Bactericidal and Bacteriolytic Activity of Serum Against Gram-Negative Bacteria

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INTRODUCTION

Exposure of many strains of gram-negative bacteria to suitable concentrations of human or animal serum results in loss of viability, and sometimes dissolution, of the bacterial cells. Since the recognition, towards the end of the last century, that the bactericidal and bacteriolytic properties of serum are destroyed by heating at 56°C, an extensive literature has accumulated indicating that the killing process is effected by deposition on or insertion into the bacterial envelope of the assembled terminal proteins of the complement cascade, the membrane attack complex (MAC). It is now clear that activation of complement by gram-negative bacteria can occur via the classical or the alternative pathway; the former usually requires for its activation recognition of bacterial surface antigens by certain antibody classes, whereas activation of the latter can be initiated and amplified, in the absence of antigen-antibody interactions, by poorly understood structural or conformational characteristics of the cell surface. Killing by serum is often, but not invariably, accompanied by bacteriolysis, an event dependent on adequate amounts of lysozyme (mucopeptide N-acetylmuramoylhydrolase; EC 3.2.1.17); this basic enzyme degrades peptidoglycan to form monomers or multiples of the disaccharide-tetrapeptide unit (265).

The majority of investigators of bactericidal and bacteriolytic phenomena have utilized serum from humans (26, 222, 328) or from domestic (194, 296) and laboratory animals (7, 47, 144) as sources of complement; the capacity to kill gram-negative bacteria is associated, however, with serum from a wide variety of warm-blooded and cold-blooded animals, including fish (218, 322), and probably reflects the distribution of immunological responsiveness and the complement system in the animal kingdom. Susceptibility to the serum bactericidal system is a widespread characteristic of gram-negative bacteria; in addition to the many well-documented instances of enterobacterial susceptibility to complement, serum is known to possess bactericidal and bacteriolytic activity against susceptible representatives of practically every gram-negative genus so far examined. In fact, any procaryote that presents a lipid bilayer membrane to the external environment would appear to be potentially susceptible to complement killing. Although outside the scope of this review, comple-
ment in conjunction with specific antibody is also capable of lysing lipid-enveloped viruses (67).

In addition to susceptible strains, there are gram-negative bacteria that appear refractory to the serum bactericidal and bacteriolytic systems; these resistant strains are frequently isolated as causative agents of infections involving tissue damage. It has therefore been suggested that serum resistance is an important determinant of virulence in at least some infections due to gram-negative bacteria; although much of the literature does tend to show a relationship between insensitivity to serum and the ability to cause infection, the precise role and relative importance of serum resistance at any stage of the infection process is at present not clear.

It has often been noted that smooth strains of gram-negative bacteria, synthesizing a lipopolysaccharide with a high degree of substitution of core units by O-specific side chain moieties, are more resistant to serum than rough isolates or mutants that have lost the ability to either synthesize or attach the O antigen component of lipopolysaccharide (214, 222, 260, 300, 329). Similarly, resistant strains can be sensitized to serum by agents known to disrupt the integrity of the outer membrane (246). Serum-resistant Escherichia coli strains are less likely to be agglutinated by anti-O antigen antibodies than susceptible strains (201); O inagglutinability is often correlated with the presence on the bacterial surface of acidic polysaccharide K antigens (227). These and many other observations make it likely that resistance to serum is determined by the presence of structures at or near the bacterial surface capable of interfering with the formation, attachment, or subsequent activity of the MAC. The recent realization that some antibiotic resistance plasmids carry a determinant which reduces the serum susceptibility of E. coli strains (244) has led to a substantial increase in interest in the mechanism of serum resistance, partly due to the fact that plasmid-determined serum resistance is an easily quantifiable parameter that lends itself to investigation by recombinant DNA techniques (165) and also because it is now important to determine whether or not administration of antibiotics may select bacteria with undesirable invasive properties (33).

The large number of studies devoted to serum bactericidal and bacteriolytic reactions have not been the subject of a full literature survey since the excellent review by Inoue (127), which appeared in 1972. Since then, a number of important advances in our understanding of the activation of complement pathways by viable gram-negative bacteria and of the nature of the bacterial surface in relation to host-parasite interactions justify a reexamination of these reactions. Bactericidal and bacteriolytic systems will also be discussed in relation to the defense of the host against infection.

MEASUREMENT OF BACTERICIDAL AND BACTERIOLYTIC ACTIVITY

Detection of Serum Bactericidal Activity

In its most simple form, determination of serum bactericidal activity involves exposure of a suspension of viable organisms to a suitable concentration of antibody and complement, incubation at the optimum temperature for complement activity, and determination, after suitable periods of time, of the absolute concentration of surviving organisms by some form of viable counting. Unfortunately, no single technique has emerged as a readily acceptable and widely applicable standard assay for estimation of serum activity, and the reader is confronted by a bewildering array of assays that frequently make meaningful comparisons between studies difficult. As a number of parameters have been defined that significantly influence the outcome of the serum bactericidal reaction, it is somewhat surprising that no definitive study of optimum conditions for bactericidal activity has so far been undertaken. It is, for example, well established that the serum susceptibility of a gram-negative strain is influenced by the growth conditions employed for preparation of the bacterial inoculum; this may be partly due to environmental influence on the biosynthesis of cell surface structures that affect the degree of serum susceptibility (302) and partly to its effect on the metabolic state of the target cell (106). In batch culture, both the nature of the medium and the phase of growth during which the cells are harvested may influence the response to serum. It has been repeatedly observed that E. coli and other gram-negative bacteria are more readily killed by serum when in the early logarithmic phase than in either the log or stationary phases (54, 55, 263). In fact, susceptible strains may appear to be completely refractory to the serum bactericidal system if the bacteria are harvested before the onset of rapid cell division (54).

Melching and Vas (180) demonstrated that logarithmic-phase E. coli O111:B4 cells were more susceptible when grown in a simple salts medium than in a nutritionally complex medium. Furthermore, cells offered glycerol or acetate as a carbon and energy source were less susceptible than glucose- or succinate-grown cells; the authors postulated that these differences might be related to the ability of the cell to repair complement-mediated membrane damage. Similar observations were made by Maaløe (163, 164), who noted that Salmonella typhimurium...
grown in diluted broth was more susceptible to human serum than when grown in undiluted broth; the experimental conditions were arranged in such a way that the growth rates in these media were identical. Addition of various monosaccharides and organic acids to the system led to a phenotypic increase in serum resistance, whereas addition of ammonium salts had the opposite effect. The serum susceptibility of chemostat-grown *E. coli* urinary isolates was markedly dependent on the nature of the limiting nutrient and, to a lesser extent, on the dilution rate (302); in addition, the reproducibility of serum bactericidal kinetics was higher with chemostat cultures than when batch-cultured cells were used. These observations make it essential that particular attention be given to the procedure for inoculum preparation for serum bactericidal assays; when batch culture is utilized, bacteria should be harvested during the early logarithmic phase of growth from, if possible, a medium that can be formulated with a minimal amount of batch-to-batch variation. Bacteria should be grown at a temperature at or near the optimum, as cultivation at suboptimal or elevated temperatures frequently results in alteration of serum killing rates (144, 229). In a comparison of three established serum bactericidal assays, DeMatteo and colleagues (55) found that the system demonstrating the highest degree of killing activity was the only one using cells in early logarithmic-phase culture. It is therefore unfortunate that many procedures use bacteria in an undefined and variable metabolic state, including those washed from overnight agar slope cultures and suspended in diluent (138, 183, 296).

The procedure adopted for harvesting and washing of cells before exposure to serum may influence the outcome of the bactericidal reaction; the frequently adopted procedure of centrifugation at 0 to 4°C is likely to temperature shock the cells and should be avoided (342). Cells that have been extensively washed with buffer may show increased susceptibility to serum, and a more accurate estimation of true serum susceptibility may be obtained by resuspension in buffer without washing (78).

Complement activation in the bactericidal reaction may depend on an initial antigen-antibody interaction at or near the surface of the bacterial cell. Classical pathway function normally requires the formation of a complex involving immunoglobulin M (IgM) or IgG molecules (28, 253, 261), and amplification of the alternative pathway may also require the participation of an immune reaction (81, 151). The presence in the reaction mixture of antibody molecules that are able to participate in complement activation is necessary, therefore, to properly assess the outcome of a bactericidal reaction, particularly as both pathways may function simultaneously to kill susceptible bacteria (242, 282, 306). Early colonization of the intestinal tract by commensal bacteria ensures that small quantities of antibodies directed against the surface antigens of many types of gram-negative bacteria are present in the blood and tissue fluids of humans (150, 168) and a variety of animals (150, 289). Thus, IgM and IgG antibodies directed against surface antigens of *E. coli* (45) and other enterobacteria (187, 189) as well as against *Neisseria* species (45, 100) and *Haemophilus influenzae* (219) can be detected in the serum of adults in the absence of any history of immunization or specific infection. In those cases in which the presence of antibodies capable of participating in complement activation can be demonstrated, the serum can be utilized in the bactericidal assay as a source of both antibody and complement. Antibodies against some overtly pathogenic gram-negative bacteria, such as *Vibrio cholerae* and *Salmonella typhi*, are generally absent from non-immune sera, and it has been common practice to supplement the bactericidal system with an antibody source in the form of heated (56°C) immune serum when investigating such organisms (261). This procedure is, however, complicated by the fact that natural and immune antibodies differ in physicochemical properties that affect their behavior in bactericidal tests (189). Also, immune sera are rich in IgG, which functions poorly in complement activation in comparison to IgM; at high concentrations, IgG molecules may inhibit the complement-mediated killing mechanism (213, 220). These factors must be taken into account when considering the design of serum bactericidal assays.

Since the realization (254) that a very high proportion of cases of neonatal meningitis are caused by *E. coli* strains carrying the K1 polysaccharide antigen, much effort has been spent on examining the possibility of a correlation between K1 carriage and serum susceptibility of clinical isolates; in these cases, it would seem particularly relevant to assess the antibody content of sera, as the K1 antigen is the major surface polymer in these strains (255), is a poor immunogen (255), and may prevent effective activation of the alternative pathway (290).

Possible sources of error may materialize when bactericidal systems containing heterogeneous sources of antibody and complement are used. Interaction between serum proteins of one animal species and antibodies of others occurs and may lead to fixation and deviation of complement from the bactericidal system (261). Large differences have also been reported when sera from different animal species have been compared. For example, Ogata and Levine (221)
compared the ability of rabbit, human, and guinea pig sera to kill *E. coli* K-12 strains harboring plasmid R100; they found that human serum killed *E. coli* K-12 J6-2 more efficiently than either rabbit or guinea pig serum and that the nature of the serum also affected the ability of the plasmid to modify the serum response. Schwab and Reeves (271) compared the bactericidal activity of sera from eight species of vertebrates and found considerable variation in the efficiency with which the sera could kill a number of enterobacterial strains, although it was not possible to put the various sera in an unambiguous order with respect to this property. Within a given animal species, however, there appears to be little variation in the bactericidal activity of serum from different healthy individuals, reflecting the ubiquity of the complement system and a shared spectrum of antibody specificities. This has been particularly well established for the human subject (222, 242, 328), and Olling and associates (223) have demonstrated that the reactivities of sera from sailors based in various European, Asian, and South American countries are identical.

The bactericidal action of serum is demonstrable over a wide range of serum concentrations, although the rate of killing is known to generally increase with increasing serum concentration (203, 259). For the study of serum bactericidal activity in relation to infection, the majority of investigators have employed systems containing relatively high concentrations of serum to ensure that killing is not limited by availability of essential components of either the classical or alternative complement pathway; thus, concentrations in the 20 to 100% range have been successfully utilized in a number of studies (26, 105, 162, 222, 236, 310). However, assembly of the alternative pathway from the 11 pathway proteins purified from human serum has revealed that its bactericidal and bacteriolytic activity is insignificant at protein concentrations equivalent to a 1:16 dilution of serum (268). Clas and Loos (43) also detected almost no alternative pathway activity when guinea pig serum was used at dilutions of greater than 1:10. Recently, *E. coli* K-12 strains have been used by a number of investigators to elucidate the nature of plasmid-determined serum resistance, and of necessity, very low concentrations, often in the region of 1 to 4%, have been utilized to achieve a demonstrable difference between isogenic pairs (25, 79, 196). It is likely, therefore, that only classical pathway activity is being observed in these studies.

Antibody is needed in such small amounts for complement activation that it is rarely a limiting factor in bactericidal systems (95). Attention has been drawn to the possibility that agglutination of bacteria during the bactericidal reaction may lead to an underestimation of survival rates, particularly when immune sera are used as a component of the test system (179). Such an effect is detectable by using a control containing serum in which complement activity has been abrogated by heating at 56°C. The use of low numbers of bacterial cells will favor the occurrence of gross antibody excess and may lead to inhibition of serum bactericidal action (339). Essentially identical rates of killing of susceptible bacteria are seen over a wide range of inoculum sizes, and inocula up to 10⁷ cells per ml may be used in bactericidal assays (310). Olling (222) found no variation in the serum response of 10 *E. coli* strains over a concentration range of 10⁴ to 10¹⁰ cells per ml.

A wide range of buffers and other diluents have been incorporated into serum bactericidal systems; that these solutions can have significant and unpredictable effects on the efficiency of complement killing was recently demonstrated by Clas and Loos (43). They found that the killing of *Salmonella minnesota* by guinea pig serum, which proceeded efficiently in the presence of 0.02 M Tris, pH 7.4, or 0.004 M thiomolybdate buffer, pH 7.1, was totally abolished in 0.1 M phosphate-buffered saline, pH 7.6, an effect which might be due to a nonspecific inhibition of complement action by high cation concentration (328; P. W. Taylor, unpublished data) or to a specific inhibition by phosphate ions (7, 172). Wardlaw (328) found that bacteriolysis by human serum of *E. coli* Lilly was markedly dependent on the pH and ionic strength of the system. Somewhat surprisingly, the pH optimum for bacteriolysis was found to be 8.4, in comparison to an optimum for hemolysis of 7.15 to 7.35 (170). Similarly, an optimum of 0.06 for ionic strength was found, with a sharp decrease in activity either side of this peak; this contrasts to the erythrocyte system, in which near-maximal rates of lysis occur at an ionic strength of 0.147 and significant hemolysis occurs at μ = 0.177. This suggests that the requirements for lysis of *E. coli* Lilly may be rather unusual, as the ionic strength of human serum is about 0.183 (95). The Tris buffer system at pH 8.4 used by Wardlaw has been widely adopted for studies of serum bactericidal activity (95, 241, 310), often without due consideration that optimum conditions required for bacteriolysis and bactericidal action may be different. A high pH is, in fact, known to promote the action of lysozyme on gram-negative bacteria (216, 217). If relatively high concentrations of serum are used, the strong buffering capacity of serum may limit the pH range of the serum-buffer mixtures and keep any effect due to pH at a minimum (310). Although the use of Tris buffer has been
criticized on the basis that divalent cations necessary for complement activity may interact disadvantageously with this buffer (244), it appears to be comparable to many buffers used in bactericidal systems (43, 310). In the absence of definitive data on optimum conditions for bactericidal activity that apply to the majority of strains of bacteria commonly investigated, it would seem advisable to use one of the buffer systems that have been developed for studies of immune hemolysis (170); gelatin–veronal-buffered saline plus Mg2+ and Ca2+, pH 7.35 (89), provides the essential divalent cations necessary for complement activity at optimum concentrations, does not affect deleteriously the viability of gram-negative bacteria, and provides an environment in which very high rates of serum killing of susceptible bacteria can be achieved (268, 306, 342).

Addition of complex nutrient solutions, such as Hanks balanced salts solution (222, 344) or nutrient medium (54, 194), to serum bactericidal systems may have adverse effects on the ability of the system to kill normally susceptible strains (55); nutrient broth is known, for example, to be anticomplementary at high concentrations (208). The modification of bactericidal assays by potentially metabolizable substrates has been studied in detail by Michael and Braun (184). They found that high concentrations of various complex nutrient media protected Shigella dysenteriae from the bactericidal action of human serum, whereas concentrations below 0.1% enhanced its susceptibility. Glucose significantly enhanced bacterial susceptibility, whereas other carbohydrates not metabolized by S. dysenteriae had no significant effect; the authors suggested a relationship between bacterial metabolism and susceptibility to serum which has subsequently been studied in detail (106, 306). A complex situation was apparent when individual amino acids were added to the system. Those amino acids known to stimulate metabolic activity also enhanced serum killing, others had a protective effect, and some were without effect on the rate of killing. Two analogs, 5-methyltryptophan and fluorophenylalanine, interfered with bacterial killing in the presence of usually bactericidal concentrations of serum. Nutrient factors necessary for high killing rates are present in adequate concentrations in the serum used for the assay (306). Therefore, addition to the system of complex nutrient solutions serves no purpose and modifies, in an incompletely understood way, the serum killing kinetics.

The majority of studies of serum bactericidal activity have used any of a number of viable counting procedures to determine bacterial survival rates after exposure to serum-buffer mixtures; variations inherent in viable counting are well known (240) and will not be reiterated here. However, it has been common to select only one point in time, usually in the 1- to 2-h range, at which to evaluate survival; the validity of this practice must be questioned, as it has been frequently observed that killing of some strains does not proceed at a constant rate. For example, many smooth, serum-susceptible strains show a characteristic response (Fig. 1) in which there is little change in the viable count during the first hour of exposure, but the number of survivors after 3 h is generally less than 1% (299). The rate of killing after alternate pathway activation is lower than that associated with the classical pathway (256), and strains of Serratia marcescens also show a delayed serum-susceptible response as a result of selective activation of the alternative pathway (320). The true nature of the interaction between bacterial cells and activated complement components can only be evaluated, therefore, when the number of bacterial survivors is estimated at frequent time intervals during the reaction.

Although measurement of the serum response in the presence of a large excess of the serum components essential for complement killing would appear to be the most direct way of determining the degree of serum killing, some workers have preferred to estimate the survival of bacteria over a range of serum dilutions, the extent of bactericidal action being expressed as the reciprocal of the highest serum dilution showing killing activity (194, 263, 271, 296, 318). It is possible that differences in bactericidal titer merely reflect different quantitative require-
ments for some complement components at high serum dilutions, and differences established under conditions of limiting complement concentrations may not be apparent when bactericidal kinetics are determined in the presence of excess amounts of all required serum factors. For example, the small increases in survival of _E. coli_ K-12 J6-2 resulting from acquisition of plasmid R100 are demonstrable only over a very limited range (0.5 to 3%) of human serum concentrations (25, 221); at these concentrations, key components of the classical pathway may be present in limiting amounts and alternative pathway components will have been diluted to such an extent as to be nonfunctional.

The inherent disadvantages of viable counting procedures for estimation of survival after serum treatment led Muschel and Treffers (208) to develop an alternative assay based on the ability of surviving bacteria to remultiply when removed from the action of antibody and complement. This widely used assay involves exposure of bacteria to suitable serum concentrations for a reaction period of 1 h followed by the addition of a standardized volume of growth medium, such as brain heart infusion broth; the complementary activity of the medium terminates the bactericidal action of the serum, and the density of the cultures after a second incubation period will be proportional to the number of organisms surviving the initial exposure to serum. The optical densities obtained are converted to percentages of the control values and plotted on a probit scale against the log of the serum volume. The resulting linear relationship enables the 50% lethal dose of serum to be determined. For obvious reasons, the assay is only valid when antibody concentration is varied against a background of constant complement concentration, or vice versa. It also depends on the assumption that surviving bacteria that have been exposed to potentially damaging amounts of complement will multiply at an identical rate to bacteria that have not been so exposed after transfer to nutrient media. However, one could envisage that the cellular activity of serum-exposed bacteria might be directed towards repair of complement-damaged sites, resulting in diminution or temporary suspension of cell division.

Michael and Braun (185) compared the photometric assay with a conventional direct plating method and consistently found a greater number of survivors with the latter procedure, suggesting a continuation of complement-mediated bactericidal or bacteriostatic activity after addition of broth.

The necessity of screening large numbers of bacterial cultures or clones has led to the development of a number of rapid assays for serum bactericidal activity. Plaque assays (77, 212, 277), involving spotting serum dilutions onto seeded agar plates, are difficult to control, and some batches of agar possess high anticomplementary activity. However, a rapid method developed by Provonchee and Zinner (241) and using the Steers-Foltz replicator (288) has been successfully used for the screening of phenotypic modifications to serum susceptibility induced by growth in sublethal antibiotic concentrations (303). A rapid colorimetric assay (195) has made possible the genetic analysis of plasmid-borne serum resistance genes by gene cloning techniques (196).

Techniques that do not rely on determination of cell viability have also been used to quantify serum bactericidal activity. Kato and Bito (144) took irreversible loss of protein (β-galactosidase) synthesis as an index of bacterial killing, because serum-treated cells may be active in both respiration and macromolecular synthesis but inactive in cell division. Fierer and colleagues (78) measured the release of $^{51}$Cr-labeled lipopolysaccharide from serum-susceptible and serum-resistant bacteria and found a correlation between serum susceptibility and release of label. However, even heat-inactivated serum released approximately 20% of $^{51}$Cr from serum-susceptible _E. coli_ cells.

**Detection of Serum Bacteriolytic Activity**

Relatively few bacterial strains are lysed by serum, even in the presence of adequate amounts of lysozyme; this contrasts with the many strains that are killed without obvious lysis (328). Lysis can be followed by measuring the optical density of serum-exposed bacteria at regular intervals (95, 328). Alternatively, the release of radiolabeled intracellular markers (29, 286) or cytoplasmically located enzymes (130) can be monitored. Amano and co-workers (7), in their extensive studies of immune bacteriolysis, developed a technique in which sodium deoxycholate is added to suspensions of serum-exposed bacteria. Only spheroplasts formed by immune bacteriolysis are solubilized by this procedure; after centrifugation and removal of protein by precipitation, released nucleic acids are measured spectrophotometrically.

**MECHANISM OF SERUM BACTERICIDAL AND BACTEROIYTIC ACTION**

**Requirements for Serum-Mediated Killing: Complement**

Evidence that serum-mediated killing of gram-negative bacteria via the classical pathway normally requires all nine complement components of that pathway was provided by Inoue et al. (134). They reacted _E. coli_ with guinea pig serum that had been depleted of the classical third
complement component, thus forming a complex of bacterial cells, antibody to an unidentified bacterial surface antigen, and the first three components of the classical pathway (BAC142 cells).

Functionally pure C3, C5, C6, C7, C8, and C9 preparations were then added in various combinations to the sensitized bacteria; only when all components were present in the reaction mixture could lysozyme-mediated bacteriolysis be demonstrated. Goldman and co-workers (98) studied the antibody and complement requirements for the killing of an E. coli K-12 strain. An antigen-antibody interaction involving IgM molecules was necessary for classical pathway-mediated killing of this rough strain. A requirement for C1, C4, C2, and C9 was directly demonstrated; addition of C1-deficient human serum to BAC1 cells, generated with functionally pure human C1, resulted in killing of the sensitized cells. BAC142 cells were killed after addition of a human C3 through C9 source, and BAC1 cells were only killed when a human C4 through C8 source and functionally pure human C9 were present; omitting C9 resulted in survival of all bacterial cells. These workers also found that treatment of antibody-sensitized bacteria with high concentrations of functionally pure guinea pig or human C1 rendered the organisms resistant to killing by whole sera; this effect was partially dependent on the amount of antibody used to sensitize bacteria. Such unpredictable effects may account for subsequent failures to generate bactericidal systems completely from functionally pure classical pathway components.

That gram-negative bacteria can also be killed by the alternative pathway of complement activation was suggested by the observation that C4-deficient guinea pig serum possessed bactericidal activity against E. coli B and C strains (256); C4 is essential for classical but not for alternative pathway function (Fig. 2). These strains were, however, killed significantly more slowly by C4-deficient than by normal guinea pig serum, and killing by C4-deficient serum was preceded by a lag phase, during which there was no change in the number of viable organisms in the incubation mixture. Using proteins purified to homogeneity (269) and at concentrations corresponding to those found in serum, Schreiber et al. (268) have shown that antibody-independent bactericidal activity can be generated from the 11 components (Fig. 2) of the alternative pathway. Comparable kinetics of activation by E. coli K-12 W1485 in the isolated component mixture and in C4-depleted serum were observed; lysis but not killing was dependent on the presence of lysozyme. Properdin (factor P) was found to be a nonessential component for bactericidal and bacteriolytic activity, but its presence in physiological amounts resulted in a threefold increase in activity. Killing was dependent on the presence of C9.

These studies strongly suggest that deposition of the assembled proteins of the MAC onto the surfaces of susceptible bacteria is responsible for serum-mediated killing and is a necessary prerequisite for lysozyme-mediated bacteriolysis. Depending on the bacterial strain selected for investigation, generation of the MAC may result from either classical or alternative pathway activation; indeed, both pathways may be activated simultaneously (242, 282, 306). Only the five terminal components, C5 through C9, are directly involved in the assembly of the macromolecular protein complex on the target membrane (152, 315) after proteolytic activation of C5 to C5b. Assembly of the MAC involves the transformation of hydrophilic molecules into a macromolecular amphiphilic complex capable of integration into lipid-containing bilayers (21, 23). The complex is a short, hollow cylinder 150 to 160 Å in length with an internal diameter of 100 Å and is rimmed by an annulus of an external diameter of 200 to 220 Å at one end (Fig. 3). The other terminus bears an apolar surface 40 Å in length that inserts into the hydrophobic membrane interior; after insertion, the MAC behaves as an integral membrane protein complex, as it resists elution from the membrane by ionic manipulations, binds nonionic detergents, and can be reincorporated into artificial lipid vesicles (21). The major part of the vertically oriented cylinder, including the annulus, is located exterior to the lipid bilayer, and it has been proposed that the MAC penetrates through the membrane, forming a channel (319) that destroys the integrity of the attached membrane; channel formation would seem likely, as the terminal complement components produce large increases in ion permeability across planar lipid bilayers (171). The MAC appears, therefore, to be identical to the classical complement-induced lesions found on sheep erythrocyte (126) and E. coli (95) membranes. Available evidence suggests that the MAC is a stable complex (molecular weight, 2,050,000) of the terminal complement proteins and has the formula (C5b, C6, C7, C8, 6C9)2 (C5b-9)2 (237). Very recently, however, Tschopp and Podack (238, 323) found that, under certain conditions, C9 undergoes spontaneous polymerization to form a dodecamer with a structure very similar or identical to the MAC. This raises the possibility that the transmembrane channel may be poly(C9) and that C5b-8 functions as a poly(C9) generator complex by reducing the activation energy of C9 polymerization. If this is the case, C5b-8 must remain attached to poly(C9) after channel formation, as

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the terminal (C5b-9) components are complexed to each other after their isolation from membranes by Triton X-100 solubilization (22).

Evidence that polymerized C5b-8 may also be capable of mediating bacterial killing has come from studies of C9-deficient serum. Although, in some cases, serum bactericidal activity may require the presence of physiological amounts of C9 (98, 268), this is clearly not always so. Serum from a patient with a selective, total deficiency of C9 possessed bactericidal activity against a clinical isolate of *E. coli*, although killing occurred at a rate of about 3% of that observed in normal serum (160). Normal serum depleted
immunochemically of C9 and serum from C9-deficient patients killed strains of Neisseria gonorrhoeae and Neisseria meningitidis but at a much slower rate than normal serum (115). Membranes of killed bacteria lacked the typical complement lesions, thus providing limited evidence in favor of poly(C9) channel formation.

A number of workers have presented data suggesting that the MAC may be formed as a result of activation of mechanisms distinct from either the classical or alternative pathways. Steel and associates (287) reported a bactericidal mechanism against V. cholerae that was activated by F(ab')2 fragments and was Ca2+ dependent and C4 independent; Ca2+ is required for the functional assembly of the C1q, C1r, and C1s subunits to give enzymatically active C1 esterase (Fig. 2). Rough E. coli K-12 derivative 200P was killed at very low efficiency by a mechanism analogous to reactive lysis of erythrocytes; exposure of unsensitized bacteria to the trimolecular complex C5b-7 in the presence of C8 and C9 produced a bactericidal effect that was enhanced by pretreatment of cells with EDTA and augmented by an unidentified component of the pseudoglobulin fraction of human serum (97). It is unlikely, however, that these pathways will be of relevance to natural mechanisms of serum killing.

**Role of Antibody**

The question as to the role of antibodies in the initiation of the serum bactericidal reaction has been the subject of long controversy (see, for example, the review by Inoue [127]) that has only recently been resolved owing to the realization that both complement pathways can cause deposition of MAC proteins onto the surface of gram-negative bacteria and that the requirements for activation of these pathways are different. For example, Wardlaw (328) was unable to show a requirement for antibody in the killing of a rough strain, E. coli Lilly, by human serum. Sterzl et al. (289) found that serum obtained from newborn piglets possessed bactericidal activity against rough, but not smooth, strains of Salmonella and E. coli; piglets do not receive gamma globulins from the mother during the

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**FIG. 3.** Side (s) and axial (a) projections of C5b-9 MACs incorporated into artificial lipid vesicles. Vesicles not carrying complexes (asterisks) do not contain stain (sodium silicotungstate). Scale bars represent 100 nm. Electron micrographs courtesy of S. Bhakdi, Giessen, Federal Republic of Germany, and J. Tranum-Jensen, Copenhagen, Denmark.
intrauterine period but only postnatally, via colostrum. These observations complemented earlier work by Turk (324), showing that precolostral calf serum possessed high bactericidal activity against rough enterobacteria, and he implicated the alternative pathway, then known as the properdin pathway, as the mediator of this activity. Other workers obtained apparently conflicting data. Goldman et al. (98) found an absolute requirement for IgM in the killing of E. coli 200P, and Rowley (260) observed that a series of rough mutants of S. minnesota required the presence of antibody directed against surface lipopolysaccharide determinants for killing to take place. Sheep serum could not kill E. coli Lilly after absorption with the homologous strain (194). It is now clear that these differences can be explained on the basis of differences in methodology, particularly those relating to serum concentration and to the efficiency of the bactericidal system employed, as rough strains are known to be susceptible to both antibody-dependent classical pathway activity and antibody-independent alternative pathway activity (26, 242, 306).

In the serum bactericidal reaction, initiation of the classical pathway generally results from formation of an antigen-antibody complex at or near sites on the bacterial surface that can interact with activated molecules of the complement cascade. Extremely small amounts of antibody are required for complement activation; for example, Michael et al. (188) estimated that 10 ng of antibody per ml can be demonstrated by a system possessing bactericidal activity via the classical pathway. Sterzl and co-workers (289) found the bactericidal reaction to be 2,500 to 10,000 times more sensitive as a means of antibody determination than the agglutination reaction. The bactericidal reaction has, therefore, been used as a procedure for the diagnosis of diseases such as typhoid fever (108) and syphilis (170).

There is wide variation in the efficiency with which different antibody classes can function in complement-mediated killing. Although natural antibodies in serum may belong to the IgM, IgA, or IgG classes (45), the predominant natural antibodies to representative enterobacterial species are contained in the IgM fraction of human plasma (187); the IgM fraction possessed 140-fold more activity against S. typhi and 560-fold more activity against E. coli than the IgG fraction on a weight basis (187). The bactericidal activity of cord serum from newborn infants was found to be low in comparison to the activity of the serum of the mothers (91); neonatal serum is markedly deficient in IgM, as only IgG molecules are transferred across the placental barrier. Similarly, Landy et al. (153) found that, after a single injection of Salmonella enteritidis polysaccharide, more than 99% of the complement-activating activity was associated with the IgM fraction of rabbit serum. Robbins et al. (253) purified to homogeneity IgM and IgG anti-S. typhimurium antibodies from the sera of immunized rabbits; they found IgM antibodies to be 18-fold more active in bactericidal assays than IgG antibodies. Very small amounts (6 to 7 ng) of purified IgM from rabbits immunized with Pseudomonas aeruginosa were found to be effective in bactericidal assays with the homologous strain, whereas little or no bactericidal activity was observed with as much as 40 μg of IgG (28). The molar ratio of the bactericidal activities of IgM to IgG was greater than 1:250,000. When compared on a molar basis, IgM purified from rabbits immunized with S. typhimurium O antigen was approximately 250 times as active as IgG in a complement-dependent bactericidal assay (270). Although these and other (116, 117) studies have emphasized the superiority of IgM antibodies over IgG as activators of the classical pathway in the bactericidal reaction, there are large differences in the relative activities of the two antibody classes. It is known, however, that during the development of the immune response there is a progressive increase in the association constant of the antigen-antibody bond (64, 112) and hence a concomitant increase in the biological activities of antibodies produced during this period; differences in avidity of antibodies in these studies are likely, therefore, to be a reflection of the adoption of differing immunization schedules and animal species for production of antibodies. Thus, Schulkind and co-workers (270) demonstrated a 5-fold increase in the specific bactericidal activity of IgM antibodies and a 12-fold increase for IgG antibodies during development of the immune response over a 70- to 80-day period. Antibodies of a single immunoglobulin class were further fractionated into populations of molecules with different activities in bactericidal assays, indicating that at any one time during the course of immunization the serum represents a pool of antibodies specific for a given antigen. Similar variations in affinities of antibodies during the immune response were found by Pike and Chandler (235). These authors determined the efficiency of IgM and IgG produced in rabbits inoculated with live V. cholerae at intervals over a period of almost 1 year. The activity of IgM in serum bactericidal assays remained relatively constant over this period; this contrasted with IgG activity, which increased markedly during the immunization period. These differences could be due to variations in the relative amounts of IgG subclasses in the IgG pools, as some subclasses, such as human IgG3 and IgG1, are known to activate
complement efficiently, whereas others are virtually inactive in this respect (151).

Early studies with purified specific excretory IgA suggested that this antibody could activate bacteriolytic mechanisms in the presence of complement and lysozyme (3). Subsequent investigations have failed to confirm this finding (63, 116, 117), and it has been suggested that the IgA fractions examined by Adinolfi et al. (3) may have contained small amounts of contaminating active immunoglobulins of other classes (63, 261). More recent reports have, however, provided evidence that IgA may activate the alternative pathway in bactericidal reactions against E. coli (281). There is clearly a need for clarification of the role of secretory antibodies in serum bactericidal reactions in the light of recent advances in the molecular biology of complement.

In classical pathway activation, IgM and certain IgG antibodies interact with the C1q component of the C1 complex; C1q is a 400,000-molecular-weight glycoprotein formed from 18 peptide chains. Electron microscopy studies (273, 295) have revealed that C1q has an unusual ultrastructure: six peripheral globular moieties are attached by fibril-like strands to a central core. C1q has six binding sites for the Fc portion of immunoglobulins, and these are situated on the six globular moieties (151). At least two of the six valencies must bind to immunoglobulins before the next stage in the complement sequence can take place. Studies of antibody-mediated erythrocyte lysis have indicated that one IgM molecule is sufficient to combine with at least two C1q binding sites, whereas the reaction with IgG requires that two IgG molecules be present in close proximity (30). As recently stressed by Atkinson and Frank (13), the attachment of one molecule of IgG to an antigenic surface does not influence attachment of other molecules, so a very large number of attached IgG molecules may be necessary for the required proximity to be achieved. It thus becomes easy to understand the relative efficiencies of IgM and IgG as mediators of bactericidal phenomena.

Antibodies mediating bactericidal activity combine with antigenic determinants on or very near the bacterial surface so that complement may interact with lipid membrane components. For this reason, antibodies directed against flagellar (H) determinants are ineffective (10). There are a large number of studies demonstrating directly and indirectly that both normal and immune sera contain complement-activating antibodies specific for antigenic determinants carried by lipopolysaccharide. Schwab and Reeves (271) examined sera from a number of species, including humans, pigs, kangaroos, lizards, owls, and toads, and found natural anti-bodies directed against heat-stable cell surface antigens of E. coli and a number of Salmonella sp. Absorption of human serum containing anti-S. typhi antibodies with a heat-killed suspension of S. typhi resulted in loss of bactericidal activity against S. typhi and other strains with cross-reacting O antigens, but not against salmonellae with unrelated O antigens (203), suggesting that antibodies were specific for the O side-chain moiety of lipopolysaccharide. Glynn and Ward (96) showed that normal and immune sera lose bactericidal activity against gonococci after absorption with erythrocytes coated with lipopolysaccharide, an observation confirmed by Traumont and co-workers, using hyperimmune rabbit antisera (318). Immunization with lipopolysaccharide gives rise to complement-activating antibody against homologous Salmonella sp. (50, 270, 333) and E. coli (282, 333). Lipopolysaccharide from a gram-negative strain inhibits the bactericidal activity of both normal and immune sera against the homologous strain (10, 50, 189, 282), but such experiments should be treated with caution, as lipopolysaccharides may activate complement pathways and deplete serum of complement in the absence of antibody (84, 88, 199). In addition, RNA, a frequent contaminant of lipopolysaccharide and endotoxin preparations (336), is able to act as a nonspecific inhibitor of serum bactericidal reactions (10). Antibody for classical pathway activation may be directed against antigenic determinants on the core moiety of lipopolysaccharide (39, 260) and against enterobacterial common antigen (59); antibody against outer membrane proteins of N. gonorrhoeae and N. meningitidis also functions in serum bactericidal systems (318). Similarly, antibodies raised against the E. coli receptor protein for phage λ participate in the killing of lamB+ E. coli K-12 populations (83).

Comparatively little work has been done on the role of anticapsular antibodies in the serum bactericidal reaction. Antibodies to the exopolysaccharide of mucoid strains of P. aeruginosa (28) and to the group-specific capsular polysaccharides of N. meningitidis (100) are known to activate complement in the bactericidal reaction.

There is, therefore, a large body of evidence indicating that, during the bactericidal reaction, the classical pathway is dependent on C1 activation by antigen-antibody complexes. The lipid A moiety of lipopolysaccharide is known to activate C1 in the absence of antibodies (199), but this region occupies a position deep in the outer membrane of gram-negative bacteria (338) and would not normally be exposed on the surface of cells; it is unlikely, therefore, to function in situ as a C1 activator. Loos and colleagues (161)
demonstrated that serum-susceptible Klebsiella pneumoniae and E. coli could bind the activated form of Cl in the absence of detectable IgM (<2 μg/ml) and IgG (<1 μg/ml). Binding of native Cl from absorbed normal human serum was also demonstrated, but the possibility exists that small amounts of IgM might remain after absorption. The question of whether or not antibody-independent Cl or C3 binding leads to demonstrable bactericidal activity has been recently investigated by Betz and Isleker (19, 20). They exposed E. coli J5, which is deficient in galactose-4-epimerase, to radiolabeled Cl; C1 was bound to cells in a dose-dependent manner and essentially all bound Cl was subsequently activated. The complex consumed purified C4 and C2 to an extent compatible with classical pathway activity. Addition of C3-9 resulted in a 90% decrease in bacterial viability, demonstrating that antibody-independent activation of the classical pathway may contribute to the killing of rough gram-negative bacteria. However, the smooth parent strain, E. coli 0111:B4, from which the mutant J5 was derived (177), was not killed by this mechanism, even though it is known to be susceptible to serum (206). This suggests that antibody-independent classical pathway activity may be more important in the killing of rough rather than smooth strains. Preincubation of cells with hyperimmune anti-E. coli J5 IgG or IgM resulted in increased Cl binding, although consumption of later components was comparable to that observed with antibody-independent activation. These studies demonstrate unequivocally that the classical pathway may be activated in the complete absence of antibody and that this system is relevant to the killing of rough gram-negative bacteria. Its relative importance in comparison with other more established mechanisms of activation awaits further investigation.

The alternative pathway may be activated by insoluble immune complexes (81, 281), but this mode of initiation is likely to be of only minor importance in serum bactericidal reactions (151). Early evidence for an antibody-independent bactericidal mechanism distinct from classical pathway activation was provided by Wardlaw and Pillemer (331, 332), whose discovery of a bactericidal system requiring properdin provided the impetus that led to the elucidation of the alternative pathway. It is now clear that subsequent demonstrations (289, 296, 324, 328) of bactericidal activity in the absence of antibody are likely to be manifestations of alternative pathway activity.

Role of Lysozyme

Although susceptible gram-negative bacteria are killed by antibody-complement systems, they lyse to a significant extent only in the presence of lysozyme (132). Lysozyme is present in blood and serum at a concentration in the range of 1.54 to 9.74 μg/ml (mean, 5.64 μg/ml; 80). Bacteriolytic activity is negligible when the enzyme is removed from serum by adsorption onto bentonite (132, 328, 342) or by neutralization with anti-human lysozyme antiserum (95, 167, 306). Bacteria treated with lysozyme-free serum retain their rod shape but are converted to spheroplasts after subsequent addition of lysozyme in physiological amounts; these spheroplasts will eventually lose their integrity and lyse unless protected from osmotic forces by hypertonic solutions (48, 70, 86, 342). The rate and extent of bacteriolysis is directly related to the lysozyme concentration (95, 132). Pretreatment of bacteria with lysozyme has no effect on the rate of lysis of serum-exposed cells (9), suggesting that lysozyme gains access to its peptidoglycan substrate only after formation of a functional C5b-9 complex has disrupted the integrity of the outer membrane (92, 134).

The role of lysozyme in the bactericidal reaction has, in contrast, been the subject of some controversy. Serum killing occurs in the complete absence of lysozyme (134, 268), and it has therefore been asserted that the enzyme has no effect on the rate of serum killing by complement (86, 167, 185). Other workers have, however, produced data that tend to contradict these findings. Inoue and co-workers (132) removed all detectable lysozyme from rabbit antiserum and guinea pig complement and found that a mixture of these reagents possessed much reduced bacteriolytic but still powerful bactericidal activity against E. coli B. The addition of 5 μg of egg white lysozyme per ml to this system both enhanced and accelerated the bactericidal effect. Glynn and Milne (95) found that neutralization of lysozyme activity with antilysozyme antiserum or removal by bentonite adsorption resulted in reduced rates of serum killing of both a rough and a smooth isolate of E. coli. The addition of egg white lysozyme restored killing of the rough strain by both sera and of the smooth strain by antiserum-treated serum. Lysozyme addition did not, however, completely restore the activity of bentonite-adsorbed serum to normal levels, and Glynn and Milne suggested that bentonite adsorption, in addition to removing lysozyme from serum, had also resulted in the loss of a factor, distinct from antibody and complement, that was necessary for maximal rates of killing of the smooth strain. Removal of lysozyme from human serum by bentonite adsorption resulted in reduced killing which could be completely restored by the addition of egg white lysozyme (70, 98). Taylor and Kroll (306) recently found that removal of all detectable lysozyme activity...
from human serum by bentonite adsorption or by neutralization with purified anti-human lysozyme IgG resulted in a relatively small but reproducible reduction in the rate of killing of a rough, encapsulated E. coli strain. Optimal activity could be restored by the addition of physiological amounts of egg white lysozyme, and the addition of larger quantities of the enzyme further increased the rate of killing. The addition of various quantities of lysozyme to unabsorbed serum also resulted in increased killing kinetics.

The reasons for these conflicting findings are unclear but may be related to methodological differences and differing requirements for the killing of various strains of gram-negative bacteria. If the differences in the rate of killing between normal and lysozyme-free sera are small, then great care is needed in constructing response curves. In many of the cases in which the influence of lysozyme on serum killing has been dismissed as negligible or nonexistent, insufficient sampling over the defined time period has been carried out. Also, it has been common practice to remove lysozyme activity by bentonite treatment of serum, but this procedure is known to remove gram-positive bactericidins (211) that may possess activity against gram-negative species (35, 61), as well as factors essential for alternative pathway activity (306). Addition of antilysozyme antibodies could, in theory, result in complement deviation and loss as a result of complement activation by antigen-antibody complexes. One possible explanation of the enhancing effect of lysozyme is that depolymerization of peptidoglycan removes a barrier to the rapid assembly of the C5b-9 complex at the inner membrane, as it is likely that the primary lesion responsible for the bactericidal effect occurs at this site.

Additional Factors

The alternative pathway has been entirely reconstructed from chemically pure components (268), and there is good evidence that classical pathway killing requires only C1 through C9 and some activating mechanism that usually involves antibodies of the IgM or IgG class (98, 134). There are a number of reports in the literature, however, suggesting that the presence of additional factors in serum is necessary for maximal rates of killing to occur. For example, Skarnes (282) examined the bactericidal activity of rabbit and guinea pig sera against a number of rough and smooth enterobacterial strains and found that complement killing of rough strains occurred in the absence of antibody and required an additional nonspecific component that was destroyed by freezer storage and by heating at 52°C. Smooth organisms were killed by complement in the presence of antibody, but the presence of the labile component was also required. Killing of rough strains presumably occurred after antibody-independent alternative pathway activation, whereas killing of smooth strains required classical pathway activation. The author speculated that the labile component might be an alternative pathway component or regulator, and this raises the interesting possibility that serum killing of some strains of gram-negative bacteria requires the participation of both pathways, or at least one pathway and a limited number of components of the other. That both pathways may be activated simultaneously is suggested by a number of studies (102, 242, 306), but the relevance of any interaction or synergy between the two in the serum bactericidal reaction is an area that has been almost completely ignored. Similar effects have been noted after zymosan adsorption of serum (306). Treatment of serum with this yeast cell wall preparation at 17°C is known to remove properdin (331) and also part of the lysozyme activity (210), but not to activate the complement cascade. Zymosan-treated serum was found to possess no bactericidal activity against a rough, encapsulated E. coli strain that was susceptible to both classical pathway- and alternative pathway-mediated killing, even though the serum retained a functional classical pathway as judged by its high lytic activity against sensitized erythrocytes. Addition of an antibody source to the system failed to restore bactericidal activity, and the authors (306) speculated that either properdin or another alternative pathway component is essential for classical pathway killing of the E. coli strain or that zymosan removed a previously unrecognized factor essential for classical pathway killing. As classical pathway-mediated killing has yet to be demonstrated by assembly of chemically pure classical components, the intriguing possibility remains that other components in addition to C1 through C9 and antibody are needed. Glynn and Milne (95) found that the reduction in the rate of serum killing resulting from neutralization of lysozyme by antilysozyme antisera could be completely restored by the addition of egg white lysozyme, whereas loss of full activity after bentonite adsorption could not. The bentonite-adsorbable factor essential for full serum bactericidal activity has, therefore, properties similar to those of other enhancers of serum killing.

Serum from a variety of animal species contains relatively low-molecular-weight cationic proteins that have been collectively termed β-lysins; the proteins possess lethal activity against gram-positive bacterial types and appear to be released from platelets during coagulation
of blood (121). Unless special care is taken, therefore, these substances are likely to be present in variable amounts in human and animal sera utilized for bactericidal tests. The primary site of action of β-lysin is considered to be the cytoplasmic membrane (60), and consequently, these proteins have no direct lethal action against gram-negative bacteria possessing an integral envelope structure. Removal of β-lysin from rabbit serum, however, was shown by Donaldson and co-workers (61) to result in decreased bactericidal activity against E. coli B cells. Highest rates of killing were obtained when β-lysin, lysozyme, and the antibody-complement system functioned in a cooperative fashion. It would appear, therefore, that disruption of outer membrane integrity by the MAC provides the means whereby β-lysin may reach their target sites at the cytoplasmic membrane.

Recently, Carroll and Martinez (35, 36) isolated and characterized the major heat-stable bactericide from rabbit serum. They demonstrated that the primary bactericidal activity of normal rabbit serum against Bacillus subtilis resides in a single, 1,800- to 2,000-molecular-weight cationic polypeptide which they designated PC-III (35, 36). They found that both E. coli ML35, a K-12 derivative, and an S. typhimurium strain were rapidly killed after exposure to 80 to 200 ng of PC-III per ml. Although gram-positive bacteria were significantly more susceptible, PC-III concentrations in normal rabbit serum were found to be about 1 μg/ml, and it is therefore likely that antibacterial activity against gram-negative strains would manifest itself in relatively undiluted serum. Any possible interrelationship between direct or indirect β-lysin PC-III activity and the antibody-complement system awaits further elucidation.

Sequence of Events After Exposure to Serum

Exposure of susceptible gram-negative cells to serum is generally followed by a period during which there is little apparent change in viability; the length of this period varies depending on the bacterial strain used, the test system, the serum concentration, and the metabolic state of the bacterial cells. For example, early-log-phase E. coli O111:B4 or O127:B8 cells grown and tested at 37°C in the system of Davis and Wedgwood (54) began to lose viability 6 to 12 min after exposure to human serum, whereas Pasteurella septica growing in undiluted horse serum survived in an identical fashion to control cells for 30 to 40 min after addition of specific antiserum (105). Rough enterobacteria, including E. coli Lilly, B, and K-12 strains, appear to be extremely susceptible to serum, and the lag phase may be so short with these organisms as to be virtually undetectable (146, 167). Consequently, the availability of an enormous amount of data relating to the genetics and metabolism of K-12 derivatives, as well as a wide range of well-characterized substrains and mutants, should be carefully weighed against the disadvantage that the extreme susceptibility of K-12 to serum provides only a limited opportunity to discern early events in the bactericidal reaction that must precede any reduction in viability.

Although direct evidence is lacking, it would seem reasonable to assume that during this latent period the components of the MAC are under assembly at the site of the primary lesion. This period may correspond to the first stage of complement attack on E. coli, as demonstrated by Michael and Braun (185), who found that cells exposed to slightly diluted serum and divalent cations for 7 min and then washed could be efficiently killed by highly diluted serum in the absence of cations. No killing could be detected during the initial period of exposure, and highly diluted serum had no bactericidal activity. Selective removal of the late-acting complement components from the second-stage treatment resulted in complete loss of activity. Therefore, once functional lesions have been assembled, loss of viability rapidly ensues, and killing proceeds in a logarithmic fashion as reflected in steep killing kinetics of susceptible organisms (54, 221, 306). Rapid killing of bacteria suggests that the bactericidal mechanism has a high degree of efficiency. It has been well established by the classic studies of Mayer (170) that lysis of sensitized erythrocytes by complement follows one-hit killing kinetics. The gram-negative cell obviously presents a more complex target for complement attack than the erythrocyte; bacteria have a more complex envelope structure, they can respond rapidly to changes in the environment, and they have a capacity for repair of damaged sites. Nevertheless, Inoue et al. (128) developed an analytical method for determination of the number of lesions necessary for killing of E. coli B that involved exposure of increasing concentrations of antibody-sensitized bacteria to a constant amount of complement; their data was comparable to theoretical curves based on one-hit killing kinetics. However, some theoretical aspects of these estimations have been criticized by Wright and Levine (343), who reexamined dose-response kinetics for killing of E. coli by using human serum lacking either C7 or C8 and then adding back, in analogy to the hemolysis experiments of Mayer, limiting amounts of the missing components. They found that killing of E. coli, as well as complement-mediated inner and outer membrane damage, required more than one hit and that the lethal
process closely approximated two-hit killing kinetics. The authors suggested that this reflected the dimeric nature of the MAC, but it does not preclude the possibility that damage to inner and outer membranes are independent events.

Serum treatment of E. coli cells results in rapid and often total release of enzymes, such as alkaline phosphatase and 5'-nucleotidase, that are associated with the periplasmic space (53, 71, 130, 306, 342); release is dependent on activation of the terminal complement attack sequence and formation of a functional MAC (130, 342). Integration of the MAC into phospholipid bilayer areas of the outer membrane therefore disrupts outer membrane integrity and removes the barrier to hydrolysis of peptidoglycan by lysozyme (92, 130, 167). It is unlikely, however, that damage confined to the outer membrane is responsible for the lethal activity of complement. For example, Feingold and co-workers (70) examined the effect of lysozyme-free human serum on E. coli K-12 cells in the presence of 0.6 M sucrose; cells plasmolyzed in this fashion were found to be refractory to the serum bactericidal system. Bacteria grown for extended periods of time in the presence of sucrose did not undergo plasmolysis when resuspended in hypertonic solutions and were susceptible to serum. The authors therefore suggested that cells were killed after complement damage to the cytoplasmic membrane and that plasmolysed cells escape this damage due to retraction of the cytoplasmic membrane. Furthermore, plasmolysed cells promptly released alkaline phosphatase (71), demonstrating the essentially nonlethal nature of outer membrane damage. These experiments have been criticized on the grounds that the high concentrations of sucrose employed would inhibit bactericidal action (127, 205); yet the data presented by Feingold et al. (70) demonstrate a high degree of bactericidal activity in the presence of sucrose.

These studies (70, 71) also provided evidence that complement damage to the cytoplasmic membrane results in irreversible loss of viability. The K-12 strain used, 200P, is inducible for the cytoplasmic enzyme β-galactosidase but lacks a transport system for β-galactosides. The loss of the permeability barrier to β-nitrophenyl-β-D-galactopyranoside paralleled the loss of viability of plasmolysed and nonplasmolysed cells after exposure to lysozyme-free serum, whereas serum-mediated periplasmic enzyme release occurred readily in the absence of lethal effects. Loss of β-galactoside crypticity after serum treatment has subsequently been observed by Martinez and Carroll (167) and by Wright and Levine (342) and found to be strongly correlated with cell death. Serum treatment also causes other perturbations that suggest damage to the cytoplasmic membrane. For example, active transport of sugars, amino acids, and 86Rb+ (a K+ analog) are inhibited by serum treatment (53). Exposure to lysozyme-free serum has also been reported to cause a rapid efflux of 86Rb+ from preloaded cells (167, 342), suggesting that serum may function by dissipating the electrical potential of the inner membrane. We have recently found, however, that significant amounts of 86Rb+ may be released by heat-inactivated serum and even by some buffers (306) and that serum-resistant strains of E. coli may release as much preloaded 86Rb+ as susceptible strains when exposed to activated complement components (P. W. Taylor and H. P. Kroll, unpublished data). It would, therefore, seem pertinent to treat data of serum-mediated 86Rb+ release with caution, particularly when inadequate information regarding nonspecific marker release is presented.

Complement-mediated damage to the inner membrane is likely to be limited and not involve gross disruption, as bacterial respiration remains relatively unaffected until late in the reaction sequence and may even be transiently stimulated (10, 272). However, it then becomes difficult to explain how complement can cause lethal damage to the cytoplasmic membrane after deposition onto and insertion into the outer membrane. Some of the possibilities have been discussed by Feingold et al. (71) and more recently by Wright and Levine (342). The limited size of the hydrophobic domain of the MAC (21) makes it unlikely that a transmembrane channel could be inserted across the double membrane system of gram-negative bacteria. One possibility is that the cytoplasmic and outer membranes are damaged by two separate complement-mediated reactions that occur during the course of bacterial killing. Insertion of the MAC into the outer membrane is known to cause release of phospholipid into the surrounding medium (129, 131, 133, 341), and extensive disassembly of the outer membrane would allow subsequent MAC insertion into the cytoplasmic membrane. Such a mechanism would, however, appear to be discounted by the observation of Wright and Levine (342); they exposed E. coli cells to C8-deficient serum, removed excess complement components by thorough washing, and then treated C5b-7 bacteria with purified C8 and C9. Cytoplasmic membrane damage occurred in the absence of any new C5b-7 deposition, thus demonstrating that damage to the cytoplasmic membrane is not a result of fresh complement activation on the cytoplasmic membrane after outer membrane disruption.

Another possibility, considered by Feingold et al. (71) and subsequently by others (167, 342), is that effective lesions occur only when terminal
complexes are deposited on the bacterial surface at the points of contact between the cytoplasmic and outer membranes described and characterized by Bayer (16). Simultaneous damage would then occur on both membranes. Evidence in favor of this mechanism has been obtained by Wright and Levine (342), who found that the kinetics of release of periplasmic enzymes and intracellular cations after exposure to lysozyme-free serum are virtually identical, indicating that cytoplasmic membrane damage and outer membrane damage are closely coupled events. They found, as have others (130, 306), that cytoplasmic marker release is selective; large intracellular molecules such as β-galactosidase (molecular weight, 540,000) and thiol-galactoside transacetylase (molecular weight, 63,500) are retained.

Insertion of the MAC at points of membrane adhesion would also account for cytoplasmic membrane damage of E. coli C5b-7 by purified C8 and C9 in the absence of any new C5b-7 deposition (342) and for the fact that stationary-phase cells, possessing few adhesion sites, are relatively less susceptible to serum (55, 342). As exponentially growing bacteria possess only 200 to 400 adhesion sites per cell (16), such a mechanism suggests that many lesions formed on the surface would not directly result in killing, making the serum bactericidal system a relatively inefficient phenomenon.

A resolution of the current controversy concerning the molecular nature of the MAC in favor of a poly(C9) cylindrical structure would present another possibility for closely linked cytoplasmic and outer membrane damage. There are indications that complement-mediated outer membrane damage differs from damage to erythrocyte membranes and to bacterial cytoplasmic membranes. MAC lesions on erythrocyte membranes restrict the free diffusion of small molecules such as sucrose and raffinose (157, 280), whereas damage to the outer membrane facilitates the rapid diffusion of macromolecules through the membrane. This latter observation indicates disruption of the outer membrane, as periplasmic enzymes are too large to diffuse through the transmembrane MAC channel. Extensive disruption is also suggested by the frequently observed release of outer membrane fragments, as lipopolysaccharide-protein-phospholipid complexes, into the surrounding environment after exposure to serum (53, 70, 340). Thus, a detergent-like action rather than ion-channel formation appears to be operative at the outer membrane. Erythrocytes (171) and E. coli protoplasts (5) undergo slow C5b-8-mediated lysis which parallels increases in ion permeability of C5b-8-treated planar lipid bilayers; this process follows single-hit kinetics, in contrast to membrane disintegration, which displays multihit characteristics (171). Poly(C9) might, therefore, have evolved to effect detergent-like disruption of the gram-negative outer membrane to allow C5b-8-mediated cytoplasmic membrane perturbation. Such a mechanism would not necessarily require fresh deposition of MAC proteins after outer membrane disruption, as C5b-8 could be translocated onto active sites at the cytoplasmic membrane subsequent to poly(C9) generation. Analysis of the distribution of the proteins of the membrane attack pathway between cytoplasmic and outer membranes of serum-treated bacteria would help resolve the various proposed mechanisms, and such experiments are presently being conducted.

Such a translocation step is suggested by the observation that, in contrast to erythrocyte lysis, the serum bactericidal reaction requires an input of bacterially generated energy. Griffiths (106) demonstrated that the lethal action of antibody and complement against P. septica could be prevented by addition of uncouplers or inhibitors of oxidative phosphorylation 5 min after initiation of the bactericidal reaction. Complete inhibition of serum killing by an inhibitor (cyanide) and an uncoupler (2,4-dinitrophenol) was also found with an E. coli strain (306). E. coli LP1092 cells were killed by human serum after a lag period of about 10 min; if KCN was added to the system 8 to 18 min after initiation of the bactericidal reaction, the degree of inhibition was much reduced. Exposure of LP1092 cells to serum was followed by a rapid and large increase in intracellular ATP levels; ATP synthesis did not occur when bacteria were exposed to dialyzed serum, which killed at a much reduced rate. Addition of glucose or serum ultrafiltrate to dialyzed serum restored optimal bactericidal activity, suggesting that optimal killing of gram-negative bacteria requires an input of bacterially generated ATP. It is not known for which early stage in the lethal process energy is required.

The precise nature of the lethal event after interaction of MAC proteins with fluid bacterial membranes (144) is unknown. A rapid efflux of intracellular cations (167, 342) without inhibition of respiration (10, 272) suggests that complement kills E. coli by dissipating the energized state of the cytoplasmic membrane and that MAC proteins form ion-permeable channels. They would then act in an analogous fashion to membrane-active colicins such as El, K, and Ia. These colicins have been found to inhibit respiration-linked active transport systems (74), to lower intracellular ATP levels (74), to cause a rapid efflux of intracellular $K^+$ (335), and to inhibit the energy-linked transhydrogenase reaction (264) as a consequence of a rapid and drastic reduction of the membrane potential (334). ATP levels fall after colicin treatment because the
bacteria attempt to maintain the concentration gradient of $K^+$ and possibly $Mg^{2+}$ through the expenditure of ATP. Serum-treated cells do not, however, lose ATP, even when metabolizable substrates have been removed from serum by dialysis (306). The effect of MAC formation on the electrochemical potential ($\Delta \psi$) across the cytoplasmic membrane has recently been evaluated by measuring the uptake of the lipophilic cation tetraphenylphosphonium and of proline (A. F. Esser, Fed. Proc. 39:1755, 1980). Both functions were inhibited by deposition of C5b-9, but formation of C5b-8 with C9-deficient serum had a similar effect. In the absence of C9 deposition, uptake and transport recovered but were irreversibly inhibited by addition of C9. These experiments illustrate that C5b-8 can transiently perturb the cytoplasmic membrane but that dissipation of $\Delta \psi$ alone is insufficient to account for the bactericidal action of serum.

Exposure to serum also results in an inhibition of macromolecular biosynthesis, but in all cases in which kinetics of precursor incorporation have been followed, reduction in cell viability was evident well before inhibition became apparent. It is likely, therefore, that inhibition of macromolecular biosynthesis occurs secondary to the lethal event and merely reflects a running down of cellular activity in cells that have already been rendered nonviable. For example, loss of viability of $E. coli$ O111:B4 cells began almost immediately in the bactericidal system of Melching and Vas (180), whereas RNA synthesis was affected after 7 min, changing from an increasing rate of synthesis to a constant rate of synthesis. In contrast, the rate of DNA synthesis was not affected until after 15 min. Total RNA accumulation began to decrease after 15 min, and after 30 min much of the intracellular RNA began to be lost. Inhibition of DNA accumulation was not apparent until after 25 min, and protein synthesis was affected even later in the reaction sequence. Inhibition of RNA synthesis followed by inhibition of protein and DNA synthesis was also noted for antibody-complement-exposed $P. septica$ (105) and for rough strains of $E. coli$ (167, 306). Martinez and Carroll (167), however, noted significant rates of DNA synthesis, even well after cell lysis had occurred; the significance of this extended lysis remains unclear. Until the point of inhibition, mRNAs and their translated products remain fully functional (167). Early suggestions (8) that inhibition of protein synthesis is responsible for loss of viability of serum-exposed bacteria appear, therefore, to be unfounded; indeed, a certain amount of protein synthesis appears to be essential for maximal rates of serum killing, as inhibitors of protein synthesis reduce the rate of viability loss (184, 306).

RESISTANCE TO SERUM BACTERICIDAL ACTIVITY

Definition of Serum Resistance

Under conditions approaching the optimum, many strains of gram-negative bacteria are rapidly killed by human and animal sera. Some strains appear to be completely refractory to the serum bactericidal system, and prolonged incubation in the presence of adequate amounts of sensitizing antibody and an excess of complement components may result in a considerable increase in viable cell numbers (125, 302). There are, however, many strains that fall between these two extremes with regard to serum susceptibility; significant reduction in viable count may be apparent only after lengthy periods of incubation (125, 299, 320). Nevertheless, these strains should be regarded as susceptible to serum, or at least significantly different from strains that are capable of replicating in serum, because decrease in viable cell numbers implies insertion of functional MACs into sites on the surface of the cell envelope from which they can effect killing, albeit slowly. It is therefore unfortunate that many studies have described as resistant strains which have undergone significant reduction in viability after incubation in serum (145, 221, 251, 279). In an extreme example (79), certain antibiotic resistance plasmids were described as conferring serum resistance on $E. coli$ K-12 strains; in fact, numbers of plasmid-carrying strains were reduced to 1% of the inoculum after 30 min of incubation in 3% human serum. Although these plasmids did reduce by a small amount the rate of serum killing, they clearly did not confer serum resistance. It is recommended that the term “serum resistance” be restricted to the description of strains that are totally refractory to serum when tested in early logarithmic phase in assays containing excess amounts of the components of the bactericidal system.

Location of Serum Resistance Determinants at the Outer Membrane

There is no evidence that resistant gram-negative bacteria circumvent the bactericidal action of serum by producing proteases or other extracellular products that neutralize or destroy the functional integrity of complement components in the fluid phase (139). As protoplasts derived from serum-resistant enterobacteria (248) and even from gram-positive bacteria (204) are rapidly lysed by complement, it appears that the outer membrane constitutes the main barrier to the serum killing of gram-negative bacteria. Serum-resistant cells may be sensitized to the bactericidal action of serum by treatment with Tris and EDTA (246, 248), a process known to lower the permeability barrier of the outer mem-
brane by effecting the release of approximately half of the complexed lipopolysaccharide (156). Similarly, serum-resistant enterobacteria become susceptible to complement after exposure to polymyxin B (75, 293); this antibiotic is known to adversely affect the integrity of the outer membrane (72, 313), perhaps as a result of its ability to complex with lipopolysaccharide (15, 198). Further, albeit indirect, evidence for cell envelope involvement in the determination of serum resistance is provided by the observation that penicillin- or cephalosporin-resistant mutants selected from serum-resistant, antibiotic-susceptible populations of gram-negative bacteria may be serum susceptible (183, 214, 252).

It appears that serum resistance does not result from a block in the activation of the complement cascade. Rather, MACs formed on the surfaces of serum-resistant strains are not effectively inserted into the bacterial membranes and are released without causing lethal damage (139, 140). Reynolds et al. (247) found that equivalent amounts of C3 were deposited on the surfaces of serum-resistant S. typhimurium cells and on the same bacteria that had been rendered susceptible to serum by treatment with Tris and EDTA. They did, however, fail to show deposition of C5 on untreated cells, an observation that contrasts with those of other workers (139, 140, 221). Ogata and Levine (221) observed comparable fixation of C4, C3, and C5 by E. coli J6-2 and by the same strain harboring plasmid R100 after treatment with low concentrations of either guinea pig or human serum. However, the degree of serum resistance conferred by this FII incompatibility group plasmid is marginal and only detectable in systems containing small amounts of serum and is, therefore, not comparable with the high levels of resistance attributable to many wild strains. C3 deposition on the surface of serum-resistant blood isolates of E. coli has been demonstrated by Fierer and Finley (75). Joiner and colleagues (139, 140) have recently studied the interaction of C3 and some of the terminal components of the complement cascade with the smooth S. minnesota strain S218 and a deep rough mutant (Re595) of S218 that synthesizes a lipopolysaccharide containing only lipid A and 3-deoxy-d-manno-octulosonic acid; S218 is completely resistant to serum, whereas Re595 is highly susceptible (139, 260). Although both strains consumed 75 to 80% of the C3 from human serum, twice as many molecules of radiolabeled C3 were bound per cell of S218 compared with Re595. C5, C7, and C9 depletion of human serum after incubation with Re595 cells was found to be relatively low; in contrast, depletion of C5 and C7 by S218 cells was 95% after 15 min of incubation, and complete inactivation of C9 occurred after 5 min. Comparatively few molecules of radiolabeled MAC components were rapidly and irreversibly bound to the surface of Re595 cells. Therefore, this serum-susceptible strain consumes relatively small amounts of C5, C7, and C9, and these components are efficiently and stably deposited on the bacterial surface. Much larger amounts of the terminal components were deposited onto S218 cells, but binding reached a peak after about 10 min, and a progressive loss of the bound components was observed with prolonged incubation. The serum resistance of S. minnesota S218 is not, therefore, attributable to a defect in MAC formation on the bacterial surface; the formed complex does not remain associated with the surface, suggesting that the complex fails to integrate into the outer membrane. Furthermore, MACs could be eluted from the surfaces of S218 cells, but not Re595 cells, after incubation in buffers of increasing ionic strength (140), indicating that MACs on the surface of the serum-resistant strain did not behave as integral membrane proteins. Significant loss of membrane phospholipids from the serum-resistant strain was not observed, demonstrating that loss of surface-bound MACs was not due to shedding of part of the bacterial surface. Other workers have also found that, in contrast to serum-susceptible organisms, phospholipid is not released from serum-resistant strains (17), emphasizing that one mechanism of serum resistance is due to a failure of amphiphilic MACs to integrate into hydrophobic domains on the bacterial envelope.

One parameter that appears to be intimately related to the ability of MACs to integrate into biological and artificial membranes is the degree of fluidity of these structures. Consequently, factors reducing the fluidity of either the outer or cytoplasmic membranes of gram-negative bacteria could be crucial in determining serum resistance. The degree of disruption of phospholipid-cholesterol liposomes varies inversely with the concentration of membrane cholesterol (275), and there is a correlation between susceptibility of sheep erythrocytes to complement attack and the ratio of membrane lecithin to sphingomyelin (171), suggesting that increased membrane fluidity enables a more efficient assembly and integration of MACs on phospholipid membranes. Incorporation of the fluidizing and fusogenic agent 2-(2-methoxy)-ethoxyethyl-8-(cis-2-n-oc-tylcyclopropyl)-octanolate into sheep erythrocyte membranes loosens phospholipid acyl-chain packing and thus increases membrane lipid disorder; cells treated in this way become extremely susceptible to complement lysis (274).

Below the phase transition temperature, membrane phospholipids are in a state of gel packing with their acyl chains in a restricted and ordered
state; above this temperature, they are in a liquid crystal state with the fatty acyl chains exhibiting a high degree of molecular motion. Kato and Bito (144) have examined the effect of the physical state of membrane phospholipids on the susceptibility of E. coli to immune lysis. They found that the phase transition temperature and the temperature at which cells became susceptible to complement were both subject to variation depending on the conditions of bacterial growth and were highly correlated. Thus, susceptibility to complement increased rapidly with increases in temperature, up to the point at which the transition to liquid crystal is considered finished (27°C for E. coli B cells grown to stationary phase in complex media at 42°C). The authors suggested that membrane fluidity is obligatory for the formation of functional complement lesions. Parenthetically, membranes become initially more rigid after insertion of the MAC (51, 90), probably as a result of reorientation of ordered bilayered lipid effected by strong binding of phospholipids to MAC proteins (68). Akiyama and Inoue (4) reported that complement-resistant variants of E. coli K-12 had a less-fluid membrane structure due to a shift of fatty acid composition, although the basis of these changes is unclear as resistance was lost during maintenance of the strains.

It therefore becomes attractive to suggest that those macromolecular components of the outer membrane that are thought to confer serum resistance do so by virtue of the fact that they reduce membrane fluidity and exclude integration of MACs. Unfortunately, relatively little information is currently available concerning the physical state of gram-negative membranes. Overath et al. (231) found that less than one-half of the outer membrane phospholipids takes part in the order-disorder transition, whereas the bulk of cytoplasmic membrane phospholipids become ordered, suggesting that many lipid molecules in the outer membrane cannot participate in the lipid-lipid interactions characteristic of the order-disorder transition owing to interaction with protein. These lipid-protein interactions would therefore have a fluidizing effect on the outer membrane. In support of this contention, Nikaido and co-workers (215) have found that isolated outer and cytoplasmic membranes from a S. typhimurium rough (Rc) mutant, incorporating spin-labeled fatty acid probes, produced remarkably similar electron spin resonance spectra, indicating minimal immobilization of probe by the Rc lipopolysaccharide, which lacks O side chains and sugar residues from the outer core (338). Other studies, however, suggest that complete, S-type lipopolysaccharide may increase the microviscosity of the outer membrane (40, 257). Outer membrane preparations containing lipopolysaccharide molecules with long O side chains were less fluid than preparations containing the same number of lipopolysaccharide molecules lacking O side chains and some core sugars (257). Furthermore, removal of about half of the S-type lipopolysaccharide from membrane preparations by EDTA treatment greatly increased the fluidity of the membranes. Although these findings are controversial (49), they would, if verified, suggest an attractive mechanism for the serum resistance of smooth gram-negative bacteria. Clearly, however, susceptibility to complement is dependent not only upon the properties of hydrophobic membrane domains. Modulation of hydrophilic structures also influences the ability of complement to interact with membranes. For example, removal by enzymatic hydrolysis of N-acetyl-neuraminic acid (NANA)-containing structures from the outer surface of the membrane increases erythrocyte susceptibility to complement lysis (154). Therefore, of the structures that have been implicated as mediators of serum resistance, lipopolysaccharides and outer membrane proteins may affect membrane viscosity, whereas some acidic exopolysaccharides (e.g., K antigens of E. coli) might function in an analogous fashion to the NANA-containing structures on the erythrocyte membrane.

Role of Lipopolysaccharides

The earliest attempt to systematically define cell envelope components involved in the determination of serum resistance can be attributed to Wardlaw (329, 330), who compared the composition of purified envelopes from a smooth, resistant E. coli strain with those from the rough, extremely susceptible strain Lilly. The author originally postulated (328) that resistant strains might contain relatively few phospholipid complement-binding sites on the envelope in comparison with susceptible strains. It was found, however, that both strains contained comparable amounts of envelope protein and lipid but that there was a ninefold difference in the amount of lipopolysaccharide that could be extracted from the envelopes by the phenol-water procedure (336). It was suggested that, in some way, an outer membrane rich in lipopolysaccharide could protect cells from complement attack. However, the phenol-water technique removes only a proportion, sometimes very small, of R lipopolysaccharide from rough strains but is considerably more efficient when applied to smooth forms (85). Efficiency of lipopolysaccharide extraction could, therefore, go some way towards explaining the disparity in yields of this macromolecule from the two strains. Both Rowley (259) and Michael and
Landy (186), using only smooth enterobacteria, showed that serum-susceptible organisms possessed less intrinsic endotoxic activity than resistant ones, a function that can be directly attributed to the lipid A moiety of lipopolysaccharide (193). However, no significant differences could be found in the amount of lipopolysaccharide extractable from 28 smooth, urinary E. coli strains of differing serum susceptibilities (301). Similarly, mutations to serum resistance occurred independently of quantitative changes in E. coli lipopolysaccharide content (300), and phenotypically induced transitions from serum resistance to susceptibility were not accompanied by differences in lipopolysaccharide yields (308). Therefore, although strains with a low lipopolysaccharide content may generally be susceptible to serum, it is unlikely that the absolute amount of polymer in the envelope is the major factor determining resistance.

Apparent differences in lipopolysaccharide yields obtained from smooth and rough strains may be due to the fact that R-type lipopolysaccharides have a significantly lower molecular weight than S-type molecules, because R lipopolysaccharides lack the side chain moiety and sometimes a portion of the core. Therefore, comparisons between smooth and rough strains with regard to serum reactivity may not be valid and in any case will provide no information concerning the nature of the serum susceptibility exhibited by many smooth enterobacteria. Lipopolysaccharides do, however, play a central role in determining the susceptibility of enterobacteria to serum. For example, mutations from the smooth to the rough colonial form, usually but not invariably associated with loss of the ability to synthesize lipopolysaccharide O side chains (137, 227, 338), are accompanied by drastic increases in serum susceptibility (127, 166, 214, 260, 300, 314). In a detailed study of this phenomenon, Dlabac (56) examined the susceptibility to piglet serum of a series of rough mutants derived from a smooth S. typhimurium strain; although the smooth strain was completely resistant, nearly all of the rough mutants were susceptible. There was a progressive increase in serum susceptibility corresponding to sequential loss of sugar residues from the lipopolysaccharide core. S. typhimurium mutants deficient in UDP-galactose-4-epimerase form incomplete lipopolysaccharides which lack both O side chains and that part of the core distal to the point of the biosynthetic lesion, unless supplied with exogenous galactose (230). Dlabac (57) found that, when grown in galactose-free medium, these mutants were susceptible to serum; when galactose was supplied to growing cells, they became increasing resistant with time. Similarly, the serum resistance of mutants deficient in epimerase increased in direct proportion to the amount of galactose added to the growth medium.

Further evidence of the influence of the O side chain moiety of lipopolysaccharide on serum susceptibility was obtained by Roantree and coworkers (214, 291), who derived serum-susceptible mutants from resistant, smooth Salmonella strains by selection for resistance to cephalosporin or penicillin. The majority of such mutants were rough or part rough as a result of partial or total loss of the O side chain. A small number, however, possessed lipopolysaccharide with an O side chain sugar-to-core sugar ratio identical to that found in the parent strains, indicating that other factors must be involved in the determination of resistance of the smooth parental strains. Feingold (69) found that serum-resistant E. coli and Pseudomonas strains became susceptible after growth in media supplemented with low concentrations of diphenylamine. Analysis of lipopolysaccharides extracted from diphenylamine-grown cells revealed a striking reduction in the ratio of O side chain sugars to core sugars when compared with cells grown in the normal way. Some serum-resistant smooth E. coli strains are converted to serum susceptibility after growth in the presence of the 6-β-amidinopenicillanic acid meccillinam (303); lipopolysaccharides from cells grown in this way contain reduced amounts of O side chain components (307).

In all of these studies, increased susceptibility to serum was accompanied by a reduction in O side chain sugars of lipopolysaccharide. However, these structural changes were relatively drastic inasmuch as serum-susceptible cells showed many of the characteristics of rough organisms. Many susceptible clinical isolates are completely smooth by cultural, morphological, and serological criteria and are indistinguishable in this respect from serum-resistant organisms. As the O side chain length and degree of substitution of core stubs by O side chains is known to be subject to phenotypic and perhaps genotypic variation (42, 46, 99, 312), the possibility arises that the degree of serum resistance expressed by smooth strains may simply be a reflection of these parameters.

In general, more O side chain material was associated with lipopolysaccharide from serum-resistant than from serum-susceptible urinary strains of E. coli, but differences did not reach levels of statistical significance (301). Similarly, no quantitative differences in lipopolysaccharide sugar composition could be found between serum-resistant mutants and their respective E. coli parent strains (300). It appears unlikely, therefore, that the serum resistance of gram-negative bacteria is determined solely by the
length and number of O-antigenic polysaccharide chains associated with lipopolysaccharide, although these studies do not discount the possibility that a subtle rearrangement of lipopolysaccharide components within the molecule might account for the observed differences in serum reactivity.

Many strains carrying a full complement of lipopolysaccharide O side chains may be susceptible to serum although, unlike rough strains, they are not usually promptly killed but exhibit a delayed sensitive response (299). That this delay is related to the presence of O side chains is indicated by observations made with colonially rough variants derived from a serum-resistant mutant (300, 305). One rough form had lost the ability to synthesize the lipopolysaccharide O side chain as a result of a mutation in the his-linked rbf gene locus that specifies sugar transferases participating in O repeat unit biosynthesis (227) and was rapidly killed by serum, whereas another colonially rough form retained serological O specificity, produced a full complement of lipopolysaccharide O side chains, and was killed in a delayed fashion. Inheritance by a promptly susceptible rough E. coli strain of the rfb locus from a serum-resistant, K-negative, smooth O8 Hfr donor resulted in smooth recombinants that displayed the delayed serum killing response (300, 311). E. coli urinary isolate LP729 (serotype O9) displays delayed serum killing kinetics (300, 302); when grown in the chemostat under conditions of carbon limitation and magnesium limitation, the degree of killing after 1 h of incubation in human serum was directly related to the ratio of the lipopolysaccharide O side chain sugar (mannose) to the core sugar L-glycero-D-mannoheptose (308). These observations make it likely that O side chains do not determine serum resistance per se but that a high degree of substitution of lipopolysaccharide core stubs by long O side chains is responsible for the delayed serum killing response characteristic of many smooth isolates. The mechanism by which O side chains may do this is unclear; the attractive possibility that low membrane fluidity, resulting from synthesis of a complete lipopolysaccharide, reduces the rate of insertion of functional MACs into the cell envelope has not so far been investigated. It has been frequently suggested that long O side chains cause the antibody-mediated activation of complement at some distance from the site of lesion formation, and intermediates therefore decay before they can be incorporated into functional MACs (204, 206, 261, 262). Such a concept could also be extended to include antibody-independent activation of the alternative pathway, as the polysaccharide moiety of lipopolysaccharide is known to activate complement (199).

Role of Acidic Polysaccharides

Muschel et al. (203) examined the acidic exopolysaccharide (Vi antigen) content of clinical isolates of S. typhi and suggested that serum-resistant S. typhi strains produced larger amounts of Vi antigen than susceptible strains. Later, a similar relationship was proposed for E. coli strains isolated from both intestinal and extraintestinal infections (201). Correlations were far from complete, however, and in each of these studies only eight strains were examined.

Other work also suggests a relationship between acid-exopolysaccharide production and serum resistance. Glynn and Howard (94) found that saline extracts of serum-resistant strains of E. coli were, in general, more able to nonspecifically inhibit the agglutination of sheep erythrocytes by antierythrocyte serum than extracts of serum-susceptible strains. The authors interpreted the inhibitory effects as being due to the presence of K antigens in the preparations; the contribution of other components in the extracts is unclear, although purified K antigen polymers from two strains were shown to possess agglutination-inhibiting activity. It was also shown that a purified hexuronic acid-containing K antigen from a serum-resistant strain had greater inhibitory activity than equal amounts of K1 antigen, a NANA homopolymer, from a susceptible strain. It has been subsequently established, however, that hexuronic acid-containing E. coli exopolysaccharides are more potent inhibitors of hemagglutination systems than NANA-containing polymers, regardless of the reactivity in serum of the strain from which the polymer was extracted (301). It has been suggested that acidic polysaccharides may exert an influence on the extent of bacterial killing by serum due to an ability to impede antibody binding and subsequent attachment of complement components to the bacterial surface (94). Current evidence would tend to suggest, however, that polysaccharide capsules do not represent a diffusion, permeability, or adsorption barrier to macromolecules such as IgG (147) or other proteins (146) and that inagglutinability frequently associated with the presence of acidic polysaccharides superficial to the bacterial surface is most likely due to surface protein components (136) or to inhibition of lattice formation between adjacent bacteria (38).

No obvious relationships were found when acidic exopolysaccharides were prepared by two methods from a large number of urine E. coli isolates of various serum susceptibilities (301). Some susceptible and resistant strains produced large amounts of K antigen with high hemagglutination-inhibiting activity, but both groups also contained strains that produced small quantities
of polymer with no detectable activity. A similar lack of correlation was found for *E. coli* bacteremic strains by McCabe et al. (174). Mutations to serum resistance did not result in increased exopolysaccharide production (300). Inheritance of the *his*-linked genes for K27 antigen production by *E. coli* recipients did not result in an altered serum response, regardless of whether recombinants produced R lipopolysaccharides or had simultaneously inherited O side chains (311). Van Dijk and co-workers (325) also failed to find a correlation between K antigen production as determined by the hemagglutination inhibition method and the serum resistance of blood and fecal isolates of *E. coli*. *Klebsiella* strains are frequently found to be susceptible to serum (77, 103), even though most members of this genus produce copious amounts of hexuronic acid-containing acidic polysaccharide (294). *P. aeruginosa* isolates originating from the respiratory tracts of patients with cystic fibrosis are known to be generally more susceptible to serum than strains of this species from other sources (122); cystic fibrosis isolates are generally extremely mucoid (58), owing to the production of a polyuronide of composition similar to alginic acid (159).

There appears, therefore, to be substantial evidence against a major role for acidic exopolysaccharides as mediators of serum resistance, at least in a general sense that can be discerned by comparative epidemiological investigation. These polymers form a distinctly heterogeneous group of bacterial products with regard to chemical composition, antigenicity, molecular size, biophysical properties, and the intimacy of their association with the cell surface. These factors are likely to distort any such complex relationship that between serum resistance and the interaction of the bacterial surface with activated complement components unless steps are taken to minimize these effects. It is in any case clear that any increase in serum resistance directly attributable to the presence of acidic polysaccharides cannot form part of a general mechanism for resistance of gram-negative bacteria, as many groups, such as *Salmonella* spp. (56, 209, 214), possess a high level of intrinsic serum resistance and yet do not produce acidic polysaccharides.

*E. coli* strains synthesizing the K1 antigen, an α-2,8-linked NANA homopolymer with a degree of polymerization of 150 to 200 sialyl residues in situ (321), are isolated with high frequency from cases of neonatal meningitis (254) and renal infection in infancy (142). Thus, the K1 antigen or the presence of closely linked traits may be a virulence factor related to the pathogenesis of these diseases. Several groups have considered the possibility that the K1-mediated pathogenicity of these strains is related to a capacity to resist the bactericidal activity of serum. The relative homogeneity of K1 strains as a group (276) makes them considerably more attractive material for comparative epidemiological studies than random *E. coli* isolates.

Two groups have reported on the cloning into plasmids of the chromosomally located, *serA*-linked *kpsA* locus determining synthesis of K1 (228) and its expression in *E. coli* strains. Silver et al. (277) used a cosmid cloning vector in combination with in vitro packaging techniques to clone the K1 genes; *kpsA* specified synthesis of a NANA polymer in *E. coli* K-12 that was indistinguishable chemically and immunologically from that of wild-type K1 strains. In contrast to the K-12 host, *E. coli* K-12 expressing the cloned K1 antigen was reported to be serum resistant on the basis of efficiency of plating on agar containing equine meningococcal group B antiserum; the non-O-acetylated form of the K1 antigen and the group B polysaccharide are identical (255). Timmis et al. (316), using an identical cosmid cloning-packaging system, introduced a number of *BamHI* or *PstI*-generated fragments into an *E. coli* K-12 strain. In 6% rabbit serum, all K-12 cells were killed after 3 h, whereas all K1+ derivatives survived; such differences were not apparent at higher serum concentrations. These results complement those of other investigators who have assigned a role for the K1 antigen on the basis of genetic studies of serum resistance. K1-negative mutants of *E. coli* K1 clinical isolates were found to be more susceptible to high concentrations of serum than their parent strains (32). Rough, K1-positive *E. coli* bacteremic isolates were resistant to 10 to 20% human serum, whereas single-step isogenic K1-negative derivatives, with an identical outer membrane protein profile, were found to be extremely serum susceptible (87). These studies therefore suggest a direct role for the K1 antigen in the protection of *E. coli* against serum killing. As Taylor and Robinson (311) had earlier failed to observe any increased serum resistance after inheritance of genes determining biosynthesis of the K27 antigen, Gemski and co-workers (225) constructed strains expressing both K1 and K27 antigenic determinants. It is possible to prepare such hybrids because gene loci for these antigens are nonallelic (227). Hybrid strains expressing both K1 and K27 antigens exhibited serum resistance, but at levels lower than those found with a K1 parental strain. An isogenic K1-negative derivative expressing only the K27 antigen was fully serum susceptible. Loss of capacity to produce K1 antigen by chemostat-grown cells is accompanied by a large increase in serum susceptibility (308).

When, however, *E. coli* K1 clinical isolates
are examined and compared, no clear picture emerges with regard to a relationship between K1 carriage and serum resistance. Björksten et al. (26) found no consistency in the way fecal, blood, or cerebrospinal fluid isolates of *E. coli* K1 reacted in a number of test procedures involving bacteria-serum interactions, including serum bactericidal systems. No significant differences could be found between the amount of acidic polysaccharide produced by urinary and fecal K1 strains and their reactivity in human serum (222). *E. coli* K1 strains from cases of neonatal meningitis were more susceptible to serum than those from cases of neonatal or adult sepsis or adult meningitis (236). In these studies, many serum-susceptible K1 isolates were reported, and as a group, K1 strains appear to be rather serum susceptible. It is therefore difficult to reconcile these data with the results of the genetic studies cited above, particularly as the latter have generally utilized *E. coli* K-12 strains as hosts for K1 genes; effects attributable to K1 carriage would therefore be seen against a background of high serum susceptibility. Discrepancies could be due, however, to the fact that anti-K1 antibodies are not usually present in reasonable amounts in normal sera or, indeed, in the sera of patients recovering from either *E. coli* K1 or meningococcal B infection (266). Sialic acid-containing polymers, including the K1 capsule (290), restrict activation of the alternative pathway, and so killing of such strains would be expected to proceed via the antibody-dependent classical route. If K1 is the only major surface antigen available for interaction with serum factors, then apparent resistance may be due to lack of a mechanism for classical pathway activation. The addition of an effective antibody source to the bactericidal system might then result in killing. The high incidence of serum-susceptible K1 isolates observed in most clinical studies emphasizes that the K1 antigen cannot be universally considered as providing protection against serum killing mechanisms.

**Role of Plasmid-Determined Factors**

The earliest report of an altered serum response after the acquisition of foreign DNA appears to be that of Muschel and associates (202, 207), who found increased serum resistance after lysogenization of *E. coli* K-12 with λ phage. In 1974, Williams Smith (338a) noted that strains of *E. coli* invasive for humans and domestic animals harbored ColV plasmids which, when transferred to K-12 and other *E. coli* strains, were responsible for increased lethality for chickens and mice and a greater ability of the host bacteria to survive in blood and serum. Binns et al. (24) have cloned various fragments of ColV, I-K94, a plasmid specifying production of colicins V and I; a BamHI-generated fragment increased the resistance to fresh rabbit serum of a Col*+* *E. coli* O78:K80 strain. The gene specifying serum resistance, the *iss* determinant, was mapped to a 5.3-kilobase sequence within the fragment and found to be closely linked, though not coincident with, genes for colicin V production. It is likely that the *iss* gene product, in addition to inhibiting insertion or activity of the MAC on the outer membrane (25), is responsible for the increased virulence associated with ColV carriage. That the *iss* gene product may be important in relation to the ecology of these organisms is suggested by the observation that a high proportion of virulent *E. coli* strains isolated from infections of hospitalized patients carry and express ColV-determined functions (52).

Reynard and Beck (244) reported that the plasmids R1 and R100 (NR1), in addition to encoding resistance against a number of antibiotics, were able to confer relatively high degrees of resistance against rabbit serum on a number of *E. coli* K-12 strains. This ability appeared to be restricted to F-like plasmids; plasmids from 4 of 16 clinical isolates examined were able to protect *E. coli* K-12 J6-2N against the bactericidal action of rabbit serum (245). Although it has been repeatedly confirmed that certain F plasmids are able to afford host protection against the bactericidal effects of serum, there has been some controversy over the degree of resistance that plasmids may confer that has its origins in the fact that, as discussed earlier, the use of widely differing techniques and the utilization of serum from a variety of sources and at different concentrations makes meaningful comparisons between such studies difficult.

Thus, a number of other workers have been unable to confirm that either R1 or NR1 protect K-12 strains against serum killing. Fietta et al. (79) could not detect increased survival after inheritance by strain J6-2 of plasmid R1 when a system employing highly dilute human serum was used; they did, however, report that 8 of 26 plasmids examined conferred relative serum resistance to *E. coli* K-12 strains. A plasmid was said to have conferred resistance if the amount of serum needed to reduce the viable count to 1% after 30 min was greater for R*+* progeny than for R*−* parents. The amounts of serum required to effect this degree of killing were extremely small, usually about 3% of the total reaction mixture, and at such concentrations classical pathway components may be present in limiting amounts and alternative pathway activity is almost certain to be nonexistent (268). Taylor and Hughes (304) examined by two assay methods the effect of carriage of a wide range of R and Col plasmids, including R1 and NR1, on the susceptibility of six *E. coli* K-12 strains to no-
mal human serum. With both methods, all K-12 strains examined were rapidly killed by serum, regardless of plasmid carriage. Survival in rabbit serum was also unaffected by the presence of these plasmids. Part of this discrepancy can be attributed to the source of serum; Reynard and Beck (244) used commercially available lyophilized rabbit serum as source of antibody and complement, and the material was reconstituted with physiological saline, whereas in other studies, fresh rabbit or human serum was used. Human serum appears to be an inappropriate source, as Reynard and co-workers have themselves reported that plasmid R1 does not provide K-12 strains with protection against human serum (245). Much of this confused situation has been clarified by Ogata and Levine (221), who examined the effect of various concentrations of rabbit, guinea pig, and human sera on E. coli strains carrying plasmid R100. They found that differences in the serum reactivity of pairs of K-12 strains with and without R100 could be detected provided a high bacterial cell density and relatively low concentrations of rabbit serum were used. At a given serum concentration, survival of plasmid-carrying strains decreased as the bacterial concentration decreased. However, in 10% rabbit serum, significant killing of all five plasmid-carrying K-12 strains was observed, and in four cases, incubation for 50 to 60 min resulted in less than 10% survival. It is therefore clear that what is being measured is not serum resistance as defined earlier but a reduction in the rate of killing that is only manifest under certain experimental conditions. One could imagine that insertion of novel plasmid-encoded proteins with unrelated cell functions into the outer membrane might fortuitously reduce membrane fluidity and hence the rate at which MACs are inserted into the membrane, especially when concentrations of serum are limiting. E. coli J6-2(R100) survived better in guinea pig serum than E. coli J6-2, but in human serum, which is a much more potent source of bactericidal activity than either rabbit or guinea pig serum, a barely discernible effect was recorded over a serum concentration range of 0.5 to 2%. The authors (221) suggested that failure of other groups (245, 304) to detect a protective effect of R100 against human serum was due to the use of serum concentrations greater than 10% and cell concentrations 10- to 100-fold lower than used in their study.

As is clear from the studies of Binns et al. (24), more readily detectable effects of plasmids on bacterial survival in serum can be achieved if strains with a certain intrinsic level of resistance are used as hosts. Taylor and Hughes (304) cured a number of enterobacteria isolated from polluted river water of antibiotic resistance and bacteriocin determinants. One strain, E. coli C8, displayed delayed human serum killing kinetics; curing of all markers resulted in a small but significant increase in serum susceptibility. Introduction into the cured derivative of plasmids from six of eight other river isolates and of plasmids R1 and R100 resulted in significant increases in survival in serum. R1 and R100 were unable to modify the serum response of a cured strain derived from a promptly susceptible isolate, and the authors therefore suggested that lipopolysaccharide O side chains, the surface components responsible for the delay in serum killing, were essential for expression of plasmid factors that modify susceptibility to serum, at least in systems containing adequate concentrations of fresh human serum. That this might be so was indicated by the results of a study of plasmid-determined factors expressed in recombinants from Hfr crosses carrying defined combinations of polysaccharide surface antigens (311). Plasmid factors encoded by R1 and R100 failed to modify the promptly susceptible response to human serum of either E. coli F470 or F470 progeny that had inherited and expressed his-linked genes for K27 antigen production. Recombinants inheriting the rfb locus for O8 side chain production showed delayed serum killing kinetics, and serum susceptibility was additionally decreased by the presence of either of the two plasmids. Although not completely serum resistant, a small but significant contribution towards the partial resistance to serum of such recombinants was made by plasmid-determined genes.

Plasmid R6-5 is, like R100, a large, low-copy-number, conjugative, multiple antibiotic resistance plasmid of the FII incompatibility group. Timmis and co-workers have recently shown (195, 196, 316) that R6-5 encodes a factor that increases the survival of the moderately serum-susceptible fecal isolate E. coli 59 in rabbit serum. Using gene cloning techniques, they localized these functions to a segment of the plasmid specifying conjugal transfer functions. Analysis of serum-susceptible deletion and insertion mutants demonstrated that the gene specifying serum resistance was coincident with the traT locus, one of two loci involved in surface exclusion, the poor recipient ability in conjugation of F factor-carrying cells (1). The traT protein is present at about 21,000 copies per cell and is situated on the outer surface of the outer membrane. It has, therefore, the capacity to prevent cell-protein interactions that would normally result in assembly and insertion of functional MACs. It is likely to do this in a highly specific way, as plasmids containing point mutations in the traT gene no longer confer protection against serum, even though bacteria
contain more of the altered gene product in their outer membrane than do bacteria harboring the parental plasmid (196). The studies cited above suggest that the presence of plasmids of incompatibility group FII may increase the rate of survival of suitable host bacteria on serum, although it is clear that the degree of resistance conferred is not enough to explain the total resistance to human serum of many clinical isolates. The bactericidal assays used in much of this work have been specifically tailored to demonstrate the maximum possible difference in serum reactivity between isogenic pairs. Differences demonstrable with rabbit serum but not apparent in human serum may be of little relevance to an understanding of the role of serum resistance as a host defense mechanism. Plasmids encoding products that decrease the serum susceptibility of suitable recipients have been found in wild strains that are completely susceptible to serum (304), indicating that the ecology of these determinants is likely to be complex. The limited amount of epidemiological data available suggests that the carriage of R and Col plasmids shown previously to be capable of conferring increased levels of serum resistance on individual E. coli strains isolated from other sources does not play an important role in determining the serum susceptibility of E. coli populations, at least in urinary tract infections (125). Indeed, the obviously multifactorial nature of serum resistance makes it unlikely that any convincing association between resistance and expression of surface structures would emerge from such an approach. Hopefully, a better understanding of the molecular nature of the bactericidal reaction will allow conclusions to be drawn about the biophysical characteristics of the cell surface that enable gram-negative bacteria to circumvent the potentially lethal action of complement.

Role of Other Outer Membrane Proteins

A serum-resistant mutant derived from a smooth, delayed-susceptible urinary isolate of E. coli produced more of an envelope protein of molecular weight 46,000 than the parent (309): the amount of this protein was subject to variation as a result of alterations in growth conditions and was well correlated with environmentally induced modification of the bacterial response to serum (308). The authors suggested that the protein factor was involved in the determination of serum resistance but that it was only functional when superimposed on a full complement of lipopolysaccharide O side chains (309).

A protein modifying the response to human serum of N. gonorrhoeae has been identified in strains producing disseminated gonococcal infection (120). Transformation of a serum-suscep-

SERUM BACTERICIDAL ACTIVITY AS AN IMMUNE DEFENSE MECHANISM

Precise in vivo evaluation of the role of bactericidal mechanisms against gram-negative bacteria has been difficult to achieve, most obviously because of the difficulties in distinguishing complement-mediated bactericidal and bacteriolytic phenomena from other specific and nonspecific host defense systems that may also modify the interaction between host and invading parasite. Furthermore, other host systems, such as immune phagocytosis by blood leukocytes, may also require at least some of the components of complement for efficient processing and elimination of invading microorganisms. It is also likely that distinct components of the host defense act in concert after a challenge with potentially pathogenic microorganisms and that complement-mediated humoral killing mechanisms will be of more importance in the pathogenesis of some infections than in others. For example, only a limited role for the serum bactericidal system can be envisaged in infections caused by bacteria elicits granulomatous tissue responses in which the ability of the microorganisms to survive in an intracellular location protected from circulating noncellular mediators is a well-established prerequisite of pathogenicity. Conversely, in infections such as gram-negative bacterial endocarditis, in which invading microorganisms colonize a site that is apparently inaccessible to phagocytic cells, resistance to serum bactericidal factors appears to be an important pathogenicity factor enabling strains to resist elimination from the host (12, 62, 109).

Further complications arise because bacterial surface determinants known to increase the serum resistance of certain gram-negative strains may also provide protection against other host defense mechanisms (27, 123, 149). However, acquisition by gram-negative bacteria of determinants known to increase serum resistance is sometimes associated with an increase in virulence (24, 214), suggesting a direct role for serum resistance as a bacterial pathogenicity factor in certain situations. The importance of the complement system in host defense is suggested by
the observation that the acute stage of many gram-negative infections is characterized by increased biosynthesis and turnover of complement proteins (13) and a reduction in the serum level of natural complement inhibitors (337); thus, in these situations complement behaves as an acute-phase reactant. In some infections, the primary mechanism of defense is provided by immune phagocytosis, and many serum-resistant strains can be efficiently phagocytosed after opsonization (135, 279, 344). Nevertheless, an extensive body of literature has steadily accumulated implicating resistance to the bactericidal action of serum as an important determinant of virulence in certain infections. Much of this data is derived from two broad sources: a large number of epidemiological studies based on clinical observations are complemented by results obtained with experimental invasive bacterial infections in laboratory animals.

Clinical Observations on the Relevance of Serum Resistance as a Bacterial Pathogenicity Determinant

Are gram-negative bacteria isolated from infected clinical material more resistant to serum than random isolates from noninfected sites? It would seem reasonable to assume that serum-resistant gram-negative bacteria would possess a significant survival advantage in the blood system, and Roantree and Rantz (251) and Fierer et al. (77) found that a much higher proportion of strains isolated from the blood of bacteremic patients than of those isolated from feces or urine was resistant to killing by human serum. Similarly, Vosti and Randall (326) found significantly fewer serum-susceptible E. coli strains isolated from blood cultures than from urine and feces. Of 46 isolates of P. aeruginosa from blood cultures of patients in a New York hospital, 42 (91%) were found to be resistant to human serum (344); these authors also found, however, that many apparently saprophytic pseudomonads were also serum resistant. In a survey of clinical isolates of S. marcescens, Simberkoff and co-workers (279) found that no blood culture isolates were serum susceptible, whereas 40% of urinary isolates were susceptible to complement-mediated mechanisms. There appears, therefore, to be a strong correlation between serum resistance and the ability of a variety of gram-negative bacteria to invade and survive in the human bloodstream. Furthermore, bacteremia caused by serum-resistant E. coli strains is more likely to be associated with shock and death than bacteremia due to susceptible strains (174). However, in a study of patients with bacteremia due to gram-negative bacteria and including a large number of elderly patients and patients with complicating disease, Elgefor and Oiling (66) found that only 37% of isolates were completely resistant to normal human serum and that 26% were markedly serum susceptible.

The survival of apparently serum-susceptible bacilli in the blood of bacteremic patients might be related to a number of factors. Serum from some bacteremic patients has been reported to be ineffective in bactericidal assays utilizing the infecting, homologous organism, even though the strain is fully susceptible to serum from healthy individuals (251). A similar phenomenon has been reported for naturally occurring bacteremia due to Brucella canis in an adult beagle dog population (124). Bacteria may circumvent the serum bactericidal system by adopting an intracellular location in the blood or reticuloendothelial system (250). For example, viable bacteria may be found within leukocytes with infections of the blood system (283). The early activation and consumption of complement before the onset of shock (82) may render bactericidal activity efficient only during the initial stage of bacterial invasion (66), when cells may be in a state of low metabolic activity and therefore not susceptible to complement-mediated killing (54). Many gram-negative bacteria capable of rapid growth in a nutrient-rich in vitro environment may achieve only very low rates of cell division when present in tissue or body fluids (169, 181, 182, 239).

Bacteria dividing at submaximal rates may be less susceptible to serum than more rapidly dividing cells (302), and this may assist survival of apparently susceptible isolates in vivo. Also, the genetic basis for virulence may be expressed completely only during growth in vivo: gonococci adapted to growth in chambers implanted subcutaneously into guinea pigs were found to be resistant to human serum, but loss of this phenotypically acquired trait occurred after a few generations of in vitro growth (249). Serum-resistant E. coli acquired phenotypic resistance to serum after multiplication in rat mononuclear cells; resistance was rapidly lost during subculture on solid medium (176). Residence in monocytes from normal guinea pigs leads to increased serum resistance of smooth strains of Brucella abortus (292). Thus, serum resistance attainable in vivo may not be apparent after in vitro assay.

Perhaps, however, the most important factor that should be considered pertains to the immune status of the infected individual. Preexisting disease is of major importance in the acquisition of bacteremia and in its outcome (173, 222, 345) and may therefore reduce the necessity for invading bacteria to express virulence determinants. For example, patients with sickle cell anemia frequently contract systemic salmonellosis, and serum from such individuals displays
deficient bactericidal function against *S. typhimurium* (114), probably as a result of abnormal alternative pathway activity. Neonates with hyperbilirubinemia as a result of blood group incompatibility are prone to infections with gram-negative bacteria, and their serum possesses significantly decreased bactericidal activity (190) due to inhibition of complement function by bilirubin (73). Sera from patients with bilirubinemia (410) and renal failure (197). Clearly, patients with bacilli of the urinary tract (41, 191) is possible, therefore, that the serum bactericidal system may constitute an important defense mechanism in those parts of the urinary tract in contact with antibody-containing fluids. Urinary isolates do not appear to constitute a particularly serum-resistant group of organisms (222, 299, 326), although Kimball et al. (145) have presented evidence indicating that *E. coli* O groups most frequently isolated from patients with bacteriuria possess a higher degree of intrinsic serum resistance than organisms belonging to O groups infrequently isolated from infections of the urinary tract. The significance of this observation is unclear, as the distribution of O groups causing urinary tract infections broadly reflects the relative frequencies of those O groups predominating in the gut flora (107). During initiation of urinary infection, urinary pathogens are thought to enter the bladder during miccitrition through the short female urethra, and the bacteria concerned are those nonanaerobes predominating at that time in the gut flora. Once inside the urinary tract, however, there appears to be some selectivity with regard to the type of pathogen that can successfully invade and colonize the upper urinary tract. For example, it has been shown that only strains of *E. coli* producing large quantities of K antigen are able to infect renal tissue (93). Gower and co-workers (103) examined the serum susceptibility of the infecting gram-negative organism from patients with infections that had been localized to either the upper (ureters and kidneys) or lower (bladder) urinary tract. A very high proportion of lower infections was due to serum-susceptible organ-

isms. In contrast, the majority of upper urinary tract isolates were not susceptible to serum obtained from the infected patient. In some cases, the infecting strains were inherently serum resistant, but others were efficiently killed by normal human serum. The serum of at least some of these patients was found to contain high amounts of IgG antibody (297) directed against determinants within the lipopolysaccharide of the infecting strain (298) that effectively inhibited the bactericidal action of both the patients’ sera and normal serum, probably by blocking cell surface sites normally available to IgM molecules. No evidence was found of a generalized decrease in serum bactericidal activity of patients with urinary tract infections. The persistence of serum-susceptible organisms in the upper urinary tracts of patients whose sera contain specific blocking factors that arise as a result of bacterial invasion and colonization suggests an active role for complement-mediated bactericidal mechanisms in defending the kidneys against infection.

It has been suggested, however, that complement-mediated mechanisms are of limited relevance to infections involving kidney tissue. As part of an investigation of the basis of the vulnerability of the kidney to infection, Beeson and Rowley (18) reported that exposure of human or rabbit serum to homogenates of renal tissue resulted in rapid loss of complement activity. They suggested that this effect could be extrapolated to the situation in vivo, and they attributed the anticomplementary action of renal tissue to the ability of the kidney to produce ammonia, which is known to inactivate the fourth component of complement at pH 8.5 to 10 (101); Beeson and Rowley, however, conducted their experiments at pH 7.4, and at this pH the effect of ammonia on complement activity is minimal (101). Contradictory findings were presented by Henkel (118), who could find no inhibition of serum bactericidal activity by kidney homogenates. The phenomenon has recently been reinvestigated by Ormrod and Miller (226), using renal tissue maintained in vitro under physiological conditions. There was a rapid decrease in the hemolytic capacity of serum after addition of kidney tissue, but C3 levels remained essentially unaltered. Liver tissue, however, had a greater complement-inactivating capacity than renal tissue. Inactivation was not due to ammonia, as the concentration of ammonia produced had no effect on the rate of complement inactivation, and liver tissue produced only marginal amounts of ammonia. The authors stressed that the conditions necessary for C4 inactivation by ammonia (101) are incompatible with renal physiology and limit the potential role of ammonia as a factor modifying
complement-mediated immune mechanisms. Furthermore, the degree of complement inactivation after even prolonged incubation of serum with renal tissue was insufficient to affect complement-mediated bacteriolysis of an E. coli strain, suggesting that the significance of complement inactivation in vitro as a factor modulating host defenses has been exaggerated. Complement-mediated bactericidal activity is inhibited by hypertonic, but not hypotonic or isotonic, urine (2), an observation likely to have significance regarding the often exaggerated (191) susceptibility of the renal medulla to infection, as the mechanisms for concentrating the urine are mainly located in this part of the kidney. In fact, the susceptibility of the renal medulla to infection is less during water diuresis (11). The relative inability of serum-susceptible strains to survive in the renal environment is compatible with present concepts of host defense in the urinary tract. The primary means of disposing of bacteria that enter the bladder is mechanical, and immune processes are probably confined to the bladder wall and are likely to be exclusively cellular in nature (44). In contrast, both humoral and cellular defense mechanisms are known to be operative in the kidney (191). Selection within the urinary tract is also implied by the observation that strains from patients with asymptomatic bacteriuria may be more serum susceptible than fecal isolates, whereas strains from patients with symptoms of urinary infection are more serum resistant than either fecal isolates or isolates from asymptomatic patients (223). In some patients with asymptomatic bacteriuria, the infecting strain becomes more susceptible to serum if the infection is left untreated (158). Spontaneous clearance of asymptomatic bacteriuria may be preceded by an increase in the serum susceptibility of the urinary pathogen (224).

Complement-mediated bactericidal mechanisms may also be important in other types of infection. The overwhelming majority of enterobacteria causing bovine mastitis were found to be serum resistant (34). Isolates of Bacteroides spp. from feces were significantly more serum susceptible than isolates from patients with clinical infections, including bacteremia (37). Strains of Bacteroides fragilis, the predominant species found in serious infections, form a group characterized by a high degree of serum resistance. Waisbren and Brown (327) recorded specific defects in the ability of patients' sera to kill the infecting organism in a variety of clinical situations that included Pseudomonas pneumonia, chronic pyelonephritis, endocarditis, peritonitis, and septicemia; sera from similar patients were subsequently shown to contain IgG directed against bacterial surface components which could block complement-mediated killing mechanisms (110). It is tempting to speculate that such blocking mechanisms are responsible for the survival of genotypically serum-susceptible bacteria in tissues from which they would normally be swiftly eradicated by the host's defense mechanisms.

Susceptibility to the serum bactericidal system seems to be of primary importance in determining the pathogenesis of infections due to Neisseria sp. Thus, gonococci causing localized genital infections are generally susceptible to human serum (65), whereas they normally invade the blood system and produce disseminated gonococcal infection only if they are resistant to killing by serum (65, 267, 285). Deficiency of antibody does not account for inability to kill disseminated gonococcal infection pathogens (42) but in some cases is related to IgG blocking mechanisms (175). Similarly, IgA-mediated bactericidal blocking mechanisms (113) may aid dissemination of N. meningitidis in the early stages of systemic meningococcal disease (104). Establishment of localized gonococcal infection in the genital tract by serum-susceptible strains might be promoted by inhibition of complement-mediated bactericidal activity due to proteases in seminal plasma (233). Similarly, absence of adequate levels of antibody and complement in cerebrospinal fluids may contribute to the maintenance of high concentrations of bacteria in untreated cases of meningitis (278).

Perhaps the most convincing evidence in favor of a role for bactericidal activity in these infections is afforded by observations on patients with total or near-total congenital deficiencies of a single complement protein. The critical role of C3 in a variety of immune reactions beneficial to the host is indicated by a generalized susceptibility to microbial infection in individuals deficient in C3 (6, 13). Patients with genetic deficiencies of the proteins of the MAC have a propensity to infections with bacteria of the Neisseria group. Thus, deficiencies of C5 (111, 232), C6 (14, 31, 111, 234), C7 (31, 155, 234), and C8 (31, 234) are associated with episodes of gonococcal or meningococcal bacteremia and meningitis. The observations imply that the late-acting complement components are necessary for normal host defense against pathogenic Neisseria spp., particularly with regard to impeding dissemination of serum-susceptible bacteria. As the absence of these components does not impair the opsonic and leukocyte chemotactic functions of the complement system, a direct role for serum bactericidal activity is suggested by these observations, particularly as phagocytic cells are often inefficient in ingesting these organisms.

Individuals deficient in C1, C4, and C2 do not
appear to be unduly susceptible to infections with gram-negative organisms; it is likely that in such individuals the alternative pathway is responsible for preventing serious recurrent infections (13).

Evidence from Experimental Infections of Laboratory Animals

A clear relationship has emerged between serum resistance and the ability of enterobacteria to produce endocarditis in experimental animals. Durack and Beeson (62) found that only serum-resistant E. coli strains were consistently able to cause infective endocarditis in rabbits prepared by prior placement of an intracardiac catheter. In contrast, all five serum-susceptible strains tested caused endocarditis in C6-deficient rabbits. Similar experimental models were used to verify this relationship with P. aeruginosa (12) and S. marcescens (109).

Evidence from animal challenge experiments suggests a relationship between virulence and serum resistance. For example, the ability of E. coli strains to multiply in the peritoneum and produce infection in mice after intraperitoneal challenge was correlated to their ability to survive in vitro in normal serum bactericidal systems (258). Serum-susceptible enterobacteria are cleared from the blood of rabbits more rapidly than resistant organisms and are recoverable from the viscera 24 h after infection at only about 10% of the concentration of resistant strains (250). A similar relationship was established for E. coli after intravenous injection into normal mice (178). In peritoneal membrane chambers in guinea pigs, serum-susceptible S. enteritidis cells were killed, whereas serum-resistant ones survived (291). Caution should be exercised, however, when extrapolating these results to natural infections in humans. Much of this work has been performed in mice, which have a relatively inefficient serum bactericidal system. Furthermore, Salmonella spp., often employed in such studies, are predominantly intracellular parasites and may be concentrated in locations where they are protected from complement-mediated bactericidal systems.

In estrogen-pretreated rats, only serum-resistant strains were consistently able to induce lesions indicative of pyelonephritis; this relationship was not maintained when a rat model of obstructive pyelonephritis was used (119). In an experimental rat model, serum-susceptible strains were able to produce kidney infection only after the animals had been depleted of complement with cobra venom factor (192).

CONCLUDING REMARKS

The weight of published evidence indicates that resistance to the bactericidal action of anti-

body-complement systems is likely to play a role in the pathogenesis of at least some infections due to gram-negative bacteria. It can therefore be inferred that the serum bactericidal system has evolved partly in response to the need of the host to efficiently eliminate invading bacteria from potential sites of infection. In suitable in vitro systems, these complement-mediated reactions often proceed at an impressive rate, although one feels that our understanding of serum killing would progress in a more orderly fashion if there was broad agreement on the basic methodology of serum bactericidal assay.

Recent work has emphasized the essentially multifactorial nature of serum resistance, and a number of distinct cell envelope polymers, such as outer membrane proteins, lipopolysaccharides, and acidic exopolysaccharides, have been implicated as mediators of resistance to activated complement components. The relative importance of these various structures is less clear, as is the precise way in which some gram-negative bacteria successfully circumvent the lethal mechanism. It is to be hoped that future work will shed some light on the biophysical properties of the outer membrane of resistant organisms that will permit a description of serum resistance in purely molecular terms.

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