HRBC represent the classical and alternative complement pathways of turkeys, respectively. Treatment of turkey serum with zymosan or inulin caused depletion of hemolytic activity against both SSRBC and HRBC, suggesting the presence of a common complement pathway in turkeys.

The third component of complement was identified and isolated from turkey plasma. Turkey C3 consisted of two peptide chains, a larger C3α chain (120,000 daltons) joined by disulfide bonds to a smaller C3β chain (75,000 daltons). Using crossed-immunoelectrophoresis, turkey C3 was found to have a distinctive mobility (movement toward the anode) in complement-activated serum. A rabbit antiserum produced against turkey C3 caused agglutination of sheep red blood cells sensitized with antibody and complement, but did not cause agglutination of sheep red blood cells sensitized with

only antibody. Therefore, the isolated protein was a complement component. Based on structural and immunological analysis, and comparison to characteristics of human C3, the isolated protein was confirmed as turkey C3.

Characterization of turkey complement and development of hemolytic assays facilitated analysis of the role of turkey complement in *Escherichia coli* infections of turkeys. Resistance to the bactericidal effects of serum mediated by complement was a characteristic of virulent *E. coli* isolated from turkeys. However, five *E. coli* strains were found to be serum-resistant/avirulent, suggesting that other factors are also required for virulence. Killing of some *E. coli* strains by turkey serum was mediated by activation of the alternative complement pathway, whereas activation of the antibody-dependent classical pathway was required for killing of other *E. coli* strains. Hence, the complement system of turkeys was found to have a significant role in *E. coli* infections of turkeys in the absence and presence of antibody.

GENERAL INTRODUCTION

There are several defense mechanisms elicited in the host in response to infectious agents. Some of the host responses are nonspecific, killing varied types of organisms, while others may be limited to organisms to which the individual has had previous exposure. The complement system, a group of at least 18 serum proteins is an example of a nonspecific host defense mechanism, and it is considered to be a first line of defense. Complement-mediated killing of organisms may occur through activation of the classical or alternative complement pathways. The classical pathway usually requires specific antibody for activation. Therefore it can have a significant role in immune individuals, but little if any effect in early infection. In contrast, the alternative pathway does not require specific antibody and is activated by direct binding of the third component of complement to the surface of an organism. The alternative complement pathway is an important element of natural resistance to many infectious agents.

Activation of complement is synonymous with proteolytic cleavage of the various complement proteins. These proteins interact sequentially and result in the deposition of a membrane attack complex on the surface of an organism. Stable insertion of this complex into the cell membrane of an organism causes irreversible membrane damage and cell death.

In addition to the formation of a lytic complex, complement activation leads to the release of several protein fragments with important biological functions. Fragments of these complement proteins function in immune adherence, chemotaxis, opsonization, and mediation of acute inflammation. Hence, activation of complement by an invasive organism can have a marked effect on the outcome of infection.

Study of the role of avian complement in disease pathogenesis has been limited because very few components of complement have been purified from avian species. Manipulation of the avian complement system using known activators of mammalian complement has identified a role for complement in several viral infections. Although complement is assumed to have an important role in bacterial infections of poultry, very little research has emphasized this area.

Escherichia coli is a commonly isolated bacterial pathogen of broiler chickens and turkeys. Severe economic losses in poultry flocks infected with *E. coli* result from high mortality and carcass condemnations. Disease caused by *E. coli* is often characterized as subacute fibrinopurulent airsacculitis or acute colisepticemia. The latter form of the disease is often fatal. In order for *E. coli* to cause septicemia, bacteria must survive and multiply in the bloodstream of the host. Therefore, virulent *E. coli* must resist the bactericidal effects of serum mediated by

complement. An understanding of the virulence mechanisms of *E. coli* may be facilitated by studying the role of complement in defense against *E. coli*.

The objectives of this research were to: 1. characterize the complement activity of turkeys; 2. isolate and characterize the third component of turkey complement; and, 3. clarify the role of complement in defense against *E. coli* infections in turkeys.

Explanation of Thesis Format

This thesis is prepared in the alternate format. A general introduction and literature review precede four manuscripts dealing with turkey complement and the interaction of complement with *Escherichia coli* isolated from turkeys. The final manuscript is followed by a general discussion section and literature cited in the literature review and final discussion.

The four sections of this thesis represent the manuscripts which have already or will be submitted for publication in scientific journals under the authorship of Marlene G. Ellis, Susan J. Lamont and Lawrence H. Arp. Marlene G. Ellis was the principal investigator of each study. Each section is prepared according to the format for the journal, Infection and Immunity.

LITERATURE REVIEW

An overview of the complement system, its functions and the specific mechanisms of regulation will be presented to familiarize the reader with the primary focus of this thesis. Research in the area of complement is extensive, and the reader is referred to several good reviews of the complement system and its activation (22, 86, 87, 89). Following this introduction to the complement system, a detailed description of avian complement will be presented. The final section in this literature review discusses the interaction of complement with bacteria. Several reviews of complement and its interaction with bacteria are available to the reader (13, 15, 30, 48).

The Complement System

The complement system consists of a group of at least 18 serum proteins that actively participate in the nonspecific mechanisms of host defense against bacteria, viruses and parasites (89). Complement mediates its effects through direct interaction with pathogens (75), or by indirect interaction via specific immunoglobulin (antibody) (14, 33). Therefore, the complement system can have an important role in both immune and nonimmune individuals. Upon activation, complement components react in a specific sequence resulting in the formation of enzyme complexes, which cleave the next

complement component in the sequence. In this sense, complement activation refers to proteolytic cleavage of the respective complement components. The final result of this cascade of proteins is the deposition of a membrane attack complex onto the target cell surface (86, 98). Insertion of this complex into the cell membrane creates water- and ion-permeable channels culminating in osmotic lysis of the target cell (71). Hence, one function of complement may be the direct killing of infectious agents by disrupting the selective permeability of the cell membrane. In addition, several peptide fragments are generated during complement activation which are known to influence host defense mechanisms. A discussion of their function in defense against infectious agents will be developed later in this review.

Complement is now recognized as a multifunctional, self-assembling system that is important in humoral defense, as well as in stimulation of cellular defense mechanisms (85). A unique feature of complement which facilitated its identification is that it is heat labile, being inactivated by heating serum to 56^{o}C for 30 minutes. Much of the information on complement derives from studies of the human or guinea pig complement systems. Purification of isolated complement components has facilitated studies on the complement activation sequence and the specific mechanisms of

regulation. The molecular weight and concentration in the serum of each of the human complement components is presented in Table 1.

Two pathways of complement activation are known to exist in mammals, specifically the classical (22) and alternative (87) pathways. These pathways converge at the cleavage of the third component of complement (C3) and continue with a common terminal pathway involved in the formation of the membrane attack complex (86, 98). The interaction of these pathways is illustrated in Figure 1. The events of the classical and alternative pathways described in the following sections are the result of cell-surface associated complement activation. Therefore, two or more components forming a multimolecular complex are assumed to be in close proximity on the cell surface.

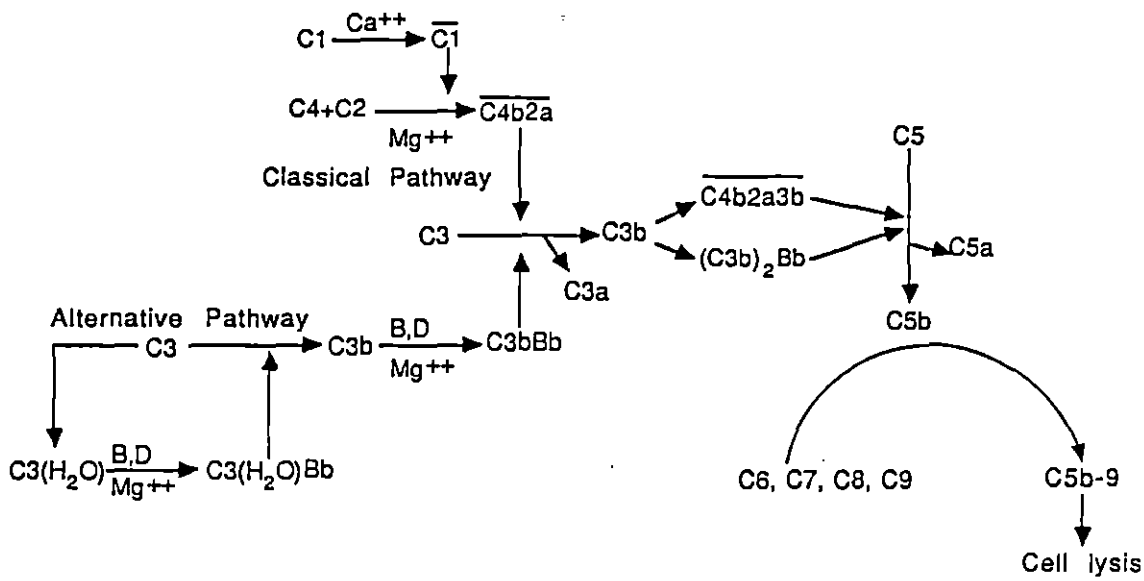
The Classical Pathway

The classical pathway, so named because it was the first to be identified and characterized, was originally observed through its bacteriolytic and bactericidal effects on specific antibody-bacterial complexes (72). Therefore, this activation pathway is important in immune individuals. Study of the reaction mechanism of the classical pathway was facilitated by the observation that complement could also lyse erythrocytes sensitized with specific antibody (11).

Table 1. Molecular weight and concentration of complement components in human serum (73)

Component	Mol. wt.	Serum concentration (ug/ml)	Chain no. x mol. wt
Classical Pathway:			
C1q	410,000	150-180	6 x 24,000 6 x 23,000 6 x 22,000
C1r	85,000	50	1 x 85,000
C1s	85,000	100	1 x 85,000
C4	210,000	400-450	1 x 90,000 1 x 78,000 1 x 33,000
C2	110,000	30	1 x 110,000
Alternative pathway:			
B	93,000	200-225	1 x 93,000
D	25,000	1.5	1 x 25,000
P	220,000	10-25	4 x 56,000
Common Pathway:			
C3	195,000	1200-1300	1 x 110,000 1 x 85,000
C5	205,000	80	1 x 120,000 1 x 85,000
C6	128,000	75	1 x 128,000
C7	121,000	55	1 x 121,000
C8	155,000	80	1 x 77,000 1 x 63,000 1 x 14,000
C9	79,000	200	1 x 79,000

Figure 1. The activation sequence of the mammalian complement system


C1
Ca++
C1
C4+C2
C4b2a
Mg++
Classical Pathway
C3
C3b
C3a
C4b2a3b
(C3b)2Bb
C5
C5a
C5b
Alternative Pathway
C3
C3b
B,D
Mg++
C3bBb
C3(H2O)
B,D
Mg++
C3(H2O)Bb
C6, C7, C8, C9
C5b-9
Cell lysis

Hence, because erythrocytes possess a single membrane and are less complex than bacteria, simplified systems were developed using sensitized sheep red blood cells to elucidate the mechanisms of the classical pathway, as well as the terminal complement pathway (4, 11, 79).

The initial step in the activation sequence of the classical pathway is the non-covalent binding of the first complement component (C1) to an antigenic surface via specific antibody (99), or in some cases, by direct non-immunological binding of C1 to complex molecules such as those listed in Table 2. The first component occurs naturally in serum as a complex of three different subunits, C1q, C1r and C1s having the molecular formula, $C1q(C1r)_2(C1s)_2$ (22). This complex is calcium-dependent with the C1q subunit binding three calcium ions and the $(C1r)_2(C1s)_2$ complex held together by four calcium ions. Chelators of calcium ions such as ethylenediamine tetraacetic acid (EDTA) or ethylene glycol-bis-βaminoethyl ether-N,N,N',N'-tetraacetic acid (EGTA) dissociate C1 thus rendering the classical pathway inactive (78). Interaction of C1 with an activating surface occurs through the C1q subunit. C1q is a highly basic protein and therefore can interact noncovalently with many polyanionic substances as shown in Table 2. The C1q molecule is composed of six peripheral globular regions, each containing a binding site

Table 2. Substances that bind and activate the first component of complement (73)

Bacteria: *E. coli* *Klebsiella* *Salmonella* *Mycoplasma*	Carbohydrates: Ant venom polysaccharide Certain di- and trisaccharides Dextran sulfate
Retroviruses	Polyanions: Heparin Polyvinyl sulfate Polyanethol sulfonate Polynucleotides Carrageenan
Parasites *Schistosoma mansoni* *Trypanosoma brucei*	
Proteins: p15E retroviral surface protein C-reactive protein complexes Myelin basic protein Immunoglobulins	Lipids: Lipid A Lipoteichoic acid
Miscellaneous: Monosodium urate crystals Mitochondrial membranes Certain cellular membranes Nitrophenylated molecules	

for immunoglobulin (89). Therefore, one C1q molecule can have up to six binding sites for immunoglobulin molecules, as well as other types of activating molecules. Only certain classes and subclasses of immunoglobulins are important in the binding of C1q and hence, classical pathway activation. In humans, IgM is a potent activator of the classical pathway, whereas IgG is less effective (89). Only one IgM molecule is needed to activate the classical pathway, whereas

at least two IgG molecules in close proximity are required for binding and activation of C1. The human IgG subclasses, IgG_3, IgG_1 and IgG_2, listed in decreasing order of C1q binding activity, are efficient complement activators, whereas IgG_4 binds complement poorly (22). The interaction of C1q with immunoglobulin occurs through the CH2 domain of IgG or the CH4 domain of IgM (22). Recent evidence suggests that the CH3 domain of IgM may also be important for the binding of C1q (109).

Upon binding to the activating surface, a conformational change occurs in C1q. By some unknown mechanism, this conformational change leads to the activation of one of the attached C1r subunits (73). This activated C1r subunit cleaves the polypeptide chain of the C1r subunit, and exposes its catalytic site (autocatalytic activation) (62). Both C1r subunits have enzymatic activity for the two C1s molecules associated with the complex. After cleavage by C1r, a catalytic site with enzymatic activity for the second (C2) and fourth (C4) components of complement is exposed on C1s. Activated C1s initially cleaves C4 into two fragments. The larger fragment, C4b binds covalently to the activating surface (89). The C2 component associates with surface-bound C4b in the presence of magnesium ions, and is subsequently cleaved by activated C1s (73). The complex, C4b2a forms an enzyme termed C3 convertase of the classical complement

pathway. The C3 convertase cleaves the third component of complement, yielding a larger fragment, C3b which interacts covalently with the activating surface (68). Interaction of C3b with the C4b2a complex results in the formation of a C5 convertase (C4b2a3b), and initiates the terminal pathway of complement activation (89).

The Alternative Pathway

Activation of the alternative complement pathway was first observed and proposed by Pillemer and colleagues in 1954 (94). It was observed that zymosan, a crude preparation of yeast cell walls could activate complement without the consumption of the initial components (C1, C2, and C4) of the classical pathway (95). Complement activation by zymosan proceeded in the absence of specific antibody, and it was suggested that an alternative pathway may be important in resistance to infections in non-immune individuals. Since this early discovery, several substances have been reported to activate the alternative complement pathway in the absence of antibody, and some of them are listed in Table 3.

The covalent binding of C3b to the activating surface is an essential requirement for activation of the alternative pathway (87, 89). However, the mechanism of formation of the initial C3b is unclear. It was proposed by Lachmann that a "C3 tickover" occurs naturally in serum (64). He suggested

Table 3. Substances that activate the alternative complement pathway

Substance	References
Polysaccharides:	
Yeast (zymosan)	95, 107
Bacterial lipopolysaccharide	51, 84
Inulin	73, 91
Immunoglobulins: guinea pig IgG_1; aggregated human IgG_4; IgA; IgE; $F(ab')_2$ fragments of IgG	18, 33, 89, 106
Cobra venom factor	21
Heterologous erythrocytes	88, 91
Dextran sulfate	73

that C3 interacts with water, forming a C3b-like molecule which can react with additional components (factors B and D) of the alternative complement pathway. A weak C3 convertase, $C3(H_2O)Bb$ is produced which is able to cleave C3, yielding the initial C3b molecules. More recent evidence suggests that this is true, and that C3 can spontaneously interact with water, forming a C3b-like molecule (29). Under normal conditions, the spontaneously formed C3b is rapidly inactivated (67, 105). Substances such as zymosan, or certain strains of bacteria present in the serum can bind C3b and hence may activate the alternative complement pathway

(24, 84). Whether a substance is able to activate the alternative complement pathway depends on the stability of C3b and its susceptibility to inactivation by factor H and C3b-inactivator (see Regulation of the Complement System, page 22). It should be noted that the classical and alternative pathways are not mutually exclusive. Molecules of C3b formed via the classical pathway may initiate the alternative pathway upon binding to the activating surface.

Once C3b is surface-bound, it interacts noncovalently with factor B. Factor D, a serine protease present in its active form in serum specifically cleaves factor B into two fragments. The larger fragment, Bb remains associated with C3b to yield the C3 convertase, C3bBb. This C3 convertase is stabilized by the serum protein, properdin (P) (81). The C3bBbP complex is able to cleave C3 and generate additional molecules of C3b. An amplification loop occurs in which newly formed C3b molecules interact with factors B, D, and P to form additional C3 convertases (87). This leads to the deposition of large amounts of C3b on the activating surface. Interaction of two or three molecules of C3b with one molecule of Bb in the presence of properdin forms the C5 convertase, $(C3b)_2BbP$ thus initiating the terminal complement pathway (89).

The Terminal Pathway

The classical and alternative complement pathways converge with the cleavage of the third component of complement, as shown in Figure 1. After C3 is cleaved, the larger fragment, C3b interacts with the C3 convertase to form the respective C5 convertase of either the classical (C4b2a3b) or alternative (C3bBbPC3b) pathways. Cleavage of C5 produces a larger fragment, C5b which initiates formation of the lytic complex of complement (86, 98). The remainder of the complement pathway does not involve enzymatic cleavages, but rather a self-assembly of the final complement components. The C5b fragment generated by the C5 convertase contains a labile binding site, and must interact with the next component, C6, within milliseconds (6). Interaction of C5b with C6 results in a stable bimolecular complex that is loosely associated with surface-bound C3b (86). Upon binding of C7, conformational changes occur, exposing hydrophobic binding sites on the C5b67 complex (3). Expression of these hydrophobic domains on the surface of the C5b67 complex allows it to partially insert into the phospholipid bilayer of the target membrane (80). At this stage, very little damage occurs to the target membrane, and the C5b67 complex is relatively stable in the membrane. Binding of C8 to the C5b67 complex creates a small membrane pore that is sufficient to lyse erythrocytes, but not nucleated cells or

bacteria (86). The C5b678 complex serves to concentrate C9 and facilitate its polymerization on the target cell membrane (128). The poly-C9 forms the hydrophilic channel of the membrane attack complex which is responsible for lysis of nucleated cells and bacteria (54, 86). The interaction of these final components (C5b-9) forms the membrane attack complex of complement which is responsible for cell lysis.

The Membrane Attack Complex and Mechanisms of Cytolysis

The actual molecular formula and weight of the membrane attack complex is not known because the C5b-9 complex has a tendency to aggregate after detergent solubilization (126). Current evidence indicates that the membrane attack complex may be composed of a dimer of C5b-8 plus a polymer of twelve to eighteen C9 molecules (8, 127). The approximate molecular weight of this $(C5b\text{-}8)_2C9_{12\text{-}18}$ complex is estimated to be greater than 1,000,000 daltons (6).

Ultrastructural analysis of the complement lesions on erythrocyte membranes shows electron-dense "holes" surrounded by ring-like structures (126). This observation was described by Borsos and colleagues and referred to as the "doughnut hypothesis" (11). Further analysis by Mayer suggested that the ring structure may be a transmembrane channel formed by the C5b-9 complex (79). The complete structure of the membrane attack complex in the target

membrane is now described as a short, hollow cylinder rimmed by an annulus exterior to the membrane (5, 126). The external and internal diameters of the ring-like structure are 20 nm and 10 nm, respectively (126). The length of the cylindrical structure of the membrane attack complex is approximately 15 nm (126). Although the size of the membrane attack complex is quite uniform, heterogeneity is observed in the functional pore size (100). The heterogeneity in functional pore size is attributed to either differences in the number of C9 molecules associated with the complex, or "fusion" of individual monomers of C5b-9 (101, 111).

The exact depth which the membrane attack complex inserts into the lipid bilayer of the membrane is uncertain. Studies with freeze-etch electron microscopy suggest insertion of the membrane attack complex to a depth of 4 to 5 nm (4, 5). Therefore, the inserted protein complex would span the external lipid monolayer and extend at least partially into the internal lipid monolayer of the target erythrocyte membrane. The remaining portion of the cylinder and annulus extend from the membrane to the extracellular environment (5). Two models have been proposed to explain the mechanisms of cytolysis by the membrane attack complex. The "mixed micelle model" described by Esser et al. suggests that the membrane attack complex has membranodisruptive characteristics (23). Insertion of the membrane attack

complex into the target membrane would cause disruption of the lipid bilayer and thus, result in membrane leakiness. In this case, the complement lesions would be poorly-defined in size, unstable in the membrane and perhaps, membrane disassembly would be observed (7). The alternative "channel model" proposed by Mayer suggests that the membrane attack complex would assume properties of an integral membrane protein and form a stable hydrophilic transmembrane pore through which ions and small molecules may pass bidirectionally (79, 80). Because of the high density of solute within the cell, water and electrolytes move into the cell via the complement pore causing cell lysis. The latter model is now generally accepted. However, the actual mechanisms of cytolysis probably involve a combination of the two models (7). Diffusion of water and small molecules into, or from the cell may occur both through the hydrophilic protein pore and along the boundary lipid.

Complement Fragments and their Function

As described previously, complement activation refers to the proteolytic cleavage of the respective complement components into two fragments. The larger fragments (e.g. Bb, C3b, C4b, C5b, etc.) usually continue to function in the assembly of the membrane attack complex, while the smaller fragments (e.g. Ba, C3a, C4a, C5a, etc.) are released into the

tissue sites of complement activation (73). These smaller fragments of complement are responsible for several important biological functions during host defense against invasive organisms (7, 89). Some of the complement fragments and their known functions are summarized in Table 4.

The overall role of the complement system is the destruction and removal of infectious agents from host tissues (15, 30). The complement system effects its action through the formation of the membrane attack complex and release of activated complement fragments. The membrane attack complex has a significant role in the killing of certain strains of gram-negative bacteria and virus-infected cells (66, 90, 121). In contrast, the membrane attack complex has very little influence on gram-positive bacteria, and some strains of gram-negative bacteria (13, 52, 53). The complement fragments released into the surrounding tissues have a more significant effect on these complement-resistant organisms.

In general, some of the complement fragments are chemotactic for phagocytic cells, some facilitate phagocytosis (opsonins) and others stimulate an inflammatory response. Opsonization of organisms by fragments of complement can be an important host defense mechanism (48). Cell surface receptors for C1q, C4b, C3b ($C3b_i$, C3d) and C5b have been found on phagocytic cells (22, 73, 87). For

Table 4. Biological functions of activated complement components (73)

Complement component	Functions
C2a	Kinin-like activity; increase of vascular permeability and contraction of smooth muscle
C3a,C4a,C5a	Anaphylatoxic peptides bind to receptors on granulocytes, macrophages, mast cells, and thrombocytes; release of vasoactive amines; enhanced vascular permeability; contraction of smooth muscle; induced release of lysosomal enzymes
C5a,C5b67,Ba	Chemotaxis; induction of migration of leukocytes into an area of complement activation; granulocyte aggregation; activation of intracellular process, such as release of oxygen metabolites and SRS-A
C3b,C4b	Immune adherence and opsonization; bridging between a complex or target cell bearing C3b or C4b and the responding cell having a receptor for C3b or C4b: phagocytic cells, macrophages, monocytes, polymorphonuclear leukocytes, B lymphocytes, primate erythrocytes, platelets

example, cells of the mononuclear phagocytic system have receptors for C3b, and hence, play an important role in the clearance of opsonized organisms from the bloodstream (13, 48). Hence, the conjoint action of the complement system and the phagocytic cells has a significant influence on survival of infectious organisms in host tissues.

Regulation of the Complement System

The effects of complement are very localized and limited to the site of complement activation (85). Rapid inactivation of complement fragments by various serum regulators is essential for preventing lysis of innocent bystander cells, a process known as "reactive lysis" (65, 125). Several steps in the complement activation sequence are subject to regulation. Three levels of regulatory mechanisms coexist.

Firstly, certain serum proteins have been identified that react specifically with activated complement components causing their inactivation, as shown in Table 5. Most of these regulators act either by cleaving the activated complement protein or by dissociating complexes of complement proteins. In each case, there is a loss in complement activity.

A second regulatory mechanism exists for certain complement proteins. Several of the activated complement fragments expose binding sites, which interact with the next complement component in the sequence, or with cell surfaces. Many of these binding sites on the activated molecules are metastable, and usually have only milliseconds to react with a receptor molecule (85). This has been identified to occur with the reactive groups in C4b, C3b, and C5b (99, 33, 98). The unavailability of receptive surfaces for the activated

Table 5. Regulators of complement activity (89, 99)

Regulator	Mechanism of Action
C1 Inhibitor	-Dissociates C1r and C1s from C1q. Thereby, indirectly inhibits activation of C1s and reduces the rate of cleavage of C4 and C2. -Inhibitor of other serum proteins (plasmin, kallikrein, Hageman factor).
C3b-Inactivator (Factor I)	-Cleaves the alpha chain of C3b, and in the presence of unidentified serine proteases forms C3c and C3d. A loss in all opsonic, immune adherence, and C5 acceptor activities occurs with C3b inactivation. -Inactivates C4b by a similar mechanism to that of C3b.
Factor H	-Cofactor; binds to C3b and facilitates inactivation of C3b by Factor I. Binding of factor H is essential for C3b inactivation.
C4b-binding protein	-Cofactor; binds to C4b and accelerates inactivation of C4b by Factor I.
S Protein	-Binds to hydrophobic regions of the C5b67 complex, and prevents its uptake by cell surfaces of bystander cells.
Anaphylatoxin Inactivator	-Removes the C-terminal arginine residue from C3a and C5a, which inactivates the anaphylatoxic activity.

complement fragments leads to hydrolysis of these fragments, and hence, their inactivation.

Thirdly, host cell surface structures are now recognized as being important in the regulation of complement activity. A common constituent on host cell surfaces is sialic acid, and evidence indicates that this molecule may have an important role in regulation of C3b deposition by the alternative complement pathway (25, 57). The classical example of this phenomenon is that of sheep red blood cells, which do not activate the alternative pathway. Removal of the sialic acid by treatment with trypsin renders the sheep red blood cells potent activators of the alternative pathway (25). Sialic acid is suggested to increase the affinity of factor H for C3b, and hence, make C3b more susceptible to inactivation by the C3b-inactivator (57). Therefore, absence of sialic acid on target cell surfaces may be required for activation of the alternative pathway. This is exemplified by zymosan and rabbit erythrocytes, which lack sialic acid on their surfaces and are potent activators of the human alternative pathway (88, 96, 107). However, other mechanisms of regulation must be in effect as the rabbit alternative pathway is not activated by homologous red blood cells. Another molecule suggested to be important in complement regulation is the C3b-receptor (CR-1) found on various host cells. This CR-1 receptor modifies C3b and C4b by some

unknown mechanism, and makes them susceptible to cleavage by C3b-inactivator (58, 82). The presence of the CR-1 receptor on host cells may be important in preventing lysis of homologous cells under normal conditions. For example, when C3b is spontaneously formed, its binding to homologous cells does not result in activation of the alternative pathway.

The Third Component (C3) of Complement

The third component of complement plays a key role in the complement activation sequence. Both the classical and alternative complement pathways converge with the cleavage of C3 (73, 89). The activated fragments of C3 include an anaphylatoxin (C3a) and a surface-bound receptor (C3b) (17, 33, 63). The membrane-associated C3b has important functions in opsonization, immune adherence, and formation of the C5 convertase for final assembly of the membrane attack complex (48, 87, 130). Hence, because C3 is multifunctional it is a good candidate for analysis during disease pathogenesis. The importance of C3 is shown by the observation that its deficiency leads to severe recurrent bacterial infections (45).

The third component of complement is the most abundant in human serum (approximately 1.2-1.3 mg/ml) (73). Isolation and characterization of C3 has been reported for several species, including human, rabbit, chicken and quail

(38, 41, 56, 59). Human C3 is characterized as a glycoprotein (195,000 mol. wt.) composed of a larger C3α chain (127,000 mol. wt.) joined by a disulfide bridge to a smaller C3β chain (75,000 mol. wt.) (38). The C3 convertases of the classical and alternative pathways activate C3 by cleaving the α chain, and releasing C3a (9,000 mol. wt.) (68). Cleavage of C3 exposes a reactive carbonyl group in the C3b fragment, which binds covalently (amide or ester linkage) to proteins or polysaccharides (18, 68, 93). One particular interaction of C3b is that with the immunoglobulin, IgG. The C3b-IgG complex is more efficient than IgG in sensitizing *E. coli* for complement-mediated killing, as will be discussed later (47).

The reactive group of C3b is believed to originate from an internal thiolester bond in C3 (46, 124). A similar group has been identified in C4 and α2-macroglobulin (110). This reactive thiolester bond is sensitive to amines (e.g. hydroxylamine, methylamine), ammonium salts, and denaturing conditions (extremes in temperature, pH, and reducing conditions with mercaptoethanol) (68, 110, 116). Sensitivity to the nitrogen nucleophiles and appearance of a free thiol group after treatment with the nucleophiles provides some evidence that the reactive group in C3 is a thiolester bond (116, 124). It is suggested that this reactive thiolester is associated with the covalent binding mechanism of C3, and

therefore has an important role in the function of C3 (68). Inactivation of the thiolester bond by amines, etc., results in a loss of C3 activity (68, 124). Endogenous substances such as ammonia may have an important role in regulating this particular complement protein (42).

The Avian Complement System

Unlike the mammalian and human complement systems, relatively little is known about the avian complement system and its role in infection. Much of this lack of knowledge is due to the unavailability of purified complement proteins from avian species. Presently, most of the assays used in the study of avian complement analyze functional activity (e.g. lysis of erythrocytes or of virus-infected cells), and therefore do not identify functions of individual complement proteins (60, 135). Progress in the isolation of avian complement proteins has been made, as shown in Table 6 and the future availability of these individual proteins will facilitate characterization of the avian complement system and its role in host defense.

The chicken complement system is the most widely studied system in avian species, followed by the turkey and quail systems. An initial feature of chicken and turkey complement to be described was their incompatibility with mammalian antibody (102). The incompatibility has been attributed to

Table 6. Complement proteins isolated from avian species

species	complement protein	reference
chicken	C1q	134
	C4	69
	C3	59
	B	61
quail	C3	56

evolutionary differences between mammalian and avian species. Researchers have acknowledged this finding, and suggest that caution should be taken when applying assays which are used for the study of mammalian complement to the study of avian complement (59, 60).

Evaluation of the hemolytic activity of chicken or turkey complement for sheep red blood cells sensitized with homologous antibody has indicated several characteristics. Firstly, hemolytic complement activity of chicken serum is much lower than that of several mammalian species including guinea pig, cat, dog and rabbit (35). A second distinct feature of chicken complement is its ability to cause hemolysis at low ionic strengths, a condition which severely limits the activity of mammalian complement (32). Thirdly, the functional complement activity of chicken and turkey sera increases with age, reaching maximal levels at approximately

six weeks of age (112). The hemolytic titers for chickens and turkeys at this age are 1:33 and 1:25, respectively.

Several studies have presented evidence suggesting the presence of an alternative pathway in avian species. Lysis of horse and rat erythrocytes is indicative of alternative pathway activity in chickens (60, 91). A role for the alternative complement pathway is well-established in some viral infections of poultry. The cytolytic activity of quail serum against Rous-sarcoma virus-infected cells and of chicken serum against fowlpox virus-infected cells is attributed to activation of the alternative complement pathway (90, 135). *In vivo* studies with fowlpox virus showed that decomplementation of chickens using cobra venom factor resulted in an enhancement of virus growth and dissemination of the virus throughout the host (92). In addition, it was noted that infiltration of polymorphonuclear cells, macrophages or lymphocytes to the site of viral infection was reduced in the cobra venom factor-treated chickens. These observations suggest that not only is complement activation important in the lysis of virus-infected cells, but may also be necessary for stimulation of cellular functions.

There has been some controversy over the presence of a functional classical pathway in poultry species, and this may perhaps explain the incompatibility between avian and mammalian systems (59, 60). An antibody-dependent, calcium-

dependent pathway is described in the chicken (91, 135). However, there is some hesitancy in defining it as the classical pathway. Although the initial component of the classical pathway (C1q) has been identified in chickens, it was suggested by one group that chicken C2 and C4 may be non-existant (60). The basis for this hypothesis was not clearly indicated. Although there is no published data available, the fourth component (C4) of chicken complement was isolated and characterized by W. H. Lee (69). At this point, the requirement for purified avian complement proteins is emphasized. Availability of these proteins will facilitate analysis of the avian complement system, and provide direct evidence for an avian classical complement pathway.

The Complement System and Defense Against Bacteria

The precise role for complement in defense against bacteria depends on several factors related to the type of organism, its cell surface composition and other virulence factors (48). The effects of complement on gram-negative bacteria are different from those described for gram-positive bacteria (13, 121). The difference is attributed to the bacterial cell wall structure. Gram-negative bacteria have a more complex cell wall structure than gram-positive bacteria. The cell envelope of gram-negative bacteria consists of four layers: the cytoplasmic membrane; the peptidoglycan layer;

the periplasmic space; and the outer membrane (47). In contrast, the gram-positive bacterial cell wall lacks the outer membrane, and has a much thicker peptidoglycan layer (13). Additional capsular polysaccharide may be present on the surface of both gram-negative and gram-positive bacteria. Because there are differences between gram-negative and gram-positive bacteria and their interaction with complement, they will be addressed separately, with emphasis on gram-negative bacteria.

Interaction of Complement with Gram-Negative Bacteria

The outer membrane of gram-negative bacteria and its surface constituents are exposed to the microenvironment of the bacteria. The outer membrane is not composed of the normal phospholipid bilayer as seen with the cytoplasmic membrane, or with eucaryotic membranes (39). Instead, the inner monolayer contains primarily phospholipid and the outer monolayer contains lipopolysaccharide (LPS). The LPS is anchored in the outer membrane by hydrophobic binding to membrane proteins. Divalent cations, calcium and magnesium are suggested to be important in maintaining stable interactions between adjacent LPS molecules, and hence the integrity of the bacterial cell envelope (76). Chelation of these cations by EDTA or EGTA releases large amounts of LPS from the membrane (39, 70). For example, up to 67% of the

total LPS in *E. coli* may be released by treatment with EDTA (39). With the release of LPS, the membrane becomes more permeable to various molecules, and susceptible to attack by colicins, antibiotics and complement (16, 39, 70).

The LPS is unique to gram-negative bacteria. It is composed of three covalently-linked domains: the lipid A, the core polysaccharide and the O-polysaccharide side chain. The lipid A is the portion through which the LPS is anchored in the membrane. The lipid A has toxic properties, therefore, LPS is often referred to as endotoxin. The core polysaccharide is invariable among gram-negative bacteria, and consists of residues unique to procaryotes. The O-side chain is the outermost region of LPS, and consists of repeating units of oligosaccharides. It is the O-side chain which determines the serological specificity of an organism. The length of the O-side chain varies from strain to strain, ranging from smooth variants with long, complete LPS molecules to rough variants which lack the O-side chain of LPS.

In general, gram-negative bacteria are more susceptible to the bactericidal effects of complement than gram-positive bacteria (20). In the absence of antibody, complement may be activated by the classical (75, 104) or alternative (103, 108) pathways upon interaction with gram-negative bacteria. The primary molecule responsible for non-immunological

activation of the complement system is LPS (27). The lipid A portion of LPS binds C1q and activates the classical pathway (75). Hence, rough bacterial strains which lack the O-polysaccharide side chain may be efficient activators of the classical pathway. This antibody-independent activation of the classical pathway by some strains of gram-negative bacteria may require an additional undefined serum protein (19). In contrast, the alternative pathway is usually activated by the O-polysaccharide side chain of LPS (84). Polysaccharides are well known acceptors of activated C3b, and it is suggested that the complex polysaccharides of bacterial LPS may serve as acceptors of the initial C3b molecules (18). Deposition of C3b on the O-side chain of LPS in a "protected site" from factor H and C3b-inactivator allows activation of the alternative complement pathway by certain strains of gram-negative bacteria. The length and composition of the O-side chain can influence the capacity of bacteria to activate complement (84).

An additional cell surface structure of gram-negative bacteria which may interact with complement is the capsular polysaccharide. Because of its high charge and hydrophilic nature, the capsular polysaccharide does not activate complement efficiently (1, 129). In addition, certain polysaccharide capsules such as the K1 capsule of *E. coli* do not activate the alternative complement pathway (114). The

K1 capsule of *E. coli* is a homopolymer of sialic acid, a molecule involved in the inactivation of surface-bound C3b, as described earlier.

Although some strains of bacteria are killed in the absence of antibody, many strains usually require the presence of specific antibody. For example, antibody markedly increased the rate of killing of K1 *E. coli* isolates (115). The bactericidal antibodies may be specific for O-antigens, outer membrane proteins or capsular polysaccharides (15, 104). Antibody serves three functions in complement-mediated defense against bacteria. Firstly, it may facilitate activation of the classical pathway. Many of the K1 *E. coli* strains require antibody for their killing by the classical pathway (115). Secondly, it may enhance C3b deposition by the alternative pathway (50, 51). The IgG-C3b complexes are more efficient in opsonization and killing of bacteria (37). The killing may be the result of C5b-9 localization near membrane-bound IgG-C3b complexes (49). Thirdly, antibody may increase the bactericidal efficiency of the C5b-9 complex by localizing the complex near IgG-C3b complexes or stabilizing it in the outer membrane of bacteria (49, 50).

The ability to survive the bactericidal effects of complement establishes the basis for serum-resistant and serum-sensitive gram-negative bacterial strains (118). The

mechanisms by which complement causes killing of certain bacterial strains are not understood. Complement-mediated killing of bacteria is quite different from the lysis of red blood cells. Gram-negative bacteria possess a more complex cell envelope, have the capacity to multiply rapidly and respond to environmental stressors (phenotypic variation), and are able to deviate energy sources for repair of damaged sites (121). Hence, it is understandable that more effective molecules of complement are needed for the killing of gram-negative bacteria, in comparison to lysis of red blood cells. For example, it has been estimated that at least 800 to 1000 C5b-9 complexes per colony-forming unit of *E. coli* are needed for killing by human serum (53).

The events which occur after interaction of complement with serum-sensitive bacterial strains are partially defined. Initially, there is a short lag phase in growth, followed by a rapid loss in viability by 80 minutes after treatment of bacteria with serum (119). Release of periplasmic proteins and phopholipids has been reported to occur during serum killing of bacteria (26, 108). The phospholipids are suggested to originate from the bacterial outer membrane or from the periplasm (108). However, loss of either material is not apparently related to bacterial killing (121).

Both bacterial-derived energy sources, and macromolecular biosynthesis (DNA, RNA, and protein) may be required for efficient killing of bacteria by complement (114, 119). The reasons for these requirements are unclear. It was suggested that serum-sensitive bacterial strains may translocate the C5b-9 complex from the outer membrane to the inner membrane by means of its own energy sources (119). However, more recent evidence suggests that this does not occur. Examination of the inner membrane from serum-sensitive *E. coli* indicated that very few of the complement components of the membrane attack complex were deposited on it (120). This would suggest that the membrane attack complex of complement mediates its killing from the outer membrane of gram-negative bacteria.

At the present time, the mechanism for complement killing is believed to be the result of perturbation of the inner membrane. Taylor and Kroll proposed that there is a transient contact of the inner membrane with the membrane attack complex (121). Several reports have suggested that this contact may occur at the bioadhesion zones where the outer and inner membranes are temporarily joined together (10, 121, 133). The function of these bioadhesion zones are not known. It has been suggested that they may have a role in transport of newly synthesized outer membrane proteins or LPS molecules from the inner membrane to the outer membrane

(76). It may be that damage of the outer membrane by the membrane attack complex stimulates protein synthesis and cell repair, resulting in the formation of bioadhesion zones. This may allow interaction of the membrane attack complex with the inner membrane at times of cell repair. A disturbance of the inner membrane may cause a potassium efflux, membrane depolarization and hence, bacterial death (10). This is supported by the fact that disruption of the outer membrane is separable from disruption of the inner membrane (26). An understanding of the biochemical and cellular events that occur during complement attack on bacteria may be necessary to elucidate the mechanisms of complement killing.

Several bacterial strains have developed virulence factors to avoid the lethal attack of complement. These organisms are usually the cause of many types of disease (43, 113, 114). Surface structures associated with complement resistance include the capsular polysaccharide (34, 43), smooth lipopolysaccharide (36, 97), and outer membrane proteins (83, 122). No one specific structure has been associated with complement resistance, and it appears that a combination of these virulence factors are needed for complete complement resistance (123). The mechanism for complement resistance centers on the interaction of the membrane attack complex with the bacterial outer membrane.

For example, stable insertion of the C5b-9 complex into the outer membranes of *Escherichia coli* and *Salmonella minnesota* is required for bacterial killing (52, 53). Release of the membrane attack complex from the outer membrane is associated with the serum-resistant strains of these organisms. In contrast, studies with gonococci indicate that both serum-sensitive and serum-resistant strains have a stable interaction with the membrane attack complex (40, 55). It is suggested that serum-resistant gonococci may alter the orientation of the membrane attack complex in the outer membrane, or they may have different sites for attachment of the complex (15). Hence, it can be seen that the mechanisms for complement resistance are complex, depend on the bacterial species, and understanding them requires molecular analysis of the bacterial surface structures and their interaction with complement components.

Interaction of Complement with Gram-Positive Bacteria

Several gram-positive bacteria, including strains of *Streptococcus pneumoniae*, and *Staphylococcus aureus* have been shown to activate either the classical or alternative complement pathways in the absence of specific antibody (13, 31, 131). The nature of the structures involved in complement activation are not well-defined. The lipoteichoic acid extending from the cytoplasmic membrane of gram-positive

bacteria may be important in direct binding of C1q and activation of the classical complement pathway (74). The peptidoglycan layer may activate the alternative complement pathway, but the extent of activation is limited (131).

Gram-positive bacteria are generally considered to be serum-resistant (13). The ability to resist killing by complement is largely due to the thick peptidoglycan layer overlying the cytoplasmic membrane. The length of the membrane attack complex is approximately 15 nm (126). In comparison to a 160 nm thickness of the peptidoglycan layer of for example, *Streptococcus pneumoniae* the effect of the membrane attack complex on gram-positive bacteria is minimal (13). Therefore, the role for complement in gram-positive bacterial infections is not one of cell lysis, but rather generation of opsonic complement fragments, such as C5b67 and C3b, as well as inflammatory stimulators, C5a and C3a (13). However, several pathogenic gram-positive bacteria, including *Streptococcus pneumoniae* and *Staphylococcus aureus* are not markedly affected by these complement fragments. The reason for this complement resistance is that most of the opsonic fragments are deposited on the bacterial surface in areas where they are not exposed to phagocytic cells. For example, studies with *Staphylococcus aureus* indicated that C3b is deposited on the cell wall "hidden beneath" the bacterial capsule (132). It has been shown that anti-capsular antibody

redistributes the C3b to the capsule, and thus makes the C3b component a more efficient opsonin (14). In summary, complement in conjunction with antibody and the phagocytic system can provide an effective means of eliminating invasive gram-positive bacteria.

SECTION I. CHARACTERIZATION OF COMPLEMENT ACTIVITY IN TURKEYS: EVIDENCE FOR CLASSICAL AND ALTERNATIVE COMPLEMENT PATHWAYS

ABSTRACT

The complement activity in turkey serum was examined by using activators or inhibitors of mammalian complement. Hemolytic test systems using sheep red blood cells sensitized with specific turkey antibody (SSRBC) and horse red blood cells (HRBC) were developed to measure complement activity of turkey sera treated with the various activators/inhibitors. Lysis of SSRBC was blocked by treatment with 6 mM EDTA, 10 mM EGTA and carrageenan. In contrast, lysis of HRBC was blocked by 6 mM EDTA, but not by 10 mM EGTA or carrageenan. Addition of magnesium ions to EGTA-chelated serum facilitated the lysis of HRBC but not the lysis of SSRBC. Treatment of serum with zymosan at 1 mg/ml and inulin at 5 mg/ml depleted hemolytic activity against both SSRBC and HRBC, suggesting depletion of components common to both pathways. Differences in the hemolytic activities of sera against SSRBC and HRBC after treatment with the various complement activators and inhibitors suggest the presence of two complement pathways. The lysis of SSRBC is antibody-dependent and requires calcium and magnesium. This pathway is analogous to the classical complement pathway of mammals. The lysis of HRBC is antibody-independent and requires only magnesium. This pathway is analogous to the mammalian alternative complement pathway. Thus, both classical and alternative complement pathways were demonstrated in turkeys.

INTRODUCTION

The complement system, a group of serum proteins, is involved in mediation of inflammation and nonspecific host defense against bacteria, parasites and viruses (4). Two pathways of complement activation are recognized in mammals, namely the classical and alternative pathways. The classical pathway is activated by specific antibody, or by certain polymeric substances such as heparin, protamine, carrageenan and lipid A of gram-negative bacteria (2). In contrast, the alternative pathway is not activated by specific antibody but by the covalent binding of the third component (C3) to aggregated immunoglobulins or complex polysaccharides such as zymosan, inulin, and lipopolysaccharide of gram-negative bacteria (12). The classical and alternative pathways converge with the cleavage of C3 as the common pathway culminating in formation of a membrane attack complex. Insertion of this complex into biological membranes results in increased permeability and subsequent cell lysis (11).

Research on chicken complement strongly suggests the presence of both classical and alternative complement pathways analogous to the mammalian complement pathways. The lysis of sheep red blood cells sensitized with antibody is evidence of classical complement activity in chickens (13). Several viruses, as well as xenogenic red blood cells are

known to activate the chicken and quail alternative complement pathways (7, 13, 14, 18). Additional support for classical and alternative complement activities is shown by the specific isolation of complement components from both pathways (6, 8, 9, 17).

Basic information on the classical or alternative pathways of turkey complement is not available. The mean hemolytic titer of 6-week old turkeys was reported to be 1:25 using the lysis of SSRBC as an indicator system (16). This titer gives a baseline measure for complement activity in turkeys. In this paper, we demonstrate the presence of classical and alternative complement pathways in turkeys. Also, concentrations of various complement activators that result in total depletion of one or both pathways of complement activity were determined.

MATERIALS AND METHODS

Turkey Serum. Blood was collected from normal adult turkeys (n=25) and allowed to clot at room temperature for 1 hour and then at 4°C for 1 hour. Serum was harvested, pooled and divided into 10 ml aliquots. All serum was frozen at -70°C until required.

Red Blood Cells. Sheep red blood cells (SRBC) and horse red blood cells (HRBC) were collected separately, and each was mixed with equal volumes of Alsever's solution. Red blood cells were washed twice in phosphate-buffered saline, pH 7.2, a third time in gelatin veronal buffer (GVB++) containing 0.15 mM calcium and 0.5 mM magnesium, pH 7.2 (5) and then resuspended in GVB++. Horse red blood cells were standardized to a concentration of 7×10^7 cells/ml for use in the hemolytic assay. Sheep red blood cells were sensitized with specific antibody by incubating a 1% SRBC suspension with an equal volume of 1:200 turkey anti-SRBC antibody at 37°C for 30 minutes. Sheep red blood cells sensitized with specific antibody (SSRBC) were then pelleted, resuspended in GVB++ and standardized to a concentration of 1.4×10^8 cells/ml for use in the hemolytic assay.

Hemolytic Complement Assays. The hemolytic activity of sera was determined using a round-bottom microtiter test plate system. Two-fold serial dilutions of test sera

(50 ul/well) were mixed with equal volumes of red blood cell suspensions (7×10^7 HRBC/ml or 1.4×10^8 SSRBC/ml). Plates were incubated at 37°C for 1 hour (SSRBC) or 2 hours (HRBC) as previously described (13). Microtiter test plates were then centrifuged to pellet unlysed cells. The dilution of serum causing an approximate 50% decrease in the red blood cell button was noted, and the hemolytic titer was then reported as the reciprocal of that dilution of serum, recorded as the $\log_2$ titer. All serum samples were tested in quadruplicate.

Adsorption of Natural Antibody to HRBC from Turkey Serum. In order to determine whether horse red blood cells were lysed by classical pathway or alternative pathway activation, anti-HRBC antibody was adsorbed from turkey serum, and then hemolytic activity against HRBC was determined. Turkey serum (1 ml) was mixed with 0.25 ml packed HRBC at 4°C for 15 minutes. Horse red blood cells were removed by centrifugation (1000 rpm, 10 minutes) and the adsorption was repeated a second time. The supernatant was collected after the second adsorption and tested for hemolytic activity against HRBC.

Chelation of Divalent Cations. Ethylenediamine tetraacetic acid (EDTA) and ethylene glycol-bis-βaminoethyl ether-N,N,N',N'-tetraacetic acid (EGTA) were prepared as 0.1 M stock solutions. Turkey serum (1 ml) was chelated by

the addition of various concentrations of EDTA or EGTA. All samples were brought to a final volume of 1.1 ml using veronal-buffered saline without calcium and magnesium, and examined for hemolytic activity.

Treatment of Turkey Serum with Complement Activators. Various concentrations of zymosan (SIGMA, St. Louis, MO) and inulin (from Chicory root, SIGMA) were mixed with 1 ml of turkey serum. Samples were incubated for 30 minutes at 37^{o}C, centrifuged (2000 rpm, 30 minutes, 4^{o}C) and supernatants tested for hemolytic activity. Various concentrations of carrageenan (Type IV, Lambda, SIGMA) in 0.5 ml volumes were added to 0.5 ml turkey serum. Samples were mixed at 4^{o}C for 20 minutes and then centrifuged (2500 rpm, 20 minutes). Supernatants were collected and tested for residual hemolytic activity.

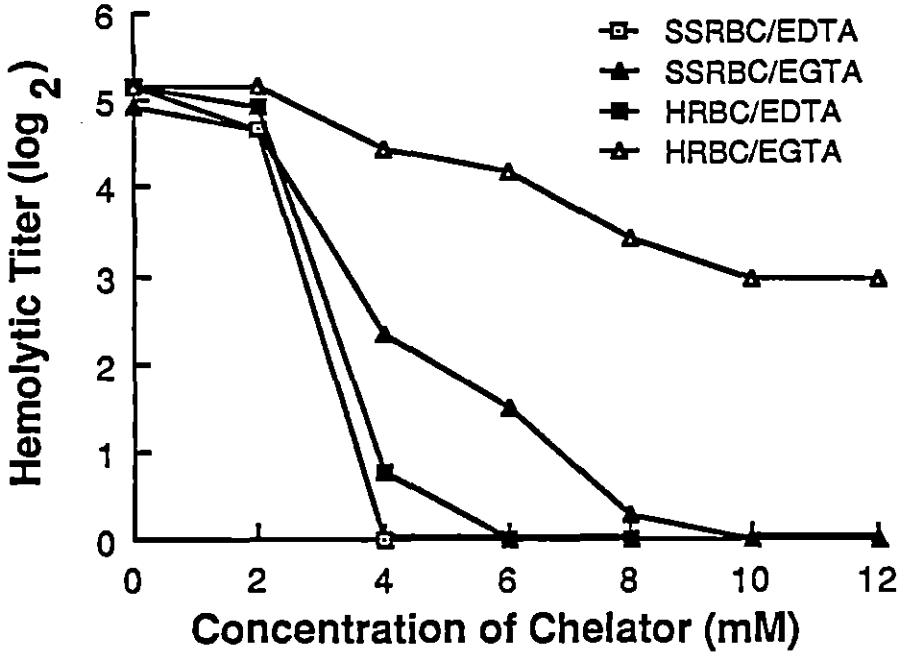
RESULTS

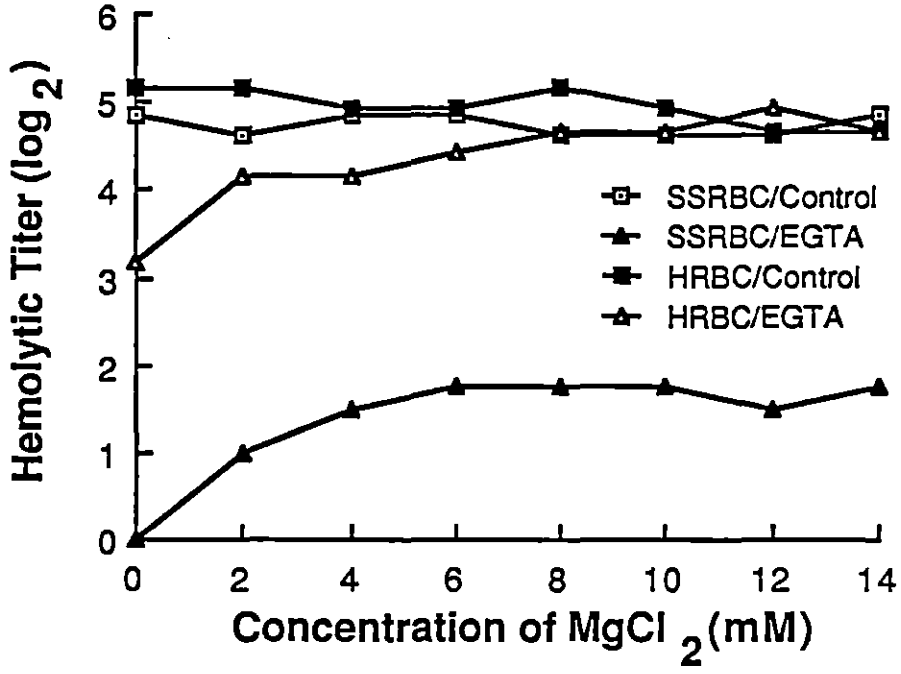
Adsorption of natural antibodies to HRBC from turkey serum had little effect ($p > 0.05$) on the hemolytic activity against HRBC. Mean hemolytic titers of nonadsorbed and adsorbed sera were 5.17 and 4.87, respectively.

The influence of calcium and magnesium ions on the complement activity of turkey serum is shown in Figure 1. Chelation of calcium and magnesium ions by EDTA at a final concentration of 6 mM blocked hemolytic activity against both SSRBC and HRBC. Chelation of calcium ions by EGTA at a final concentration of 10 mM blocked hemolytic activity against SSRBC, but did not block all hemolytic activity against HRBC. The hemolytic activity against HRBC decreased from 5.17 in normal serum to 2.96 in serum chelated with 10 mm EGTA. Addition of magnesium to serum treated with 10 mM EGTA restored hemolytic activity against HRBC to normal control values, as shown in Figure 2. Although hemolytic activity against SSRBC was increased by addition of magnesium, titers did not reach normal control values. Addition of magnesium to normal turkey serum is also shown in Figure 2, and the results indicate that hemolytic activity against either SSRBC or HRBC was not significantly affected by addition of magnesium ($p > 0.05$).

Figure 1. Residual hemolytic activity against SSRBC or HRBC after chelation of calcium and magnesium ions from turkey serum by EDTA or EGTA. Data points represent the mean of four samples

Figure 2. Influence of additional magnesium ions on hemolytic activity of normal turkey serum (control) and EGTA-chelated turkey serum. Data points represent the mean of four samples

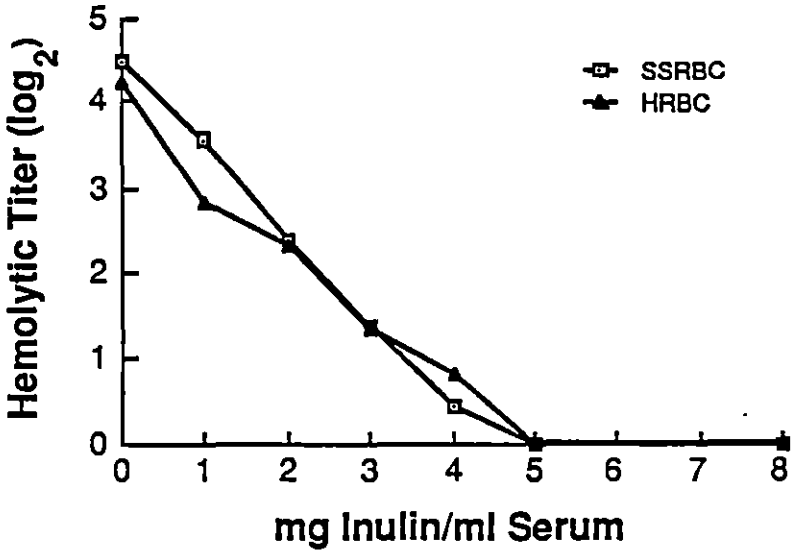

SSRBC/EDTA
SSRBC/EGTA
HRBC/EDTA
HRBC/EGTA
Hemolytic Titer (log 2)
0
1
2
3
4
5
6
0
2
4
6
8
10
12
Concentration of Chelator (mM)


SSRBC/Control
SSRBC/EGTA
HRBC/Control
HRBC/EGTA
Hemolytic Titer (log 2)
0
1
2
3
4
5
6
0
2
4
6
8
10
12
14
Concentration of MgCl 2 (mM)

Treatment of turkey serum with zymosan or inulin depleted hemolytic activity against both SSRBC and HRBC as shown in Figures 3 and 4, respectively. However, turkey complement was more sensitive to zymosan than to inulin. Whereas zymosan at only 1 mg/ml serum abolished hemolytic activity, at least 5 mg of inulin per milliliter of serum was required to abolish hemolytic activity. The effect of carrageenan treatment on turkey complement activity is shown in Figure 5. With increasing concentrations of carrageenan, hemolytic activity against SSRBC decreased significantly, whereas hemolytic activity against HRBC was not significantly affected ($p > 0.05$). The hemolytic titers of normal serum and carrageenan-treated serum (1.5 mg carrageenan/0.5 ml serum) against HRBC were 4.75 and 4.25, respectively.

Figure 3. Depletion of hemolytic activity against SSRBC and HRBC by inulin treatment of turkey serum. Data points represent the mean of four samples

Figure 4. Depletion of hemolytic activity against SSRBC and HRBC by zymosan treatment of turkey serum. Data points represent the mean of four samples


Hemolytic Titer (log_2)
5
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3
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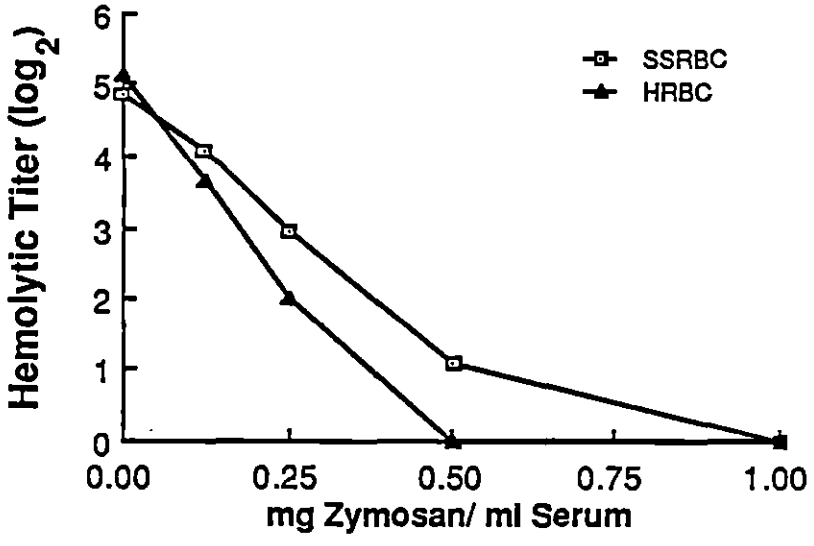
0 1 2 3 4 5 6 7 8
mg Inulin/ml Serum
SSRBC
HRBC


Hemolytic Titer (log_2)
6
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4
3
2
1
0
0.00 0.25 0.50 0.75 1.00
mg Zymosan/ ml Serum
SSRBC
HRBC

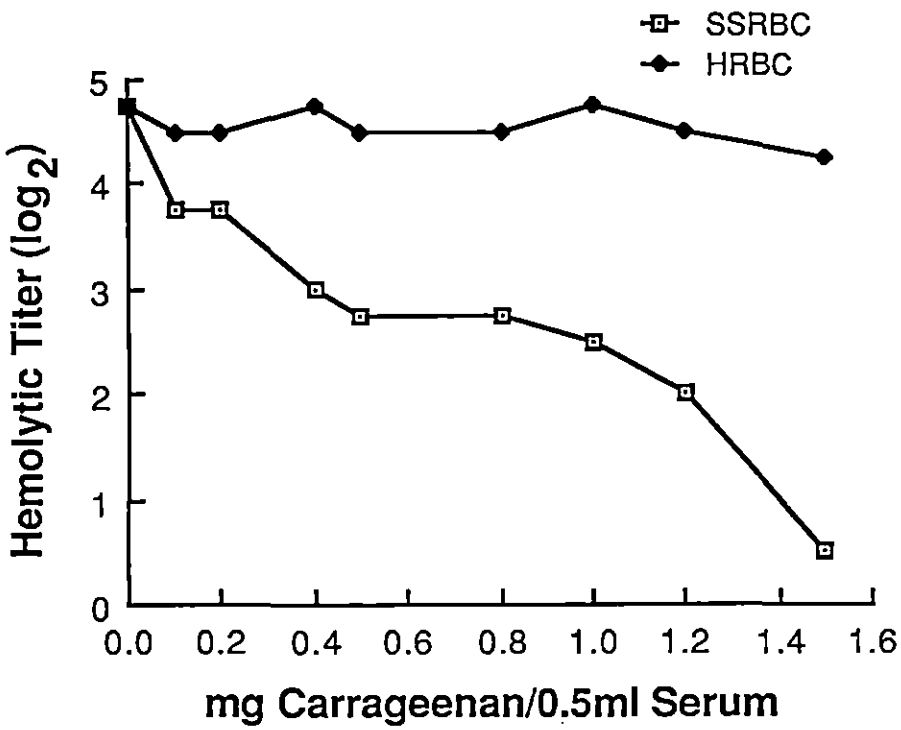

Figure 5. Depletion of hemolytic activity against SSRBC and HRBC by carrageenan treatment of turkey serum. Data points represent the mean of four samples

DISCUSSION

The results of this study indicate that two complement pathways exist in turkeys. Lysis of HRBC by turkey complement is mediated through an antibody-independent pathway, as similarly described for chickens (13). A commonly used procedure to separate alternative pathway activation from classical pathway activation is differential chelation of calcium and magnesium ions by EDTA or EGTA. Both calcium and magnesium ions are chelated by EDTA, whereas only calcium ions are chelated efficiently by EGTA. Hence, chelation of serum by EGTA inhibits classical complement pathway activity but allows expression of alternative pathway activity (3). Treatment of turkey serum with EGTA did not inhibit all hemolytic activity against HRBC, suggesting that lysis of HRBC is via the alternative complement pathway.

In contrast, lysis of SSRBC probably occurs through activation of the classical complement pathway. Evidence for activation of the classical complement pathway by SSRBC is two-fold. Firstly, both EDTA and EGTA, chelators of calcium ions, blocked the hemolytic activity against SSRBC. These chelators inhibit the mammalian classical pathway (3, 10). Secondly, carrageenan, which precipitates the first component of the classical complement pathway (1), depleted hemolytic activity against SSRBC, but not HRBC. The results of carrageenan treatment of turkey serum also suggest that

lysis of HRBC does not occur through the classical pathway.

Zymosan and inulin activate the alternative complement pathway of mammals (3, 10, 15). In this study, depletion of hemolytic activity against both SSRBC and HRBC was observed after zymosan and inulin treatments. This would suggest depletion of a component(s) common to both complement pathways, for example components of the terminal membrane attack complex. The terminal complement pathway in avian species has not been characterized. Zymosan and inulin treatments of turkey serum suggest that a common complement pathway arises from the classical and alternative pathways, as similarly described for the mammalian system (11).

Application of assays utilized in the study of mammalian complement has facilitated characterization of complement activity in turkeys. The present study shows convincing evidence that turkey complement may be activated through two pathways, these being the analogues of the mammalian classical and alternative complement pathways. Lysis of SSRBC occurs through an antibody-dependent pathway, which requires calcium and magnesium ions for efficient functioning. In contrast, the pathway effecting lysis of HRBC is antibody-independent and requires only magnesium ions for efficient functioning. Hence, hemolytic activity against SSRBC and HRBC, respectively, represents the classical and alternative complement pathways of turkeys.

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SECTION II. ISOLATION AND CHARACTERIZATION OF THE THIRD COMPONENT OF TURKEY COMPLEMENT

ABSTRACT

The third component (C3) of complement was isolated and characterized from turkey plasma. Turkey C3 consists of two chains joined by disulfide bonds. The molecular weights of the α and β chains of turkey C3 are 120,000 and 75,000 daltons, respectively. By crossed-immunoelectrophoresis, turkey C3 was identified to change in electrophoretic mobility after complement activation by inulin treatment of turkey serum. A monospecific antiserum to turkey C3 caused agglutination of sheep red blood cells sensitized with antibody and complement proteins, but did not cause agglutination of sheep red blood cells sensitized with antibody alone. Based on its structural similarity to C3 of other mammalian and avian species, its distinct electrophoretic mobility in complement-activated serum and its association with antibody-coated erythrocytes, the isolated turkey serum protein is identified as the third component of turkey complement.

INTRODUCTION

The complement system consists of a group of serum proteins which have a significant role in defense against infectious agents, in particular, gram-negative bacteria (6, 15). Complement-mediated defense may occur through activation of either the classical or alternative pathways (14, 16). A critical step in the complement sequence is the cleavage and activation of the third component (C3) of complement. Both the classical and alternative complement pathways converge with the cleavage of C3, releasing a small fragment (C3a) with anaphylatoxic activity and a large fragment (C3b) which can bind covalently to target cell surfaces or immunoglobulins (1, 9, 10). The activated molecule, C3b may serve as an acceptor for additional complement components during the assembly of the membrane attack complex (20), or it may act as an opsonin recognized by macrophages or polymorphonuclear cells (6, 11). The significance of C3 in host resistance is exemplified by the occurrence of severe recurrent bacterial infections in humans with C3 deficiency (5).

The third component of complement is the most abundant in human serum (1.2-1.3 mg/ml) (11). Human C3 is characterized as a glycoprotein (195,000 daltons) composed of two chains joined by disulfide bonds (3). Under reducing

conditions, the molecular weights are 127,000 and 75,000 daltons for C3α and C3β, respectively. Analogues of human C3 have been identified and characterized in the chicken and quail (7, 8). These proteins are structurally similar to human C3, and have molecular weights of 190,000 and 184,000 daltons for chicken C3 and quail C3, respectively. In addition to structural similarities to human C3, the avian analogues may possess the same functional group involved in covalent binding of C3b to cell surfaces. Both chicken and quail C3 are sensitive to methylamine, suggesting that the reactive group is a thiolester bond as reported for human C3 (19).

Research in turkey complement is limited to analysis of functional activity by various assays described for the measurement of human and mammalian complement (2, 17, 18). None of the individual turkey complement components have been identified or characterized. In the present study, we describe the first isolation of a turkey complement protein, which is analogous to the third component of human complement.

MATERIALS AND METHODS

Experimental design. The first part of this experiment was a two step procedure which involved the production of a rabbit anti-turkey C3 antiserum. A suspension of zymosan with bound turkey complement proteins was used as the initial antigen to produce an antiserum reacting with several of the turkey complement proteins. The antiserum which resulted from this procedure and is subsequently referred to as an anti-turkey complement antiserum was then used in a modification of the crossed-immunoelectrophoresis procedure to precipitate a turkey serum protein, identified as C3. The precipitated rabbit antibody/turkey C3 complex was removed from the agarose, and used to immunize rabbits for production of the anti-turkey C3 antiserum. To confirm that the rabbit antiserum reacted with a complement protein (C3), the antiserum was tested for its ability to hemagglutinate sheep red blood cells sensitized with antibody (EA) or sheep red blood cells sensitized with antibody and complement (EAC).

The anti-turkey C3 antiserum was then used in the second part of this experiment to examine sequential steps in the isolation of turkey C3 from plasma. Fractions collected during the isolation procedure were utilized in immunoelectrophoresis and analyzed with the anti-turkey C3 antiserum.

Turkey serum and plasma. Blood was collected from normal adult turkeys and allowed to clot at room temperature for 1 hour and at 4°C for 1 hour. Serum was harvested by centrifugation (1500 rpm, 20 minutes), pooled and stored in 10 ml aliquots at -70°C until required. For turkey plasma, blood was collected in heparinized tubes and immediately centrifuged. Plasma was pooled and stored in 30 ml aliquots at -70°C until required.

Complement-activated turkey serum was produced by treatment with inulin. Turkey serum was mixed with inulin (from Chicory root, SIGMA) at a ratio of one ml of serum to 20 mg of inulin, and then incubated at 37°C for 30 minutes. Treated serum was centrifuged, diluted 1:8 and analyzed by crossed-immunoelectrophoresis.

Production of rabbit antiserum to complement proteins. The initial antiserum to turkey complement proteins was produced by a procedure previously described for isolation of quail C3 (7). Briefly, 100 mg zymosan (SIGMA), an activator of the mammalian alternative complement pathway was incubated with 20 ml of turkey serum at 37°C for 1 hour. The serum-treated zymosan was collected by centrifugation (2000 rpm, 15 minutes), washed three times in phosphate-buffered saline, pH 7.2 (PBS) and then resuspended in PBS to one-half the original serum volume. Two rabbits were each injected intradermally with three doses (10 mg/dose) mixed with equal

volumes of Freund's adjuvant, at 10 day intervals. Rabbit sera were collected 10 days after the last immunization and immunoglobulins were purified by ammonium sulfate precipitation (50% saturation).

Production of rabbit anti-turkey C3 antiserum. A modification of the crossed-immunoelectrophoresis procedure was used to isolate a precipitation band formed by the rabbit anti-turkey complement antiserum and normal turkey serum. The procedure involved removing some of the contaminating precipitation bands in the second-dimension agarose by incorporating a rabbit anti-turkey immunoglobulin antiserum[1] at a 20% final concentration in the first-dimension agarose. The isolated precipitation band observed in the second-dimension agarose was cut from the gel, and used as an antigen to immunize rabbits. Rabbit immunizations were similar to those previously described.

Purification of turkey C3. The initial purification step of turkey C3 was similar to that described for isolation of rabbit C3 (4). Turkey plasma (300 ml) was taken to final concentrations of 10 mM ethylenediamine tetraacetic acid (EDTA) and 6.4 mM benzamidine-HCl. Turkey plasma was

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[1]The anti-turkey immunoglobulin antiserum was produced in our lab by immunizing rabbits with a preparation of turkey serum IgG (H and L chains).

equilibrated with 4% (w/v) polyethlyene glycol (P4000, SIGMA) for 1 hour at 4°C, and then centrifuged (2500 rpm, 30 minutes). The supernatant was collected, and the precipitation repeated, taking the sample to 12% (w/v) polyethylene glycol. The precipitated proteins were collected by centrifugation and dialyzed against a 0.01 M Tris-HCl buffer containing 0.03 M NaCl, 6.4 mM EDTA, and 6 mM benzamidine-HCl, pH 8.0. Twenty milliliters of the dialyzed sample were applied to a DEAE-cellulose column (Whatman-52, 2 x 20 cm) equilibrated with the same buffer. A stepwise elution of the plasma proteins was performed using the same buffer system and increasing NaCl molarities (0.05 M, 0.1 M, 0.15 M, 0.2 M, and 0.3 M). The protein fraction eluting with 0.05 M NaCl, was concentrated by ultrafiltration and applied to a sephadex G-200 column (3 x 90 cm). Contaminating turkey immunoglobulin was removed from the C3 sample by affinity chromatography using the rabbit anti-turkey immunoglobulin antiserum linked to cyanogen bromide activated-sepharose 4B (SIGMA).

Immunoelectrophoresis and crossed-immunoelectrophoresis. Electrophoresis of samples was performed in 1% agarose in a tris-tricine buffer (0.024 M tricine, 0.062 M tris-HCl, 0.003 M sodium azide, 0.003 M calcium lactate, pH 8.6). For immunoelectrophoresis, antigen samples were added to the wells (10 ul/well) and electrophoresed at 6 volts/cm for 2 hours.

Troughs were removed, and 150 ul of appropriate antibody were added to each trough. Samples were allowed to diffuse for 24 hours, and then gels were washed in PBS to remove non-precipitating proteins. For crossed-immunoelectrophoresis, 10 ul of turkey serum (diluted 1:8) were added to each well, and electrophoresed in the first dimension agarose at 6 volts/cm for 4 hours. Second-dimension electrophoresis was carried out at 2 volts/cm for 18 hours. Rabbit antibodies were incorporated into the second-dimension agarose at 20% final concentrations. Agarose gels from both procedures were washed in PBS for 24 hours, and then stained for protein using Coomassie blue R250.

Polyacrylamide gel electrophoresis (PAGE). Protein samples were analyzed by discontinuous PAGE in the presence of 1% sodium dodecyl sulfate. Non-reduced samples were analyzed in 7% polyacrylamide slab gels, and samples reduced with 2% β-mercaptoethanol were analyzed in gradient slab gels of 5 to 15% polyacrylamide. Electrophoresis was carried out at 25 mAmps, constant current for 4 hours. Gels were stained for protein with Coomassie-blue R250.

Erythrocyte-Antibody and Erythrocyte-Antibody-Complement production. Sheep red blood cells sensitized with specific antibody (EA) were prepared by mixing 5 ml of a 5% SRBC suspension with 10 ml of gelatin-veronal-buffered saline containing 10 mM EDTA, 1:200 turkey anti-SRBC antibody and

1:16 normal turkey plasma. Sheep red blood cells sensitized with specific antibody and complement proteins (EAC) were prepared similarly, except that the EDTA was removed to allow complement activation. Red blood cell suspensions were incubated at 37^{o}C for 1 hour, and then centrifuged (1000 rpm, 15 minutes). Red blood cells (EA and EAC) were resuspended to 2% final concentrations with PBS for use in the hemagglutination assay.

Hemagglutination Assay. Two-fold dilutions of rabbit antisera (50 ul/well) were prepared in a microtiter test plate. Fifty microliters of red blood cell suspension (EA or EAC) were added to the serum dilutions, samples shaken and then allowed to settle for 2 hours. The hemagglutinating titer was recorded as the last serum dilution causing agglutination of the red blood cells. The rabbit antisera were tested in duplicate for each red blood cell suspension.

RESULTS

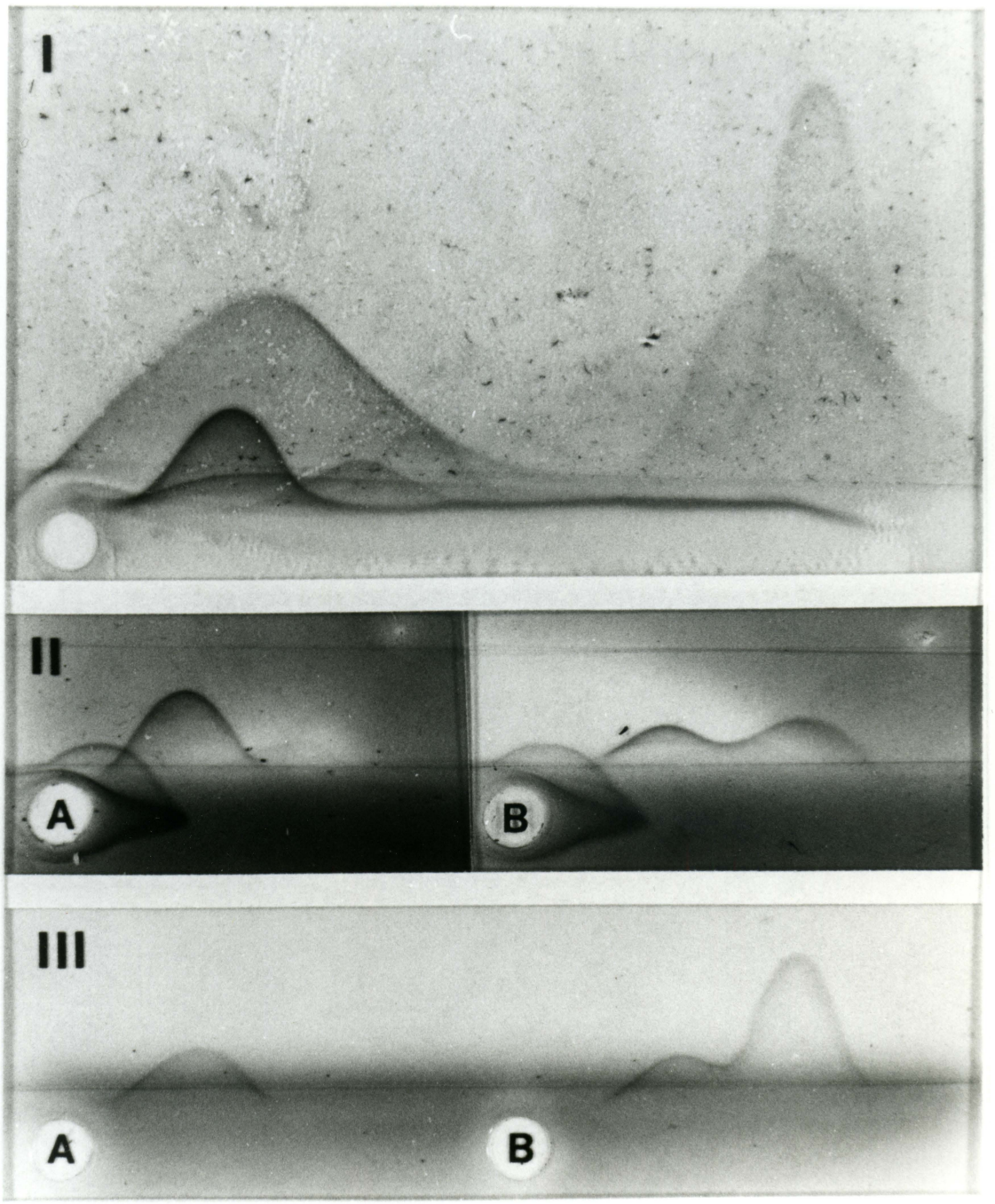
The initial rabbit antiserum which was produced against serum-treated zymosan was found to react with several turkey serum proteins as demonstrated by crossed-immunoelectrophoresis in Figure 1-1 . Two distinct precipitation bands were formed by proteins which predominated in the gamma-globulin fraction of turkey serum. One of these bands was observed to change in electrophoretic mobility (movement toward the anode) after activation of turkey complement by inulin treatment (data not shown). This precipitation band assumed to be C3, was selectively isolated by incorporating a rabbit anti-turkey immunoglobulin antiserum into the first-dimension agarose of the crossed-immunoelectrophoresis procedure. The isolated precipitation bands resulting from normal turkey serum and inulin-treated turkey serum are shown in Figures 1-11A and 1-11B, respectively. By cutting the isolated precipitation band from the agarose and immunizing rabbits, a monospecific antiserum was subsequently produced which reacted with a protein in normal turkey serum, as shown in Figure 1-111A. This turkey serum protein was observed to change in electrophoretic mobility after treatment of turkey serum with inulin, as shown in Figure 1-111B.

The monospecific antiserum, subsequently referred to as a putative anti-turkey C3 antiserum was tested for its

Figure 1-1. Identification of turkey serum proteins by crossed-immunoelectrophoresis which react with the anti-turkey complement antiserum

Figure 1-11. Adsorption of contaminating turkey serum proteins from normal turkey serum (A) and inulin-treated turkey serum (B) by incorporation of an anti-turkey immunoglobulin antiserum in the first-dimension agarose of the crossed-immunoelectrophoresis procedure

Figure 1-111. Identification of turkey C3 by crossed immunoelectrophoresis in normal turkey serum (A) and inulin-treated turkey serum (B), using the anti-turkey C3 antiserum


I
II
A
B
III
A
B

hemagglutinating characteristics. The anti-turkey C3 antiserum hemagglutinated sheep red blood cells sensitized with turkey immunoglobulin and complement (EAC) with a titer of 1:32. In contrast, the anti-turkey C3 antiserum did not hemagglutinate sheep red blood cells sensitized with turkey immunoglobulin (EA).

Purified turkey C3 was obtained following a four-step isolation procedure. Turkey plasma was fractionated by precipitating proteins between 4 and 12% (w/v) polyethylene glycol. This sample of turkey serum proteins reacted with the anti-turkey C3 antiserum in immunoelectrophoresis (data not shown). The second step for C3 isolation was anion exchange chromatography using DEAE-cellulose. The elution profile of the proteins from the DEAE-cellulose column is shown in Figure 2. The protein fraction which eluted with 0.05 M NaCl (shaded area) was found to contain the putative turkey C3, as determined by immunoelectrophoresis (data not shown). In the third step, high and low molecular weight protein contaminants were removed by gel filtration using Sephadex G-200. At this point, the sample containing turkey C3 was also found to contain turkey immunoglobulin. The contaminating turkey immunoglobulin was adsorbed from turkey C3 by affinity chromatography. The various protein fractions obtained during the isolation procedures, as well as the putative turkey C3 sample are shown in Figure 3.

Figure 2. Elution profile of turkey plasma proteins from the DEAE-cellulose column, using increasing salt concentration. Arrows indicate changes in NaCl molarities starting with 0.03 M, and proceeding with 0.05 M, 0.1 M, 0.15 M, 0.2 M and 0.3 M. The fraction eluting with 0.05 M (shaded) was found to contain turkey C3

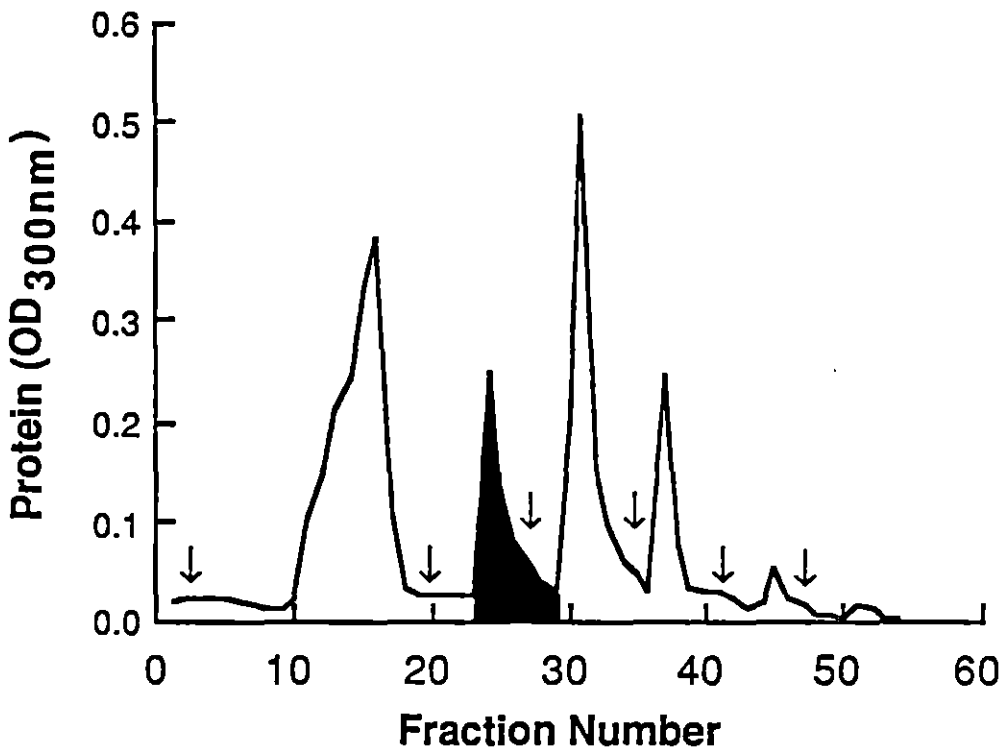
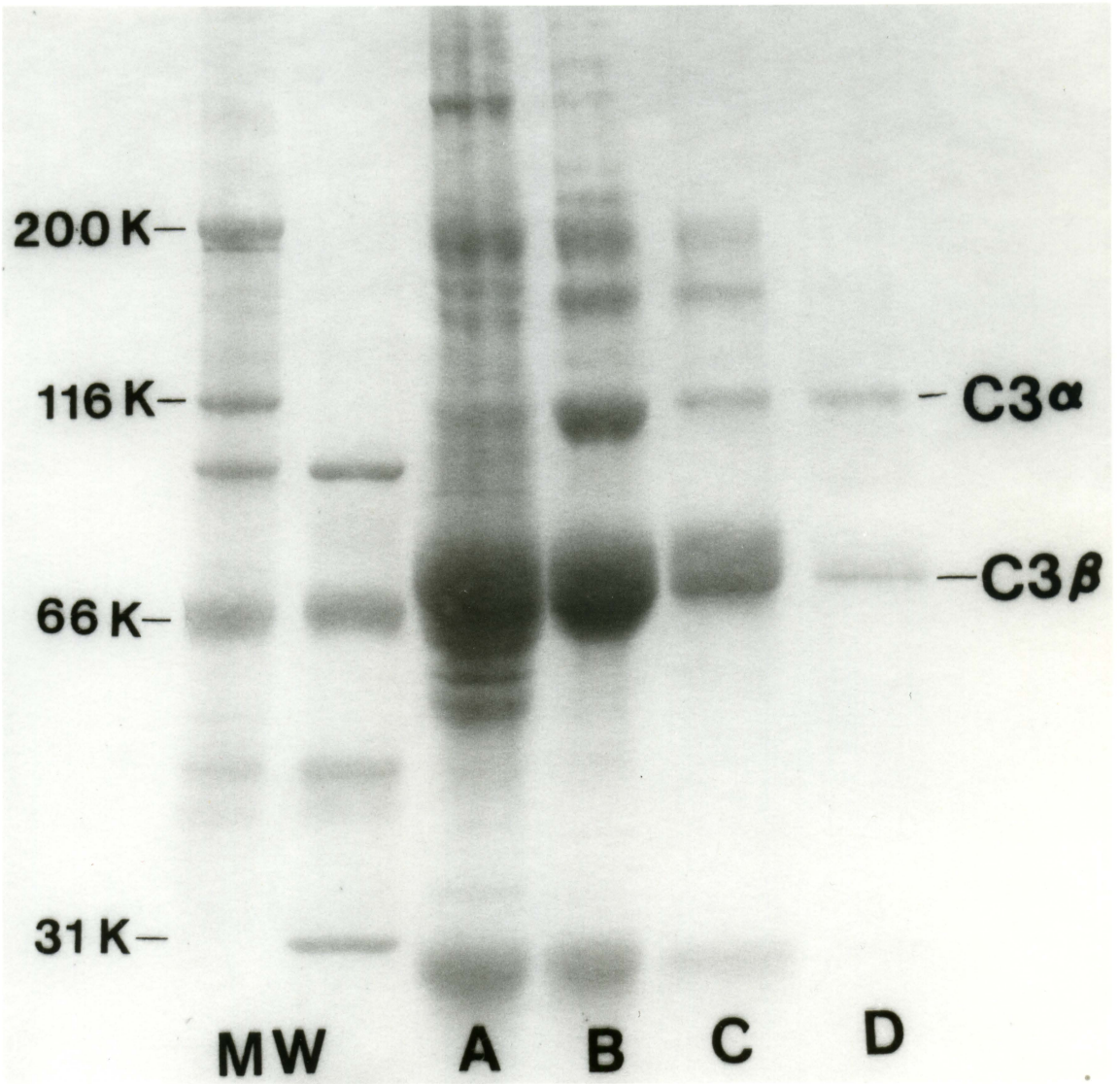

Protein (OD_{300nm})
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0
10
20
30
40
50
60
Fraction Number

Figure 3. Analysis of fractions obtained during isolation of turkey C3 by SDS-PAGE (5 to 15% acrylamide gel). Samples were solubilized in buffer containing 0.1% SDS and 2% mercaptoethanol, and boiled at 100^{o}C for 3 minutes. Lanes represent molecular weight markers (MW), proteins precipitated by polyethylene glycol (4 to 12% W/V) (A), proteins eluted with 0.05 M NaCl from DEAE-cellulose column (B), proteins eluted from Sephadex G-200 column (C), and purified turkey C3 (D).


200 K–
116 K–
66 K–
31 K–
C3α
C3β
MW
A
B
C
D

Treatment of turkey C3 with β-mercaptoethanol reveals that it consists of two polypeptide chains, as shown in Figure 3. Examination of these chains in a 5 to 15% polyacrylamide gradient gel indicates molecular weights of 120,000 and 75,000 for C3α and C3β, respectively.

DISCUSSION

In the present study, the third component of turkey complement was identified and isolated using various immunological and biochemical techniques. Utilization of zymosan, a suspension of yeast cell walls to activate turkey complement facilitated the production of a rabbit antiserum to turkey complement proteins. Zymosan activation of complement has been used for the isolation of both mouse C3 and quail C3 (7, 12). The antisera produced against the complement-bound zymosan in these previous reports were found to react primarily with the third component of complement of each of the respective species. The results of this study suggest that the rabbit anti-zymosan/turkey complement antiserum reacts with several turkey serum proteins, although two distinct precipitation bands were formed by proteins in the gamma-globulin fraction of serum. One of these proteins was observed to have a characteristic mobility in complement-activated serum, as similarly described for the third component of other animal species (7, 8, 12). The second serum protein was precipitated by an anti-turkey immunoglobulin antiserum in the first-dimension agarose of the crossed-immunoelectrophoresis procedure, suggesting that this protein was turkey immunoglobulin. Further evidence indicating that the second protein was immunoglobulin is shown by its predominance in normal turkey serum.

To maintain the isolated turkey C3 as an active molecule, it was essential to add EDTA to turkey serum, as well as to subsequent buffers to prevent complement activation. In addition, benzamidine-HCl was included in buffers to prevent spontaneous activation of the purified product isolated from its serum inhibitors (3). Failure to use these inhibitors during C3 isolation resulted in high molecular weight complexes believed to be covalently-bound turkey IgG-C3 complexes (9). During isolation of C3, turkey immunoglobulin was a common contaminant of the isolated C3, as similarly described for human C3 isolation (3). For purification of human C3, a procedure was developed in which contaminating proteins in the C3 sample were removed by affinity chromatography (13). This procedure was successfully used for removing the contaminating turkey immunoglobulin, requiring two to three adsorptions for complete removal of the immunoglobulins.

The purified turkey C3 obtained by the procedures outlined in this study was structured similarly to the third component of humans, chickens and quail (3, 7, 8). The isolated turkey protein is composed of two chains with molecular weights of 120,000 and 75,000 daltons, respectively. The third component of complement from humans, chickens and quail is composed of two chains linked by disulfide bonds. In these species, the larger chain has a

molecular weight range of 110,000 to 127,000 daltons, whereas the smaller chain has a molecular weight range of 68,000 to 75,000. Hence, we have identified the isolated turkey serum protein as C3 based on its molecular similarity to C3 of other species, its characteristic mobility in complement-activated serum, and its association with antibody-coated erythrocytes.

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SECTION III. SERUM RESISTANCE AND VIRULENCE IN *ESCHERICHIA COLI* ISOLATED FROM TURKEYS

ABSTRACT

Twenty-five strains of *Escherichia coli* isolated from turkeys were characterized for their serum resistance and virulence. An *in vitro* bactericidal assay was used to determine the serum resistance of *E. coli*. Virulence was determined by survival time following intravenous inoculation of each strain into 3-week old turkeys. Serum-resistant *E. coli* were generally found to be virulent for turkeys, whereas serum-sensitive *E. coli* were avirulent. Of the 25 strains, 18 strains were placed in the two categories of serum-resistant/virulent and serum-sensitive/avirulent. Five strains were serum-resistant and avirulent and two strains were serum-sensitive and virulent. Serum resistance appears to be an important determinant of virulence for *E. coli* in turkeys. However, the requirement for other virulence factors, in addition to serum resistance, is suggested by the finding that 5 serum-resistant strains were avirulent in turkeys.

INTRODUCTION

Escherichia coli is an economically important bacterial pathogen of broiler chickens and young turkeys. In turkeys, *E. coli* infection is most commonly characterized by subacute fibrinopurulent airsacculitis or acute septicemia (7). The septicemic form of *E. coli* infection, colisepticemia, is often fatal; survivors may be unthrifty due to pericarditis, perihepatitis, airsacculitis and arthritis. The majority of *E. coli* infections of turkeys and broilers are caused by the serotypes O1, O2 and O78, but a large number of other serotypes have been isolated in a limited number of disease outbreaks (7, 11, 18).

Some strains of *E. coli* resist clearance from the bloodstream by the liver and spleen, replicate to large numbers in blood, and cause death or severe lesions in infected turkeys (2). In contrast, *E. coli* strains which are avirulent for turkeys are readily cleared from the bloodstream and cause minimal lesions or disease. Few determinants of virulence have been characterized for *E. coli* isolated from poultry. Adherence of *E. coli* to the respiratory epithelium of turkeys may be important for initial infection (4, 15). Recently, virulence has been correlated with the efficient acquisition of iron from host tissues by some *E. coli* strains isolated from turkeys

(5). However, the molecular basis for survival of virulent *E. coli* strains in the bloodstream of turkeys remains largely unknown.

The composition of the bacterial cell surface may influence the ability of bacteria to survive within the host tissues and body fluids. Components associated with the *E. coli* outer membrane are known to influence the activity of antibody, complement and phagocytes (22). Strains that cause bacteremia in humans commonly resist the bactericidal effects of serum (23), whereas enhanced virulence of serum-resistant *E. coli* for mice is largely attributable to inefficient opsonization by complement and resistance to phagocytosis (13). The objective of the present study was to determine the serum resistance and virulence of *E. coli* strains isolated from a variety of turkey tissues.

MATERIALS AND METHODS

Bacteria. All strains of *E. coli* were isolated from turkey flocks in central Iowa by N. F. Cheville[1] and H. J. Barnes[2]. Twenty-five isolates were obtained from various tissues of turkeys including trachea, heart, liver and blood. Each isolate was confirmed as *E. coli* by standard bacteriological methods, and O, K, H serotypes were determined[3]. The tissue source and serotype for each *E. coli* strain is given in Table 1. For the present study, each strain was streaked for purity on solid media and then grown 18 hours in brain heart infusion (BHI) broth[4] at 37°C. For each *E. coli* strain, a 3-week old turkey was inoculated intravenously with 0.1 ml of the broth culture, and a 0.1 ml blood sample was collected 2 hours later. Blood samples were mixed with 10 ml of BHI broth and incubated overnight at 37°C. Isolates from blood cultures were tested for purity, mixed with sterile glycerin (1:1, V/V), and stored at -80°C as stock until required. Passage of the *E. coli* strains *in vivo* under standard conditions was done to ensure that

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[1]National Animal Disease Center, Ames, IA.

[2]North Carolina State University, Raleigh, NC.

[3]National Veterinary Services Laboratory, Ames, IA.

[4]Difco Laboratories, Detroit, MI.

Table 1. The serotype and tissue source of *E. coli* isolates from turkeys

Strain	Serotype[a]	Source
1	O78:K80:H9	liver
2	O1ab:K*:H7	liver
3	O2a:K-:NM	liver
4	O36:K-:NM	liver
5	O143:K*:H27	liver
12	O78:K80:H9	pericardium
13	O11:K*:H4	nasal sinus
20	O78:K*:H9	unknown
26	O36:K-:NM	unknown
27	O78:K3:H9	heart blood
28	O78:K*:NM	trachea
36	O*:K?:H4	trachea
38	OX9:K*:H4	liver
39	O2a:K2:NM	liver
40	O2a:K2:H5	unknown
42	Rough:K?:NM	trachea
43	O78:K80:H9	unknown
44	O11:K*:H4	trachea
48	O8:K*:H4	trachea
53	O78:K*:NM	heart blood
54	O2a:K-:H5	liver
66	O78:K80:H9	pericardium
68	O78:K*:H9	heart blood
79	O2a:K2:H5	liver
80	O88:K93:H8	liver

[a]K* = K antigen present, but not identifiable with standard K antisera; K- = no K antigen present; K? = no tests made to determine the antigen; O* = O antigen present, but not identifiable with standard O antisera; NM = nonmotile.

differences observed in serum resistance and virulence were not due to effects of *in vitro* culture on expression of virulence factors. By examining strains of *E. coli* from a variety of turkey tissues, it was anticipated that a broad spectrum of virulence and serum resistance patterns would occur.

Turkeys. Nicholas strain broad-breasted white turkeys (n=130) were obtained from a commercial hatchery[1] and raised to three weeks of age in battery cages. Feed and water were supplied *ad libitum*.

Serum. Pooled turkey serum was used as the source of complement. Blood was obtained from healthy, 4-week old turkeys and allowed to clot at room temperature for 1 hour and then at 4°C for 1 hour. Serum was separated by centrifugation, pooled, divided into 2 ml aliquots, and frozen at -70°C until required. Serum was tested for natural antibodies to all *E. coli* isolates by a microtiter agglutination assay (3). Two-fold serial dilutions of the serum were mixed with equal volumes of *E. coli* suspension (1×10^9 colony-forming units (CFU)/ml) in a microtiter test plate, and bacterial agglutination was recorded. The antibody titer of serum samples was recorded as the last dilution causing bacterial agglutination.

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[1]Midwest Turkey Hatchery, Dike, IA.

Bactericidal assay. The serum resistance of each *E. coli* strain was determined following the guidelines of Taylor (21). A 10 ul sample of a 24 hour BHI broth culture was used to inoculate 10 ml of sterile BHI broth prewarmed to 37°C. The BHI broth subcultures were incubated for 2 hours at 37°C to produce bacteria in the logarithmic phase of growth. After incubation, the bacterial suspensions were centrifuged for 20 minutes at 3000 x g, and bacterial pellets were resuspended in 10 ml gelatin-veronal buffer (GVB++) containing 0.15 mM $CaCl_2$ and 0.5 mM $MgCl_2$, pH 7.4 (8). For the assay, reaction mixtures of 250 ul containing 25 ul of bacterial suspension (1×10^7 CFU/ml) and 225 ul of undiluted turkey serum were incubated at 37°C for 3 hours. Fifty microliter samples, collected initially and at the end of the three hour incubation, were placed in 9 ml GVB++. Numbers of viable bacteria were determined by plating 10-fold serial dilutions of these samples on blood agar plates. The bactericidal activity of turkey serum for each *E. coli* strain was determined in quadruplicate.

Virulence assay. A 1:10 dilution (in phosphate-buffered saline, pH 7.4) of a 24 hour BHI broth culture of each *E. coli* strain was used as the inoculum (approximately 2×10^8 CFU/ml). For each *E. coli* strain, five 3-week old turkeys were injected intravenously with 0.5 ml of the inoculum. Control birds were similarly injected with 0.5 ml

phosphate-buffered saline. Mortality was recorded 6, 12, 24, 36, 48, 60, and 72 hours post-inoculation. Serum samples were collected from each turkey prior to challenge for determination of specific antibody against the *E. coli* strain used in the inoculum, as previously described.

RESULTS

Serum resistance of *E. coli* isolates. The turkey serum used to test serum resistance did not contain detectable antibody specific for the *E. coli* isolates, except for strains 3 and 44. With these two strains, the agglutinating antibody titer of the serum was 1:2. The strains of *E. coli* differed markedly in serum resistance as shown in Figure 1. Data are presented as the change in number of viable *E. coli* between the 3 hour and 0 hour samples, expressed as $\log_{10}$ CFU/ml. Isolates of *E. coli* were classified as serum-resistant when the number of viable *E. coli* in 3 hour samples was greater than in 0 hour samples. Isolates from the cardiac blood or pericardium were generally serum-resistant. In contrast, strains isolated from the liver or trachea varied in their degree of serum resistance. All strains (n=9) with the O78 serotype were serum-resistant. The additional O-serotypes associated with serum resistance included O1, O143, and O11 (one strain per serotype).

Virulence of *E. coli* in turkeys. Antibodies specific for the *E. coli* challenge strains were not detectable in any of the poults before challenge (data not shown). The results of the *E. coli* challenge and comparison to serum resistance of each *E. coli* strain are presented in Figure 2. Virulence data are presented as the sum total in hours that poults

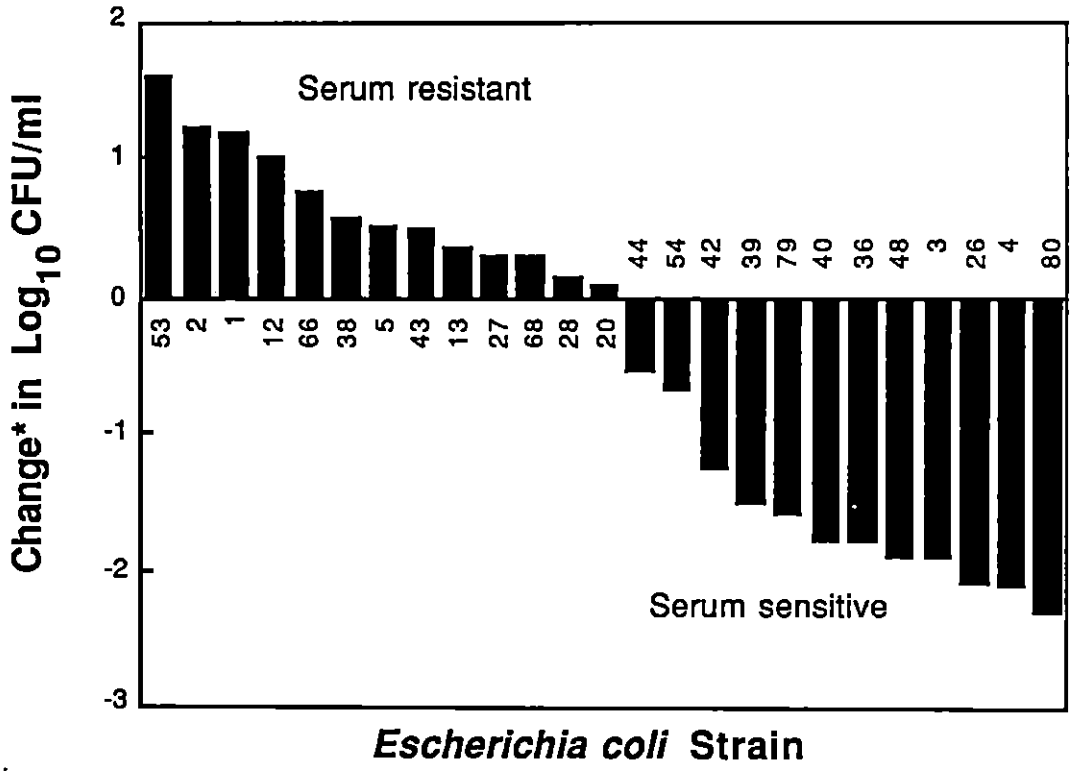

Figure 1. Resistance of Escherichia coli strains to the bactericidal effects of turkey serum. The value for each strain represents the mean of four replicates. *Change equals viable bacteria (log_{10} CFU/ml) in 3 hour sample minus viable bacteria in 0 hour sample. Strain numbers (see Table 1 for description) are displayed adjacent to each bar. Strains are plotted in order of decreasing serum resistance

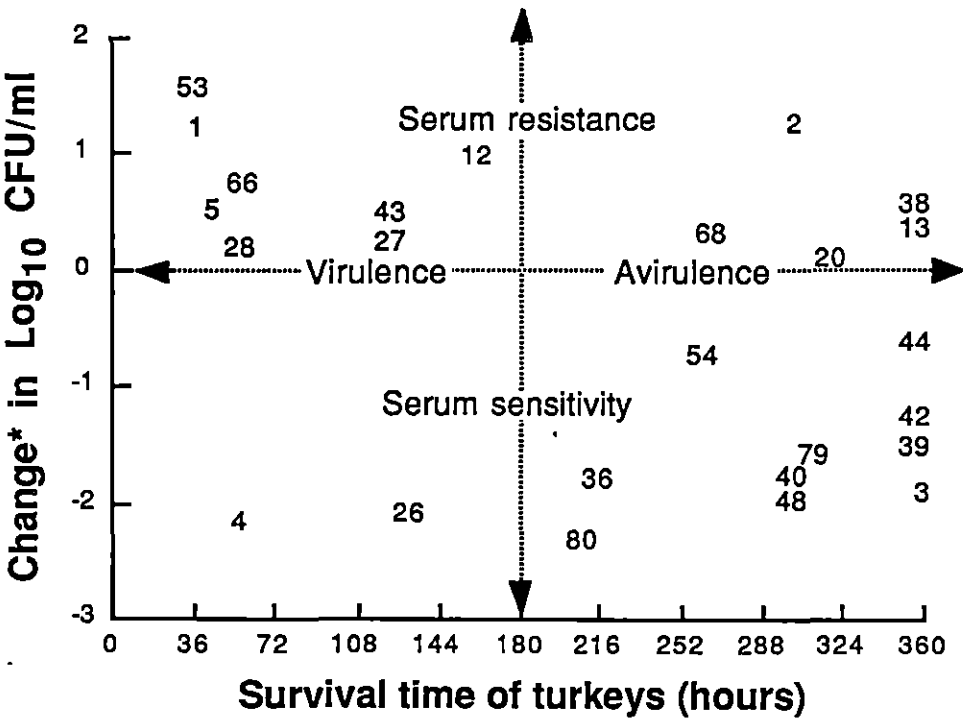

Figure 2. Relationship of serum resistance and virulence of Escherichia coli isolates from turkeys. Strain numbers represent data points for serum resistance and virulence in turkeys. *Change equals viable bacteria in 0 hour sample. Survival time is the cumulative survival time of 5 turkeys per group over a 72 hour period

within a group survived after challenge, with the maximum survival time recorded as 360 hours (5 birds per group x 72 hours). For the purpose of comparison, an intermediate value of 180 hours was chosen to differentiate virulent and avirulent *E. coli*. However, virulence of *E. coli* strains varied greatly, ranging from death of all poults within 12 hours for virulent strains to no deaths within 72 hours for avirulent strains. Strains with the O78 serotype were confirmed as highly virulent. Additional serotypes found to be virulent were O36 and O143. Serum-resistant *E. coli* tended to be virulent (n=8), whereas serum-sensitive *E. coli* were avirulent (n=10). However, five strains were serum resistant but avirulent, and one serotype, O36:K-:NM was serum sensitive but highly virulent for turkeys.

DISCUSSION

In a previous study, it was shown that virulent *E. coli*, when injected intravenously into turkeys multiplied in the blood and caused rapid mortality (7). Subsequently, it was found that virulent *E. coli* were able to resist clearance by the liver and spleen (2). The results of the present study indicate *E. coli* that are virulent for turkeys are likely to be serum-resistant. For example, all strains of the O78:K80 serotype, which is commonly associated with colisepticemia, were serum-resistant. Virulent *E. coli* are often found in the blood and localized in certain tissues, including the pericardial sac (12). The ability to survive and multiply in these tissues requires several virulence factors, one of which is serum resistance. Isolation of virulent *E. coli* from liver would be expected in turkeys dying of colisepticemia, but avirulent strains could also colonize the liver post mortem by extension from the intestines. Therefore, depending on the circumstances of liver culture, either virulent or avirulent *E. coli* strains could be recovered. In contrast, culture of blood is likely to yield virulent *E. coli* strains as found in this study. All serum-sensitive/avirulent strains identified in the present study were originally isolated from tissues other than blood.

Bacterial virulence is suggested to be multifactorial (19). The ability to resist the bactericidal effects of serum is one example of a characteristic associated with virulent organisms, as evident in this study. At the same time, there are multiple host defense mechanisms which respond to pathogenic organisms. Although some strains of *E. coli* in this study were serum-resistant, they were also avirulent for turkeys. The specific bacterial and host mechanisms responsible for the serum-resistant/avirulent *E. coli* phenotype are not known. One possibility may be the limitation of iron, and possibly other nutrients during an infection. Because iron is essential for bacterial growth, the ability to sequester iron from host tissues influences the pathogenicity of bacteria (6). For turkeys, virulence of *E. coli* is markedly enhanced by addition of exogenous iron (5), suggesting the importance of iron limitation by the host. The aerobactin iron-chelation system has been shown to be present in virulent avian *E. coli* strains and absent from most avirulent strains (14). Although five avirulent strains of *E. coli* in this study were serum-resistant, they may have inadequate iron-chelation systems and hence, are unable to survive in the bloodstream of turkeys. An alternative explanation for the serum-resistant/avirulent strains is based on interaction of the bacteria with humoral components and cells of the mononuclear

phagocytic system. The role for humoral factors, such as antibody and complement in enhancing bacterial clearance by the liver and spleen is well established. Hepatic clearance of virulent *E. coli* from the bloodstream of turkeys is markedly enhanced by passive immunization with specific antibody (1). *In vivo* decomplementation of squirrel monkeys and mice resulted in decreased clearance of *E. coli* from the blood (9, 20). The differences in virulence of serum-resistant *E. coli* may reflect variations in complement activation or deposition of opsonic complement fragments such as C3b onto the bacterial surface. The availability of surface-bound C3b on serum-resistant strains of *E. coli* may facilitate clearance by the liver and spleen in turkeys.

The mechanisms for virulence associated with the serum-sensitive serotype, O36:K-:NM are not understood. The response of the bacteria *in vitro* may not always reflect the events occurring *in vivo* due to the complexity of the host response. In the hostile *in vivo* environment, *E. coli* O36:K-:NM may undergo phenotypic change from serum-sensitive to serum-resistant. Alterations in the bacterial surface composition can have a significant effect on serum resistance (16). Hence, although serum-sensitive *in vitro*, the O36:K-:NM *E. coli* serotype may express surface structures *in vivo* that result in resistance to the bactericidal effects of serum. This type of observation is not limited to *E. coli* in

turkeys. *Salmonella cholerae-suis var kunzendorf* (strain 38) has also been found to be serum-sensitive but virulent for mice (10).

The importance of serum resistance as a determinant of virulence for *E. coli* in turkeys has been demonstrated in the present study. Restriction of iron by the host or deposition of opsonically active complement fragments on the bacterial surface may be important host defense mechanisms against serum-resistant *E. coli* strains. Serum resistance as a determinant of virulence for gram-negative bacteria has been suggested for *Pasteurella multocida* in turkeys and *Salmonella cholerae-suis var kunzendorf* in mice and pigs (10, 17). The significance of serum resistance in *E. coli* from turkeys is exemplified by strains of the O78:K80 serotype which is a prevalent cause of colisepticemia.

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SECTION IV. INTERACTION OF TURKEY COMPLEMENT WITH *ESCHERICHIA COLI* ISOLATED FROM TURKEYS

ABSTRACT

The bactericidal effects of turkey complement were determined using serum-sensitive and serum-resistant strains of *Escherichia coli* isolated from turkeys. Inactivation of turkey complement by heating (56°C for 40 minutes) or by chelation with 10 mM ethylenediamine tetraacetic acid (EDTA) eliminated bactericidal activity. Serum-sensitive *E. coli* were killed by turkey serum treated with 10 mM ethylene glycol-bis (β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA) and 5 mM magnesium chloride. Exposure of normal turkey serum to either serum-sensitive or serum-resistant *E. coli* resulted in equivalent reductions in hemolytic activity of the serum. Pretreatment of serum-resistant *E. coli* with antibody rendered the bacteria sensitive to the bactericidal effects of normal turkey serum. These results indicate that serum-sensitive *E. coli* are readily killed by the alternative complement pathway, serum-sensitive and serum-resistant *E. coli* activate the complement system equally well, and antibody is required for complement-mediated killing of certain serum-resistant strains of *E. coli* from turkeys.

INTRODUCTION

Gram-negative bacterial strains which cause disseminated infections and bacteremia are usually found to be resistant to the bactericidal effects of serum. Such is the case with gonococcal infections and K-1 *Escherichia coli* infections in humans (24, 27). A similar situation exists with *E. coli* in poultry. *Escherichia coli* infection of poultry is often an extension from the respiratory tract, resulting in acute colisepticemia (5). In a previous study, virulent *E. coli* strains isolated from turkeys were usually found to be serum-resistant, whereas avirulent strains were serum-sensitive (7). Therefore, serum resistance appears to be an important virulence determinant shared by several gram-negative bacterial pathogens across divergent host species.

The bactericidal activity of serum against gram-negative bacteria is primarily mediated by antibody and complement (28, 32). The role of lysozyme is unclear, but it may have a minor role in bacteriolysis (19). The complement system consists of a group of serum proteins which function in the formation of a membrane attack complex (C5b-9) responsible for complement-mediated killing of certain bacterial strains (16, 29). In the absence of antibody, complement activation may proceed through the classical (6, 18) or alternative (25) pathways. Specific antibody not only facilitates classical

pathway activation, but may also be important in enhancement of C3b deposition by the alternative pathway, and stabilization of the terminal components of C5b-9 (11, 12). The virulence factors that allow certain strains of bacteria to resist the bactericidal effects of complement are not well understood. Bacterial surface structures which may be important for serum resistance include smooth lipopolysaccharide, acidic capsular polysaccharide and plasmid-determined outer-membrane proteins (2, 21, 30). The molecular basis for serum resistance centers on interaction of C5b-9 with the bacterial outer membrane. For example, current evidence suggests that serum resistance of the human pathogens, *E. coli* and *Salmonella minnesota*, is due to release of C5b-9 from the outer membrane of these bacteria (13-15). In contrast, stable insertion of C5b-9 into the outer membranes of their serum-sensitive counterparts results in complement-mediated killing.

Little is known of the interaction of turkey complement with bacterial pathogens. The present study was conducted to determine the role of turkey complement in the killing of *E. coli* isolated from turkeys and to examine the interaction of antibody and complement with serum-resistant strains of *E. coli*.

MATERIALS AND METHODS

Experimental Design. This study consisted of two parts, corresponding to each of the objectives. The first part examined the role of complement in the bactericidal activity of turkey serum for *E. coli*. Three strains of *E. coli*, described in Table 1 are known to be sensitive to turkey serum and were therefore used to analyze complement function. Turkey serum was depleted of complement activity by heat treatment, or by differential chelation of calcium and magnesium ions using ethylene diamine tetraacetic acid (EDTA) or ethylene glycol-bis-β aminoethyl ether-N,N,N',N'-tetraacetic acid (EGTA). EDTA inhibits both the classical and alternative pathways of complement activation, whereas EGTA supplemented with magnesium ions inhibits only the classical pathway (8). The ability of serum-sensitive *E. coli* to survive in treated sera was determined in triplicate using the bactericidal assay.

The interaction of antibody and complement with serum-resistant *E. coli* was examined in the second part of this study. Four strains of serum-resistant *E. coli*, described in Table 1 were individually mixed with turkey serum. Serum samples treated with the *E. coli* strains were then examined for residual complement activity using hemolytic assays. This procedure was used to determine whether inability to

TABLE 1. Serotype, serum resistance and virulence of *E. coli* strains isolated from turkeys

strain	serotype[a]	serum resistance[b]	virulence[c]
1	O78:K80:H9	resistant	virulent
3	O2a:K-:NM	sensitive	avirulent
4	O36:K-:NM	sensitive	virulent
13	O11:K˚:H4	resistant	avirulent
20	O78:K˚:H9	resistant	avirulent
39	O2a:K2:NM	sensitive	avirulent
53	O78:K˚:NM	resistant	virulent

[a]K- = no K antigen present. K˚ = K antigen present, but not identifiable with standard K antisera. NM = nonmotile.

[b]Serum resistance was previously reported as the change between the 3 hour and 0 hour samples in the bactericidal assay, recorded as $\log_{10}$ CFU/ml (7).

[c]Virulence was reported as the sum total of hours turkey poults (n=5) survived after an intravenous challenge with *E. coli* strains (7).

activate complement was a mechanism for serum resistance of *E. coli* isolated from turkeys. In addition, two serum-resistant strains of *E. coli* were sensitized with specific turkey antibody produced against each of the respective live bacteria to determine the influence of antibody on the bactericidal activity of turkey serum. Immune sera were

heat-inactivated and chelated with EDTA to prevent complement activation during the sensitization procedure. Therefore immune sera served only as sources of antibody. Because EDTA and turkey serum components other than antibody were present during sensitization of *E. coli*, EDTA, or EDTA and a normal heat-inactivated turkey serum were included as controls to ensure that the effects observed were due to the antibody and not other external factors. *Escherichia coli* strains sensitized with antibody were then tested for serum resistance in duplicate using the bactericidal assay.

Bacteria and culture conditions. *Escherichia coli* strains, originally isolated from turkey flocks in central Iowa, were previously examined for serum resistance and virulence in turkeys (7). Each of the *E. coli* strains used in this study are representatives of the categories, serum-resistant/virulent, serum-resistant/avirulent, serum-sensitive/virulent or serum-sensitive/avirulent. The characteristics for each *E. coli* strain are shown in Table 1.

To reduce effects of original isolation procedures and *in vitro* culture conditions, each bacterial strain was passaged in a three-week old turkey and reisolated from the bloodstream, as previously described (7). *Escherichia coli* reisolated from blood were grown in brain heart infusion broth, mixed in glycerol (1:1, v/v), and stored at -70^{o}C. *Escherichia coli* strains 3, 4, and 39 were used in part one

of this study to determine the role of complement in the bactericidal activity of turkey serum, and strains 1, 13, 20, and 53 were used in the second part of the study to identify mechanisms associated with serum resistance of *E. coli* isolated from turkeys.

Serum. Blood was obtained from healthy 4-week old turkeys and allowed to clot at room temperature for 1 hour and then at 4°C for 1 hour. Serum was harvested, pooled and frozen in aliquots at -70°C until required. Serum was tested for specific agglutinating antibodies to each of the *E. coli* strains using a microtiter agglutination assay (1). Two-fold dilutions of serum were mixed with equal volumes of bacterial suspension (1×10^9 colony-forming units (CFU)/ml) in a microtiter test plate. The antibody titer of serum was recorded as the last dilution causing agglutination of bacteria.

For part one of this study, 5 ml aliquots of serum were either heat-inactivated (56°C, 40 minutes), treated with 10 mM EDTA, or treated with 10 mM EGTA and 5 mM $MgCl_2$. An additional 5 ml aliquot remained untreated and served as a positive control for indication of serum sensitivity of the *E. coli* strains.

Bactericidal assay. The serum resistance of each *E. coli* strain was determined as previously described (7). Briefly, bacteria in the logarithmic phase of growth were

resuspended in 10 ml gelatin-veronal buffer (GVB++) containing 0.15 mM $CaCl_2$ and 0.5 mM $MgCl_2$, pH 7.4 (9), yielding a bacterial suspension of approximately 1 x 10^7 CFU/ml. For the assay, reaction mixtures of 250 ul containing 25 ul of bacterial suspension and 225 ul of undiluted turkey serum were incubated at 37°C for 3 hours. Fifty microliter samples collected initially, and at the end of the three hour incubation, were placed in 9 ml GVB++. Numbers of viable bacteria were determined by plating 10-fold serial dilutions of these samples on blood agar plates.

Sensitization of *E. coli* with antibody. Immune sera against two serum-resistant strains of *E. coli* (strains 1 and 53) were produced in turkeys. Four-week old turkeys were immunized with 10^6 live organisms, and subsequently boosted one week later with 10^8 organisms. Serum was collected two weeks after the last immunization and the complement was heat-inactivated (56°C, 40 minutes). For sensitization, dilutions of immune sera were chosen that would not result in agglutination of the bacteria. Serum-resistant *E. coli* were resuspended in 9 ml of each of the following buffers: GVB++; GVB++ containing 10 mM EDTA; GVB++ containing 10 mM EDTA and an appropriate dilution of immune sera (ITS); and GVB++ containing 10 mM EDTA and the same dilution of heat-inactivated normal turkey serum (NTS). Bacterial suspensions were incubated for 30 minutes at 37°C, centrifuged and

bacterial pellets resuspended in 10 ml GVB++ (approximately 10^7 CFU/ml). A one milliliter sample of each bacterial suspension was serially diluted ten-fold, and dilutions were plated on blood agar plates for bacterial counts. The effect of sensitization of *E. coli* with specific antibody was then determined using the bactericidal assay.

Residual complement activity. A block titration was performed using a serum-sensitive strain of *E. coli* (strain 3) to determine a concentration of bacteria, as well as serum that yielded minimum residual hemolytic complement activity. These concentrations of bacteria and serum were used to study complement activation by serum-resistant *E. coli* (strains 1, 13, 20 and 53). Final reaction mixtures were 1 ml containing 0.1 ml bacteria (10^9 CFU/ml) and 0.9 ml of 70% turkey serum. Samples were incubated for 1 hour at 37°C, centrifuged and supernatants were collected for measurement of residual hemolytic complement activity. Bacteria were tested in duplicate.

Hemolytic complement assays. Sheep red blood cells (SRBC) sensitized with turkey anti-SRBC antibody (5×10^8 cells/ml) were prepared as previously described (9) for the measurement of total complement activity. Horse red blood cells (2.5×10^8 cells/ml) were prepared for measurement of complement activity by the alternative pathway (20). Two-fold dilutions of serum were mixed with equal volumes of red

blood cells with a final volume of 1.0 ml. Suspensions were incubated at 37°C for 1 hour for sensitized SRBC and 2 hours for HRBC, as previously described (20). The hemolytic complement titer was spectrophotometrically determined and recorded as the reciprocal of the dilution yielding 50% hemolysis of red blood cells (9).

RESULTS

Role of complement in the bactericidal activity of turkey serum. Undiluted normal turkey serum was bactericidal for *E. coli* strains 3, 4, and 39, as shown in Figure 1. The level of bactericidal activity of serum varied with the strain, with strain 4 being highly susceptible to serum, and strains 3 and 39 being intermediate in serum sensitivity. This bactericidal activity was not attributed to antibody, as serum was negative for agglutinating antibodies (data not shown). Treatment of serum by heating to 56^{o}C for 40 minutes resulted in a loss of bactericidal activity against all three *E. coli* strains. Chelation of calcium and magnesium ions from turkey serum by EDTA inhibited bactericidal activity against strains 4 and 39, but not against strain 3. Each *E. coli* strain remained serum-sensitive in turkey serum treated with EGTA and $MgCl_2$.

Effects of antibody on serum-resistant *E. coli*. Strains 1 and 53 were resistant to the bactericidal effects of turkey serum in the absence of antibody, indicated in Figure 2. Sensitization of the bacteria with antibody did not affect bacterial numbers (data not shown). In contrast, both strains when sensitized with antibody were highly susceptible to the bactericidal effects of turkey serum. Bacterial numbers decreased by two logarithmic units during the three hour incubation when serum-resistant *E. coli* were sensitized

Figure 1. Role of complement in the bactericidal activity of turkey serum. Normal turkey serum (NTS), heat-inactivated turkey serum (HITS), serum treated with 10 mM EDTA, and serum treated with 10 mM EGTA and 5 mM $MgCl_2$ were examined for bactericidal activity against *E. coli*. Shown is the mean $\log_{10}$ CFU/ml change between the 3 and 0 hour samples in the bactericidal assay plus the standard deviation of triplicate samples

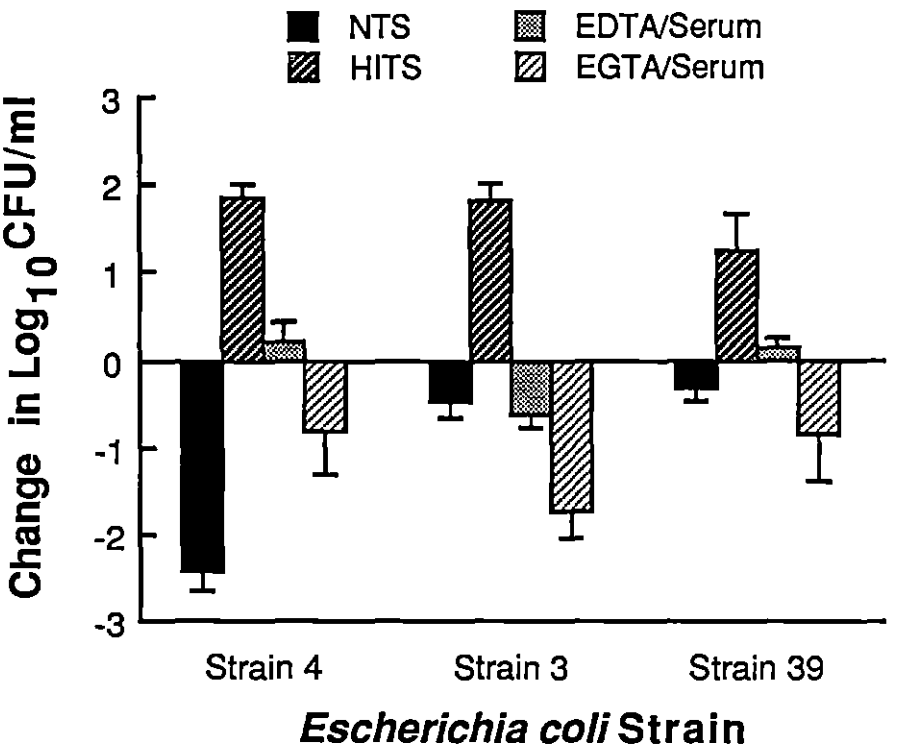
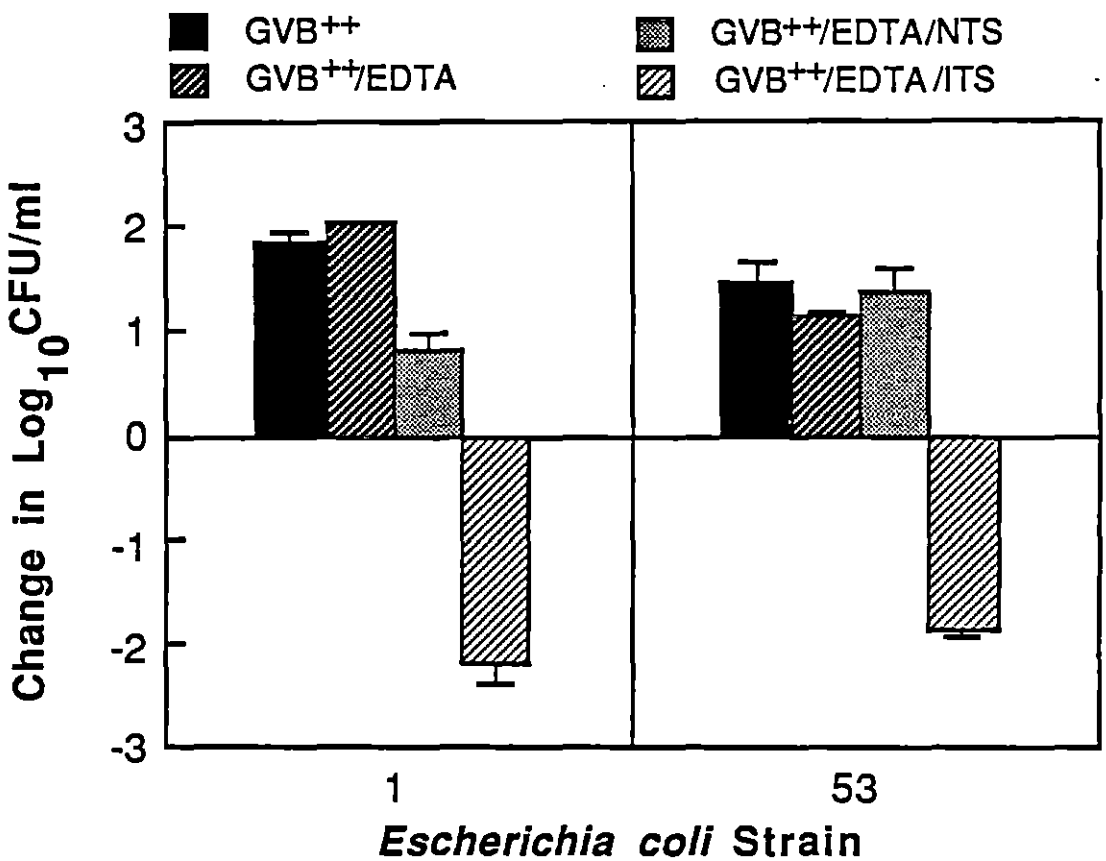

NTS
HITS
EDTA/Serum
EGTA/Serum
Change in Log10CFU/ml
3
2
1
0
-1
-2
-3
Strain 4
Strain 3
Strain 39
Escherichia coli Strain

Figure 2. Effect of antibody to serum-resistant *E. coli*. Strains of serum-resistant *E. coli* were pre-treated with GVB++, GVB++ containing 10 mM EDTA, GVB++ containing 10 mM EDTA and heat-inactivated normal turkey serum (NTS), or GVB++ containing 10 mM EDTA and heat-inactivated immune serum (ITS). Values represent the mean $\log_{10}$ CFU/ml change between the 3 and 0 hour samples in the bactericidal assay plus the standard deviation of duplicate samples


GVB++
GVB++/EDTA
GVB++/EDTA/NTS
GVB++/EDTA/ITS
Change in Log10CFU/ml
3
2
1
0
-1
-2
-3
1
53
Escherichia coli Strain

with specific antibody. The presence of EDTA or other serum components during sensitization with antibody did not markedly affect the serum resistance of strains 1 and 53. However, with strain 1, there was a $\log_{10}$ CFU/ml difference between incubation in GVB++ and incubation in normal heat-inactivated turkey serum chelated with EDTA.

Residual hemolytic complement activity. Treatment of serum with either serum-sensitive or serum-resistant *E. coli* caused a decrease in hemolytic activity, as indicated in Table 2. Lysis of both sensitized sheep red blood cells and horse red blood cells was decreased by treatment of serum with strains 1, 13, and 20. In contrast, treatment of serum with strain 53 did not affect the lysis of sensitized sheep red blood cells, but did affect hemolytic activity against horse red blood cells. Virulent/serum-resistant strains 1 and 53 or avirulent/serum-resistant strains 13 and 20 did not differ in ability to deplete serum complement. However, the virulent/serum-resistant strain 53 only affected the hemolytic activity against horse red blood cells.

Table 2. Hemolytic complement activity of turkey serum treated with various *E. coli* strains

E. coli strain	Residual complement activity	
	SSRBC	HRBC
control	14.8	10.5
3	6.1	5.4
1	4.4	4.2
53	13.2	5.1
13	5.8	6.6
20	5.9	5.4

DISCUSSION

In the present study, the bactericidal activity of turkey serum for *E. coli* was found to be the result of complement activation. Inactivation of complement by heat treatment or by chelation of calcium and magnesium ions using EDTA abolished the bactericidal activity of serum against *E. coli*. This would suggest that complement may have an important role in infection. Antibody-independent killing by complement may proceed through the classical (18, 31) or alternative (22, 25) pathways. In this study, the alternative pathway which is expressed in EGTA-chelated serum had a predominant role in the killing of serum-sensitive strains of *E. coli*. The alternative complement pathway is reported to have a significant role in the host against several types of infectious agents (25, 26, 3). Activation of the alternative complement pathway in the absence of antibody provides the host with an early non-specific defense mechanism.

In contrast, some strains of gram-negative bacteria may require specific antibody for complement-mediated killing (23, 11, 27). In the present study, this is indicated by the observation that *E. coli* strains usually resistant to the bactericidal effects of turkey serum were killed when sensitized with specific antibody. The specificity of this

antibody is not known. Previous work suggests that antibodies specific for bacterial lipopolysaccharide, outer membrane proteins or capsular polysaccharide facilitate complement-mediated killing (4, 23).

Treatment of serum with EDTA failed to completely abolish bactericidal activity, and with strain 3, bacteria were killed by the chelated serum. EDTA is reported to have a negative effect on the growth of bacteria and their ability to resist serum (6, 17). For example, EDTA has a bactericidal effect on rough bacterial strains and a bacteriostatic effect on smooth forms (6). The effect produced by EDTA is attributed to release of lipopolysaccharide and hence, alteration in the bacterial cell wall (10). The results of the present study confirm previous reports, and suggest that EDTA may influence survivability and growth of *E. coli* in turkey serum. This observation only holds true for serum-sensitive *E. coli*. In this study, serum-resistant *E. coli* strains were not affected by EDTA treatment. Therefore, different mechanisms may exist between serum-resistant and serum-sensitive *E. coli* in their ability to resist the effects of EDTA, and subsequently grow in serum. The differences between these strains may reflect variations in sequestering the divalent cations from EDTA for bacterial growth. Alternatively, if EDTA treatment releases lipopolysaccharide as previously described, then factors

other than the presence of lipopolysaccharide on the surface of *E. coli* may be necessary for serum resistance.

The mechanisms for serum resistance of *E. coli* isolated from turkeys are not associated with an inability to activate serum complement. The four serum-resistant *E. coli* strains examined in this study were found to activate and consume turkey complement equally as well as a serum-sensitive strain. Complement activation by the serum-resistant strains primarily occurred through the alternative pathway, as indicated by the reduction in hemolytic activity against horse red blood cells. Hence, serum resistance appears to be determined at the level of the individual complement components and their interaction with the bacterial surface. Studies of *E. coli* and *S. minnesota* and their interaction with human complement suggest that serum resistance is determined by the instability of the membrane attack complex (C5b-9) in the bacterial outer membrane (13-15). Release of the membrane attack complex from the bacterial outer membrane was associated with the serum-resistant strains. Study of the components of the membrane attack complex of turkey complement and their interaction with the outer membrane of *E. coli* may determine mechanisms for serum resistance of *E. coli* isolated from turkeys.

In conclusion, many *E. coli* strains isolated from turkeys are readily killed by activation of the alternative

complement pathway. However, some strains of *E. coli* require the presence of antibody for complement-mediated killing. This is particularly true for strains that normally resist the bactericidal effects of turkey serum. Although serum-resistant, *E. coli* strains 13 and 20 were previously found to be avirulent for turkeys (7). The host mechanisms causing avirulence of these strains are unclear. It may be that complement activation by these serum-resistant strains results in the stable deposition of opsonic complement fragments on the bacterial surface, with incomplete formation of the membrane attack complex. In contrast, virulent *E. coli* from turkeys may require mechanisms to resist both the bactericidal and opsonic effects of complement.

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GENERAL SUMMARY AND DISCUSSION

This research was conducted to obtain an understanding of turkey complement and its role in *Escherichia coli* infections of turkeys. The three major objectives were to:

1. characterize the complement activity of turkeys
2. isolate and characterize the third component of turkey complement
3. clarify the role of complement in defense against *E. coli* infection in turkeys

In humans and other selected mammals, activation of the complement system occurs through a classical or alternative pathway (22, 87). Avian complement has received little research emphasis; therefore, knowledge of individual avian complement components is limited. Because assays for detecting avian complement activity are limited, there existed some controversy as to whether avian species have a classical pathway (60, 61). In this research, two hemolytic assays were developed to measure distinct pathways of complement activation in turkey serum. The lysis of sheep red blood cells sensitized with specific turkey antibody identified a pathway in turkey serum which required antibody and the divalent cations, calcium and magnesium. This pathway is analogous to the human classical pathway. In contrast, lysis of horse red blood cells by turkey serum was antibody-independent and only required magnesium ions.

Heterologous erythrocytes are lysed in the absence of antibody by activation of the human alternative pathway (88, 96). Therefore, lysis of horse red blood cells by turkey serum identified complement activity analogous to the alternative pathway in humans. Carrageenan, an inhibitor of mammalian classical activity (12), prevented lysis of the SSRBC, but not the lysis of HRBC. In contrast, zymosan and inulin, both of which activate the mammalian alternative complement pathway (28, 95), depleted complement activity against both SSRBC and HRBC. Treatment of turkey serum with activators of mammalian complement confirms the differences observed in the two hemolytic test systems and demonstrates that both classical and alternative complement activity are present in turkeys.

Isolation of the third component of turkey complement was facilitated by the initial production of a putative rabbit anti-turkey C3 antiserum. Because there is an incompatibility between avian and mammalian complement components (35), and very few of the avian complement components are isolated, characterization of turkey C3 was based on structural and immunological techniques.

The rabbit anti-turkey C3 antiserum agglutinated sheep red blood cells sensitized with antibody and turkey complement, but did not agglutinate sheep red blood cells sensitized with antibody alone. Therefore the turkey protein

reacting with the anti-turkey C3 antiserum was a complement protein. In humans, mice, chickens and quail, C3 has a characteristic electrophoretic mobility in complement-activated serum (56, 59, 77, 117). The anti-turkey C3 antiserum reacted with a turkey protein which moved toward the anode after complement activation by inulin, as similarly described for the third component of the previously mentioned species. Hence, a possible C3 molecule of turkey complement was identifiable by the crossed-immunoelectrophoresis procedure.

The protein identified as turkey C3 was similar in structure to C3 of humans, chickens and quail. It was composed of two chains (120,000 and 75,000 molecular weights) joined by disulfide bonds. The reactive functional group of turkey C3 was not identified. However, it was found that turkey serum was sensitive to methylamine, suggesting that the active group in turkey C3 was a thiolester bond, similarly described for human C3 (116).

An alternative means for isolation of turkey C3 would be to use the rabbit anti-turkey C3 antiserum in affinity chromatography. However, there was a tendency for turkey immunoglobulin to predominate the sample eluted from the affinity column. The reason for this is unclear. Although the antiserum reacts specifically with a complement protein identified by crossed-immunoelectrophoresis, it binds turkey

immunoglobulin more efficiently in the affinity column. It may be that the rabbit antiserum reacts with an antigenic determinant found on turkey C3 and turkey immunoglobulin.

The final objective of this research was to characterize the role of turkey complement in *E. coli* infections of turkeys. Turkey complement has a significant role in the bactericidal activity of serum, as similarly described for human complement (118, 121). In the absence of antibody, activation of the alternative pathway of turkey complement can lead to killing of *E. coli* strains isolated from turkeys. However, antibody-dependent complement killing was required for some *E. coli* strains. The requirement for antibody to render these *E. coli* strains sensitive to complement-mediated killing may enable the bacteria to replicate in the blood and tissues during the pre-antibody phase of infection. For example, two *E. coli* strains, normally serum-resistant and virulent for turkeys were readily killed by turkey serum when sensitized with specific antibody.

The results from this research suggest that virulent *E. coli* isolated from turkeys are more likely to resist the bactericidal effects of serum mediated by complement. In contrast, avirulent *E. coli* strains are usually serum-sensitive. Hence, serum resistance can be considered an important virulence factor for *E. coli* in turkeys. However, some *E. coli* strains require other virulence factors, in

addition to serum resistance for survival in turkeys. Although serum-resistant, five *E. coli* strains were avirulent for turkeys. In a previous report, efficient acquisition of iron from host tissues was necessary for virulence of *E. coli* in turkeys (9). The iron acquisition mechanisms of the *E. coli* strains used in this study were not characterized. It is speculated that the serum-resistant/avirulent *E. coli* strains identified in this research may lack efficient iron acquisition mechanisms or other essential virulence factors. Alternatively, the presence of opsonic complement fragments on the surface of these serum-resistant *E. coli* may lead to opsonophagocytosis of the bacteria, and hence avirulence. For example, some bacterial strains which are usually virulent are readily cleared by hepatic phagocytes when opsonized by antibody or complement (2, 44).

Although this research has provided an understanding of turkey complement and has identified a role for turkey complement in *E. coli* infections, several areas remain unclear. Is there a common terminal pathway of complement activation in turkey serum? Zymosan and inulin treatments of turkey serum suggest that there is a common complement pathway. Does turkey C3 have the same functional group - a thiolester bond - as that of human C3? What are the host and bacterial mechanisms responsible for avirulence of serum-resistant *E. coli*? What virulence factors are associated

with the serum-sensitive/virulent *E. coli* strains? Why do some strains of *E. coli* resist the bactericidal effects of complement? Utilization of purified complement components has facilitated analysis of human complement-bacterial interactions. Unfortunately, few avian complement components have been isolated. Purified turkey C3 and an anti-turkey C3 antibody can be used to clarify the role of C3b as an opsonin in host defense mechanisms of turkeys to serum-resistant *E. coli*.

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