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Killing of an Encapsulated Strain of *Escherichia coli* by Human Serum

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Changes in cell viability and in factors affecting metabolic integrity were examined after exposure of *Escherichia coli* LP1092 to human serum. Antibody-dependent classical pathway activity accounted for the rapid killing of strain LP1092 by complement. Removal of serum lysozyme by bentonite absorption or by neutralization with anti-human lysozyme immunoglobulin G resulted in a reduction in the rate of killing; optimal activity could be restored by the addition of physiological amounts of egg-white lysozyme. The pattern of $^{86}Rb^{+}$ and alkaline phosphatase release obtained after serum treatment did not support the view that complement simultaneously disrupts cytoplasmic and outer membrane integrity. Macromolecular synthesis was affected late in the reaction sequence; complete inhibition of precursor incorporation into RNA, DNA, and protein occurred only after almost total loss of bacterial colony-forming ability. Addition of chloramphenicol, an inhibitor of protein synthesis, to the bactericidal system resulted in a marked reduction in the rate of serum killing. Killing was completely inhibited by an inhibitor (KCN) and an uncoupler (2,4-dinitrophenol) of oxidative phosphorylation. 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 LP1092 cells at a much reduced rate. Addition of glucose or serum ultrafiltrate to dialyzed serum restored optimal bactericidal activity. We suggest that optimal killing of gram-negative bacteria is an energy-dependent process requiring an input of bacterially generated ATP.

There is a large body of evidence indicating that the complement system is an important component of the host defense against infection with gram-negative bacteria. For example, it has frequently been shown that strains causing severe infections involving tissue penetration and damage are more likely to be resistant to the complement-mediated serum bactericidal system than commensals or noninvasive strains (39, 45, 47). Inherited complement deficiencies in humans have been associated with a predisposition to infection with gram-negative bacteria, most frequently of the genus *Neisseria* (1, 37) but including other organisms (21).

Killing of susceptible bacteria by complement is mediated by either the classical pathway (25) or the alternative pathway (43). The classical pathway may be activated by the interaction of antibody and bacterial surface antigens (27) or directly by the lipid A region of lipopolysaccharide (28). Alternative pathway activation can be effected in the absence of antibody by a variety of surface polysaccharides (27). Activation of either pathway results in the generation of the functional lesion, the C5b-9 complex (2), at the bacterial surface; this complex is presumably responsible for the irreversible damage to the cytoplasmic membrane that leads to cell death. Bacterioloysis, but not the bactericidal effect, requires the presence of lysozyme (29). Current knowledge of the mechanism of cell damage by complement derives almost exclusively from studies of red cell lysis (27, 30). Gram-negative bacteria, with their complex cell envelope structure, ability to respond rapidly to environmental change, and capacity for repair of damaged sites, undoubtedly present a more complex target for complement attack than the red cell surface, and there is at present little understanding in molecular terms of the sequence of events that occurs after complement activation and culminates in the death of the bacterial cell.

In the present study we investigated the killing of a susceptible urinary *Escherichia coli* strain by human serum. We attempted to define serum components essential for rapid killing and examined some changes in metabolic parameters occurring after exposure of bacteria to serum.

MATERIALS AND METHODS

Bacteria. *E. coli* LP1092 was isolated from the urine of a patient with a severe upper urinary tract infection. This strain is rough, with a complete lipopolysaccharide R2 core structure, and synthesizes a K-antigen polysaccharide consisting of ribose and 3-deoxy-D-*manno*-2-octulosonic acid (P. W. Taylor, Abstr. IXth Int. Symp. Carbohydr. Chem. 1978, F27, p. 411–412).

Serum. Blood was obtained from healthy donors and allowed to clot at room temperature, and serum was separated by centrifugation. Pooled serum from four to six individuals was either used fresh or stored in small samples in liquid nitrogen until required.

Serum bactericidal assay. Bacteria were grown to the late logarithmic phase in 50 ml of DO medium (16) at 37°C on an orbital incubator (120 orbits per min) in Erlenmeyer flasks. A sample of cells was removed and washed at room temperature with gelatin–Veronal-buffered saline plus magnesium and calcium ions (pH 7.35) (GVB^{2+}) (13). The bacteria were resuspended in GVB^{2+} to give a concentration of 1.5×10^7 cells per ml; 500 μl of the bacterial suspension was then added to 250 μl of GVB^{2+} containing 50 μl of serum in Eppendorf K-Cuvettes (Eppendorf GmbH, Hamburg, Federal Republic of Germany) and, after being mixed, incubated in a 37°C waterbath. Viable counts were prepared at regular intervals after the withdrawal of 10-μl samples from the cuvettes.

The system was also modified by addition of the following inhibitors and uncouplers in 50-μl amounts: nalidixic acid, chloramphenicol, 2,4-dinitrophenol and KCN (all from Sigma Chemical Co., St. Louis, Mo.).

Treatment of serum. Aliquots (1 ml) of serum were absorbed with 10^{11} Formalin-treated or heat-killed (100°C, 1 h) LP1092 cells at 0°C for 24 h. The bacteria were removed by centrifugation followed by filtration through a 0.45-μm Swinnex-type Millipore filter. The process was repeated, and absorbed sera were stored at −80°C until required. Absorption had no effect on the hemolytic complement titer of the serum. Two methods were used to obtain lysozyme-free serum. Fresh serum was treated with bentonite (Sigma) as described by Wardlaw (48). The bentonite was washed repeatedly with water and then added to the serum to give a concentration of 5 mg/ml. The mixture of bentonite and serum was stirred for 15 min at 0°C and centrifuged at 0°C, and the supernatant was decanted and filtered (Millipore 0.45-μm filter). Lysozyme activity was also neutralized with specific anti-human lysozyme immunoglobulin G (IgG). The IgG, kindly supplied by Rafael J. Martinez, University of California, Los Angeles, was dialyzed extensively against GVB^{2+}; 10-μl amounts were then added to 50 μl of human serum in 250 μl of GVB^{2+} and incubated for 2 min at 37°C before initiation of the bactericidal reaction by addition of bacterial cells. The two methods were equally effective in removing all lysozyme activity as judged by the failure of treated serum to reduce the optical density of a commercially prepared lysozyme substrate of *Micrococcus luteus* (ATCC 4698) cells (Sigma Feinbiochemica, Heidelberg, Federal Republic of Germany) over a 4-h incubation period at room temperature.

Serum was treated with inulin according to the method of Götze and Müller-Eberhard (17) and was depleted of properdin as described by Traub and Fukushima (46) with zymosan (Sigma). Classical pathway activity was selectively inhibited by chelation of serum with 0.01 M Mg ethylene glycol-bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid (Mg EGTA) according to the method of Fine and co-workers (8). Bactericidal activity of serum was abolished either by heating at 56°C for 30 min or by treatment with 0.01 M dithiothreitol.

Determination of macromolecular biosynthesis with radioactively labeled precursors. *E. coli* LP1092 cells were prepared as described above for the serum bactericidal assay, and 500 μl was added to 250 μl of GVB^{2+} containing 50 μl of human serum or heated serum and either 1 μCi each of [^{3}H]proline and [^{14}C]glycine or 1 μCi each of [^{3}H]thymidine and [^{14}C]uracil. The mixtures were incubated at 37°C, and at regular intervals 50-μl samples were taken and mixed with an equal volume of 10% trichloracetic acid (TCA). Precipitates were collected by centrifugation in an Eppendorf minifuge, dissolved in water, and reprecipitated by addition of an equal volume of 10% TCA. The precipitates were dissolved in 200 μl of water, and 4 ml of scintillation fluid (Pico-Fluor 15; Packard Instrument Co., Rockville, Md.) was added. Counting was performed with an LKB 1216 Rackbeta liquid scintillation counter.

Release of ^{86}Rb. Cells were harvested from DO medium, washed with GVB^{2+}, and preloaded with $^{86}Rb^+$ by incubation of 10^8 cells per ml with 20 μCi of ^{86}RbCl per ml at room temperature for 30 min. Extracellular $^{86}Rb^+$ was removed by washing the cells five times with GVB^{2+}. Loaded bacteria were added to the serum bactericidal system containing 50 μl of serum and incubated at 37°C. At intervals, 50-μl samples were removed and briefly centrifuged in an Eppendorf minifuge, and 40 μl of supernatant was transferred to a scintillation vial; 4 ml of Pico-Fluor 15 was then added to each vial, and radioactivity was counted in the Rackbeta counter.

Release of β-galactosidase. Production of β-galactosidase by *E. coli* LP1092 was induced by addition to the culture in DO medium of 5×10^{-3} M isopropyl-β-D-thiogalactoside (Sigma) 2 h before harvesting. Induced cells were exposed to 50 μl of serum in a total volume of 750 μl as described above, and at regular intervals samples were briefly centrifuged in an Eppendorf minifuge; 0.25 ml of supernatant was then added to 1 ml of *o*-nitrophenyl-β-galactoside (1 mg/ml) in GVB^{2+}. The tubes were incubated at 37°C until sufficient color had developed, and the absorbance at 405 nm was measured. Total intracellular β-galactosidase was determined with supernatants from freeze-thaw-fractured LP1092 cells.

Release of alkaline phosphatase. Cells were grown overnight in the limited phosphate medium of Garen and Levinthal (11), washed, and exposed to lysozyme-free serum as described for the serum bactericidal assay. At various times, samples were centrifuged (Eppendorf minifuge), and 125 μl of 5 mM *p*-nitrophenylphosphate in 1.0 M Tris (pH 8.0) was added to each 500 μl sample of supernatant. Color was allowed to develop at 37°C, and the absorbance was measured at 405 nm. Freeze-thaw-fractured cells served as controls. The periplasmic location of the enzyme was confirmed by the procedure of Neu and Heppel (36).

Complement assay. Quantitative assay of hemolytic complement activity, based on the 50% hemolytic

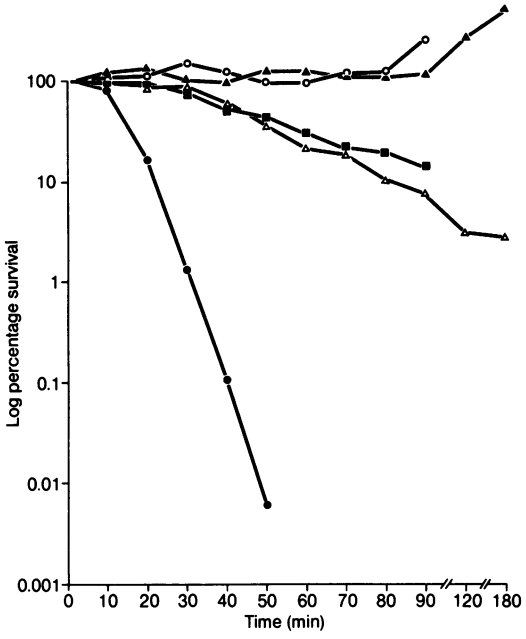

FIG. 1. Kinetics of killing of *E. coli* LP1092 by 6.67% human serum (50 μl in a total volume of 750 μl). Symbols: ●, human serum; ○, heated (56°C, 30 min) serum; ▲, serum plus 0.01 M dithiothreitol; △, serum plus 0.01 M Mg EGTA; ■, serum absorbed twice with heat-killed (100°C, 1 h) LP1092 cells. Serum absorbed twice with Formalin-treated LP1092 cells gave a response identical to that of serum absorbed with heat-killed cells. All points in this and subsequent figures of serum killing kinetics represent the means of at least three independent determinations.

complement unit of Mayer, was performed as described by Gewurz and Suyehira (13).

Measurement of ATP. Samples (10 μl) of the reaction mixture from the serum bactericidal assay were pipetted into a plastic vial containing 100 μl of Lumac NRB releasing agent. The vial was placed in a Luminometer 1250 photometer, and 100 μl of freshly prepared CLS Luciferin/Luciferase reagent (Boehringer Mannheim Corp.) was added. Measurements were taken after a constant signal was obtained.

RESULTS

Requirement for rapid killing. After exposure to 50 μl of human serum in a total volume of 750 μl, the viability of *E. coli* LP1092 cells was reduced at a constant rate; killing was preceeded by a 10-min lag period during which there was little change in the viable count (Fig. 1). Bactericidal activity was completely abolished by heat treatment (56°C, 30 min) of serum or by addition of 0.01 M dithiothreitol. Removal by absorption of antibodies directed against surface antigens of strain LP1092 resulted in a marked reduction in serum bactericidal activity. Similar killing kinetics were obtained when calcium ions, essential for classical pathway activity, were chelated with 0.01 M Mg EGTA (Fig. 1). Significant, rapid killing was obtained over a serum concentration range of 12.5 to 200 μl (total volume, 750 μl). Serum treated with zymosan possessed no bactericidal activity despite retaining a functional classical pathway as determined by hemolytic assay. The addition of heated (56°C, 30 min) serum to zymosan-treated serum did not restore bactericidal activity. Treatment of serum with inulin, an alternative pathway activator (17), resulted in an extension of the lag phase in the bactericidal reaction to 20 min and a reduction in the rate of killing (0.1% survivors after 70 min) in comparison with untreated serum. The hemolytic complement titer of inulin-treated serum was slightly reduced. Removal of all detectable serum lysozyme by either absorption with bentonite or neutralization with specific anti-human lysozyme IgG resulted in a small but reproducible reduction in the rate of serum killing of *E. coli* LP1092 (Fig. 2). The addition of 2 μg of egg-white lysozyme (EWL) per ml to bentonite-absorbed serum resulted in killing kinetics identical to those of untreated human serum, and the addition of higher concentrations of EWL further increased the rate of killing (Fig. 2). Bentonite absorption clearly removed, in addition to

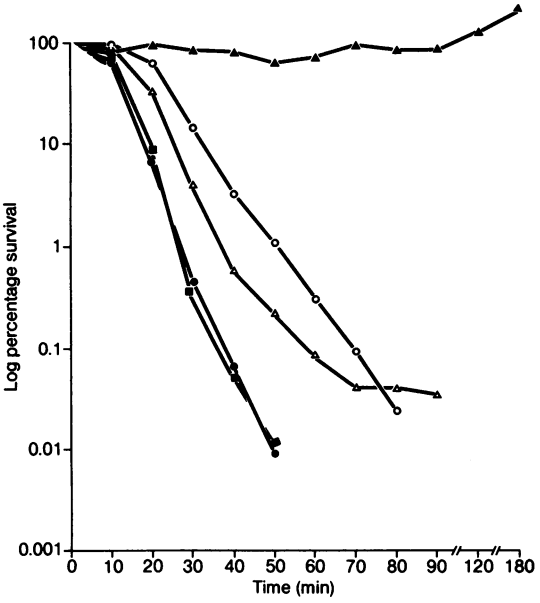

FIG. 2. Kinetics of killing of *E. coli* LP1092 by 6.67% human serum (50 μl). Symbols: ●, human serum; ○, bentonite-absorbed serum; ▲, bentonite-absorbed serum plus 0.01 M Mg EGTA; △, serum plus 10 μl of anti-human lysozyme IgG; ■, bentonite-absorbed serum plus 2 μg of EWL per ml.

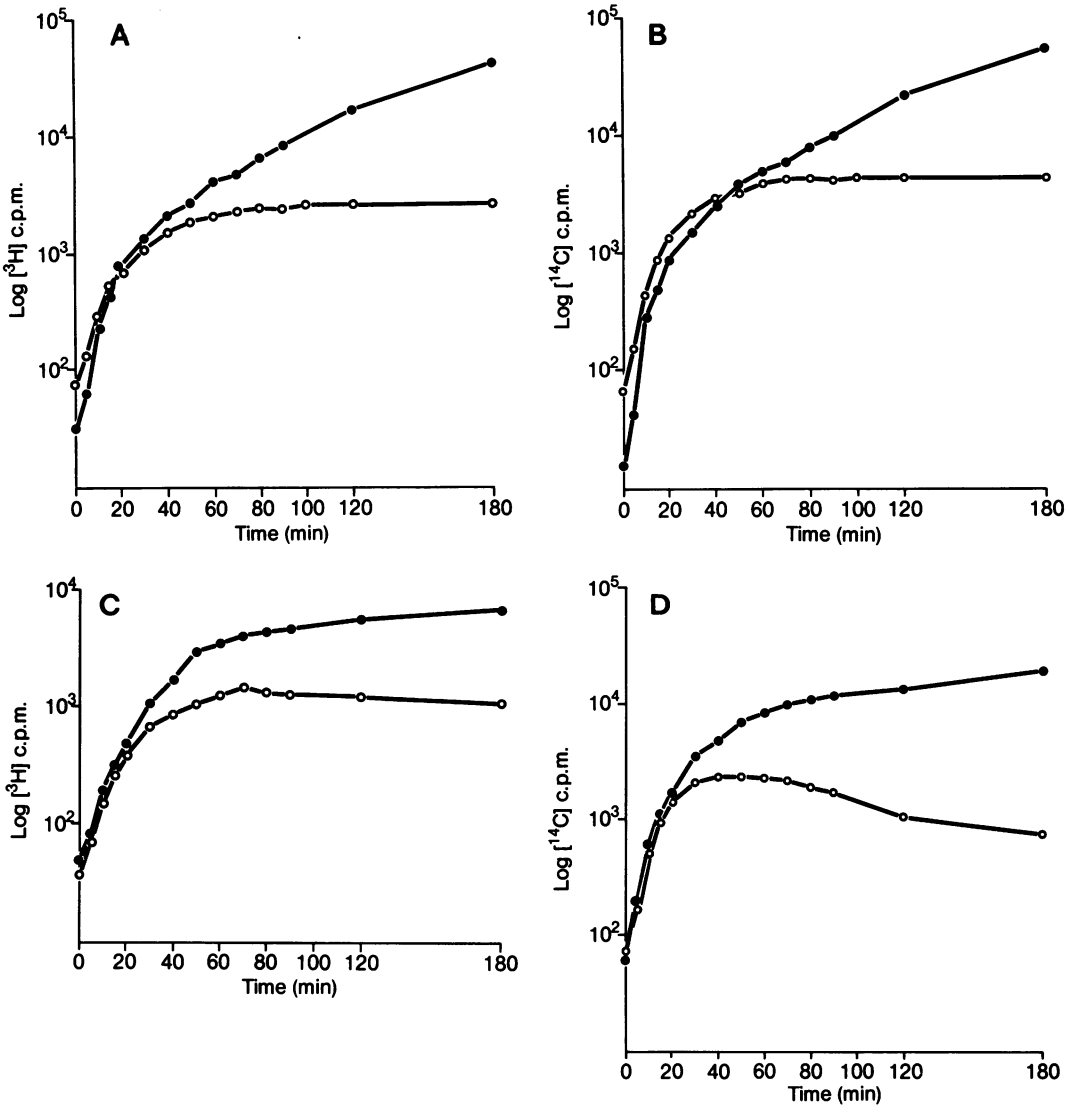

FIG. 3.Incorporation of [^{3}H]proline (A), [^{14}C]glycine (B), [^{3}H]thymidine (C), and [^{14}C]uracil (D) into TCA-precipitable material by *E. coli* LP1092 in the presence of 6.67% heated (56°C, 30 min) human serum (●) or 6.67% untreated human serum (○).

lysozyme, components essential for alternative pathway activity, as bentonite-absorbed serum possessed no bactericidal activity after the addition of 0.01 M Mg EGTA (Fig. 2). The addition of various concentrations of EWL to unabsorbed human serum also resulted in increased serum killing kinetics (data not shown).

Effects of serum on macromolecular biosynthesis. The effects of exposure to human serum on the ability of *E. coli* LP1092 to synthesize protein, DNA, and RNA were examined by following the incorporation of [^{3}H]proline, [^{14}C]glycine, [^{3}H]thymidine, and [^{14}C]uracil into TCA-precipitable material. A reduction in the rate of incorporation of [^{3}H]proline in the presence of human serum, as compared with incorporation in the heated serum control, became evident after 40 min, and incorporation ceased completely after 70 min (Fig. 3A). Similar data were obtained for incorporation of [^{14}C]glycine into protein (Fig. 3B). In both cases there was no loss of radioactive label from TCA-precipitable material, indicating no breakdown of labeled protein over the 180-min incubation period. Inhibition of [^{3}H]thymidine incorporation into DNA could be detected after 30 min and was complete

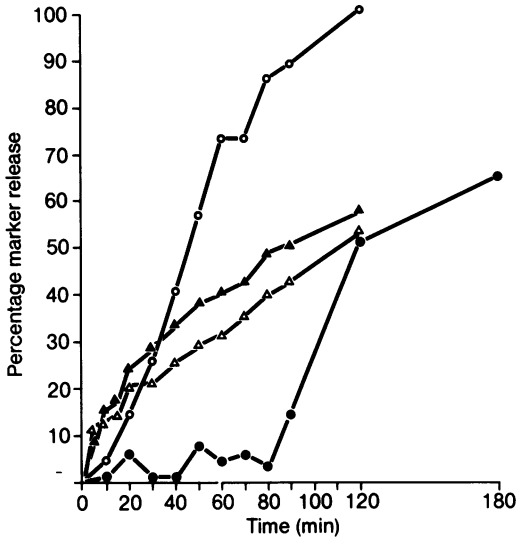

FIG. 4, Kinetics of marker release from *E. coli* LP1092 after treatment with 6.67% lysozyme-free human serum (50 μl). Symbols: ●, β-galactosidase; ○, alkaline phosphatase; ▲, $^{86}Rb^+$; △, $^{86}Rb^+$ released by heat-inactivated (56°C, 30 min) serum.

after 60 min (Fig. 3C). The rate of incorporation of [^{14}C]uracil into RNA in the presence of human serum deviated from the control rates after 20 to 30 min of incubation, and the amount of label in TCA-precipitable material reached a maximum after 40 min (Fig. 3D); the reduction in the amount of [^{14}C]uracil-labeled TCA-precipitable material after further incubation suggested a breakdown of RNA.

Inhibition of macromolecular synthesis was also seen when human serum was depleted of lysozyme by treatment with anti-human lysozyme IgG; the inhibition of [^{3}H]proline, [^{14}C]glycine, [^{3}H]thymidine, and [^{14}C]uracil incorporation into TCA-precipitable material was similar to that observed with untreated human serum except that the effect was slightly delayed. Differences in the rate of incorporation of [^{14}C]uracil between test and control experiments were again detectable at a slightly earlier stage than with incorporation of precursors into DNA and protein.

Release of markers from serum-treated bacteria. In agreement with the results of other workers using *E. coli* B or K12 strains (6, 23, 51), treatment of LP1092 cells with lysozyme-free serum did not cause a significant release of β-galactosidase, an intracellular enzyme, during the first 60 to 90 min of the reaction (Fig. 4). Prolonged incubation (up to 3 h) resulted in the release of 50 to 70% of the enzyme. The release of β-galactosidase by untreated human serum was not much greater than that observed with lysozyme-free serum, probably indicating that, in the system used in this study, little lysozyme-dependent bacteriolysis occurred. The kinetics of release of alkaline phosphatase by lysozyme-free serum are also shown in Fig. 4. After a lag of about 10 min, enzyme appeared in supernatants, and the amount steadily increased over the reaction period; 100% of the enzyme was released during the first 2 h of reaction. No release of alkaline phosphatase could be detected when cells were treated with heated (56°C, 30 min) serum. The release of $^{86}Rb^+$, a K^+ analog, from LP1092 cells was also determined over the course of the serum bactericidal reaction (Fig. 4). $^{86}Rb^+$ release occurred more slowly than alkaline phosphatase release; after 2 h, only 50 to 60% of the analog was released from preloaded cells. The pattern of release obtained with heated (56°C, 30 min) serum did not differ significantly from that found with lysozyme-free (IgG-treated) serum.

Effect of inhibitors on the rate of serum killing of strain LP1092. The addition of nalidixic acid, a selective inhibitor of DNA-dependent DNA biosynthesis (4), to the serum bactericidal assay system in concentrations of up to 20 μg/ml had no detectable effect on the rate of killing of *E. coli* LP1092. In contrast, the rate of killing of

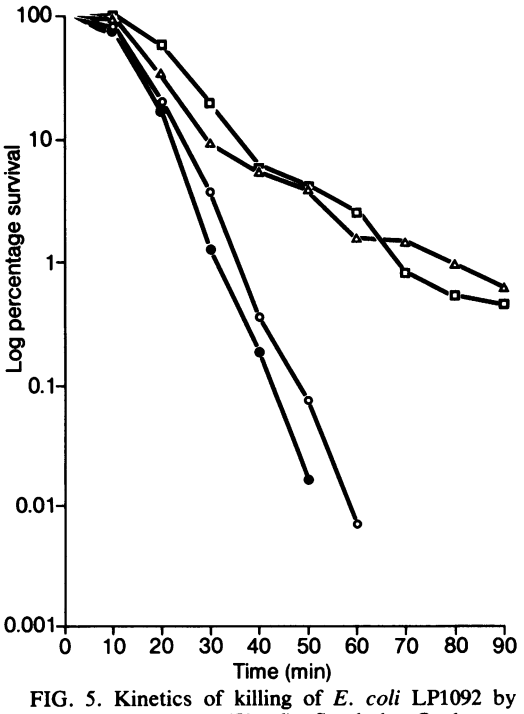

FIG. 5. Kinetics of killing of *E. coli* LP1092 by 6.67% human serum (50 μl). Symbols: ●, human serum; ○, serum plus chloramphenicol (1 μg/ml); △, serum plus chloramphenicol (10 μg/ml); □, serum plus chloramphenicol (100 μg/ml).

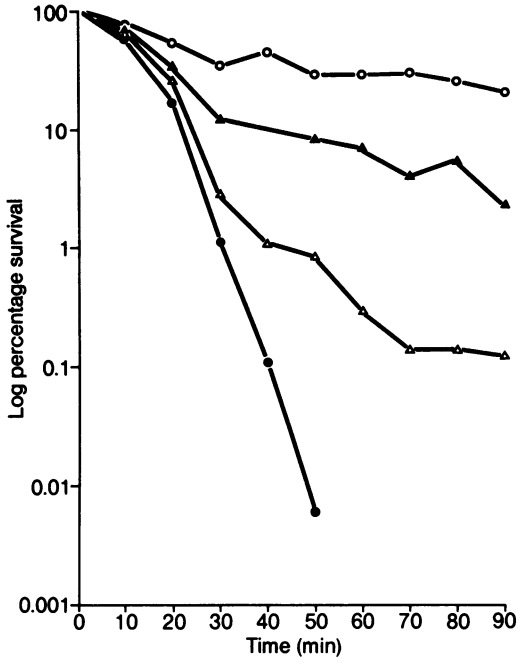

FIG. 6. Kinetics of killing of *E. coli* LP1092 by 6.67% human serum (50 μl). Symbols: ●, human serum; ○, serum plus 5 mM KCN added at the beginning of the experiment; ▲, serum plus 5 mM KCN added after 8 min; △, serum plus 5 mM KCN added after 18 min.

strain LP1092 was reduced in the presence of chloramphenicol (Fig. 5), an antibiotic that specifically inhibits protein biosynthesis by binding to the 50S subunit of the 70S ribosome (9). The degree of inhibition of serum killing was related to drug concentrations up to 10 μg/ml, a concentration coincident with the minimal inhibitory concentration. In the presence of 10 μg of chloramphenicol per ml, significant killing of strain LP1092 occurred, with less than 1% of the cells surviving 90 min of exposure to serum; the rate of killing was, however, much less than that observed in control experiments. No further reduction in the rate was observed over the concentration range of 10 to 100 μg of chloramphenicol per ml (Fig. 5). When bacteria were exposed to serum in the presence of 5 mM KCN, almost complete inhibition of complement killing occurred (Fig. 6); this was so whether untreated or bentonite-absorbed human serum was used. When the KCN was added to the system after 8 min of incubation at 37°C, the degree of inhibition was much reduced; an even greater reduction was seen when cyanide was added after 18 min (Fig. 6). KCN had no effect on the hemolytic complement titer of human serum, nor did it interfere with the binding of antigen to antibody in the assay. A similar degree of inhibition of serum killing of strain LP1092 was found with 5 mM 2,4-dinitrophenol.

The finding that an inhibitor (KCN) and an uncoupler (2,4-dinitrophenol) of bacterial oxidative energy metabolism could practically abolish the antibacterical action of human serum suggested to us that the killing mechanism of serum might have similarities to the mechanism of action of certain colicins, such as E1, K, and Ia. These proteins are thought to kill susceptible *E. coli* strains by depolarization of the membrane potential through the formation of ion-permeable channels in the bacterial cytoplasmic membrane (26, 42). Membrane-active colicins therefore inhibit respiration-linked active transport systems (7), cause a rapid efflux of intracellular K^+ (50), and lower cellular ATP levels (7). We measured ATP levels associated with serum-exposed LP1092 cells, and the results from a typical experiment are shown in Fig. 7. In the bactericidal system containing untreated human serum, there was a rapid rise in intracellular ATP levels during the first 30 min of the incubation period, followed by a less rapid decrease to a level below that found at time zero. Essentially identical curves were found when bentonite-

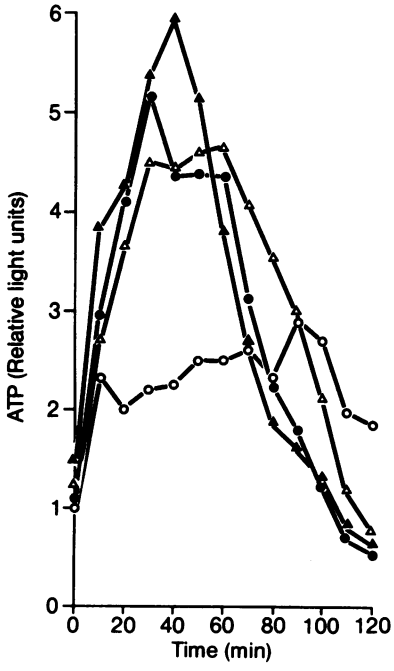

FIG. 7. Effect of 6.67% human serum (50 μl) on ATP levels of *E. coli* LP1092. Symbols: ●, human serum; ○, human serum dialyzed against GVB^{2+}; ▲, dialyzed human serum in bactericidal system containing GVB^{2+} plus 2.5% glucose; △, dialyzed human serum plus 50 μl of serum ultrafiltrate. Heated (56°C, 30 min) human serum produced an ATP response identical to that found with untreated human serum.

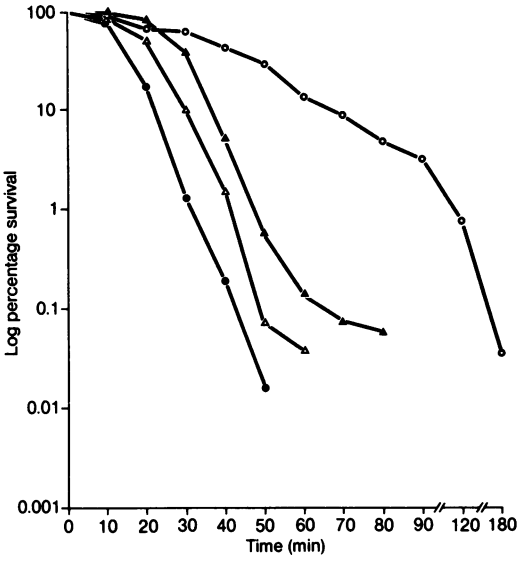

FIG. 8. Kinetics of killing of *E. coli* LP1092 by 6.67% human serum (50 μl). Symbols: ●, human serum; ○, human serum dialyzed against GVB^{2+}; ▲, dialyzed human serum in bactericidal system containing GVB^{2+} plus 2.5% glucose; △, dialyzed human serum plus 50 μl of serum ultrafiltrate.

absorbed serum or heat-inactivated serum was used; almost all (>95%) detectable ATP cosedimented with the serum-treated cells and was therefore most likely to be located intracellularly. Incubation of solutions of ATP with human serum resulted in no detectable reduction in measurable ATP, indicating that failure to detect extracellular ATP was unlikely to be due to hydrolysis of ATP by serum. The rapid rise in intracellular ATP levels did not occur when strain LP1092 was exposed to human serum that had been extensively dialyzed against GVB^{2+} at 0°C (Fig. 7), suggesting that the ATP was generated from low-molecular-weight energy sources, such as glucose, present in serum. When assayed for bactericidal activity in the presence of GVB^{2+}, dialyzed serum was found to kill LP1092 cells only very slowly (Fig. 8). However, when killing was assayed with dialyzed serum by using GVB^{2+} containing 2.5% glucose (13), the rapid increase in intracellular ATP was again observed (Fig. 7), and much of the bactericidal activity of the system could be restored (Fig. 8). An even greater killing effect was noted when 50 μl of an ultrafiltrate (Amicon PM10 membrane; molecular weight, <10,000) of human serum was added to the bactericidal system containing dialyzed serum (Fig. 8).

DISCUSSION

Although the complement-mediated bactericidal action of serum was first described nearly a century ago (reviewed by Inoue [22]), the molecular basis of the phenomenon has not been subjected to intensive investigation, partly due to the commonly held view that complement action against gram-negative bacteria is strictly analogous to that against erythrocytes (see, for example, reference 41). However, as recently emphasized (29, 51), there are clear differences between the two types of target cell that must be taken into account when trying to explain complement action. The present investigation was undertaken, therefore, to attempt to throw some light on the sequence of events that occurs after exposure of susceptible *E. coli* cells to human serum and that results in the loss of cell viability.

E. coli LP1092 was rapidly killed by human serum after a lag of about 10 min. It would appear that the major part of this bactericidal effect is contributed by antibody-dependent activation of the classical complement pathway, as both absorption of antibody and chelation of calcium, an essential cation for C1 function and hence classical pathway activity (27), produced a very large reduction in the rate of killing. The antibody is directed against a heat-stable component of the bacterial cell surface, because heat-killed and Formalin-treated cells were equally effective as absorbents (Fig. 7). Although in the majority of our experiments serum was used at a 15-fold dilution, it still possessed significant antibody-dependent alternative pathway activity against strain LP1092; previous workers have also found the rate of killing of gram-negative bacteria via alternative pathway activation is much reduced in comparison with classical pathway activity, especially when diluted serum is used (38, 40). It is unclear whether both pathways are activated simultaneously, although there is some indication that this is likely to be so (10, 38). Incubation of serum with inulin, a treatment known to cause depletion of factor B and hence alternative pathway activity (17), resulted in a reduction in the rate of killing, confirming previous work with inulin-treated serum (17, 38). However, inulin treatment also reduces the serum concentration of some of the late-acting components common to both complement pathways (17), and the effect of this on the rate of serum killing is difficult to assess. An attempt to deplete serum of properdin, a nonessential enhancer of alternative pathway bactericidal activity (43), by incubation at 17°C with zymosan (46) surprisingly resulted in the loss of all detectable bactericidal activity. Zymosan absorption at this temperature removes properdin (49) and also some lysozyme (34), but does not deplete serum of hemolytic complement (classical pathway) activity; zymosan-treated serum was, in fact, as hemolytically active as untreated serum. Zymosan had, therefore, removed an

essential factor for serum bactericidal activity that was not a component of the classical pathway. The addition of heat-inactivated serum to zymosan-treated serum did not restore bactericidal activity, indicating that the essential factor was not antibody. Either properdin is an essential factor for classical pathway killing of strain LP1092 or, more likely, zymosan removed a previously unrecognized factor essential for classical pathway killing of *E. coli* LP1092.

It is well established that lysozyme (mucopeptide *N*-acetylmuramyl hydrolase) is essential for serum lysis of gram-negative bacteria (24, 29, 48) and that it gains access to its peptidoglycan substrate after the formation of a functional C5b-9 complex (14, 25). Far less clear is the role, if any, of lysozyme in the bactericidal reaction. Serum killing occurs in the complete absence of lysozyme (25, 43), and it has frequently been asserted that the enzyme does not influence the rate of serum killing (12, 29, 33). Other studies have, however, tended to contradict these findings. For example, Inoue and co-workers (24) found that the addition of 5 μg of lysozyme per ml to antibody and complement both accelerated and enhanced the bactericidal effect; this lysozyme concentration is within the normal range of serum values. Lysozyme enhanced the bactericidal activity of complement in the presence of sheep IgG (20), and Glynn and Milne (15) found a large reduction in the rate and extent of killing after neutralization of human serum lysozyme activity with anti-human lysozyme antiserum. Killing rates could be restored by addition of EWL. They noted an even larger reduction in killing when lysozyme was absorbed from serum with bentonite. The reasons for these conflicting findings are unclear, but there are differences in methodology that may go some way toward explaining these data. It has been common practice to remove lysozyme activity from serum by absorption with bentonite (15, 24, 46, 48), but this procedure is known to remove gram-positive bactericidins (35) that may possess activity against gram-negative species (3, 5). Our data demonstrate that bentonite absorption also removed a factor(s) essential for alternative pathway function, as Mg EGTA-treated, bentonite-absorbed serum possessed no bactericidal activity (Fig. 2).

To assess the contribution of lysozyme to serum bactericidal activity against strain LP1092, therefore, we used two methods for neutralization of enzyme activity; serum was absorbed with bentonite and also treated with anti-human lysozyme IgG. Both procedures removed all detectable lysozyme activity and also resulted in sera with reduced killing activity which could be completely restored by addition of small, physiological amounts of EWL. The addition of EWL to untreated serum resulted in an enhancement of killing activity that was related to EWL concentration. The results clearly demonstrate that lysozyme can, to a limited extent, affect the rate of serum killing of strain LP1092, but the observed differences are not of the same order of magnitude as those described by Glynn and Milne (15). As the primary complement lesion responsible for the killing of cells occurs at the cytoplasmic membrane (6, 51), depolymerization of peptidoglycan might remove one barrier to the rapid assembly of the functional lesion at this site, and this would almost certainly be reflected in an increased rate of killing.

Exposure to serum results in an inhibition of macromolecular biosynthesis. As noted by other workers (18, 29, 31), inhibition of RNA biosynthesis seems to precede inhibition of either DNA or protein biosynthesis. Inhibition would appear, however, to be secondary to the primary event responsible for the bactericidal effect, as a logarithmic reduction in viability was evident well before the inhibition of incorporation became apparent. Our data do not, therefore, support the view of Griffiths (18) that cell death is an event subsequent to the cessation of RNA biosynthesis. On the other hand, the observed reduction in the rate of killing in the presence of chloramphenicol (Fig. 5) suggests that a certain amount of protein biosynthesis is essential for optimum killing.

It has frequently been noted that bacteria in the logarithmic phase of growth are more readily killed by serum than those in the lag or stationary phases, and this has given rise to the idea that a certain amount of cellular biosynthetic activity is necessary for serum killing. For example, addition of metabolizable carbohydrates to serum bactericidal systems enhances serum sensitivity (32). Our data would seem to indicate that cell division is not necessary for killing, as nalidixic acid, an inhibitor of DNA-dependent DNA biosynthesis, had no effect on the rate of serum killing over a wide concentration range. However, at least one stage of the killing process appears to be dependent on cell-generated energy sources, as the addition of an inhibitor or an uncoupler of oxidative phosphorylation to our bactericidal system resulted in complete inhibition of killing. Inhibition was much reduced when KCN was added to the reaction mixture after 8 or 18 min, indicating that the energy-dependent step occurs early in the reaction, before loss of viability becomes apparent. If the analogy to certain colicins is pursued, the step could involve the translocation of the membrane attack complex from the outer membrane to a site where it can interact with the cytoplasmic membrane. The bacteria would therefore

take an active part in their own death, as suggested by Griffiths (19).

E. coli LP1092 cells synthesize large amounts of intracellular ATP during the first 30 min of the bactericidal reaction; the size of the intracellular pool is then steadily diminished by a process that does not appear to involve leakage. Increased metabolic activity was also observed by Sevag and Miller (44), who noted that stimulation of oxygen consumption followed exposure to serum. In our system, removal by dialysis of carbon and energy sources from serum resulted in very little intracellular ATP generation and loss of a large part of serum killing activity; both of these functions were restored by the addition of glucose, thus providing additional evidence of an energy requirement for serum killing. A rapid rise in intracellular ATP levels was also recorded after exposure of bacteria to heat-inactivated serum; this effect appears, therefore, to be related to metabolism of utilizable substrates and is not a consequence of complement activity.

It recently has been suggested by Wright and Levine (51) that complement simultaneously damages both the outer and cytoplasmic membranes of *E. coli*, as evidenced by identical release kinetics of an intracellular cation (the K^+ analog $^{86}Rb^+$) and a periplasmic enzyme (alkaline phosphatase). They suggested that the coordinate loss of cytoplasmic and periplasmic markers results from the formation of a C5b-9 terminal dimer at a point of adhesion (or fusion) of cytoplasmic and outer membranes. We were unable to confirm simultaneous damage to both membranes (Fig. 4). The release of all cellular aklaline phosphatase occurred after exposure to serum but lagged behind the decrease in viability; $^{86}Rb^+$ efflux, however, proceeded at a much lower rate and was not dependent on a functional complement pathway. These observations led us to undertake a study of the complement-induced release of $^{86}Rb^+$ from a number of *E. coli* strains of various serum sensitivity, and these data will be reported elsewhere. We suggest, however, that the analogy that has recently been drawn between complement killing and the mechanism of action of some colicins may be too simplistic. After colicin treatment, there is a rapid efflux of K^+, dissipation of the membrane potential, rapid lowering of intracellular ATP levels, and recovery of viability after plating out on media containing high K^+ concentrations. We have been unable to detect a significant complement-induced efflux of $^{86}Rb^+$ from strain LP1092, and intracellular ATP levels increase rather than decrease after exposure to serum. Plating of serum-treated LP1092 cells on plates containing 0.2 M KCl offers no protection against serum bactericidal activity (P. W. Taylor, unpublished data). The loss of small molecules from the cytoplasm of strain LP1092 must be selective, as very little ATP could be found outside the outer membrane of serum-treated cells. The question of the degree of membrane perturbation could be directly resolved by monitoring the state of membrane energization of *E. coli* exposed to serum.

We suggest, therefore, that after exposure of susceptible *E. coli* LP1092 cells to serum, the late-acting components of complement are assembled into the C5b-9 complex on the outer membrane. The complex is then rapidly translocated, by a mechanism requiring an input of bacterially generated energy, to a site at the inner membrane, causing damage resulting in the release of periplasmic markers and certain low-molecular-weight cytoplasmic constituents. The membrane potential may be dissipated, but the cell makes no attempt to recover lost cations by exploiting intracellular reserves of ATP. The ability of the cells to form colonies on agar plates begins to be rapidly lost after a 10-min lag period, but nonviable cells continue to generate ATP, and synthesis of RNA, DNA, and protein continues at near maximal rates. After about 40 min, RNA synthesis ceases and is followed by loss of protein and DNA-synthesizing ability. Cells that are unable to translocate the C5b-9 complex escape damage to the cytoplasmic membrane and remain viable.

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