

# BACTERICIDAL ACTIVITY OF THE ALTERNATIVE COMPLEMENT PATHWAY GENERATED FROM 11 ISOLATED PLASMA PROTEINS\*

By ROBERT D. SCHREIBER,<sup>‡</sup> DAVID C. MORRISON,<sup>§</sup> ECKHARD R. PODACK,<sup>||</sup>  
and HANS J. MÜLLER-EBERHARD<sup>¶</sup>

*From the Departments of Molecular Immunology and Immunopathology, Research Institute of Scripps Clinic,  
La Jolla, California 92037*

It has recently been demonstrated in this laboratory (1) that the composite mixture of the isolated alternative pathway proteins (C3, factor B, factor D,  $\beta$ 1H, C3b inactivator, and properdin) and the isolated membrane attack pathway proteins (C5, C6, C7, C8, and C9) constitutes an intact cytolytic alternative pathway quantitatively comparable to that of human serum. In these studies, mammalian cells were used as activating target cells. Because of apparent implications for our understanding of nonspecific resistance to infections, we tested the isolated component system with respect to its ability to eradicate gram-negative bacteria. Using *Escherichia coli* K12 W1485 we found that the 11 component system was capable of killing the bacteria in the total absence of immunoglobulins or other serum factors. Killing was associated with distinct morphological changes of the bacteria, but not with disintegration. Disintegration or lysis required, in addition to the 11 proteins, lysozyme.

Information regarding the bactericidal activity of the alternative pathway is scarce. That the proteins of the alternative pathway can be involved in bactericidal reactions against certain bacterial strains has been demonstrated through the use of factor B-depleted human serum (2) and C4-deficient guinea pig serum (3). Whether specific antibody is required or not has remained uncertain (4-11). The requirement of lysozyme for complement-dependent lysis of gram-negative bacteria has been well documented (12, 13). However, all requirements of accessory factors and of late acting complement components known to date have been elaborated by studying bacteriolysis via the classical pathway and serum reagents rather than isolated proteins.

## Materials and Methods

*Buffers.* VB: veronal-buffered physiological saline, pH 7.4; VB<sup>++</sup>: VB containing 0.15 mM CaCl<sub>2</sub> and 0.5 mM MgCl<sub>2</sub>; HBS: Hank's balanced salt solution; and GHBS:<sup>1</sup> HBS containing 0.1% gelatin.

*Purified Components.* C3 (14), factor B (2), factor D (15), C3b inactivator (16),  $\beta$ 1H (16),

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<sup>||</sup> Recipient of Established Investigatorship 79-149 of the American Heart Association.

<sup>¶</sup> Cecil H. and Ida M. Green Investigator in Medical Research, Research Institute of Scripps Clinic.

<sup>1</sup> Abbreviations used in this paper: ICM, isolated complement mixture; GHBS, Hank's balanced salt solution containing 0.1% gelatin; TSB, trypticase soy broth.

native properdin (17), C5 (14), C6 (18), C7 (18), C8 (19), and C9 (20) were prepared as described. Purity of the isolated proteins was assessed, in part, by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Representative gel patterns have been published previously (1). In this study, at least two different preparations of each protein were used. Egg white lysozyme [three times recrystallized] was purchased from Sigma Chemical Co. (St. Louis, Mo.). Human lysozyme was purchased from Worthington Biochemical Corp. (Freehold, N. J.). Proteins were radioiodinated using the lactoperoxidase method of David and Reisfeld (21).

*Preparation of the Isolated Component Mixture.* The mixture of purified components at their physiological concentrations was prepared as described (1). Possible C3b contamination of C3 preparations was eliminated by incubation of C3 with appropriate amounts of  $\beta$ 1H and C3b inactivator just before the addition of the other eight isolated proteins. Final component concentrations were: 1,200  $\mu$ g/ml C3, 200  $\mu$ g/ml factor B, 2  $\mu$ g/ml factor D, 470  $\mu$ g/ml  $\beta$ 1H, 34  $\mu$ g/ml C3b inactivator, 20  $\mu$ g/ml native properdin, 72  $\mu$ g/ml C5, 64  $\mu$ g/ml C6, 54  $\mu$ g/ml C7, 54  $\mu$ g/ml C8, and 58  $\mu$ g/ml C9. Before use in the bactericidal assays, the isolated component mixture was tested for the ability to lyse rabbit erythrocytes as described previously (1) and compared with C4-depleted serum.

*C4-Depleted Human Serum.* Normal human serum was immunochemically depleted of C4 by utilizing a Sepharose 4B column (Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Piscataway, N. J.) to which the IgG fraction of monospecific anti-human C4 serum had been coupled (22).

*Bacteria.* *E. coli* K12 W1485 were kindly supplied to us in a lyophilized state by Dr. John Spizizen (Research Institute of Scripps Clinic). Organisms were cultured and maintained on 1.3% agar slants containing 1.0% Difco protease peptone No. 3 (Difco Laboratories, Detroit, Mich.), 0.1% Difco bacto beef extract (Difco Laboratories), and 0.5% NaCl. Broth cultures were established by transferring 25  $\mu$ l of slant culture to 5 ml of 3% trypticase soy broth (TSB) (BBL Microbiology Systems, Becton, Dickinson & Co., Cockeysville, Md.) and by incubation for 18 h at 37°C.

*<sup>33</sup>P-Labeling of E. coli.* The medium used for labeling was a Tris buffered, minimal phosphate medium, pH 7.4, containing per liter the following materials: 0.022 g  $K_2HPO_4$ , 0.48 mg NaCl, 7.6 g KCl, 0.096 g  $MgSO_4$ , 11.6 g Trizma base (Sigma Chemical Co., St. Louis, Mo.), 0.096 ml 1 M  $CaCl_2$ , 1.0 g  $NH_4Cl$ , 2.0 g glucose, and 20 ml of 5% casamino acids (23). 10  $\mu$ l of TSB culture containing  $\approx 10^8$  *E. coli* was added to 10 ml of Tris buffered, minimal phosphate medium containing 50  $\mu$ Ci of <sup>33</sup>P-labeled orthophosphate (New England Nuclear, Boston, Mass.). Bacterial growth was allowed to proceed in a shaking incubator for 16 h at 37°C at 400 rpm. Bacteria were harvested by centrifugation at 10,000 g for 10 min at 4°C in a Sorvall RC2B centrifuge (DuPont Instruments-Sorvall, DuPont Co., Newtown, Conn.). After washing three times with phosphate-buffered saline and two times with GHBS, bacteria were resuspended to a concentration of  $5 \times 10^8$  *E. coli*/ml and standardized spectrophotometrically at 650 nm where  $5 \times 10^8$  bacteria/ml = 0.8 adsorbance units. Incorporation of <sup>33</sup>P was 37-40% of total, with the resulting *E. coli* exhibiting a specific activity of 300,000 cpm/ $10^7$  bacteria. Distribution of <sup>33</sup>P in the labeled *E. coli* was determined using the method of Roberts et al. (24). Approximately 83% of the radiolabel was incorporated into nucleic acids, 5% into phospholipids, and the remaining 12% into non-trichloroacetic precipitable material which includes lipopolysaccharides.

*Deposition of Complement Proteins onto Bacteria.* Reaction mixtures of 400  $\mu$ l contained 200  $\mu$ l of bacteria ( $1 \times 10^9$ /ml) in GHBS, 200  $\mu$ l of either C4-depleted serum or the isolated component mixture, and 0.5  $\mu$ Ci of radio-iodinated C3 or nP or C9. After periods of time at 37°C, 40- $\mu$ l aliquots were withdrawn and specific binding determined after separation of cell bound and free radioactive protein as described elsewhere (25). Controls contained heat-inactivated serum or heat-inactivated isolated component mixture (56°C, 30 min), bacteria and radiolabeled proteins.

*Assays of Bactericidal and Bacteriolytic Activity.* Total reaction mixtures of 45  $\mu$ l which contained  $8 \times 10^8$  <sup>33</sup>P *E. coli*, 25  $\mu$ l GHBS, 0.15 mM  $CaCl_2$ , 0.5 mM  $MgCl_2$ , and varying amounts of C4-depleted serum or isolated component mixture were incubated at 37°C. Controls included heat-inactivated (56°C, 30 min) C4-depleted serum, heat-inactivated (56°C, 30 min) isolated component mixture and buffer. Effects on viability were determined by plating on agar. 5- $\mu$ l samples of the reaction mixtures were diluted 20,000-fold and 50- $\mu$ l aliquots applied to agar

plates containing 3% TSB. Triplicate plates were incubated for 18 h at 37°C and the number of colonies determined.

Bacteriolysis was determined as a function of  $^{33}\text{P}$  release into the supernate. 30  $\mu\text{l}$  of the reaction mixture was layered onto a 300  $\mu\text{l}$  cushion of 20% sucrose dissolved in  $\text{VB}^{++}$  in a 400  $\mu\text{l}$  polyethylene microfuge tube. Separation of cell associated and released radioactivity was achieved by centrifugation at 13,000  $g$  for 3 min in a Beckman microfuge 152 (Beckman Instruments, Inc., Clinical Instrument Div., Fullerton, Calif.). Tubes were clamped with a hemostat 5 mm above the bottom and the pelleted cells discarded by sectioning the tube at the lower edge of the hemostat with a razor blade. The tubes were inverted into 20-ml scintillation vials and the hemostat released. The sucrose solution which contained released  $^{33}\text{P}$  was forced out the top of the tube by pressure exerted with a 12-ml syringe from the tube bottom. Total radioactivity was determined by addition of 30  $\mu\text{l}$  of reaction mixture and 300  $\mu\text{l}$  of sucrose solution directly into a scintillation vial. Radioactivity of  $^{33}\text{P}$  was assessed using a Beckman LS-230 liquid scintillation system (Beckman Instruments, Inc., Clinical Div.) employing a scintillation cocktail which consisted of 5% acetic acid, 8%  $\text{H}_2\text{O}$ , and 87% Scintiverse (Fisher Scientific Company, Pittsburgh, Pa.).

*Transmission Electron Microscopy.* Washed samples were prepared by negative staining with 12 mM uranyl oxalate, pH 6.8, as described elsewhere (26). Electron microscopy was performed with a Hitachi 12 A electron microscope (Perkin-Elmer Co., Mountain View, Calif.) operated at 75 kV with a direct magnification of 50,000.

*Scanning Electron Microscopy.* Washed samples containing  $2 \times 10^6$  treated *E. coli* were applied to a 0.4- $\mu$  nucleopore filter (Nuclepore Corp., Pleasanton, Calif.) which had been pretreated with 1% poly-L-lysine (70,000–100,000 dalton mol wt, Sigma Chemical Co.). Filter-bound bacteria were fixed in a modified Karnofsky fixative containing 0.72% glutaraldehyde (EM grade), 1.02% paraformaldehyde and 0.125 M cacodylate buffer, pH 7.0. Samples were dehydrated and dried in a Bomar SPC-900/EX critical point drier (The Bomar Co., Tacoma, Wash.) using Freon 13 (Matheson Co., Cucamonga, Calif.) as the transition fluid. Electron microscopy was performed with a Hitachi 500-SEM at 25 kV at a 5-mm working distance.

*Light Scattering.* Reaction mixtures of 200  $\mu\text{l}$  contained  $1.75 \times 10^7$  *E. coli* K12 W1485, 50  $\mu\text{l}$  GHBS and 150  $\mu\text{l}$  of either the isolated component mixture or heat inactivated (56°C, 30 min) isolated components or buffer. Reactions were performed at 37°C in 400  $\mu\text{l}$  microcuvettes in a Gilford 240 spectrophotometer equipped with a thermostatically controlled cuvette changer (Gilford Instrument Laboratories Inc., Oberlin, Ohio). Light scattering changes were measured as the change in adsorbance at 650 nm as a function of time. At the end of the reaction period, a sample of bacteria was removed from each cuvette, diluted, and viability determined by plating.

## Results

*Activation of the Alternative Pathway by E. coli K12 W1485 in Absence of Immunoglobulins.* The bacteria were incubated at 37°C for 30 min with either isolated component mixture or C4-depleted serum. 400  $\mu\text{l}$  of reaction mixture contained  $2 \times 10^8$  *E. coli* and one-half physiological concentrations of either isolated component mixture or C4-depleted serum. 40- $\mu\text{l}$  samples were withdrawn at timed intervals and after removal of unbound protein, specific uptake of radiolabeled C3b, properdin, and C9 was quantitated. Fig. 1 shows the time-course of uptake of the three proteins by the bacterial cells. After an initial lag of  $\approx 5$  min, C3b and properdin deposition commenced and reached a maximum at 20–25 min. At that time, the number of molecules bound per bacterium was 35,000 for C3b and 12,000 for properdin. These values represent 1% and 25% of C3 and properdin input, respectively. For technical reasons, C9 deposition was measured at 15-min intervals. Therefore, the time-course does not reveal an initial lag, but shows that in C4-depleted serum deposition is maximal only at 30 min. A total of 40,000 C9 molecules were bound per bacterium, which represents 17% of input and is equivalent to  $\approx 10,000$  C5b-9 dimers (27). These results show that

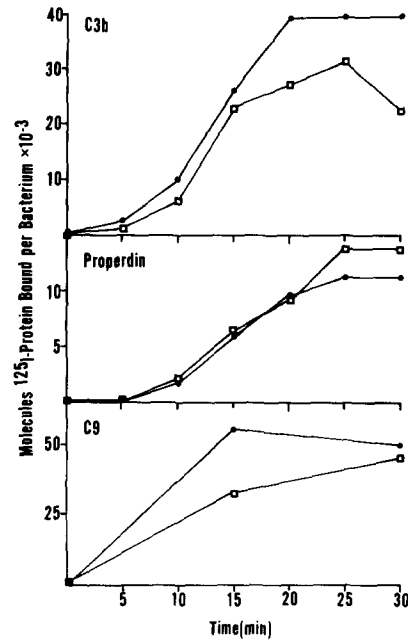


FIG. 1. Comparable kinetics of activation by *E. coli* K12 W1485 in the isolated component mixture and in C4-depleted human serum, as evidenced by quantitative measurements of C3b, properdin, and C9 deposition. Reactions were performed as described in Materials and Methods. □, C4-depleted serum; ●, isolated component mixture.

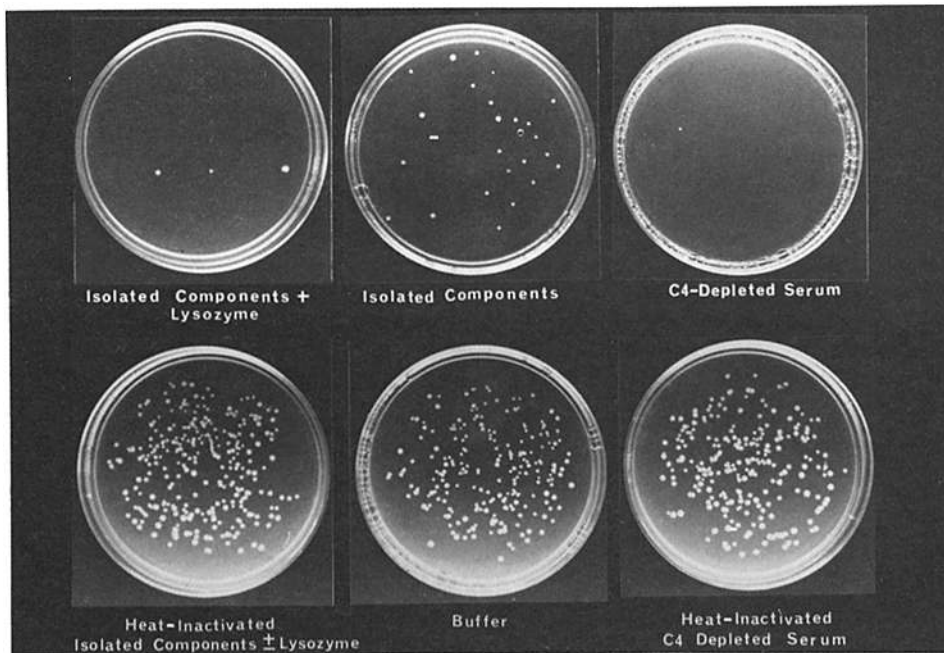


FIG. 2. Loss of viability of *E. coli* K12 W1485 by treatment with the isolated component system: Lack of lysozyme requirement. Reactions were performed as described in Methods. Loss of viability was determined by plating. The concentration of egg white lysozyme used was 10  $\mu$ g/ml of isolated component mixture.

TABLE I  
*Differentiation between Bactericidal and Bacteriolytic Activity of the Isolated Component Mixture*

Treatment	No. of colonies	<sup>33</sup> P release
		%
Buffer	217 ± 7	14.6
ICM + Lysozyme*	3 ± 1	62.6
ICM	23 ± 5	19.3
ICM (56°C, 30 min) + Lysozyme	284 ± 11	13.6
C4-Depleted Serum	0	55.5
C4-Depleted Serum (56°C, 30 min)	220 ± 18	6.9

ICM, isolated component mixture.

\* Lysozyme concentration was 10 µg/ml.

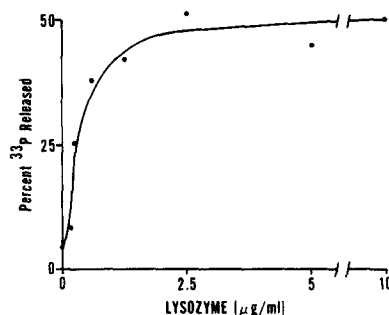


FIG. 3. Lysozyme requirement for the lysis of *E. coli* K12 W1485 by the isolated component mixture. Reaction mixtures of 45 µl contained 10 µl isolated component mixture,  $8 \times 10^5$  <sup>33</sup>P-labeled *E. coli*, GHBS, and concentrations of egg white lysozyme varying from 0.16 to 10 µg/ml of component mixture. Incubations were allowed to proceed at 37°C for 60 min, after which time lysis was assessed by measurement of <sup>33</sup>P release as described in Methods. Controls for each concentration of lysozyme involved utilization of heat inactivated (56°C, 30 min) isolated components. None of these controls effected <sup>33</sup>P release which was greater than the buffer control.

this strain of *E. coli* was capable of activating the cytolytic alternative pathway and that such activation can be achieved in the total absence of immunoglobulins.

*Differentiation between Killing and Lysis of E. coli K12 W1485 by the Isolated Component Mixture.* The effects of activation of the component mixture on the bacterial cells were measured by determining viability and release of intracellular <sup>33</sup>P (lysis). Fig. 2 and Table I show the following results: (a) the isolated components, per se, killed the bacteria without causing specific <sup>33</sup>P release; (b) killing and <sup>33</sup>P release occurred when the bacteria were incubated with the component mixture containing lysozyme; (c) heated components plus lysozyme caused no killing and no <sup>33</sup>P release, indicating that lysozyme alone was insufficient to induce lysis; (d) C4-depleted serum, which contains lysozyme, effected killing and lysis to an extent comparable to the isolated component mixture.

Fig. 3 depicts the dependence of <sup>33</sup>P release on the concentration of lysozyme in the isolated component mixture. The plateau of the dose-response curve is reached at 2.5-5 µg/ml, while 7 µg/ml correspond to the activity of lysozyme in human serum. That 4% of <sup>33</sup>P is released in absence of lysozyme may be related to the extraction of some cell wall phospholipids by the membrane attack complex (26, 28) or to the complement dependent escape of low molecular weight phosphorus compounds from the periplasmic space (13).

TABLE II  
Control Experiments of the Bactericidal and Bacteriolytic Isolated Component System  
(Including Lysozyme)

Components deleted	No. of colonies	<sup>33</sup> P release*
		%
None	0	47.4
Lysozyme	1	1.7
Six alternative pathway proteins	79	0.0
C5-C9	76	0.0
C8, C9	76	4.0
C9	80	0.0
Factor B	84	0.1
All (buffer)	86	0.0

\* Corrected for spontaneous <sup>33</sup>P release.

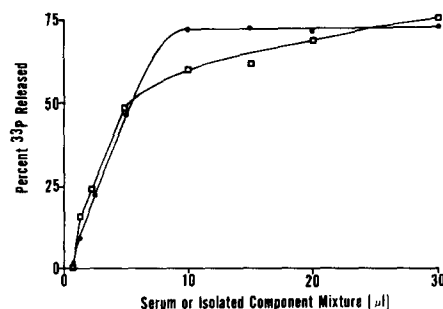


FIG. 4. Dose-dependent lysis of *E. coli* K12 W1485 by the 11 isolated proteins of the cytolytic alternative pathway and lysozyme. Incubations at 37°C for 60 min were performed as described in Methods using the isolated component mixture or C4-depleted serum. Lysozyme concentrations used were 10 μg egg white lysozyme/ml isolated component mixture. ●, isolated component mixture plus lysozyme; □, C4-depleted serum.

The control experiments are listed in Table II. No killing and no lysis was observed when the reaction mixture lacked the six alternative pathway proteins or the five membrane attack pathway proteins, or when either factor B, or C8 and C9, or C9 were deleted.

*Quantitative Comparison of the Bacteriolytic Activity of the Isolated Component Mixture and C4-Depleted Serum.* Both the component mixture and the serum contained comparable concentrations of the respective complement proteins and lysozyme. Fig. 4 demonstrates that the bacteriolytic activity of the component mixture is virtually identical to that of C4-depleted serum, 50% of maximal <sup>33</sup>P release was obtained with 4.0 μl and 3.6 μl of the mixture or serum, respectively. Similarly, the kinetics of lysis were comparable for both reaction mixtures, with lysis approaching completion after 45 min at 37°C (Fig. 5).

Because the function of the alternative pathway is highly sensitive to dilution (29, 30), we tested the dependence of the bacteriolytic activity on protein concentration using the isolated component mixture. Fig. 6 demonstrates that at a dilution of 1:11 the activity was reduced to 50% and that at 1:16 it became insignificant.

*The Nonessential Role of Properdin in the Lysis of E. coli K12 W1485.* Because properdin was originally viewed as a key component of the alternative pathway (31), we tested its requirement for lysis of *E. coli* K12 W1485. Fig. 7 shows that the isolated

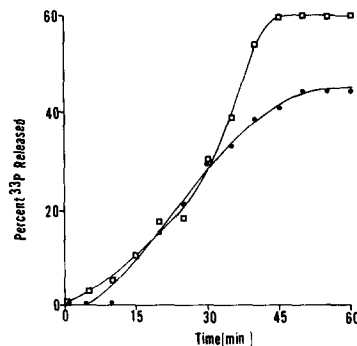


FIG. 5. Kinetics of alternative pathway lysis of *E. coli* K12 W1485: comparison of the isolated component mixture and C4-depleted human serum. Reaction mixtures of 446  $\mu$ l contained  $2.2 \times 10^7$  <sup>33</sup>P-labeled *E. coli*, and 221  $\mu$ l of either the isolated component mixture containing 4.5  $\mu$ g egg white lysozyme or C4-depleted serum. Controls contained either heat-inactivated C4-depleted serum or heat-inactivated isolated component mixture or buffer. Reaction mixtures were brought to 37°C, and at designated times, 30- $\mu$ l samples were removed and <sup>33</sup>P release determined.  $\square$ , C4-depleted serum;  $\bullet$ , isolated components + lysozyme.

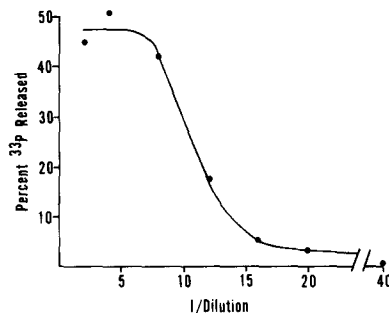


FIG. 6. Effect of dilution of the lysis of *E. coli* K12 W1485 by the isolated component mixture and lysozyme. Reaction mixtures contained 10  $\mu$ l isolated component mixture, 100 ng egg white lysozyme,  $8 \times 10^5$  <sup>33</sup>P-labeled *E. coli* and varying amounts of GHBS to yield the indicated dilutions. Incubations at 37°C were performed for 60 min. A constant percentage of the volume of each reaction mixture was used to determine release of intracellular <sup>33</sup>P. Separate controls for nonspecific radiophosphorus release and total radioactivity were performed for each dilution.

components lacking properdin are capable of generating bacteriolytic activity. Therefore, properdin cannot be considered an essential component in this system. Its presence, in physiological amounts, however, led to an apparent threefold increase in bacteriolytic activity.

*Distinct Electron Microscopic Appearances of Killed and Lysed E. coli K12 W1485.* The results presented so far indicate that dependent on the presence or absence of lysozyme, the isolated component system effected either killing or lysis of the exposed bacteria. Because killing was C9 dependent and because adsorption of C9 results in formation of the membrane attack complex (C5b-9)<sub>2</sub>, the killed bacteria were examined for the presence of the characteristic complement lesions by transmission electron microscopy and compared with lysed bacteria. Fig. 8 demonstrates that both killed and lysed bacteria exhibit comparable numbers of complement lesions which, in both cases, are of similar size and appearance. Both preparations also showed similar irregularities in surface structure which are most apparent along the edges of the cells

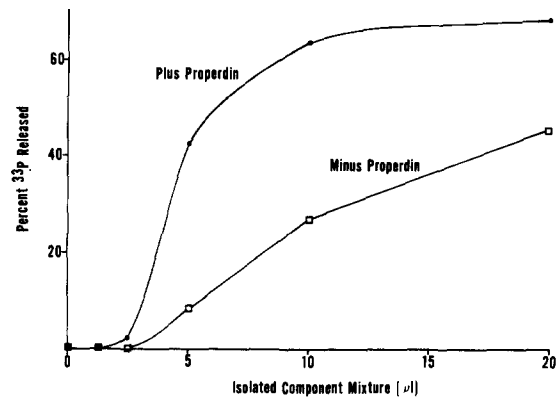


FIG. 7. The enhancing, regulatory role of properdin for lysis of *E. coli* K12 W1485. Dose-dependent lysis of <sup>33</sup>P-labeled *E. coli* with the isolated component mixture either containing or lacking properdin were performed as described in Methods. Lysozyme concentrations used were 10 μg egg white lysozyme/ml of isolated component mixture. Incubations were performed at 37°C for 60 min.

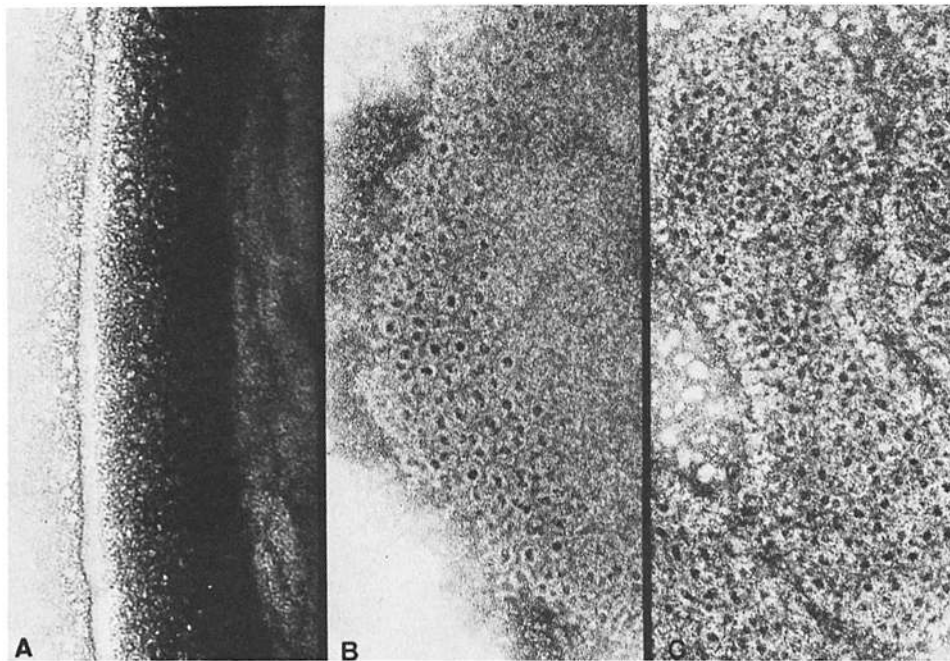


FIG. 8. Transmission electron microscopy of *E. coli* after treatment with the isolated component mixture lacking (B) or containing (C) lysozyme. Control (A) was treated with heat-inactivated isolated components and lysozyme. Magnification: × 250,000.

or cell fragments. These structures may be a result of the marked reorganization of surface phospholipid molecules known to be caused by the membrane attack complex (26).

By scanning electron microscopy (Fig. 9) the killed bacteria exhibited a larger size than the controls. The size had increased approximately twofold and irregular surface protrusions became evident. In addition, the killed bacteria displayed sizable depressions of the cell wall. These marked morphological changes correlated with killing



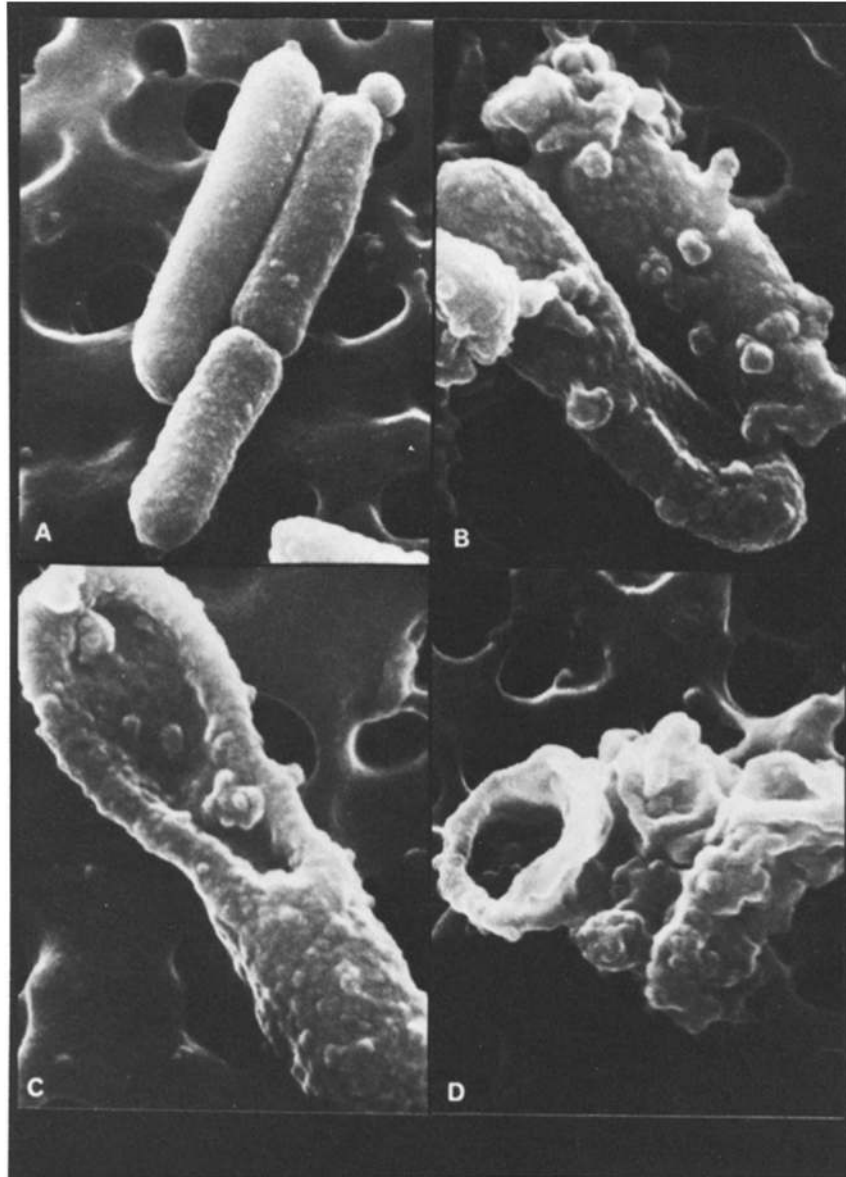


FIG. 9. Scanning electron microscopy of alternative pathway killed or lysed *E. coli* K12 W1485. Shown are *E. coli* treated at 37°C for 60 min with buffer or the heat-inactivated (56°C, 30 min) isolated component mixture (A), the isolated component mixture (B, C), or the isolated component mixture containing 10 µg/ml egg white lysozyme or C4-depleted serum (D). Concomitant analysis of viability and lysis revealed that compared to the control (A), samples B and C were killed and not lysed, whereas sample D was killed and lysed. Magnification: × 30,000.

and were strictly dependent on C9. In contrast, the lysed cells had lost any resemblance to their controls and had assumed a random polymorphic appearance.

The apparent swelling of the killed bacteria compared to controls was verified by light scattering measurements. Fig. 10 shows an analysis of the effect of the isolated component mixture on the adsorbance at 650 nm as a function of time. After 60 min

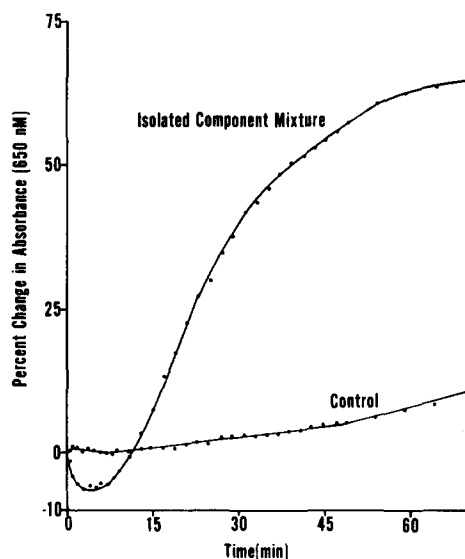


FIG. 10. Increased light scattering by *E. coli* K12 W1485 during treatment with the isolated component mixture. *E. coli* were incubated either with the isolated component mixture or with the heat-inactivated isolated component mixture (control) at 37°C as described in Methods. Adsorbance changes were followed at 650 nm.

at 37°C an increment of 65% was recorded. At the end of these measurements, the viability of the treated bacteria was determined and found to be 30% of control. The data are consistent with the conclusion that killing increases light scattering of a bacterial suspension approximately twofold.

### Discussion

This study constitutes the first demonstration of the generation of bactericidal activity through interaction of the 11 isolated plasma proteins which comprise the cytolytic alternative pathway. It also provides a clear example that the alternative pathway is capable of expressing bactericidal activity in the complete absence of immunoglobulins. The results are not at variance with observations made by others (8–11) that suggest immunoglobulins are involved in the bactericidal function of the alternative pathway. Our preliminary results indicate that with certain strains of *E. coli* the addition of immunoglobulins to the isolated component system has a positive effect on killing. According to current views, the ability to differentiate between cells that can or cannot activate the alternative pathway, however, resides in C3b (25). It has been proposed that a low magnitude fluid phase C3 convertase, the initial enzyme of the alternative pathway, deposits C3b in a random fashion onto available surfaces of biological particles. Deposition of C3b occurs via its enzymatically exposed metastable binding site. Subsequently, bound C3b interacts with critical surface constituents of activators through a second, discriminating site. This interaction allows uncontrolled formation of the activator-bound C3 convertase, C3b,Bb and thus amplification. When the discriminating interaction involving C3b does not occur, such as on nonactivating particles, C3b is cleaved and inactivated by  $\beta$ 1H and C3b inactivator and formation of the C3 convertase is prevented.

Activator-bound C3 convertase catalyzes the binding of many C3b molecules and

the formation of multiple C3-convertase complexes. As a result, C5 convertase is formed, properdin is recruited as a stabilizer of the enzyme, and membrane attack is initiated. Although properdin is the protein that originally revealed the existence of the alternative pathway and although it was thought to be the key component of the pathway (31), the results presented here shows that it fulfills no essential role in the particular bactericidal or bacteriolytic reaction examined in this study. This observation is in agreement with previous work utilizing different systems which assigned to properdin the function of an enhancing regulator of the alternative pathway (22, 25). That Pillemer et al. (31) considered properdin an essential component of the system may be explained in part by its enhancing activity which, as shown with the isolated component mixture, is most apparent at low component concentrations (Fig. 7). Although properdin appears to have only a minor effect on the bactericidal reaction, it does have a marked effect on C3b deposition which varies with the strain of bacteria tested as preliminary results have shown. In this manner properdin may influence the fate of susceptible bacteria *in vivo*.

To confer on the bactericidal alternative pathway bacteriolytic activity, the addition of lysozyme to the component mixture was necessary. The electron microscopic analyses showed that in absence of lysozyme, the membrane attack complex clearly formed within the outer lipid bilayer: typical complement membrane lesions were observed which have been shown to constitute the C5b-9 dimer (27). These analyses also revealed additional changes, namely an uneven structure of the cell surface which is similar to that of membrane attack complex modified liposomes (26) and which has been attributed to the unusual phospholipid binding capacity of the complex. Concomitantly, the unlysed bacterium increases the surface area of its outer membrane approximately twofold. In spite of these drastic changes, no efflux of  $^{33}\text{P}$ -containing cytoplasmic constituents occurred. These same changes of the outer phospholipid membrane probably afford lysozyme access to the underlying peptidoglycan layer, enzymatic degradation of which eventuates in lysis.

Although our results shows that *E. coli* K12 W1485 can be killed by the 11 cytolytic alternative pathway proteins, we do not wish to imply that susceptibility to killing by these proteins is a general property of gram-negative bacteria. Future work will have to determine the extent to which this property is shared by other strains. Even though certain strains may resist killing by the membrane attack complex, they may allow activation of the alternative pathway and opsonization by C3b and thus become susceptible to phagocytosis. It is conceivable on the basis of kinetic considerations that in the presence of phagocytic cells, i.e., polymorphonuclear leukocytes, ingestion precedes killing and lysis in the fluid phase. Work is underway to determine the fate of susceptible bacteria in the isolated component system into which phagocytic cells have been introduced.

### Summary

Exposure of *E. coli* K12 W1485 to the cytolytic alternative pathway assembled from the 11 isolated pathway proteins resulted in killing of the bacteria, as evidenced by loss of viability. Lysis of the bacteria required introduction of lysozyme into the reaction mixture. The time-course and dose dependency of bacteriolysis in the isolated system were identical to those in C4-depleted serum. The bacteriolytic activity of the pathway was highly dependent on the concentration of the pathway proteins and

became insignificant at 1:16 physiological concentration. Electron microscopic visualization of killed and of lysed bacteria revealed numerous complement membrane lesions and partial disintegration of the outer phospholipid membrane. Scanning electron microscopy showed that killed bacteria were enlarged, partially collapsed and exhibited irregular surface protrusions. Lysed bacteria were fragmented and appeared polymorphic. This study demonstrates that the alternative pathway, in absence of immunoglobulins, has the potential of eradicating gram-negative bacteria.

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