Sensitivity of *Salmonella cholerae-suis* to different animal species complements

by

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GENERAL INTRODUCTION

The study of complement (C) was initiated in the late 1800s when investigators observed the ability of fresh serum to kill bacteria. Since that time, the majority of C research has been involved with its effects on bacteria. The term alexin was given to the bactericidal activity of serum. Alexin was thought to be an enzyme because it was inactivated by heat. Pfeiffer and Bordet demonstrated that antibody was required for bacteriolysis by alexin. Ehrlich and Morgenroth used the term complement to describe the ability of enzymes present in normal serum to lyse red blood cells in conjunction with antibody (Osler 1976). Complement was not a single enzyme but a mixture of enzymes. Early attempts to understand the nature of C resulted in identification of four components. The results from later studies indicated that C was made up of nine components. Further work with the first C component indicated that it actually was a complex of three proteins, thus raising the component numbers to eleven. The discovery of the alternate C pathway and C inhibitors gave a total of nineteen components involved in the C system.

Complement is activated by two major pathways, both of which are characterized by the formation of enzymes that cleave C components to produce new enzymes, initiating a cascade reaction. The classical pathway is activated by antigen-antibody complexes. When antibody binds to antigen, there is a conformational change in the antibody structure which exposes C receptor sites. Complement component C1 is

a complex made up of three individual molecules: Clq, Cls, and Clr. The Clq component of Cl binds to the antibody. The other two Cl components, Clr and Cls, are then also bound. Together, they produce Cl esterase. This enzyme cleaves C4 to produce C14, which in turn cleaves C2. The C142 enzyme is known as C3 convertase (Mayer 1973, Vogt 1974, Muller-Eberhard 1977, Fine 1981).

The other major pathway of C activation is the alternate pathway. Pillemer et al. (1954) described a new serum protein, which they named properdin, and which reacted with zymosan (yeast cell wall polysaccharide) to inactivate C3. Further investigations have determined that two other factors, factors B and D, are also involved in the alternate pathway. A number of substances, such as zymosan, viruses, lipopolysaccharide (LPS), rabbit erythrocytes, aggregated and nonaggregated antibodies, and some gram-positive bacteria, are capable of activating the alternate pathway. Fearon (1979) attributed alternate C pathway activation to the presence of protected C3b receptor sites lacking in sialic acid. Sialic acid is required for an inhibitor, factor H, to degrade C3b. Substances that activate the alternate C pathway interact with native C3b or with C3 to produce C3b. Factor B is bound to C3b and is cleaved by factor D to form C3bBb, otherwise known as C3 convertase. Properdin acts to stabilize the complex (Fearon 1979, Fine 1981, Pangburn 1983). The enzyme C3 convertase cleaves C3 to form C3a and C3b. The C3b fragment is bound to the enzyme to produce C5 convertase

The terminal sequence is identical for both pathways after the formation of C3 convertase. The component C5 is cleaved by C5 convertase to produce membrane-bound C5b and C5a. The two cleavage products, C3a and C5a, are anaphylactic and chemotactic factors important in processes such as endotoxic shock and inflammation. The membrane-bound C5b binds C6. Then, C7 is bound by the C5bC6 complex. Once C7 is bound, C8 binds to C5b, and in turn binds C9. Lysis of the membrane begins after C8 is bound, and the addition of C9 accelerates the process (Fine 1981, Mayer 1973).

An additional mechanism of classical pathway activation has been demonstrated. Lipopolysaccharide can directly interact with C1, independent of antibody, to initiate the classical pathway. Loos et al. (1974) observed that LPS, specifically lipid A, bound the C1q portion of C1. Further studies by Loos et al. (1978) indicated that serum C1 as well as activated C1 was bound by LPS. Morrison and Kline (1977) demonstrated that lipid A initiated the classical pathway independent of antibody, and the polysaccharide region of LPS activated the alternate pathway. Betz and Isliker (1981) confirmed that C1 bound to LPS, and demonstrated that LPS bound and activated precursor C1, which was capable of cleaving C4 and initiating hemolytic and bactericidal activities.

The elucidation of complement components and activation has arisen from studies of their bactericidal effects. Gram-negative bacteria have been the primary focus of these studies. The frequency of nosocomial gram-negative infections has been steadily increasing.

Increased hospitalization, surgery, and immunosuppressive drugs have contributed to the rise. A number of gram-negative bacteria are common in the intestinal tract and are considered normal flora. Pathogenic and virulent bacteria have developed resistance to both host and bacterial defenses. The presence of large concentrations of gram-negative bacteria in the intestine is controlled and balanced by host mechanisms. In addition, there are two major defenses against invasive bacteria, the mucosal-epithelial cell barrier and the hepatic reticulendothelial system. Despite this, there is continuous absorption of endotoxin from the intestine. Small amounts of endotoxin are constantly released as bacteria die or are killed. Increased blood levels of vasoactive substances, such as serotonin, norepinephrine, and histamine, enhance intestinal permeability. Other factors, such as the absence of food or bile, or the presence of milk, may enhance transmucosal transport (Mims 1982, Walker and Porvaznik 1978, Walker 1978).

Many of the effects of gram-negative infection can be attributed to endotoxin present on the bacterial cell wall. Endotoxin is a complex of lipopolysaccharide (LPS) with a protein component bound to it. The term endotoxin was first used by Richard Pfeiffer in 1892 when he observed that cholera vibrios produced a toxin associated with the bacterial cell that was unaffected when the bacteria were killed. Endotoxic activity was associated with the lipopolysaccharide component of gram-negative bacteria and was related to the O antigen of Salmonella by Andre Boivin. Walter Morgan separated purified O

antigen into three components: a polysaccharide component, a conjugated protein, and a lipid component (Westphal et al. 1981). Westphal obtained a pure, protein-free lipopolysaccharide which was pyrogenic by using a phenol-water extraction method. Further separation with acid yielded lipopolysaccharide split into polysaccharide and lipid fractions. The lipid portion, lipid A, was responsible for endotoxic activity (Figure 1).

Lipid A is a hydrophobic glucosamine disaccharide to which saturated fatty acids are attached (Figure 2). The polysaccharide component consists of the core oligosaccharide, which is characterized by unusual sugars such as 2 keto-3-deoxyoctonate and heptuloses, and the polysaccharide side chains. These side chains have repeating units of oligosaccharides and determine the O antigen specificity for each gram-negative strain (Stryer 1981, Westphal et al. 1981).

Damage to the intestine will increase the uptake of endotoxin. An endotoxemia as well as a bacteremia can develop when the barrier function of the intestinal wall is damaged, whether by kinins, poor peristaltic flow or from lysosomal enzymes resulting from injury. Jacob Fine (1972) injected normal rabbits and rabbits that had no gram-negative flora with lethal doses of endotoxin. The normal rabbits died, but the rabbits without gram-negative flora survived and had no traces of endotoxin in the liver. The livers of the normal rabbits contained more endotoxin than what was initially injected. Walker and Porvaznik (1978) used mice to investigate the route of endotoxin from the intestine to the liver. Their results indicated

that endotoxin-induced inflammation disrupted the tight junctions between epithelial cells, providing a channel for bacteria to enter the host tissues and become systemic. In this manner, endotoxemia can become self-sustaining and can produce fatal shock. The liver is the primary organ for endotoxin clearance and prevention of systemic infection. Endotoxin is carried by the portal vein from the intestinal tract to the liver. There fixed macrophages, or Kupfer cells, take up the endotoxin and degrade it. Bacteria and endotoxin can become systemic by overwhelming the Kupfer cells with the introduction of large concentrations of material. Endotoxin may also be taken up by the lymphatic system and become systemic when introduced to the venous system (Berry 1975, Walker 1978, Nolan 1979).

The effects of lipopolysaccharide have been intensely studied for many years. Different methods of introducing LPS into a host have been used. Animal models as well as human models have been studied. Investigations into a variety of enzymes, cell systems and immune responses have been performed.

Injection of endotoxin i.v. into cattle causes salivation, diarrhea, coughing, severe dyspnea and collapse. Neutropenia, lymphopenia and granulocytosis occur along with hemorrhages in the brain, heart and lung. Disseminated intravascular coagulation also occurs, preceded by a drop in fibrinogen levels (Thompson et al. 1974, Nagaraja et al. 1979). Injection of endotoxin into ponies does not cause diarrhea, but does induce transient arterial hypotension, neutropenia, and hyperglycemia. Respiratory and peripheral vascular

perfusion also occurs (Burrows and Cannon 1970). In goats, endotoxin causes biphasic fever, decreased plasma iron levels, neutropenia, and lymphopenia. Kurtz and Quast (1982) studied the effect of endotoxin on swine. The reactions of the swine were similar to those of cattle. The pigs fell into three groups: normal animals, animals with neurological disturbances, and animals with renal necrosis.

The classical animal model of endotoxic shock is the dog. Intravenous injection of lethal doses of endotoxin causes systemic hypotension, decreased cardiac output, and decreased central venous pressure. Bi- or mono-phasic fever and increased peripheral resistance occur, and there is formation of small thrombi which induce coagulation. The decreased perfusion causes tissue damage. Leukopenia and neutropenia followed by leukocytosis also occur. Platelets, leukocytes, and granulocytes have endotoxin receptors and are induced to release vasoactive substances such as histamine and serotonin. These substances trigger blood clotting, disseminated intravascular coagulation, and vascular collapse (Hawiger et al. 1975, Berry 1975, Muller-Berghause 1978, McNulty 1983).

The production of many of these substances is induced by the interaction of LPS with complement. The cleavage products C3a and C5a are chemotactic factors for lymphocytes, neutrophils, and macrophages and anaphlylactic factors for mast cells and basophils. The C3b fragment acts as an opsonization factor with or without antibody. Although conflicting results from in vivo studies have cast some question on the significance of complement during infection, the high

in vitro bactericidal activity of complement and involvement with other systems qualify it as an integral part of the host defense system.

It was reported that complement varies in activity with each animal species, and that components from one species are often not interchangeable with components of another species. This study was performed to examine the bactericidal effect of complement on gram-negative bacteria and the variation of such effects between animal species complements. Part 1 of this study was concerned with the bactericidal effects of complement with and without antibody on *Salmonella cholerae-suis* and *Escherichia coli*, and to determine if susceptibility varied with the animal species complement used. Bactericidal complement may kill bacteria by two major pathways. Differences in cation requirements were used as a tool for studying the pathways in Part 2 to determine which pathway was activated by susceptible bacterial strains.

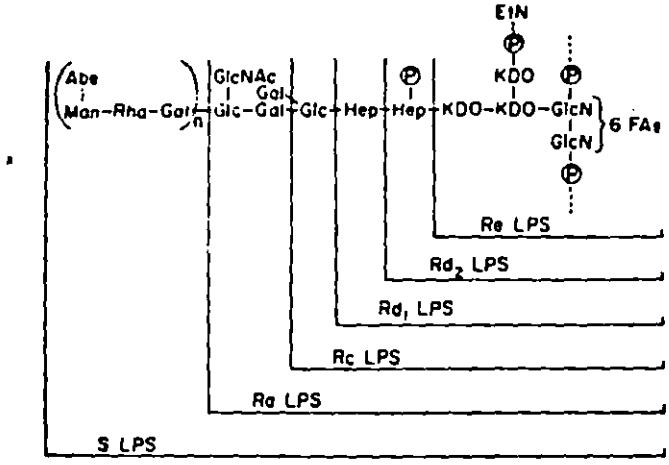

Figure 1 The structure of lipopolysaccharide of Salmonella typhimurium (Nikaido and Nakoe 1979)

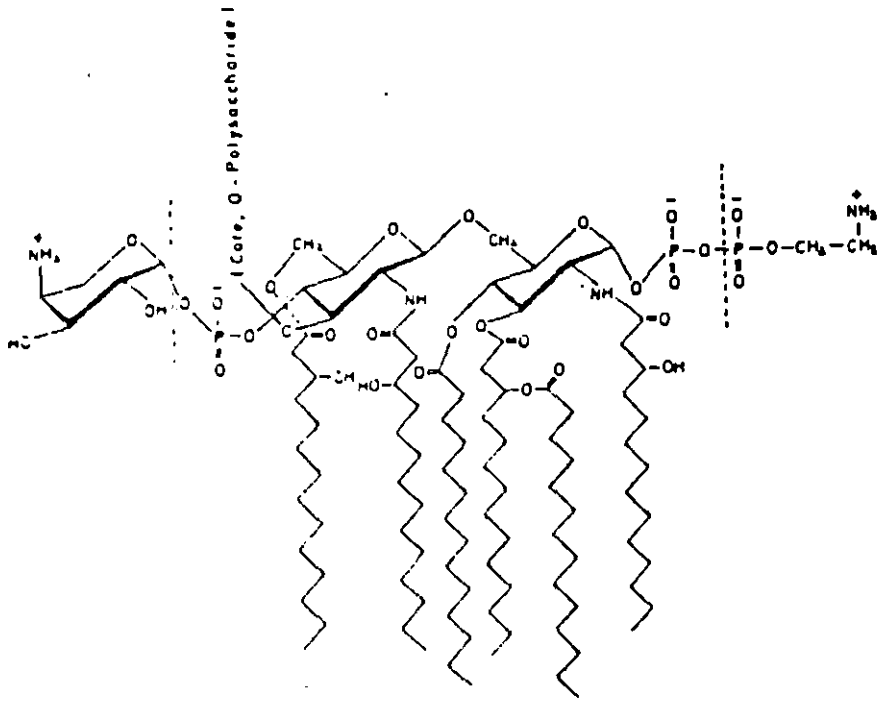

Figure 2 The structure of lipid A from Salmonella minnesota (Nikaido and Nakoe 1979)

PART 1 BACTERICIDAL EFFECTS OF COMPLEMENT AGAINST *SALMONELLA CHOLERAE-SUIS* AND *ESCHERICHIA COLI*

INTRODUCTION

A characteristic of gram-negative bacteria is the presence of lipopolysaccharide (LPS) in the outer membrane of the bacterial cell wall. Lipopolysaccharide is a complex molecule divided into hydrophobic and hydrophilic regions. Lipid A is a hydrophobic glycolipid, the backbone of which consists of a glucosamine dissaccharide. Six saturated fatty acids are attached to the glucosamine backbone; the tails of the fatty acids are embedded in the outer membrane. Lipid A is responsible for many of the effects of LPS: endotoxicity, pyrogen production, vascular changes, macrophage and complement activation, etc. (Stryer 1981, Westphal et al. 1981).

The other two components, the R core and the O side chains, are hydrophilic polysaccharides. The R core, or core oligosaccharide, is characterized by 2 keto-3-deoxyoctonate (KDO) and heptulose sugars. The O side chains have repeating units of oligosaccharides which, by the variability of types and number of sugars attached, determine the O antigenic specificity used for grouping gram-negative bacteria into serotypes. The R core varies very little between bacterial species, and lipid A is quite consistent for most genera. Studies of LPS have suggested that there is crosslinking of LPS monomer units by divalent cations such as Ca^{+2} and Mg^{+2}, possibly at the O side chains (Stryer 1981, Westphal et al. 1981) or at pyrophosphate bridges on KDO units (Nikaido and Nakoe 1979), and that the structure and attachment of LPS is dependent on these divalent cations.

Bacteria that have complete LPS components are referred to as wild-type or smooth (S) strains. Strains that have oligosaccharide deletions in the O side chains and/or the R core are called rough (R) mutants. The S and R designations were derived from distinct differences in colony appearances of these bacterial types. Rough mutations occur when polysaccharide biosynthesis is blocked at one or more stages, producing shorter polysaccharide chains. Wardlaw (1963) compared the cell-wall composition of smooth and rough strains of E. coli and found that the major difference in composition between the two types was the amount of LPS; smooth strains had a higher percentage than rough strains of LPS. The transition from smooth to rough occurs by successive deletions of sugar residues. Deep rough mutants have only lipid A and three molecules of KDO.

Another characteristic of the transition from smooth to rough is an increase in susceptibility to antibody and complement. Many strains of bacteria are readily killed by antibody and complement. Rough strains are generally more sensitive than smooth strains to killing, and many smooth strains are totally resistant. The polysaccharide side chains of S strains can cover and block antibody and complement receptors, inhibiting lysis by antibody and complement. Rough strains have shorter side chains than smooth strains, and antibody and complement have easier access to the cell wall.

Smooth bacteria that are resistant to antibody-complement lysis can be made susceptible by exposure to substances that remove some of the LPS from the cell wall. Voss (1967) demonstrated that gram-

negative bacteria resistant to killing in physiological saline would become more sensitive to killing when treated in tris(hydroxymethyl) aminomethane (Tris) buffer. He also observed that ethylene diaminetetraacetic acid (EDTA) also would render resistant strains more susceptible to killing. When the bacteria were exposed to both Tris and EDTA, Tris enhanced the bactericidal effect of serum and EDTA. The Tris buffer contains organic cations and is a mild chelator of inorganic divalent cations. The action of EDTA is to chelate inorganic divalent cations, such as Ca^{+2} and Mg^{+2}. When bacteria were exposed to both agents simultaneously, resistant bacteria became sensitive to killing by serum due to the removal of LPS from the cell wall (Voss 1967, Hedstrom et al. 1981, Reynolds and Pruul 1971a).

Reynolds and Pruul (1971a,b) postulated that EDTA removed the divalent cations that crosslinked the phosphate groups on the LPS monomer units. The univalent cations, supplied by Tris, replaced the divalent cations and were unable to form crosslinkages. The substitution by univalent cations altered the LPS structure and caused the release of LPS. The concentration of Tris and EDTA required for removal of LPS did not affect viability of the bacteria, but high concentrations of EDTA, variations in pH (Hedstrom et al. 1981) and the duration of exposure to Tris and EDTA (Griffith and Kramer 1982) did affect the action of the two agents so that they were bactericidal.

Lipopolysaccharide prevents binding of antibody to specific antigens and complement to cell receptors or to bound antibody. The

treatment of bacteria with Tris and EDTA caused the release of LPS from the cell wall which allowed antibody to reach the cell wall and bind to its specific antigen. Complement is activated by antigen-antibody complexes. The exact mechanism of complement recognition of bound antibody is unknown, but when the antibody is bound to the antigen, a conformational change in the structure of the antibody occurs which reveals the Clq receptor on the Fc portion of the antibody (Vogt 1974, Osler 1976). The component Cl is then fixed and activated to initiate the classical complement pathway.

For many years, bacteria were thought to activate only the classical pathway. But complement is activated by bacteria in antibody-free serum (Wardlaw 1962, Muschel et al. 1964, Gerwurz et al. 1968). Lipopolysaccharide activates complement by three mechanisms: antibody-dependent and -independent classical pathways and the alternate pathway. Evidence for alternate pathway activation by LPS was given in studies using agammaglobulinemic serum, precolostral serum and C4-deficient serum (Sterzl et al. 1964, Muschel et al. 1964, Fine 1981). The levels of the late components, C5-9, were reduced after incubation with LPS, but the levels of the early C components were relatively unchanged (Gerwurz et al. 1968). It was reported that complement is bactericidal without antibody. Schreiber et al. 1979 demonstrated that a mixture of purified alternate pathway components killed E. coli K12. Sterzl et al. (1964) found that precolostral piglet serum was bactericidal for gram-negative bacteria, and that the sensitivity to piglet complement increased with the degree of cell

wall roughness. But the results from other studies have reported that C1 was involved, and that the classical pathway was activated, possibly through the involvement of antibody. Fine (1974) coated LPS onto sheep erythrocytes (E-LPS). He found that LPS activated only the classical pathway, and a factor was required which was probably antibody to LPS. Lipopolysaccharide, but not E-LPS, activated the alternate pathway. Fine (1974) postulated that association with the erythrocyte membrane may have interfered with the ability of LPS to activate the alternate pathway.

Lipopolysaccharide can bind C1 and activate the classical pathway independently of antibody (Fine 1974, Morrison and Kline 1977). Loos et al. (1974) determined that lipid A was the component which interacted with C1q, but the polysaccharide components could affect the interaction. A later study by Loos et al. (1978) confirmed these results and demonstrated that activated C1 also bound to LPS and was capable of binding C4. The binding of C1 by lipid A, with the subsequent cleavage and activation of the other components, was bactericidal for serum-sensitive bacteria (Betz and Isliker 1981).

Lipopolysaccharide can activate complement by both pathways, but investigators have shown that activation of either pathway requires different LPS components. Bjornson and Bjornson (1977) tested three strains of Salmonella minnesota for their ability to activate complement. The smooth strain activated the alternate pathway. The Rb mutant, which lacked O side chains, activated the alternate pathway to a lesser extent. The deep rough mutant did not activate the

alternate pathway, but did activate the classical pathway. These results confirmed the findings of Loos et al. (1978) by demonstrating that the polysaccharide components of LPS activated the alternate pathway and lipid A interacted with C1 to initiate the classical pathway. Morrison and Kline (1977) observed that the length of the polysaccharide and the carbohydrate content also affected the activation of the alternate pathway.

Therefore, it was demonstrated that LPS activated both complement pathways independently of antibody. But does complement activation lead to lysis and become bactericidal? Complement was effective in killing serum-sensitive bacteria, and sometimes serum-resistant bacteria when high enough concentrations of complement were used (Sterzl et al. 1964, Leist-Walsh and Bjornson 1979, Betz and Isliker 1981). Schreiber et al. (1979) also demonstrated that C was bactericidal by the alternate pathway. Eleven proteins involved in the alternate pathway were isolated and purified. The isolated protein mixture and C4-deficient serum were each incubated with E. coli K12. The isolated protein mixture, which was free of antibody, killed E. coli K12 to the same extent as the C4-deficient serum.

Seventy-two field strains of Salmonella cholerae-suis var kunzendorf were tested by Griffith and Kramer (1982) for sensitivity to porcine antibody and complement. The bacteria were pretreated in Tris and EDTA (Tris-EDTA) and in saline, and were incubated with antibody and complement (Ab-C). Treatment with Tris-EDTA enhanced killing by Ab-C as compared to saline pretreatment. The seventy-two

strains varied widely in sensitivity to killing. Some strains were identified as susceptible and other strains as resistant. In the present study, selected strains of S. cholerae-suis obtained from Griffith and Kramer (1982) were tested for sensitivity to antibody-independent complement killing. The assay used in the present study was a modification of the assay previously described (Griffith and Kramer 1982). The bacteria were pretreated in Tris-EDTA and in saline and then were incubated with Ab-C and with complement alone (C). Killing by Ab-C and by C and the effect of pretreatment with Tris-EDTA were compared. Six animal species were used as sources of C: bovine, equine, porcine, human, mouse, and guinea pig. These complements were tested to determine if the complement from each species was bactericidal, and if sensitivity of S. cholerae-suis to complement varied with the species of complement used.

MATERIALS AND METHODS

Bacteria

Six strains of Salmonella cholerae-suis var kunzendorf (SC) were obtained from the seventy-two strains previously tested by Griffith and Kramer (1982). The six strains were selected on the basis of their responses to killing by porcine antibody and complement. Salmonella cholerae-suis var kunzendorf strains 9 (SC9), 33 (SC33), and 38 (SC38) exhibited a high degree of sensitivity to killing (>85% killed). Salmonella cholerae-suis var kunzendorf strains 2 (SC2), 5 (SC5), and 10 (SC10) were partially sensitive to killing (10-85% killed). Two strains of Escherichia coli K88 and K99 (EC K88, EC K99) obtained from H. W. Moon, National Animal Disease Center, Ames, Iowa, were laboratory-adapted strains. All strains were streaked once onto trypticase soy agar plates from the original agar slants, and were frozen in egg yolk at -70 C.

Each strain was inoculated from the frozen egg yolk onto trypticase soy agar plates. Smooth colonies were transferred to nutrient broth (NB) and incubated eighteen hours at 37 C. The strains were standardized with NB to an absorbance of 0.20 O.D. at 550 nm. The strains were then diluted to 10^{-3} in nutrient broth. The bacteria were pretreated in 0.1 M Tris containing 100 ug/L ethylene diaminetetraacetic acid (Tris-EDTA) or in saline for 10 minutes by adding 1 ml 10^{-3} bacterial dilution to 9 ml Tris-EDTA or saline. After incubation, the bacteria were diluted directly to 10^{-6} in NB.

Antibody

Porcine S. cholerae-suis antibody was prepared in gilts and used at a final dilution of 1:8000 in nutrient broth as previously described (Griffith and Kramer 1982). Porcine anti-E. coli K88 and K99 antisera were prepared in pigs by repeated injection of formalized bacteria. The titer was similar to that of the porcine anti-Salmonella antibody and was used at a final dilution of 1:8000 in nutrient broth. The 1:8000 dilution of each porcine antibody (Ab) was nonagglutinating. The antisera were heat-inactivated at 56 C for 30 minutes.

Complement

Bovine, equine, and porcine complements were obtained from a pool of sera from a minimum of three animals of each species. The domestic animals were bled by venipuncture, and the blood was allowed to clot for four hours at room temperature. Human C came from a pool of sera from a minimum of three volunteers. The sera were removed and kept frozen at -70 C. Guinea pig C and mouse C were obtained from Pel Freeze Co., Rodgers, Arkansas. Aliquots of each species complements were heated at 56 C for 30 minutes to inactivate the complement and were used as C controls in the assay. Complement (C) and heat-inactivated C were absorbed three times at 4 C with $1x10^{12}$ bacteria heat-killed (60 C for 1 hour) before the assay to remove natural antibody. Complement and heat-inactivated complement were used at final dilutions of 1:30 in Nβ.

Nutrient Broth

Nutrient broth was used as a growth medium for bacteria and as a diluent for the assays. Nutrient broth was prepared by adding 5.0 grams NaCl, 5.0 grams peptone, and 2.5 grams beef extract to one liter of water. The pH was adjusted to 7.2.

Bactericidal Assay

The assay consisted of adding 0.25 ml of the 10^{-6} dilution, containing approximately $1x10^4$ bacteria/ml pretreated in saline or in Tris-EDTA, to 0.25 ml each of porcine Ab (1:8000) + C (1:30), or C (1:30) + NB to give 0.75 ml total volume. The 1:8000 dilution of porcine Ab was nonagglutinating. The bacteria were also added to heat-inactivated C + NB and to NB as controls. After 5 hours incubation at 37 C, 10 ul samples were plated in triplicate onto Tergitol-7 TTC agar plates. Each assay was performed three times. Killing was determined by the formula

$$\frac{(100 - \text{CFU})\ 100}{\text{CFU control}} = \%\ \text{killed}$$

Susceptibility was categorized into three groups.

>85% killed = susceptible

10-85% killed = partially susceptible

<10% killed = resistant

RESULTS

The results presented in Table 1 demonstrated how each strain was affected by Ab-C after pretreatment with Tris-EDTA. The objective was to determine if the strains varied in susceptibility to killing with each complement used. Every complement except mouse and guinea pig complement was effective against SC33 and EC K99. Results obtained by using SC9 were inconsistent; SC9 was susceptible to bovine, equine, and human C, but was resistant to the other complements. Strains SC2, SC5, and SC1Ø varied very little in susceptibility to Ab-C killing. Equine, porcine, and human complements were highly bactericidal for SC38, but bovine, mouse, and guinea pig complements were only partially effective. E. coli strain K88 was sensitive to bovine and equine C in the presence of Ab.

The results presented in Table 2 indicated that C alone will kill strains pretreated with Tris-EDTA. Every C except mouse C was bactericidal for EC K99. Strain EC K99 was not susceptible to Ab and guinea pig C, but it was susceptible to guinea pig C alone. The presence of Ab may have affected activation of guinea pig C by the strain. Strains SC33 and SC 9 were susceptible to every C, with the exceptions of mouse and guinea pig complements. Bovine, equine, and human complements were effective for four of the eight strains. Mouse C was ineffective for all of the strains.

Table 1 Susceptibility of S. cholerae-suis and E. coli strains pretreated with Tris-EDTA to killing by porcine Ab and C

	Source of Complement					
	Bovine	Equine	Porcine	Human	Mouse	Guinea pig
S. cholerae-suis						
33	>85[a]	>85	>85	>85	10-85	10-85
9	>85	>85	<10	>85	<10	10-85
2	<10	10-85	10-85	<10	<10	<10
5	10-85	10-85	10-85	<10	<10	>85
10	10-85	10-85	10-85	<10	<10	10-85
38	10-85	>85	>85	>85	10-85	10-85
E.coli						
K88	>85	>85	10-85	10-85	10-85	<10
K99	>85	>85	>85	>85	<10	<10

[a]Percent bacteria killed by Ab-C.

Table 2 Susceptibility of *S. cholerae-suis* and *E. coli* strains pretreated with Tris-EDTA to killing by C alone

	Source of Complement					
	Bovine	Equine	Porcine	Human	Mouse	Guinea pig
S. cholerae-suis						
33	>85[a]	>85	>85	>85	10-85	10-85
9	>85	>85	<10	>85	10-85	>85
2	10-85	10-85	10-85	10-85	<10	<10
5	10-85	10-85	10-85	10-85	10-85	>85
10	10-85	10-85	10-85	10-85	<10	<10
38	>85	10-85	10-85	>85	<10	10-85
E. coli						
K88	10-85	>85	10-85	10-85	<10	10-85
K99	>85	>85	>85	>85	<10	>85

[a]Percent bacteria killed by C.

The effects of C and Ab on bacteria pretreated with saline were presented in Table 3. Bovine, equine, porcine, and human complements were very effective in the presence of antibody in killing SC9, SC33, and EC K99. Guinea pig C also killed EC K99. None of the complements were effective against SC2, SC5, and SC1Ø. Equine and human C were the most effective bactericidal complements, and again, mouse C was ineffective against all strains.

Some strains pretreated with saline were susceptible to killing by C alone (Table 4). Bovine, equine, porcine, and human complements were bactericidal for SC33 and EC K99. Equine and human C killed SC9; bovine, porcine, mouse, and guinea pig complements had little effect. Most of the complements, with the exception that guinea pig C killed SC5, were ineffective toward SC2, SC5, SC1Ø. Again, mouse C had no killing effect on the bacteria.

The results presented in Table 5 indicated that killing by C alone approximated killing by Ab-C, whether strains were pretreated with Tris-EDTA or with saline. Every C, with the exception of mouse C, was effective for a minimum of one bacterial strain. The results indicated that C from these species was bactericidal in the presence or absence of antibody. All strains were resistant to killing by mouse complement.

No single complement was bactericidal for every strain. Equine C was the most effective C, followed by bovine, porcine, and human complements. Mouse and guinea pig complements were relatively

Table 3 Susceptibility of *S. cholerae-suis* and *E. coli* strains pretreated with saline to killing by porcine Ab and C

	Source of Complement					
	Bovine	Equine	Porcine	Human	Mouse	Guinea pig
S. cholerae-suis						
33	>85[a]	>85	>85	>85	10-85	10-85
9	>85	>85	>85	>85	<10	<10
2	<10	10-85	<10	<10	<10	<10
5	10-85	10-85	<10	<10	<10	<10
10	10-85	10-85	<10	<10	<10	<10
38	10-85	10-85	10-85	>85	<10	<10
E. coli						
K88	10-85	>85	<10	<10	10-85	<10
K99	>85	>85	>85	>85	<10	>85

[a]Percent bacteria killed by Ab-C.

Table 4 Susceptibility of *S. cholerae-suis* and *E. coli* strains pretreated with saline to complement alone

	Source of Complement					
	Bovine	Equine	Porcine	Human	Mouse	Guinea pig
S. cholerae-suis						
33	>85[a]	>85	>85	>85	10-85	10-85
9	<10	>85	<10	>85	<10	10-85
2	<10	10-85	<10	<10	<10	<10
5	10-85	10-85	<10	<10	<10	>85
10	10-85	10-85	<10	<10	<10	<10
38	10-85	10-85	10-85	>85	<10	<10
E. coli						
K88	10-85	10-85	10-85	<10	10-85	<10
K99	>85	>85	>85	>85	<10	<10

[a]Percent bacteria killed by complement.

Table 5 Summary of susceptibility of strains to species complements with and without Ab

Bacterial pretreatment	Source of Complement					
	Bovine	Equine	Porcine	Human	Mouse	Guinea pig
Tris-EDTA						
Ab-C	4/8[a]	5/8	3/8	3/8	Ø/8	1/8
C	4/8	4/8	2/8	4/8	Ø/8	3/8
Saline						
Ab-C	3/8	4/8	3/8	4/8	Ø/8	1/8
C	2/8	3/8	2/8	4/8	Ø/8	1/8

[a]Number of strains killed >85% / total number of strains.

ineffective; the strains remained resistant or partially susceptible (with the exception of SC5 to guinea pig C).

The effects of Tris-EDTA or saline pretreatment and the presence or absence of Ab were demonstrated in Table 6. A comparison was made between two variables (Ab-C vs C, or saline vs Tris-EDTA) when results were conflicting between the two variables, i.e., susceptible in the presence of one variable and resistant in the presence of the other variable. The partially susceptible category was not included in this comparison. The strains affected by the variables were presented in parentheses in Table 6. The comparison was made to determine if the differences in susceptibility were random, or were associated with certain strains, or were associated with the species of complement used. Strain SC9 was associated with 50% of the differences in susceptibility, and it was the source of differences associated with bovine, equine, and porcine complements. Strains SC5 and EC K99 differed in their susceptibility to guinea pig complement. Human, and mouse complements had no differences. Guinea pig C was the main source of overall variability; three strains changed susceptibility between variables when guinea pig complement was used.

Eight strains were pretreated with Tris-EDTA or with saline and tested for susceptibility to six species complements in the presence or absence of Ab (Table 7). The strains that were partially susceptible (10-85% killed) were not included. The results in Table 7 demonstrated that pretreatment with Tris-EDTA enhanced C killing such that C killing approximated killing by Ab-C. Fewer strains pretreated

Table 6 Summary of strains pretreated with Tris-EDTA or saline differing in susceptibility to Ab-C and C killing

	Source of Complement					
	Bovine	Equine	Porcine	Human	Mouse	Guinea pig
variable						
			Tris-EDTA pretreated strains			
AB-C vs C	Ø[a]	Ø	Ø	Ø	Ø	1(K99)[b]
			Saline pretreated strains			
Ab-C vs C	1(9)	Ø	1(9)	Ø	Ø	2(5,K99)
			Strains killed by Ab-C			
Tris-EDTA vs saline	Ø	Ø	1(9)	Ø	Ø	2(5,K99)
			Strains killed by C			
Tris-EDTA vs saline	1(9)	Ø	Ø	Ø	Ø	1(K99)
			Saline or Tris-EDTA pretreated strains			
Ab-C vs C	Ø	Ø	1(9)	Ø	Ø	2(5,9)
			Saline or Tris-EDTA pretreated strains			
C vs Ab-C	1(9)	Ø	Ø	Ø	Ø	Ø

[a] Number of strains.

[b] Identification of strains.

Percentage of susceptibility differences caused by strains

SC9	50%	total differences (involving bovine and porcine C)
SC5	21%	(involving guinea pig C only)
EC K99	29%	(involving guinea pig C only)

Table 7 The effect of pretreatment with Tris-EDTA on susceptibility of strains to Ab–C and to C

		Tris-EDTA pretreatment			Saline pretreatment		
		Ab–C			Ab–C		
		>85%[a]	<10%		>85%	<10%
C	>85%	14[b]	1	C	>85%	11	1
	<10%	0	6		<10%	3	18

[a]Percent bacteria killed.

[b]Number of strain combinations affected.

with saline were susceptible to Ab-C or to C, and the number of strains resistant to Ab-C or C was increased.

The effects of Tris-EDTA and saline pretreatment on sensitivity to Ab-C were presented in Tables 1, 3, and 7. The effect of Tris-EDTA on susceptible strains was minimal: many of the susceptible strains pretreated with Tris-EDTA were also killed by Ab-C when the strains were saline pretreated. But the effect of Tris-EDTA on resistant strains was more evident. Only six strains pretreated with Tris-EDTA remained resistant to Ab-C and to C. Eighteen strains after pretreatment with saline were resistant to Ab-C or to C.

The eight strains were grouped into three categories of sensitivity to killing by Ab-C or by C. Sensitivity to killing varied by pretreatment and by species of complement used, although the strains could be identified overall as susceptible, partially susceptible or resistant. The susceptible strains, SC9, SC33, and EC K99, were the major strains of interest.

Sensitivity to C alone was enhanced by Tris-EDTA for bovine, equine, and guinea pig complements. Killing by porcine, human, and mouse complements was not affected by pretreatment. The sensitivity of SC33 and EC K99 remained unchanged, regardless of pretreatment, but the sensitivity of SC9 pretreated with Tris-EDTA was increased. The effect of Tris-EDTA on resistant strains was even more dramatic. Eighteen strain combinations pretreated with saline were resistant to both Ab-C and C alone, however, only six strain combinations remained resistant after pretreatment with Tris-EDTA (Table 7). Pretreatment

with Tris-EDTA did not enhance susceptibility to Ab and mouse C or to mouse C alone.

Equine C was the most bactericidal complement. The bactericidal activities of the species complements in decreasing order were: equine; human; bovine; porcine; guinea pig; and mouse.

Sensitivity to equine C was affected by Ab and pretreatment with Tris-EDTA. No single strain was susceptible (>85% killed) to mouse complement, but mouse C was not totally devoid of bactericidal activity. Two strains, SC33 and EC K99, were partially susceptible (1Ø-85%). Strain EC K99, which was highly sensitive to C, was completely resistant to mouse complement. Mouse and guinea pig complements were obtained commercially. When these two complements were tested in a hemolytic system (Part 2), mouse C was nonhemolytic for sensitized sheep and rabbit erythrocytes, but guinea pig C was highly hemolytic for sensitized sheep erythrocytes. It is possible that the mouse complement used for the present study was devoid of bactericidal complement activity.

Heat-inactivated C was included as a C control. Only strain SC33 was killed by heat-inactivated complement (Table 8). When pretreated with Tris-EDTA, SC33 was highly susceptible to heat-inactivated bovine, equine, and porcine complements, and partially to heat-inactivated human and mouse complements. When SC33 was pretreated with saline, it was susceptible to heat-inactivated equine complement.

Table 8 Susceptibility of SC33 to C and heat-inactivated C

	Source of Complement					
Tris-EDTA[a]	Bovine	Equine	Porcine	Human	Mouse	Guinea pig
C	97[b]	98	96	97	68	76
*C[c]	85	94	91	28	58	78
Saline[a]						
C	96	99	85	92	49	64
*C	82	97	77	16	51	47

[a]Bacterial pretreatment.

[b]Percent bacteria killed.

[c]Heat-inactivated C (56 C, 30 minutes).

DISCUSSION

Lipopolysaccharide activates complement by both the classical and the alternate pathways. The traditional concept for the bactericidal activity of serum was that antibody bound to the bacterial cell wall and activated complement by the classical pathway. However, a number of investigators have observed that complement, without antibody, could be bactericidal against serum-sensitive gram-negative bacteria (Sterzl et al. 1964, Bjornson and Bjornson 1977, Loos et al. 1978, Schreiber et al. 1979). Betz and Isliker (1981) demonstrated that antibody-free and Clq-free sera were bactericidal for E. coli, and the addition of Cl greatly increased killing. Lipopolysaccharide present in the cell wall was responsible for the antibody-independent activation of the classical complement pathway. The classical pathway was activated by lipid A, and the polysaccharide portion activated the alternate pathway (Loos et al. 1974, Fine 1974, Bjornson and Bjornson 1977, Morrison and Kline 1977, Loos et al. 1978, Pangburn 1983).

Smooth strains of gram-negative bacteria are resistant to Ab-C because intact O side chains of LPS prevent access of Ab and/or C to the cell wall. Rough strains have oligosaccharide deletions and are serum-sensitive (Reynolds and Rowley 1969, Nikaido and Nakoe 1979, Westphal et al. 1981). Some of the LPS is removed when bacteria are exposed to Tris and EDTA, and many resistant strains become susceptible to antibody and C after removal of LPS (Reynolds and

Rowley 1969, Hedstrom et al. 1981, Wright and Levine 1981).

Griffith and Kramer (1982) tested seventy-two field isolates of Salmonella cholerae-suis var kunzendorf for sensitivity to porcine antibody and complement. Their results indicated that Tris-EDTA enhanced sensitivity to Ab-C. Some strains were consistently susceptible or resistant to Ab-C. Three susceptible strains (SC9, SC33, SC38) and three resistant strains (SC2, SC5, SC1Ø) of S. cholerae-suis and two strains (EC K88, EC K99) of E. coli were used to determine if C alone could be bactericidal, and if susceptibility to C varied with the species of C used. The results presented here indicated that C was bactericidal and that sensitivity to C approached that of Ab-C after bacteria were exposed to Tris-EDTA.

Pretreatment with Tris-EDTA did not greatly enhance killing by Ab-C of the eight strains tested. The results do not conflict with other studies because selection of the strains was not a random process. The S. cholerae-suis strains were selected for resistance or sensitivity to Ab-C. The two E. coli strains had not been previously tested; EC K99 was highly sensitive to killing regardless of pretreatment, and EC K88 was affected somewhat by Tris-EDTA.

In many studies, serum-resistant bacteria were exposed to Tris-EDTA to investigate the bactericidal effects of C and Ab-C. Reynolds and Rowley (1969) and Reynolds and Pruul (1971a,b) tested smooth strains of Salmonella typhimurium with Tris-EDTA and found that removal of LPS uncovered C receptors, which may have been located in lipid A. The removal of LPS allowed sensitization of resistant

bacteria. In the present study, susceptibility to C was enhanced by Tris-EDTA, but some strains pretreated with saline were also killed by complement. The number of strains resistant to Ab-C and to C dropped from eighteen after pretreatment with saline to six when pretreated with Tris-EDTA (Table 7). Schreiber et al. (1979) reported that the mixture of purified alternate pathway components was bactericidal for E. coli K12. The components, however, were not bacteriolytic; lysozyme was required for bacteriolysis.

Complements from six species were chosen to test the susceptibility of the eight selected bacterial strains. The species complements were compared with each other to determine the effectiveness of each C in the presence or absence of antibody against pretreated bacteria. Overall, equine C was the most bactericidal C, especially in the presence of antibody against bacteria pretreated with Tris-EDTA. Bactericidal complements in decreasing activity were equine, human, bovine, porcine, guinea pig, and mouse.

Equine C has not been as well-studied as some of the other complements. It is commonly used as the complement source for bovine conglutination tests, because it is not a hemolytic complement. Rice and coworkers did extensive work on testing hemolysis of sensitized sheep (Rice and Crowson 1950) and rabbit erythrocytes (Rice and Boulanger 1952) by different animal species complements. They reported that equine complement had very low titers of C2, C4, and C5. Barta and Barta (1978) also observed low or undetectable levels of C2, C4, and C5 in equine complement. In a different study by Barta and

Hubbert (1978), equine complement was bactericidal for E. coli. The presence of low levels of classical pathway components may have indicated that equine C killed bacteria through activation of the alternate pathway. It may also indicate that very low levels of early complement components, which are capable of initiating the conglutination reaction, are sufficient for initiation and activation of the classical pathway.

Bovine complement has also been reported to contain low levels of C2 and C4 (Rice and Crowson 1950, Sterzl et al. 1964, Barta and Barta 1978). Early work by Shrigley and Irwin (1937) demonstrated that bovine C was not hemolytic, however, it was very bactericidal for Brucella suis, either alone or in the presence of antisera. Bovine C was very effective for some of the Salmonella strains and for one strain of E. coli used in the present study. Sterzl et al. (1964) reported that calf serum was much more bactericidal for gram-negative bacteria than piglet serum. Low concentrations of piglet serum were effective for rough strains, but higher concentrations of sera were required to kill smooth strains. In the present study, bovine C was also found to be more bactericidal than porcine complement.

Human and guinea pig complements have been used extensively for hemolytic and bactericidal assays. Human C was a highly bactericidal complement. Only three strains, SC5, SC9, and EC K99, were susceptible to guinea pig complement. Shrigley and Irwin (1937) tested guinea pig C in tne presence and absence of antibody against

B. suis and found that it was not bactericidal, but it was fixed by the antigen-antibody complex.

None of the eight strains tested were susceptible to mouse complement. A few strains were partially susceptible. The presence of antibody and Tris-EDTA pretreatment was not sufficient to render the bacteria susceptible, which indicated a lack of bactericidal activity of mouse complement. Brown (1943) and Muschel and Muto (1956) used human and guinea pig sera deficient in specific complement components to determine if complement from normal mice was lacking an specific component(s). Their results indicated that C2 was no present. Muschel and Muto (1956) reported C2 and C3 to be absent. These deficiencies may account for the low bactericidal activity of mouse complement. Both guinea pig C and mouse C were obtained from commercial sources. These two complements may have lost activity during processing and shipping, which may have accounted for the low bactericidal activities.

The results from Table 6 indicated that differences in susceptibility occurred when two variables were compared. The differences were strain-related rather than random or species complement-related. A total of fourteen differences occurred. At the start of the present study, a larger number were expected because of enhancement of bactericidal C activity by Tris-EDTA pretreatment and antibody. Strain SC9, when pretreated with saline, was susceptible to bovine C in the presence of antibody, but it was resistant to bovine C in the absence of antibody (Tables 3 and 4). Strain SC9 was resistant

to Ab and porcine C when pretreated with Tris-EDTA and susceptible when pretreated with saline (Tables 1 and 3). The selective resistance of Tris-EDTA pretreated bacteria to Ab and porcine C was a characteristic of SC9. The irregular growth characteristics of SC9 may account for the variability of results associated with this strain. Eight of the fourteen susceptibility differences occurred with guinea pig complement, and involved three strains. The majority of these were observed with resistance of SC5 and EC K99 to Ab-C.

The conclusions drawn from Table 6 were that SC9 differed in susceptibility to bovine and porcine complements. Differences in susceptibility to guinea pig C were influenced by pretreatment with Tris-EDTA and the presence of antibody. Equine and human complements, which were highly bactericidal, and mouse C, which was nonbactericidal, had no susceptibility differences.

Heat-inactivated sera were only bactericidal for SC33. Each heat-inactivated serum killed SC33 almost as well as its respective complement, with the exception of human complement. These results indicated that SC33 was highly susceptible to other serum component(s). The ability of SC33 to activate and consume complement was tested in Part 2. The results indicated that SC33 activated and consumed complement to the same level as the other three strains. Joiner et al. (1982 a,b) studied bacterial resistance to complement killing. Their results indicated that smooth bacteria consumed C3 and the terminal complement components, but that the C5b-9 complex formed on the cell surface was released without causing membrane damage. It

may be possible that other serum components such as lysozyme or transferrin are capable of killing or inhibiting growth of SC33.

CONCLUSIONS

Complement alone killed some smooth strains of S. cholerae-suis var kunzendorf and E. coli to the same extent as antibody and complement.

Pretreatment with Tris-EDTA enhanced killing by complement, but it was only slightly effective for enhancing antibody-complement killing.

Equine complement was the most effective complement, followed by human, bovine, porcine, guinea pig and mouse complements.

Mouse complement was not bactericidal as demonstrated by the lack of strains susceptible to it, but it may have lost complement activity during lyophilization, processing and shipping.

Equine and human complements were consistently effective against susceptible strains.

Strain SC33 was susceptible to heat-inactivated serum and to complement from each species, with the exception of human complement. This effect may have been caused by other bactericidal or bacteriostatic factors present in serum, such as transferrin or lysozyme.

Differences in susceptibility and variations in test outcomes were traced to certain bacterial strains, and not to species complements or test methods.

PART 2 REDUCTION OF HEMOLYTIC COMPLEMENT LEVELS BY *SALMONELLA CHOLERAE-SUIS* AND *ESCHERICHIA COLI*

INTRODUCTION

The results of various studies have shown that some bacteria are killed by complement independently of antibody, by activation of either the classical or the alternate pathway (Part 1 - Introduction). Lipopolysaccharide, a cell wall carbohydrate of gram-negative bacteria, activates the bactericidal activity of complement. Lipid A activates the classical pathway, and the polysaccharide portion of LPS activates the alternate pathway (Loos et al. 1978, 1974, Morrison and Kline 1977). In Part 1 of the present study, the sensitivity of six strains of Salmonella cholerae-suis var kunzendorf and two strains of Escherichia coli were tested against complements obtained from six animal species. Equine C was the most bactericidal of the six complements, followed by human C, bovine C, porcine C, guinea pig C, and mouse C. Sensitivity to C was enhanced when bacteria were pretreated with Tris-EDTA to the extent that sensitivity to C was equal to sensitivity to antibody and complement.

Complement is often used with antibody in bactericidal and hemolytic studies. The study of complement is usually performed by using hemolytic assays. The standard hemolytic assay consists of sheep erythrocytes sensitized with rabbit antibody. A standard procedure commonly used or modified is the procedure described by Kabat and Mayer (Mayer 1971) for the complement fixation test. Guinea pig C is most often used in hemolytic assays and complement fixation tests. Sheep erythrocytes are sensitized with rabbit anti-sheep

antibody. Dilutions of pooled guinea pig C are added to the sensitized cells and incubated for one hour at 37 C. The degree of lysis is determined photometrically.

Many investigators have attempted to titrate complement from different animal species by using the standard sheep erythrocyte-rabbit antibody assay (Rice and Crowson 1950, Rice and Boulanger 1952, Grant 1976, Barta and Hubbert 1978). Equine, bovine, and sheep complements could not be titered with this assay and were considered to be nonhemolytic. Rice and Crowson (1950) performed an extensive study of complements from different animal species, using the standard system. In their study, components of species complements were interchanged, using the crude R reagents prepared from guinea pig complement. The R reagents were thought to contain three of the four known complement components, but they also contained additional components unknown at that time. Their results indicated that each of the three nonhemolytic complements had very low or undetectable levels of C2 and C4. In another study, Rice and Boulanger (1952) used rabbit erythrocytes sensitized with sheep anti-rabbit erythrocyte antibody and compared the two hemolytic systems using the same series of complements. Equine, bovine, and sheep complements were hemolytic for sensitized rabbit erythrocytes. Rice also indicated that these three complements had all four of the known C components.

Barta and Barta (1978) also tested species complements for hemolytic activity. They used purified components from human and guinea pig complements instead of R reagents, and used radial

immunodiffusion to detect components. Their results confirmed earlier reports (Rice and Crowson 1950, Rice and Boulanger 1952) that bovine and equine complements had low or undetectable levels of C2, C4, and in addition, C3. Barta and Barta (1978) and Leon and Norden (1959) suggested that the method of interchanging species C components to detect missing components was inappropriate because of incompatibility of some species complements.

Rice and Crowson (1950) suggested that every vertebrate C would be hemolytic if tested in the appropriate system. Guinea pig and porcine complements are very hemolytic against sensitized sheep erythrocytes (Oykean and Barta 1980, Barta et al. 1970, Rice and Boulanger 1952). Bovine C is often tested in conglutination assays using sensitized sheep erythrocytes, and it is most hemolytic for sensitized guinea pig erythrocytes (Lachmann 1962, Triglia and Linscott 1980, Linscott and Triglia 1980). Rice and Boulanger (1952) reported that equine C was nonhemolytic for sensitized sheep erythrocytes, but it was hemolytic for sensitized rabbit erythrocytes. Leon and Norden (1959) reported that the early components of equine C reacted with sensitized sheep cells which were lysed after the addition of guinea pig C chelated with EDTA. Mouse C is reported to be nonhemolytic for sheep erythrocytes. Early studies by Brown (1943) and Muschel and Muto (1956), using R reagents, indicated that mouse C was lacking C2. But studies by van Dijk et al. (1980) indicated that mouse C was hemolytic for sensitized rabbit erythrocytes.

The hemolytic assay of sensitized erythrocytes is used to study the classical pathway. Many assays have been developed to study the alternate pathway. Since the alternate pathway does not involve the early C components, methods have been devised to block or inhibit these components. A number of substances activate the alternate pathway. Pillemer et al. (1954) used zymosan, a polysaccharide component of yeast cell walls, to absorb properdin from serum in his studies. Bacterial cell walls also activated the alternate pathway. Antibody aggregates, guinea pig and rabbit erythrocytes (Platt-Mills and Ishizaka 1974, Martin et al. 1976, Gadd and Reid 1981), cobra venom factor, and heat-killed cultured human kidney cells (Baker and Osofsky 1980) also activate the alternate pathway.

Since the early C components C1, C4 and C2 are not required for the alternate pathway, sera that are naturally deficient in these components, such as C4-deficient guinea pig C and C2-deficient human C, or sera that have been processed to remove one of the components, are often used for C pathway studies. Absorption of serum with zymosan at 17 C to remove Factor B, and heating serum to 50 C for 30 minutes are methods of blocking the alternate pathway.

A simpler method of blocking the two C pathways is the selective use of chelators. Both the classical and the alternate pathways are dependent on divalent cations. The classical pathway requires Ca^{+2} for C1q to bind to its receptor and Mg^{+2} for binding C2. The alternate pathway requires only Mg^{+2} to stabilize the complex formed by alternate pathway factors. Ethylene diaminetetraacetic acid (EDTA)

chelates inorganic divalent cations, such as Ca^{+2} and Mg^{+2}, and blocks both pathways. Ethylene glycol tetraacetic acid (EGTA) chelates Ca^{+2}; EGTA also chelates Mg^{+2} but to a much lesser extent than EDTA. Supplementing EGTA with Mg^{+2} overcomes this effect. The EGTA supplemented with Mg^{+2} is used to distinguish the alternate pathway by chelating Ca^{+2} to block the classical pathway. The alternate pathway is not blocked because of the presence of Mg^{+2} (Fine 1977). The EGTA was used to study the alternate pathway of some complements from different species: human (Platt-Mills and Ishizaka 1974, Fine et al. 1972, Fine 1977), guinea pig (Fine 1974), bovine (Triglia and Linscott 1980, Linscott and Triglia 1980, 1981) and porcine (Traub and Kleber 1976).

Fine et al. (1972) described a method in which EDTA and EGTA were used to study C activation by different substances. The substances, sensitized sheep erythrocytes, zymosan, and E. coli, were incubated with C and C chelated with EDTA or EGTA for one hour. After incubation, the substances were removed, and the chelators saturated by the addition of Ca^{+2}. The complements were titered to determine if the complement levels were reduced by the substances, and if any of the substances activated the alternate pathway. Their results indicated that the alternate pathway of human C was activated by zymosan and by E. coli.

The results of earlier studies have indicated that the antibody-independent bactericidal activity of complement can occur by either pathway. The method described by Fine et al. (1972) was used in this

study to determine which pathway was activated by each bacterial strain, and if each species complement was activated by the same pathway. Fine's method was selected because earlier attempts to measure C activation by C3 conversion (Baker and Osofsky 1980) and by gel diffusion (Martin et al. 1976, Gerwurz et al. 1978) were unsuccessful. The four complements which were most bactericidal (Part 1), equine, human, bovine, and porcine, were selected. Four strains of bacteria were chosen as test reagents: S. cholerae-suis strains 9, 33, and 38, and E. coli K99. Bovine C was chelated with different concentrations of EDTA and EGTA to determine if lysis of sheep erythrocytes by bovine C was caused by the alternate pathway or by incomplete chelation of Ca^{+2}.

MATERIALS AND METHODS

Erythrocytes

Sheep and rabbit erythrocytes were collected into Alsever's solution and aged for a minimum of four days. The erythrocytes were washed three times in Veronal buffered saline (VBS) and standardized to 2.8% by adjusting the cell suspension with VBS at an absorbance of 540 nm. Fresh 2.8% erythrocyte suspensions were prepared prior to each assay.

Antibody

Rabbit anti-sheep erythrocyte antibody was obtained from Gibco Laboratories Co., St. Louis. Sheep anti-rabbit erythrocyte antibody was prepared by repeated injection i.v. of a 10% suspension of washed rabbit erythrocytes. Both antisera were heat-inactivated at 56 C for 30 minutes. The rabbit antibody was titrated with sheep erythrocytes and guinea pig complement. Sheep antibody was titrated with rabbit erythrocytes and bovine complement. Both titrations were performed by the method described by Gustafson and Pearson (1981). The optimum antibody dilution of each antibody was 1:1500. Sensitized erythrocytes for the hemolytic assay were prepared by incubating equal volumes of a 2.8% suspension of washed erythrocytes and a 1:1500 antibody dilution for 15 minutes at 37 C.

Diluent

Veronal buffered saline was prepared as 5X VBS stock solution by adding 83 grams NaCl, 10.19 grams Na-5,5-diethyl barbituate, and 34.58 ml 1 N HCl to 2 liters water. The stock solution was supplemented with 5 ml 1 M Mg^{+2} - 0.3 M Ca^{+2} and stored at 4 C. Fresh VBS was prepared by dilution in 0.1% gelatin water before each assay.

Color standards

Hemoglobin was prepared by lysing 1 ml of a 2.8% suspension of erythrocytes with 7 ml water, then adding 2 ml of 5X VBS stock solution. Erythrocytes (2.8% suspension) were diluted 1 ml into 9 ml VBS and mixed with hemoglobin in the manner presented below. The color standards were compared visually to determine the percent hemolysis after centrifugation. Fresh color standards were prepared prior to each assay.

Color standard preparation

% hemolysis	0	10	20	30	40	50	60	70	80	90	100
hemoglobin	0	.1	.2	.3	.4	.5	.6	.7	.8	.9	1
erythrocytes	1	.9	.8	.7	.6	.5	.4	.3	.2	.1	0

Chelators

Solutions of EDTA and EGTA-Mg were prepared as described by Fine et al. (1972) and stored as 100 mM stock solutions. The EGTA-Mg stock solution and saline were supplemented with 10 mM Mg^{+2}. The stock

solutions of the chelators and saline-Mg each were added (Ø.1 ml) to Ø.9 ml complement to yield C-saline-1 mM Mg, C-1Ø mM EDTA, and C-1Ø mM EGTA-1 mM Mg.

Test Reagents

Sensitized erythrocytes

Cell suspensions were adjusted to $2x1Ø^8$ cells/ml with VBS as determined by Coulter counter. Sensitized erythrocytes (EA) were prepared by mixing $2x1Ø^8$ washed erythrocytes with equal volumes of 1:15ØØ antibody, and incubating for 15 minutes at 37 C. The cells were then washed three times in saline to remove excess antibody and VBS. Ten milliliters of sensitized erythrocytes were packed by centrifugation, and the cells were resuspended in 1 ml C to yield $2x1Ø^9$ cells/ml C. The EA were prepared prior to each assay.

Zymosan

Zymosan (Z) was homogenized and washed three times in saline, and kept frozen in aliquots of 2 mg/ml saline. Prior to each assay, the zymosan was thawed, packed by centrifugation, and resuspended in 1 ml complement to yield 2 mg Z/ml C.

Bacteria

S. cholerae-suis var *kunzendorf* strains 9 (SC9), 33 (SC33), 38 (SC38), and *E. coli* strain K99 (EC K99) were streaked from frozen egg yolk onto tryptose soy agar. Smooth colonies were transferred to

tryptose soy broth, and incubated eighteen hours at 37 C. Viable bacteria were determined by counting bacterial colony-forming units (CFU) on Tergitol-7 with TTC agar plates: SC9 5 $x10^{11}$ bacteria/ml, SC33 4 $x10^{12}$ bacteria/ml, SC38 4 x 10^{12} bacteria/ml, and EC K99 1 x 10^{12} bacteria/ml. The bacteria were killed by boiling for 30 minutes, washed three times in saline, and frozen in 1 ml aliquots. One day prior to the assay, aliquots of each strain were thawed, washed in saline, and dried overnight. The cells were resuspended in 1 ml complement.

Incubation Procedure

Complement was thawed in an ice bath. Complement (0.9 ml) was incubated with the chelators (0.1 ml) and saline-Mg (0.1 ml) for 10 minutes at room temperature. The test reagents each were resuspended in 1 ml of complement as described under Test Reagents, and incubated for one hour at 37 C with shaking. The test reagents were removed by centrifugation from the complement, and 0.1 ml of 100 mM Ca^{+2} was added to each of the complements to saturate the EDTA or EGTA. Controls consisted of C, C-saline-Mg, C-EDTA, and C-EGTA-Mg without test reagents. Controls were incubated for one hour at 37 C with shaking. The controls were included to determine if complement was inactivated after incubation.

Complement Titration

After the addition of Ca^{+2}, the complements and controls were diluted in VBS and refrigerated for 20 minutes. During C refrigeration, sensitized erythrocytes for the hemolytic assay were prepared. Each C dilution, sensitized erythrocytes, and VBS were added to duplicate sets of four tubes in the following manner.

	Tubes			
	1	2	3	4
VBS ml	.6	.55	.5	.4
C dilution	.2	.25	.3	.4
sens. erythrocytes	.2	.2	.2	.2

The tubes were incubated for one hour in a 37 C water bath, then the cells were packed by centrifugation. The percent hemolysis in each tube was determined by comparison to hemoglobin color standards.

CH50 Determination

The C titration and CH50 determination were performed by the procedure previously described (Gustafson and Pearson 1981). The percent hemolysis was converted to y/100-y value (y=% hemolysis), which was plotted against C volume/tube on log paper. The C dilution that gave two points on either side of the midpoint of the log paper was used to determine CH50. A line was drawn from the midpoint of tubes 1 and 2 to the midpoint between tubes 3 and 4. The point at

which the line intersected with the 50% hemolysis value (y/100-y=1) determined the volume of that C dilution which would lyse 50% of the sensitized erthrocytes. The CH50 was determined by the formula

$$\frac{\text{C dilution}}{\text{vol. C}} = \text{CH50/ ml undiluted C}$$

Bovine, porcine, and human C results were expressed as percent in reductions of CH50 values as compared to control CH50 values. Bovine, porcine, human, and equine complements were tested three times. Bovine complement was incubated and titrated with rabbit erythrocytes and sheep antibody. Human and porcine complements were incubated and titrated with sheep erythrocytes and rabbit antibody. Mouse complement was assayed for hemolytic C activity to determine if C activity could be detected. Mouse complement was incubated and titrated with sheep and rabbit antibody and with rabbit erythrocytes and antibody.

Chelation of Bovine Complement with EDTA and EGTA

Aliquots of bovine C were chelated with 0.1 ml of 100, 200, and 400 mM EDTA and EGTA. The EGTA was supplemented with respective concentrations of 10, 20, and 40 mM Mg^{+2}. The chelated complements were incubated with rabbit EA. After removal of test reagents, 0.1 ml of 100mM, 200 mM, and 400 mM Ca^{+2} was added to the respective complements. The complements were titrated as previously described (Table 11). Bovine C was also chelated with 200, 400, and 600 mM EGTA

supplemented with 20 mM Mg^{+2} or with their respective higher Mg^{+2} concentrations to determine the effects of different EGTA and Mg^{+2} concentrations on bovine C (Table 12).

Conglutination Assay

Conglutinin

Bovine blood was obtained by venipuncture and allowed to clot at room temperature overnight. Nine ml of serum were chelated with 1 ml 0.6 M EGTA supplemented with 0.06 M Mg^{+2}, then incubated with 2 g zymosan/ml for one hour at 37 C. The zymosan was removed by centrifugation. The serum was dialyzed against VBS at 4 C for two days, then heat-inactivated at 56 C for 30 minutes, and was absorbed three times with washed sheep erythrocytes. The conglutinin had no hemolytic activity for rabbit erythrocytes. The optimal conglutinin dilution of 1:8 was selected by using a block conglutination test.

Conglutination assay

The determination of reduction of equine complement by the test reagents involved two steps. Aliquots of equine complement were used as C-saline-1 mM Mg^{+2} and C-10 mM EGTA-1 mM Mg^{+2}, and incubated with sheep erythrocytes and zymosan as previously described. After the addition of Ca^{+2}, equine C and C-EGTA levels were measured by a conglutination assay. Equine C and C-EGTA were diluted in two-fold

serial dilutions; Ø.25 ml of each C dilution was added to a 13 x 1ØØ tube. Sensitized sheep erythrocytes (Ø.25 ml) and VBS (Ø.25 ml) were added to each tube. The tubes were shaken vigorously. Conglutinin (Ø.25 ml of 1:8 dilution) was added to each tube to give a total volume of 1 ml/tube, and the tubes were again shaken. Sensitized erythrocytes were added to VBS, C and VBS, and conglutinin and VBS as nonconglutinating controls. The tubes were incubated 1 hour at 37 C, and refrigerated eighteen hours.

The conglutination endpoints were read visually. The erythrocytes were counted on a Coulter counter after the visual endpoints were determined. Percent conglutination was determined by

(1ØØ-count)1ØØ/nonconglutinating control count.

The percent reduction of equine C and C-EGTA was determined by comparing percent conglutination of reduced complement to controls not incubated with test reagents by the formula

1ØØ-% conglutination/control % conglutination.

RESULTS

Bovine C and C-EGTA-Mg CH5Ø units were both reduced to approximately equal levels by EA, Z (Table 9), and the bacterial strains (Table 1Ø). None of the test reagents affected C-EDTA. The test reagent EA activated the alternate pathway of C-EGTA (Table 9). Since C-EDTA was not reduced by EA or Z, there was no excess Ca^{+2} present in the C to account for the reduction of C-EGTA-Mg by EA.

The bacterial strains and Z consumed porcine C and C-EGTA-Mg and reduced the CH5Ø titer to approximately equal levels, with the exception of SC38, which was not as effective in reducing the CH5Ø level to the same extent as Z (Tables 9 and 1Ø). The C-EDTA was not reduced by any of the incubation substances. The test reagent EA reduced the CH5Ø level of C, but did not reduce C-EGTA-Mg.

Human complement CH5Ø units were reduced to equal levels by Z and the bacterial strains. The C-EDTA was not reduced by any of the test reagents. The C-EGTA was not reduced by EA. All four strains of bacteria activated the alternate pathway in each of the complements, equal or slightly below the level of reduction by Z. The test reagent EA activated the alternate pathway of bovine C, as demonstrated with C-EGTA-Mg, but had no effect with porcine or human C-EGTA. Human and porcine complements were tested with sheep erythrocytes; bovine complement was tested against rabbit erythrocytes. The results may indicate that rabbit erythrocytes activated the alternate pathway, and

Table 9 Reduction of C levels by EA and Zymosan

		Bovine	Porcine	Human
control titer		35 CH50	207	207
	C	78[a]	84	100
EA	C-EDTA	0	0	0
	C-EGTA	78	0	0
	C	63	64	62
Z	C-EDTA	0	0	0
	C-EGTA	69	70	65

[a]CH50 reduction.

Table 1Ø Reduction of C levels by bacteria

		Bovine	Porcine	Human
	C	57[a]	69	66
SC9	C-EDTA	Ø	Ø	Ø
	C-EGTA	54	66	66
	C	56	68	66
SC33	C-EDTA	Ø	Ø	Ø
	C-EGTA	57	69	61
	C	56	51	63
SC38	C-EDTA	Ø	Ø	Ø
	C-EGTA	5Ø	52	56
	C	57	67	64
ECK99	C-EDTA	Ø	Ø	Ø
	C-EGTA	59	65	56

[a]Percent CH5Ø reduction.

sheep erythrocytes activated the classical pathway. Porcine and human C-EGTA were not reduced by EA.

Aliquots of bovine C were chelated with increased concentrations of EDTA, EGTA and Mg^{+2} to assure complete chelation of Ca^{+2}. Complement chelated with any concentration of EDTA was not affected by EA. However, EA depleted C-EGTA-Mg at each concentration of EGTA-Mg (Table 11). When 600 mM EGTA and different concentrations of Mg were tested against EA, similar results were observed (Table 12). The discrepancy of results between Tables 9 and 11 were caused by different dilutions of bovine complement. A 1:2 dilution of bovine C (Table 9) was reduced 78% by EA. Later results of the effect of EA on bovine C-EGTA indicated that, when bovine C was diluted to 1:3 in all tests, no subsequent hemolytic C activity was detected (Tables 11 and 12).

Equine C and C-EGTA were reduced to approximately equal levels by sheep erythrocytes in the conglutination assay (Table 13). Equine C was not reduced when incubated with zymosan, but it was reduced, when chelated with EGTA, by zymosan. It may be possible that equine C was reduced but not to the same extent as C-EGTA, and the C reduction was not detected by the conglutinin assay. A great deal of variability was observed with this test. Although actual numbers differed from test to test, the percent reduction were consistent. The results indicated that equine C was activated by the alternate pathway by both sheep erythrocytes and zymosan. Similar results were observed with bovine complement.

Table 11 Reduction of Bovine C-EDTA and C-EGTA-MG by EA

	Chelator Concentrations		
	100 mM	200 mM	400 mM
EDTA	0[a]	0	0
EGTA	100	100	100

[a]Percent CH50 reduction.

Table 12 The Effect of EA on Bovine C-EGTA-Mg

EGTA-Mg Molarity		
.2 M EGTA-.Ø2 M Mg	.4 M EGTA-.Ø2 M Mg	.6 M EGTA-.Ø2 M Mg
1ØØ[a]	1ØØ	1ØØ

.4 M EGTA-.Ø4 M Mg	.6 M EGTA-.Ø6 M Mg
1ØØ	1ØØ

[a]Percent CH5Ø reduction.

Table 13 Reduction of Equine Complement Levels by Test Reagents

	Erythrocytes		Zymosan	
C dilution	C	C-EGTA	C	C-EGTA
1:32	64[a]	7Ø	Ø[b]	5Ø
1:64	5Ø	58	Ø	55
1:128	28	nd[c]	31	nd

[a]Percent reduction of conglutinating complement levels.

[b]No reduction at this dilution, erythrocytes were too conglutinated to count.

[c]Not done.

Incubation with EA is the standard procedure to activate the classical pathway of complement. Zymosan activates the alternate pathway. The reductions of CH5Ø units by bacteria were compared to reductions by EA and zymosan to determine which pathway the bacteria activated, if each strain activated the same pathway, and if the same pathway was activated by the complement of each species.

The alternate and classical pathways were blocked by chelation of Ca^{+2} and Mg^{+2} by EDTA. The C-EDTA was not reduced by any of the incubation substances. The classical pathway was blocked in complement chelated with EGTA, but the alternate pathway could be activated. The hemolytic C levels of C-EDTA and C-EGTA-Mg were compared to C-saline-Mg to determine which C pathway was activated in the unchelated complement.

There was some variation of reduction of C levels between the test reagents, but the comparison of C and C-EGTA-Mg levels after incubaton with each test reagent indicated that C and C-EGTA-Mg were reduced to equivalent levels by Z and the bacterial strains (Tables 9 and 1Ø). Unchelated C activated either the classical or the alternate pathway. Each bacterial strain reduced CH5Ø values to the same extent as Z, indicating that all four strains activated the alternate pathway. The bacterial strains also activated the alternate pathway in each species complement tested (Table 1Ø).

Undiluted commercial and fresh mouse complements were incubated with and without sensitized sheep and rabbit erythrocytes, and then titrated with both sensitized erythrocytes to determine if mouse

complement had hemolytic activity. Bactericidal activity of mouse C was not detected (Part 1). No lysis of erythrocytes was observed after incubation, and no hemolytic activity by commercial or fresh mouse complements was observed. Lysis of rabbit erythrocytes (van Djik et al. 1980) and sheep erythrocytes (Berden et al. 1978) by mouse C has been reported. The mouse C used in the present study, however, was nonhemolytic.

DISCUSSION

Fine et al. (1972) used EDTA and EGTA to study activation of human and guinea pig complements by the alternate pathway. The EDTA blocked both classical and alternate C pathways by chelating Ca^{+2} and Mg^{+2}, while EGTA blocked only the classical pathway by the chelation of Ca^{+2}. Studies with human (Fine et al. 1972, Fine 1977) and guinea pig complements (Fine 1974) indicated that E. coli activated the alternate pathway in normal complement and in complement chelated with EGTA.

Other investigators have indicated that not all gram-negative bacteria activate the alternate pathway (Fine 1974, Traub and Kleber 1976, Roberts and Philips 1981, 1983, Hoffman and Houle 1983). Some gram-positive bacteria, which lack LPS, also activate complement via the alternate pathway. Lipopolysaccharide activates complement by either the classical or the alternate pathway. In the present study, three strains of S. cholerae-suis var kunzendorf and one strain of E. coli were tested for their ability to reduce complement levels by either pathway. Three of the strains, SC9, SC33 and EC K99 were susceptible to complement killing. One strain, SC38, was partially susceptible to complement.

Activation of the alternate pathway was determined by two procedures. Zymosan is a known activator of the alternate pathway. Zymosan binds Factor B at 17 C and both Factor B and C3b at 37 C (Nicholson et al. 1974). The complements in the present study were

not treated to remove anti-Z antibody, indicating that anti-Z antibodies may have been present to activate the classical pathway. Blockage of the classical pathway by EGTA prevented classical pathway activation. A comparison of C and C-EGTA CH50 levels after Z incubation indicated that Z activated only the alternate pathway. The reduction of bovine, porcine and human C and C-EGTA by bacteria were comparatively equal; C-EGTA reduction was sometimes slightly greater with certain strains. The four bacterial strains reduced C and C-EGTA to the same extent as zymosan. The reduction of C levels by EA was greater than reduction by Z or by bacteria. The results indicated that the three S. cholerae-suis strains 9, 33, and 38 and E. coli K99 activated the alternate pathway in bovine, porcine, and human complements. The results concurred with reports of gram-negative bacteria activating the alternate pathway.

Human C was completely depleted by EA (Table 9). Human complement chelated with EDTA and EGTA was not affected by EA. These results agreed with results obtained previously (Fine et al. 1972). However, zymosan and the bacterial strains only reduced complement levels by 50-60%. Fine et al. (1972) reported that human C was completely depleted by the test reagents. The concentration of zymosan in the present study was the same concentration used by Fine et al. (1972), and the bacterial concentrations were 10^3 greater than concentrations used by Fine. Their results indicated that Z and E. coli also completely depleted human complement. The discrepancy in results between Fine and this study may be due to C dilution by the test

reagents. Fine's report did not specify the concentration of the test reagents and of the complements. In the present study, test reagents were packed by centrifugation, resuspended in complement and removed by centrifugation with very little dilution of complement, which enabled hemolysis to be detected.

Equine C was incubated with sensitized sheep and rabbit erythrocytes to determine which erythrocyte system would be optimal. The results from earlier studies (Rice and Crowson 1950, Rice and Boulanger 1952) indicated that equine C was nonhemolytic for sensitized sheep erythrocytes, but it was hemolytic for sensitized rabbit erythrocytes. Barta and Hubbert (1978) also reported that equine complement was hemolytic for rabbit erythrocytes. Leon and Norden (1959) reported that the early components of equine C reacted with sheep erythrocytes. Undiluted equine C was only slightly hemolytic (<50% hemolysis) for sensitized rabbit erythrocytes, and it was nonhemolytic for sheep erythrocytes. Another method was used in the present study to determine equine C reduction by test reagents. Equine C is routinely used as the source of complement in bovine conglutination assays. The conglutination test was modified to measure reduction of equine C levels by standardizing conglutinin and sensitized sheep erythrocytes. Activation of the alternate pathway of equine C by EA was demonstrated by the reduction of C-EGTA levels. Sensitized sheep erythrocytes, which activated the alternate pathway in bovine C, produced similar results with equine C.

Mouse C was nonbactericidal for each of the bacterial strains tested. Attempts were made to determine C activity in mouse C by hemolysis of sensitized erythrocytes. Commercial and fresh mouse C were incubated with sensitized sheep and rabbit erythrocytes. No hemolysis was observed with either complement or erythrocytes. Muschel and Muto (1956) and Brown (1943) also reported that mouse C was nonhemolytic and indicated that mouse C lacked C2. But van Dijk et al. (1980) reported that mouse C was hemolytic for sensitized rabbit erythrocytes by the classical pathway. They reported that mouse hemolytic C levels exceeded even guinea pig C levels. They also indicated that mouse C was hemolytic via the alternate pathway for higher concentrations of complement. Berden et al. (1978) reported hemolysis of sensitized sheep erythrocytes by mouse C using ^{51}Cr-labelled erythrocytes. Both of these procedures were more sensitive in detecting hemolysis than the procedure used in the present study. The greater test sensitivity may have accounted for the differences in results.

Incubation with sensitized erythrocytes has been the traditional method of studying the classical pathway. Some studies have indicated that unsensitized erythrocytes of some species can activate the alternate pathway of some species complements, and that antibody may have a role in activation of the alternate pathway. Platts-Mills and Ishizaka (1974) reported hemolysis of rabbit erythrocytes by human C via the alternate pathway. Human C was chelated with EGTA and incubated with washed rabbit erythrocytes. Hemolysis was determined

photometrically. Their results indicated that EGTA blocked C1 binding, but not C2 and C4. Their results also indicated that human C was hemolytic via the alternate pathway for rabbit erythrocytes, but not for sheep erythrocytes. The results of the present study and the study by Fine et al. (1972) have agreed with the results obtained by Platts-Mills and Ishizaka in indicating that human C was not hemolytic for sheep erythrocytes when chelated with EGTA.

Renshaw and Gilmore (1980) studied the alternate pathway in porcine complement using 0.10 M EDTA and EGTA, and unsensitized species erythrocytes. Their results indicated that guinea pig erythrocytes were optimal for porcine complement. Rabbit erythrocytes were lysed to some extent. Goat, sheep, cat, dog, and bovine erythrocytes were insensitive to lysis by porcine complement. Oykean and Barta (1980) tested several species of erythrocytes to determine the optimal conditions required for titering porcine complement. Their results indicated that sheep and bovine erythrocytes were optimal for porcine complement. Rice and Crowson (1950) also reported that porcine C to be highly hemolytic for sheep erythrocytes.

Pang and Aston (1977) reported that bovine serum was hemolytic for human erythrocytes. In their study, bovine sera, either chelated with EGTA or depleted of C1, were hemolytic for human erythrocytes. But sera, absorbed with Z at 17 C or heat-inactivated at 50 C to remove Factor B, were not hemolytic for human erythrocytes. In the present study, bovine C and C-EGTA were hemolytic for sensitized rabbit erythrocytes. Rice and Boulanger (1952) reported that bovine C was

hemolytic for sensitized rabbit erythrocytes, but it was not hemolytic for sheep erythrocytes.

Linscott and Triglia (1980) and Triglia and Linscott (1980) studied C components of adult and fetal bovine sera. They indicated that the concentrations of EDTA and EGTA commonly used (10mM/ml C) were not sufficient to block all C1 activation. Their results indicated that a minimum of 40 mM EDTA/ml C was required to completely block C1 from binding to the erythrocyte. In the present study, bovine C was chelated with EDTA to yield 10 Mm, 20 mM, and 40 mM of each chelator/ml C, and then incubated with sensitized rabbit erythrocytes. In addition, EGTA was also included as a separate test. Complete hemolysis occurred with each concentration of EGTA, and no hemolysis was observed with any concentration of EDTA. Brock et al. (1975) also reported that 10 mM EGTA/ml C was sufficient to block the classical pathway of bovine complement. If 10 mM EDTA/ml C had been insufficient to block C1, some lysis would have been observed. The method to detect and determine hemolysis used in the present study was not as sensitive as the method used by Linscott and Triglia (1980). However, their method involved guinea pig C components and isolated bovine C components. They reported that EAC1 was readily lysed by guinea pig C in 0.10 M EDTA. If EA had been lysed by bovine C in 0.10 M EDTA, hemolysis would have been detected by the method described in the present study.

It is possible that other factors may have contributed to activation of the alternate pathway by EA. Antibody aggregates are

known activators of the alternate pathway. Polhill et al. (1978) studied the effect of antibody on the alternate pathway. Their results indicated that fixation of C3 was slower by the alternate pathway than by the classical pathway, and that antibody enhanced fixation. Other studies have reported that the $F(ab')_2$ region of antibody, not rabbit eryhtrocytes, was responsible for hemolysis by activation of human C by the alternate pathway (Gadd and Reid 1981, Schenkin and Ruddy 1981). Excessive Mg^{+2} also has been demonstrated to activate C by the alternate pathway. A report by Fine (1977) indicated that EGTA-Mg was preferred over EGTA. The EGTA-Mg itself could activate the alternate pathway, but excess test reagent (2 mg zymosan) and prolonged incubation time (1 hour) would overcome this effect. In the present study, both these conditions were met. There is a possibility that the excessive Mg^{+2} levels used to study the effect of different concentrations of EGTA on bovine complement may have affected the results.

CONCLUSIONS

Residual nonchelated C and C-EGTA-Mg levels were comparatively equal after incubation with Z and each of four bacterial strains.

The alternate C pathway was activated in bovine, equine, porcine and human complements by SC9, SC33, SC38 and EC K99.

Bovine C and C-EGTA-Mg levels were reduced to approximately equal levels by EA, indicating that EA activated the alternate pathway of this complement. The concentration of Ø.ØlØ M EDTA/ml C was sufficient to block C1 from binding to the erythrocytes and initiating the classical pathway. The concentration of Ø.Øl M EGTA/ml C blocked the classical pathway, but not the alternate pathway of bovine complement.

Equine complement was nonhemolytic for sheep and rabbit erythrocytes. When equine complement was tested in a conglutination assay, C-EGTA-Mg levels were reduced by Z and EA. Reduction of equine C by Z was not observed. More study of equine complement is required.

Mouse complement was nonhemolytic for both sensitized sheep and rabbit erythrocytes.

SUMMARY

The bactericidal activity of complement was demonstrated when it was observed in the late nineteenth century that fresh serum was capable of killing bacteria. Since that time, many complement studies have involved its bactericidal activity. New surgical techniques and immunosuppressive drugs in both human and veterinary medicine have contributed to a rise in the frequency of gram-negative bacterial infections; infections that are costly in terms of time and money. Endotoxin, specifically LPS, is responsible for many of the symptoms associated with these infections. The interaction of LPS with complement has been well-studied *in vitro* to better understand *in vivo* processes. Guinea pig and human complements are most frequently used for *in vitro* studies. Complement, though, varies from species to species, each having different properties.

Three complements from domestic animals, bovine, porcine, and equine, were selected for the present study to determine susceptibility of bacterial strains to complement. In addition, three complements commonly used for *in vitro* studies, human, guinea pig, and mouse, were also included. The bacterial strains were selected from a group of *S. cholerae-suis* strains previously tested. Two *E. coli* strains were also included.

Part 1 of the present study examined the susceptibility of these strains to each complement with and without antibody. The results from these experiments indicated that equine, bovine, human, and

porcine complements, in the absence of antibody, were capable of killing strains susceptible to antibody and complement. Pretreatment of strains with Tris-EDTA enhanced the bactericidal effect of these complements. Few strains were susceptible to guinea pig complement; none were susceptible to mouse complement.

Lipopolysaccharide activates complement by either the classical or alternate pathway, in the presence or absence of antibody. Reduction of complement levels by selected strains of bacteria were compared in Part 2 to determine which pathway was activated. Four complements, bovine, porcine, human, and equine, were selected because of their bactericidal activity. The chelators EDTA and EGTA were used to selectively inhibit one or both pathways. The results indicated that each of the four bactericidal complements were activated by the alternate pathway. Strains that reduced complement levels were not always killed by that complement. Each of the four strains consumed and reduced complement to approximately the same level as zymosan. But strain susceptibility varied for each complement. The results indicated that complement activation and consumption did not necessarily lead to bacterial killing as suggested by Joiner et al. (1982a,b). Strain SC33 was susceptible to both complement and heat-inactivated complement (Part 1). Complement activation and reduction by this strain occurred to an extent similar to reduction by other strains. It is possible that other bacteriostatic or bactericidal factors present in serum may have affected SC33.

Bovine complement was reported to lyse rabbit erythrocytes in the presence of EGTA, indicating activation of the alternate pathway by the erythrocytes. Earlier reports of Linscott and Triglia (1980) and Triglia and Linscott (1980) indicated that 10 mM EDTA/ml C was insufficient to block C1 activation; thus, the classical pathway was responsible for hemolysis. The results of the present study have demonstrated that the classical pathway of bovine complement was blocked by all concentrations of EGTA tested, and that bovine complement lysed rabbit erythrocytes by the alternate pathway.

Complement chemistry and function has been studied for many years. Complement serves as an important defense against infection, either alone or in cooperation with other immune systems. The most vital function of complement is the ability to kill bacteria, and though much work has been done, there is still much more to discover. The present study was an extension of the work of Griffith and Kramer (1982), involving the susceptibility of smooth strains of S. cholerae-suis to porcine antibody and complement. The initial objectives were to determine if susceptibility varied with the species of complement, and if complement alone was bactericidal. The results have demonstrated much more. Susceptibility varied for each complement, and some species complements, in the absence of antibody, were very bactericidal. Equine complement, which was the least studied complement, was the most bactericidal. Other unexpected results were the susceptibility of SC33 to heat-inactivated complement and the activation of the alternate pathway by erythrocytes.

Early work in complement studies interchanged components from different species complements to determine hemolytic and bacteriolytc activity of complement. Later studies have indicated that components from some species complements are not interchangeable and that each complement has different properties. The variability demonstrated in this and other studies has indicated that there is justification for examining an important immunological phenomenon such as complement from different animal species.

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