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Requirements for Immunoglobulin and the Classical and Alternative Complement Pathways for Phagocytosis and Intracellular Killing of Multiple Strains of Gram-Negative Aerobic Bacilli

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The requirements for immunoglobulin and the alternative and classical complement pathways for phagocytosis and intracellular killing of clinical isolates of *Escherichia coli, Proteus mirabilis, Klebsiella pneumoniae,* and *Serratia marcescens* by normal human polymorphonuclear leukocytes were determined. Human sera deficient in immunoglobulin or classical pathway activity, or both, were compared for their ability to promote phagocytosis and killing of 13 bacterial strains by the polymorphonuclear leukocytes. Seven of the thirteen microorganisms required immunoglobulin for phagocytosis and killing and utilized only the classical complement pathway. Three required immunoglobulin and utilized both the classical and alternative pathways. The other three microorganisms required minimal immunoglobulin and utilized the alternative or classical pathway, or both. None of the microorganisms utilized the alternative pathway in immunoglobulin-deficient sera or could be forced to utilize this pathway in sera deficient in both immunoglobulin and classical pathway activity. These results demonstrated a heterogeneity in the requirements for immunoglobulin and the alternative and classical complement pathways for phagocytosis and intracellular killing by polymorphonuclear leukocytes among various genera of gram-negative aerobic bacilli, as well as among strains of the same species. In addition, the results suggested that a mechanism of classical pathway activation dependent upon minimal immunoglobulin participates in phagocytosis and intracellular killing of certain gram-negative aerobic bacilli.

The widespread use of antibiotics has failed to decrease the incidence of infections caused by gram-negative aerobic bacilli (7, 10, 23). These microorganisms are the predominant etiological agents of urinary tract infections and endogenous and nosocomial infections (7, 10, 23). Patients receiving immunosuppressive therapy and those whose defense mechanisms have been compromised by disease or trauma are unusually susceptible to severe infections caused by these microorganisms (3, 4, 7, 37).

Phagocytosis and intracellular killing by polymorphonuclear leukocytes (PMNs) are the host's primary defense mechanism against infections caused by gram-negative aerobic bacilli. However, knowledge of the serum proteins which participate in these events is fragmentary. In several studies, participation of the alternative complement pathway in phagocytosis and intracellular killing of *Escherichia coli* (12, 18, 19, 33), *Pseudomonas aeruginosa* (1, 2), *Serratia marcescens* (11), and *Proteus mirabilis* (19) by PMNs was demonstrated. In other studies, classical pathway activity was shown to be required for phagocytosis and killing of *P. aeruginosa* (11, 41). The requirement for immunoglobulin in normal serum for phagocytosis and killing of gram-negative aerobic bacilli by PMNs is also controversial. Immunoglobulin was shown to be required for phagocytosis and killing of *E. coli* (40), *P. aeruginosa* (1, 2, 40), and *Proteus vulgaris* (40) by PMNs, but not for *S. marcescens* (40). In another study, immunoglobulin was not shown to be required for phagocytosis and killing of *E. coli* (18).

In all of the studies cited above, single strains of the microorganisms were used, and no studies have been performed utilizing multiple strains of the same species. The purpose of the present investigation was therefore to determine the requirements for immunoglobulin and classical and alternative pathway activities for phagocy-

tosis and intracellular killing of multiple strains of *E. coli*, *P. mirabilis*, *S. marcescens*, and *Klebsiella pneumoniae* by human PMNs. *K. pneumoniae* was included in our study because the frequency of isolation of this microorganism from patients with hospital-acquired pneumonias has increased (37), and virtually no information is available regarding its opsonic requirements. Most of the microorganisms used in this study were isolated from burn patients, although other clinical isolates were included to determine if differences in serum protein requirements for phagocytosis and intracellular killing between the isolates could be demonstrated.

MATERIALS AND METHODS

Bacteria. Strains of *E. coli*, *P. mirabilis*, *K. pneumoniae*, and *S. marcescens* were isolated from cultures obtained from surgical and medical patients at the Cincinnati General Hospital or Shriners Burn Institute, Cincinnati, Ohio. *E. coli* (A), (P), and (H), *P. mirabilis* (H), *K. pneumoniae* (Wo) and (W), and *S. marcescens* (W) and (S) were isolated from blood cultures. *E. coli* (F), *P. mirabilis* (A), and *K. pneumoniae* (H) were isolated from burn wound cultures, *K. pneumoniae* (B) was from a sputum culture, and *P. mirabilis* (C) was from a urine culture. Stock cultures were maintained in brain heart infusion broth at −70°C. Frozen cultures were inoculated into brain heart infusion broth and incubated for 18 h at 37°C. Bacteria were harvested and washed twice in Hanks balanced salt solution (HBSS) containing 0.0013 M calcium ions and 0.001 M magnesium ions (Microbiological Associates, Walkersville, Md.). The microorganisms were then resuspended in HBSS to a final concentration of 1.0×10^7 cells per ml.

Sera. Pooled normal human serum (PNHS) was obtained by pooling sera from 25 healthy adult donors. Individual normal sera were obtained from three adult volunteers. Individual hypogammaglobulinemic sera (HS_{1-3}) were kindly provided by John Partin, Childrens Hospital, Cincinnati, Ohio. HS_{1-3} contained 185 to 475 mg of immunoglobulin (Ig) G per 100 ml and undetectable levels of IgA and IgM. C2-deficient human serum (C2dHS) was kindly provided by Paul Quie, University of Minnesota School of Medicine, Minneapolis, Minn. Hemolytic C3 to C9, C3 conversion by inulin and cobra venom factor, and immunochemical concentrations of IgG, IgA, and IgM were found to be normal in C2dHS; total hemolytic complement and immunochemical C2 were not detectable in this serum. PNHS was treated with 10 mM ethyleneglycol-bis-(β-amino-ethyl-ether)-*N*,*N*′-tetraacetic acid (EGTA) (Sigma Chemical Co., St. Louis, Mo.) and supplemented with 10 mM $MgCl_2$ to block classical complement pathway activity (MgEGTA-PNHS) (9). In some experiments, HS_{1-3} were treated with MgEGTA and referred to as MgEGTA-HS_{1-3}. Hemolytic C3 to C9 and C3 conversion by inulin and cobra venom factor in MgEGTA sera and untreated sera were found to be equivalent. Total hemolytic complement in MgEGTA sera was undetectable, indicating that treatment of the sera with MgEGTA blocked classical pathway activity. Decomplemented serum (ΔPNHS) was prepared by heating PNHS at 56°C for 30 min. All sera were stored in small volumes at −70°C.

Preparation of human serum further depleted of IgG by immunoadsorption (HS-A). The IgG fraction of rabbit antiserum to human IgG (Behring Diagnostics, Somerville, N.J.) was prepared by ammonium sulfate precipitation followed by chromatography on diethylaminoethyl-cellulose (35). The eluted immunoglobulin fraction migrated as a single arc in the gamma region, as determined by immunoelectrophoretic analysis using rabbit anti-human serum.

The IgG fraction of rabbit anti-human IgG was coupled to Sepharose 4B (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.) by a minor modification of the method of March et al. (26). Sepharose 4B was washed three times with distilled water, mixed with 1 volume of distilled water, and added to 1 volume of 2 M sodium carbonate. A 0.05-ml volume of cyanogen bromide solution (2 g of cyanogen bromide to 1 ml of acetonitrile) was added to the slurry and stirred vigorously at 4°C. The preparation was washed on a coarse sintered-glass funnel with 10 volumes each of cold 0.1 M sodium carbonate (pH 9.5), distilled water, and coupling buffer (0.1 M borate–0.5 M NaCl, pH 9.0). The preparation was then filtered to a cakelike consistency. The prepared gel was added to 1 volume of cold coupling buffer in a plastic bottle which contained the IgG fraction of anti-human IgG (10 mg of protein to 1 ml of gel). The preparation was tumbled end over end on a rotating platform for 20 h at 4°C. Unreacted groups were blocked with 1 M ethanolamine (pH 9.0) for 4 h at 4°C. The prepared gel was washed several times with 20 volumes each of coupling buffer and acetate buffer (0.1 M sodium acetate–0.5 M NaCl, pH 4.0). The prepared gel was washed three times with starting buffer (0.1 M borate–0.5 M NaCl–0.01 M ethylenediaminetetraacetic acid, pH 7.4), and poured into a 0.9- by 15-cm column. The column was equilibrated overnight at 4°C with starting buffer.

Hypogammaglobulinemic serum was dialyzed overnight against starting buffer and applied to the immunoadsorbent column at a flow rate of 10 ml/h at 4°C. The sample was eluted with starting buffer and concentrated to the original volume. The sample was chromatographed two additional times over the same column and then dialyzed extensively against 0.01 M phosphate-buffered saline, pH 7.0. HS-A contained 22 mg of IgG per 100 ml and undetectable levels of IgA and IgM. Total hemolytic complement, hemolytic C3 to C9, and C3 conversion by inulin and cobra venom factor in HS-A and PNHS were equivalent.

Preparation of human PMNs. Blood samples from normal adult volunteers were collected in plastic tubes containing 10 U of heparin per ml and 2 ml of 6% dextran in saline, pH 7.0. After sedimentation of the erythrocytes at room temperature for 1 h, the leukocyte-rich plasma was removed and centrifuged at $200 \times g$ for 5 min. Leukocytes were washed three times and suspended in HBSS to contain 10^7 PMNs per ml.

Bactericidal assays. A minor modification of the method of Hirsch and Strauss was used to measure PMN bactericidal activity (16). Reaction mixtures consisted of 1.0×10^6 bacteria, 5.0×10^6 PMNs, serum,

and HBSS in a final volume of 1 ml in plastic-capped tubes. HBSS was substituted for the leukocytes or serum in the controls. The reaction mixtures and controls were incubated at 37°C on a rotating platform, and samples were removed at 0 time and after 60 and 120 min of incubation. Three 10-fold dilutions of the samples were made in distilled water to rupture the leukocytes, and the dilutions were plated on brain heart infusion agar. Colonies were counted after overnight incubation at 37°C to determine the total number of surviving bacteria in each reaction mixture. Serum concentrations used in the assays were based on the minimal amount of PNHS which promoted maximal phagocytosis and intracellular killing of each bacterial strain during the shortest incubation period.

For leukocyte bactericidal assays in which Mg-EGTA sera were tested, the diluent used for the experiments was HBSS lacking calcium and magnesium ions (Grand Island Biological Co., Grand Island, N.Y.) to which 0.001 M $MgCl_2$ was added. This diluent was also used for preparation of PMNs and bacteria in these experiments. Untreated PNHS and hypogamma-globulinemic serum were always included in these experiments to insure that their activities were identical to that in the bactericidal assays in which HBSS containing calcium and magnesium ions was utilized.

In initial experiments, the total number of viable bacteria surviving in the reaction mixtures after lysis of the PMNs was compared to the number of viable extracellular bacteria surviving in the supernatants of the reaction mixtures. Similar counts were obtained indicating that phagocytosis of the test strains was rapidly followed by intracellular killing.

Radial immunodiffusion. A minor modification of the single radial immunodiffusion method of Mancini et al. was used to measure concentrations of immunoglobulins, B antigen of C3, and C2 (25). For assaying IgG, IgA, and IgM, agarose (1%) was dissolved in 0.01 M phosphate buffered saline, pH 7.0. For assaying B antigen of C3 and C2, agarose (1%) was dissolved in Veronal buffer ($\mu = 0.05$, pH 8.6) containing 0.04 M ethylenediaminetetraacetic acid. Agarose and the appropriate concentration of antiserum were poured into plastic plates; 2-mm-diameter wells were filled, and diffusion was carried out at room temperature for 24 h. Standard reference sera for IgG, IgA, and IgM were purchased from Behring Diagnostics. Reference serum for B antigen of C3 was kindly provided by Clark West, Childrens Hospital Research Foundation, Cincinnati, Ohio. PNHS was used as the reference serum for C2.

Rabbit antisera to human IgG, IgA, and IgM were purchased from Behring Diagnostics. Antiserum to B antigen of C3 was prepared as previously described (39). Antiserum to human C3 was raised in goats by multiple subcutaneous injections of human C3 (Cordis Laboratories, Miami, Fla.) in saline containing 0.01 M ethylenediaminetetraacetic acid, pH 7.0, mixed with complete Freund adjuvant. The antiserum was absorbed with aged PNHS to remove antibodies to the A and D determinants of C3. Antiserum to human C2 was raised in goats by multiple intravenous injections of human C2 (Cordis Laboratories) in saline containing 0.04 M ethylenediaminetetraacetic acid, pH 7.0. The antisera yielded single lines against PNHS in immunodiffusion and immunoelectrophoresis and gave complete identity in immunodiffusion with reference goat antisera to C2 and B antigen of C3. The reference antisera were kindly provided by C. West.

Measurements of total hemolytic complement, C3 conversion, and hemolytic C3 to C9. Total hemolytic complement was titrated by the method of Kabat and Mayer (21). C3 conversion utilizing reduction in the B antigenic determinant of C3 by radial immunodiffusion was performed as described by Ruley et al. (34). Inulin (100 mg/ml) or cobra venom factor (500 U/ml) in saline was added to sera at a ratio of 1:10, and the sera were incubated at 37°C for 1 h. After centrifugation, supernatants were tested for residual B antigen of C3. Results were compared to the concentration of B antigen of C3 in saline-treated sera.

Hemolytic C3 to C9 was determined by a minor modification of the method of Borsos and Rapp (5). $EAC1_{gp}4_{hu}$ cells (Cordis Laboratories) were washed three times and suspended in Veronal buffer containing 2.5% glucose, 0.1% gelatin, 0.00015 M $CaCl_2$, and 0.0005 M $MgCl_2$ to a final concentration of 10^8 cells per ml. Equal volumes of EAC14 cells and 1,000 U of human C2 (Cordis Laboratories) in Veronal buffer containing 2.5% glucose, 0.1% gelatin, 0.00015 M $CaCl_2$, and 0.0005 $MgCl_2$ were incubated at 30°C for 2.5 min (t_{max}). A 0.5-ml volume of EAC142 cells was added to 0.25 ml of serum dilutions in Veronal buffer containing 0.025 M ethylenediaminetetraacetic acid and 0.1% gelatin at 4°C. After addition of 0.5 ml of Veronal buffer containing 0.025 M ethylenediaminetetraacetic acid and 0.1% gelatin, the tubes were incubated at 37°C for 60 min. After centrifugation, free hemoglobin was determined spectrophotometrically at 415 nm. Results were expressed in total hemolytic complement units per milliliter.

RESULTS

The ability of the various sera to promote phagocytosis and intracellular killing of *E. coli* by the PMNs was first determined. Hypogammaglobulinemic sera were unable to support phagocytosis and intracellular killing of *E. coli* (F) in comparison to PNHS or individual normal sera (Fig. 1). MgEGTA-PNHS, C2dHS, and ΔPNHS also did not promote phagocytosis and intracellular killing of this strain. In contrast, hypogammaglobulinemic sera and HS-A supported normal phagocytosis and intracellular killing of *E. coli* (P) (Fig. 2). MgEGTA-PNHS, MgEGTA-HS_{1-3}, C2dHS, and ΔPNHS were unable to promote phagocytosis and intracellular killing of this strain. These results indicated that the requirement for immunoglobulin was minimal for effective phagocytosis and intracellular killing of *E. coli* (P), but that immunoglobulin was required for phagocytosis and killing of *E. coli* (F). Although complement was required for phagocytosis and intracellular killing of these strains, the alternative complement pathway was not utilized. In addition, the results indi-

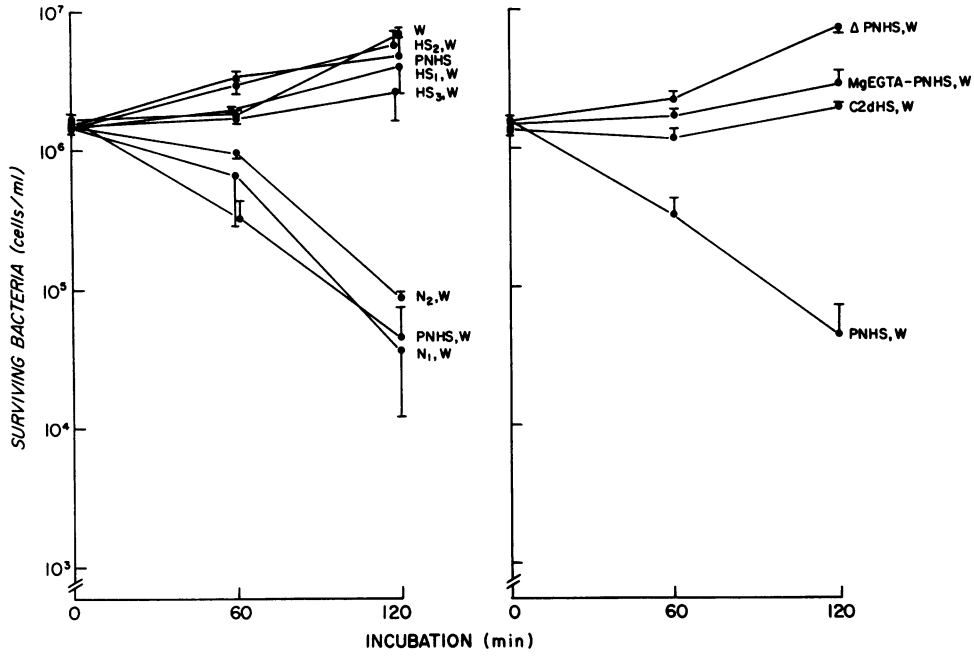

FIG. 1. *Comparison of the abilities of normal human sera and sera deficient in immunoglobulin or classical pathway activity to promote phagocytosis and intracellular killing of E. coli (F). Abbreviations not identified in the text are as follows: W, polymorphonuclear leukocytes; N_{1-2}, individual normal sera. The concentration of sera used in the reaction mixtures was 10%. The points represent mean values of two to four determinations, and each vertical bar represents the standard error of the mean.*

cated that *E. coli* (P) was unable to utilize the alternative complement pathway in sera deficient in both immunoglobulin and classical pathway activity.

Differences in immunoglobulin requirements and complement pathway utilization among strains of *P. mirabilis* were also demonstrated. Hypogammaglobulinemic sera, MgEGTA-PNHS, C2dHS, and ΔPNHS did not promote phagocytosis and intracellular killing of *P. mirabilis* (H) (Fig. 3). Hypogammaglobulinemic sera supported phagocytosis and intracellular killing of *P. mirabilis* (A) but showed reduced activity in comparison to PNHS and individual normal sera (Fig. 4). Furthermore, HS-A was unable to promote phagocytosis and intracellular killing of this strain. MgEGTA-PNHS and C2dHS promoted phagocytosis and killing of *P. mirabilis* (A), although the activity of these sera was decreased in comparison to the activity of untreated PNHS. In addition, MgEGTA-HS_{1-3} have reduced activity in comparison to untreated HS_{1-3}. These results indicated that *P. mirabilis* (H) and (A) required immunoglobulin for phagocytosis and intracellular killing. Complement was required for phagocytosis and killing of both strains; however, only *P. mirabilis* (A) utilized the alternative complement pathway. In addition, further utilization of the alternative complement pathway by *P. mirabilis* (A) did not occur in sera deficient in both immunoglobulin and classical pathway activity.

A diversity in the capacity of various sera to promote phagocytosis and killing of the *K. pneumoniae* strains was also demonstrated. PNHS and normal sera at concentrations of 5% promoted phagocytosis and intracellular killing of *K. pneumoniae* (Wo) (Fig. 5). Hypogammaglobulinemic sera promoted minimal phagocytosis and intracellular killing, and HS-A was unable to support killing of this strain. MgEGTA-PNHS and C2dHS promoted phagocytosis and intracellular killing, although not as efficiently as PNHS. MgEGTA-HS_{1-3} were less efficient than untreated HS_{1-3} in promoting phagocytosis and killing, and ΔPNHS was unable to support phagocytosis and killing of this strain. These results indicated that immunoglobulin and complement were essential for phagocytosis and intracellular killing of *K. pneumoniae* (Wo), and that the alternative complement pathway was partially utilized. Further utilization of the alternative pathway by this strain did not occur in sera deficient in both immunoglobulin and classical pathway activity.

Thirty percent of PNHS was required to pro-

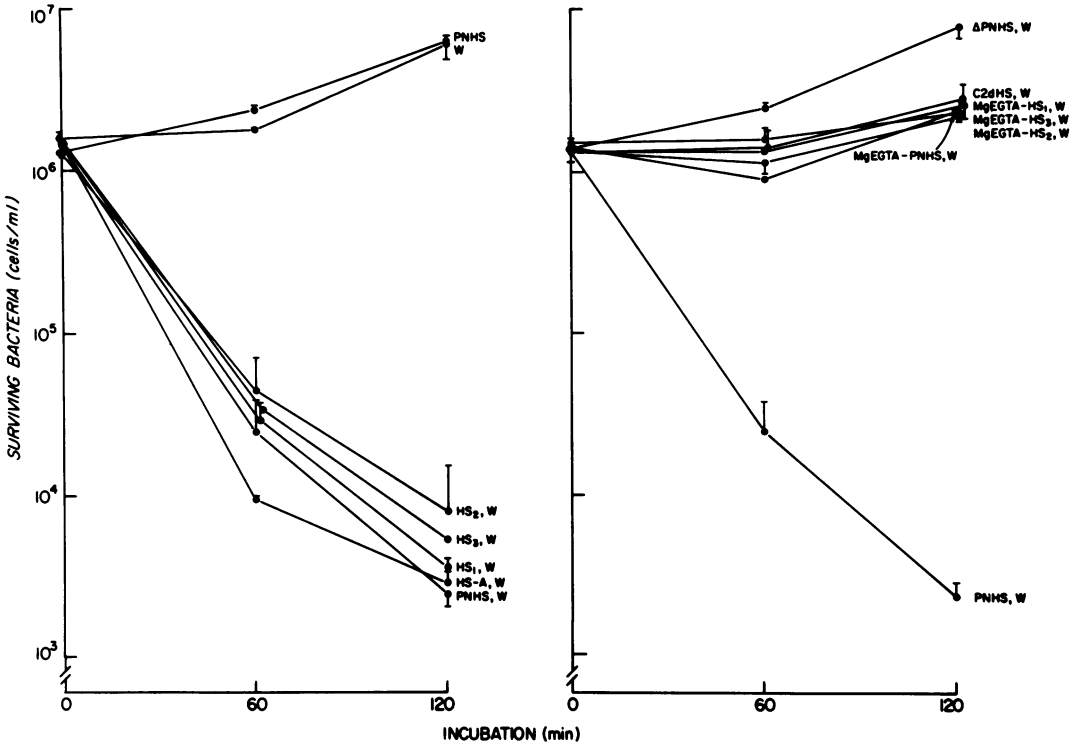

FIG. 2. *Comparison of the abilities of normal human serum and sera deficient in immunoglobulin and/or classical pathway activity to promote phagocytosis and intracellular killing of E. coli (P). Abbreviation not identified in the text is as follows: W, polymorphonuclear leukocytes. The concentration of sera used in the reaction mixtures was 5%. The points represent mean values of two to four determinations, and each vertical bar represents the standard error of the mean.*

mote over a one-log reduction in bacterial counts of *K. pneumoniae* (B) (Fig. 6). Although not shown in Fig. 6, hypogammaglobulinemic serum, MgEGTA-PNHS, and ΔPNHS were unable to support phagocytosis and intracellular killing. These results indicated that *K. pneumoniae* (B) required immunoglobulin and classical pathway activity for phagocytosis and intracellular killing. These results also indicated that higher levels of serum proteins were required to promote efficient phagocytosis and intracellular killing of *K. pneumoniae* (B), in comparison to the amount required for efficient killing of the other test strains.

Two *S. marcescens* strains were included in this study. Hypogammaglobulinemic sera and HS-A were unable to promote phagocytosis and intracellular killing of *S. marcescens* (W) (Fig. 7). MgEGTA-PNHS, MgEGTA-HS_{1-3}, C2dHS, and ΔPNHS were also unable to promote phagocytosis and killing of this strain. These results indicated that immunoglobulin and complement were required for phagocytosis and intracellular killing of *S. marcescens* (W), and that the alternative pathway was not utilized in the presence or absence of immunoglobulin. In contrast, hypogammaglobulinemic sera and HS-A promoted phagocytosis and intracellular killing of *S. marcescens* (S) equal to that of normal serum (Fig. 8). A slight reduction in bacterial counts occurred when MgEGTA-PNHS, C2dHS, and MgEGTA-HS_{1-3} were used as serum sources. These results indicated that *S. marcescens* (S) had a minimal requirement for immunoglobulin for phagocytosis and intracellular killing. This microorganism partially utilized the alternative pathway for intracellular killing in normal serum and could not be forced to further utilize this pathway in immunoglobulin-depleted sera.

DISCUSSION

In addition to the bacterial strains presented in Results, five additional strains were studied. The requirements for immunoglobulin and the classical and alternative complement pathways for phagocytosis and intracellular killing of all of the test strains by PMNs are summarized in Table 1. All strains were phagocytosed and killed

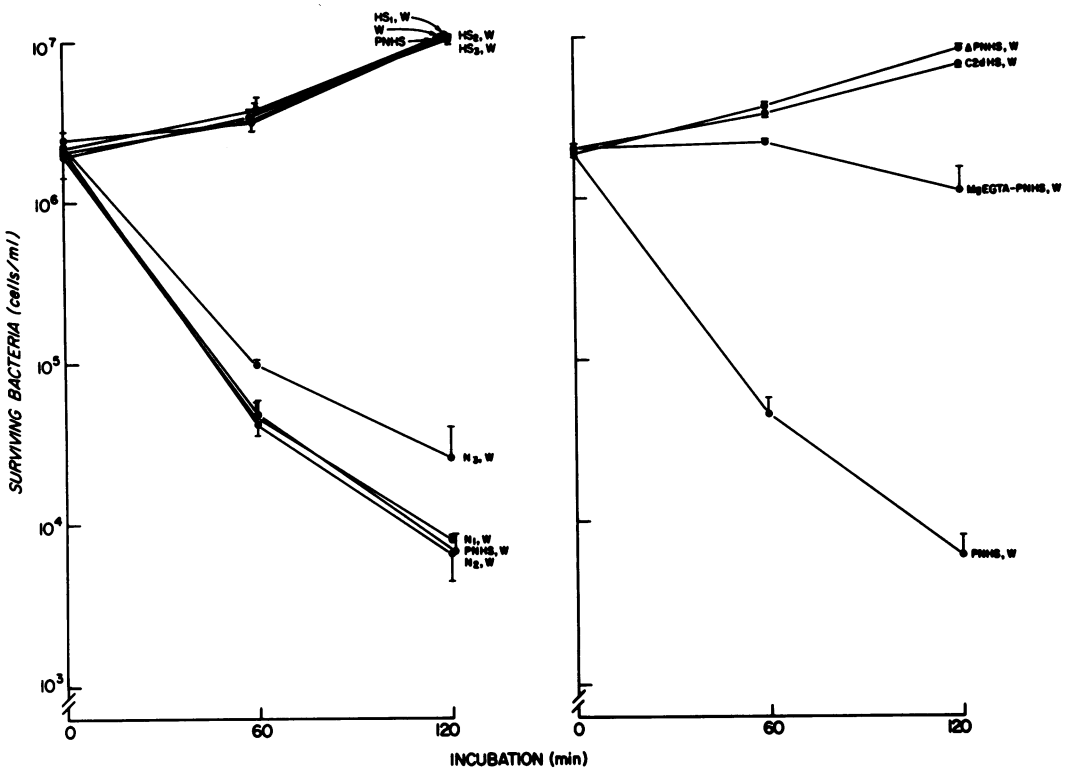

FIG. 3. *Comparison of the abilities of normal human sera and sera deficient in immunoglobulin or classical pathway activity to promote phagocytosis and intracellular killing of P. mirabilis (H). Abbreviations not identified in the text are as follows: W, polymorphonuclear leukocytes; N_{1-3}, individual normal sera. The concentration of sera used in the reaction mixtures was 5%. The points represent mean values of two to four determinations, and each vertical bar represents the standard error of the mean.*

intracellularly only in the presence of serum and PMNs and not by leukocytes alone. Killing of the strains was also not demonstrated in the presence of serum alone. In addition, none of the strains was phagocytosed and killed intracellularly by PMNs in the absence of complement, as evidenced by the inability of decomplemented PNHS to promote phagocytosis. Seven of the thirteen microorganisms included in this study demonstrated a requirement for immunoglobulin for phagocytosis and intracellular killing and utilized only the classical complement pathway or some other pathway dependent upon calcium ions and C2. Three of the microorganisms required immunoglobulin for phagocytosis and intracellular killing and utilized both the alternative and the classical pathways. Two of the microorganisms required minimal immunoglobulin and utilized only the classical pathway for phagocytosis and intracellular killing. One of the microorganisms required minimal immunoglobulin for phagocytosis and intracellular killing and utilized both complement pathways.

The minimal concentration of PNHS required to promote maximal phagocytosis and intracellular killing by PMNs was found to vary among the test strains. Eight of the microorganisms required 5% PNHS, and three microorganisms required 10% PNHS for optimal phagocytosis and killing. The other two microorganisms, *K. pneumoniae* (B) and (H), were phagocytosed and killed only when the concentration of PNHS was increased to 20 or 30%. This observation indicated that the level of serum proteins required for effective phagocytosis and intracellular killing by PMNs varied quantitatively with different strains of bacteria, even within the same species. This interpretation is in agreement with the concept of a quantitative variability in opsonic requirements for optimal phagocytic killing of bacteria, which was recently presented by Guckian et al. (14).

Opsonic requirements for phagocytosis of different strains within the same gram-positive species were recently investigated. Four *S. pneumoniae* serotypes were shown to be phagocy-

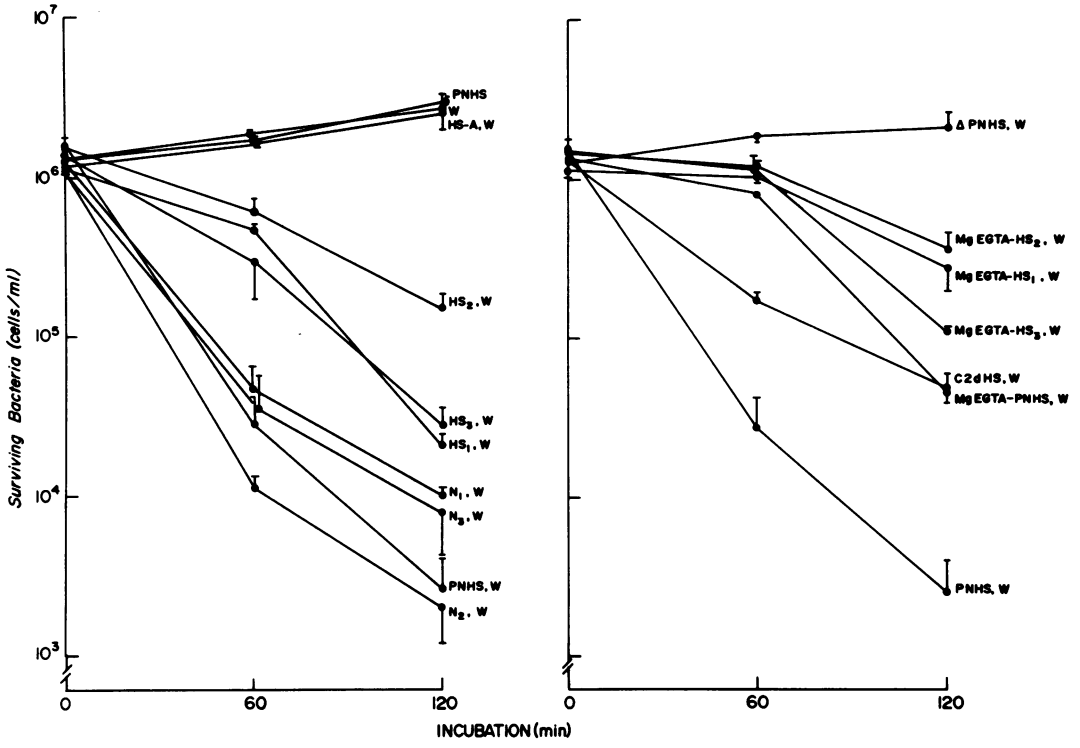

FIG. 4. *Comparison of the abilities of normal human sera and sera deficient in immunoglobulin and/or classical pathway activity to promote phagocytosis and intracellular killing of P. mirabilis (A). Abbreviations not identified in the text are as follows: W, polymorphonuclear leukocytes; N_{1-3}, individual normal sera. The concentration of sera used in the reaction mixtures was 5%. The points represent mean values of two to four determinations, and each vertical bar represents the standard error of the mean.*

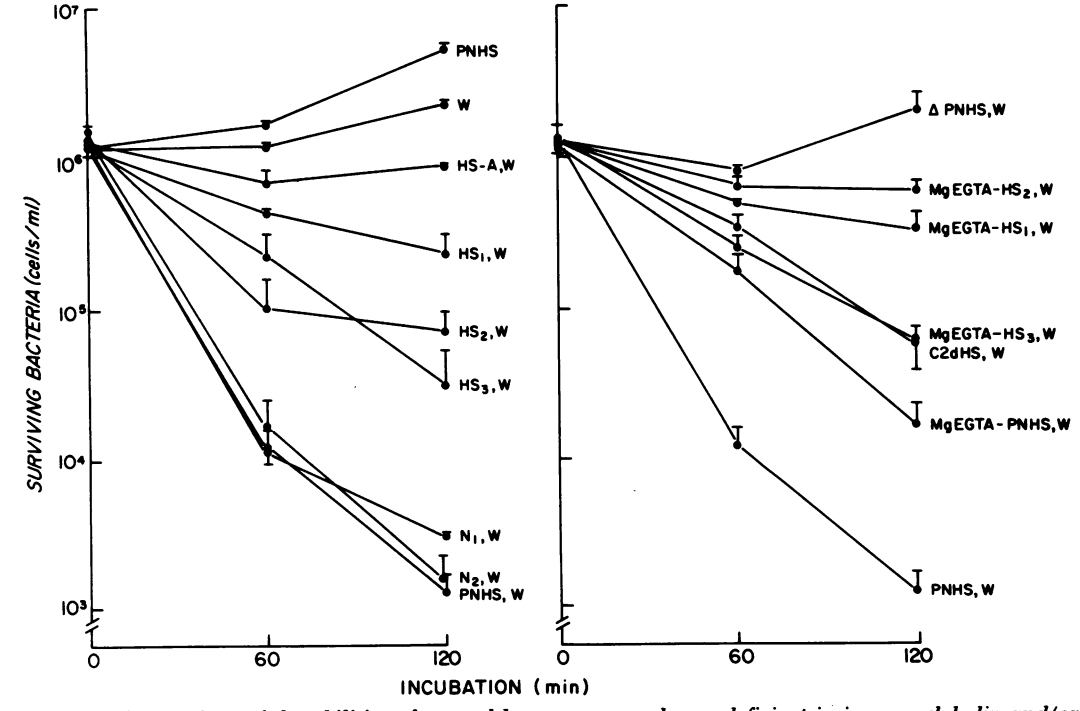

FIG. 5. *Comparison of the abilities of normal human sera and sera deficient in immunoglobulin and/or classical pathway activity to promote phagocytosis and intracellular killing of K. pneumoniae (Wo). Abbreviations not identified in the text are as follows: W, polymorphonuclear leukocytes; N_{1-2}, individual normal sera. The concentration of sera used in the reaction mixtures was 5%. The points represent mean values of two to four determinations, and each vertical bar represents the standard error of the mean.*

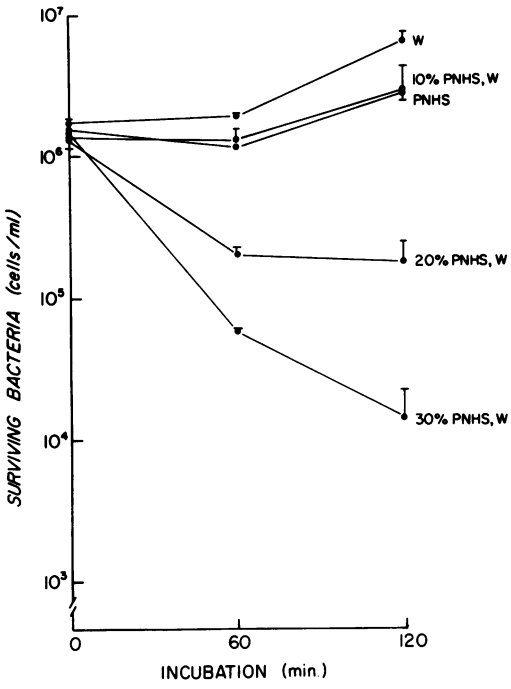

FIG. 6. *Ability of increasing concentrations of pooled normal human serum to promote phagocytosis and intracellular killing of K. pneumoniae (B). Abbreviations not identified in the text is as follows: W, polymorphonuclear leukocytes. The points represent mean values of two to three determinations, and each vertical bar represents the standard error of the mean.*

tosed in hypogammaglobulinemic serum, but to varying degrees of efficiency (13). Similarly, all four serotypes could be phagocytosed to some extent in human serum treated with MgEGTA and in C2-deficient serum. A heterogeneity of opsonic requirements among strains of *S. aureus* has also been reported (38). Two strains were shown to be phagocytosed in hypogammaglobulinemic serum and were only slightly phagocytosed via the alternative complement pathway. A third strain demonstrated a requirement for immunoglobulin, and efficient phagocytosis proceeded via the alternative pathway. It was proposed that the heterogeneity among *S. aureus* strains might be related to the presence or absence of the protein A moiety, which could preferentially activate the classical complement pathway via nonspecific interaction with the Fc fragment of IgG.

Our data support the observation that a heterogeneity in the requirements for immunoglobulin and complement exists among different strains of the same species, not only among gram-positive microorganisms as discussed above, but among gram-negative aerobic bacilli as well. In our study, the test strains isolated from burn patients did not demonstrate a unique pattern of serum protein requirements in comparison to the same species isolated from nonburned patients. Rather, a heterogeneity of serum protein requirements for all strains regardless of their source was demonstrated.

In recent studies, capsular material has been implicated as a factor contributing to differences in opsonic requirements among various bacterial strains. Stevens et al. demonstrated that the opsonic requirements for *E. coli* strains possessing the K-1 capsular antigen differed from the opsonic requirements for *E. coli* strains lacking the K-1 antigen (36). K-1-positive strains were shown to require the classical complement pathway and antibody for phagocytosis and intracellular killing by PMNs, whereas K-1-negative strains could be phagocytosed and killed via the alternative complement pathway without an apparent requirement for antibody. Peterson et al. investigated the opsonic requirements of encapsulated and nonencapsulated *S. aureus* strains (30). The presence of capsular material was shown to interfere with the opsonization of *S. aureus* via the classical and alternative pathways and with opsonization by heat-stable factors. Preliminary results from our laboratory suggest that the differences in the requirements for immunoglobulin and alternative and/or classical complement pathway activities for phagocytosis and killing of our test strains were not related in the presence or absence of capsular material. In addition, no specific pattern of serum protein requirements was observed for our K-1-negative or K-1-positive *E. coli* strains.

It is well known that antibacterial antibodies trigger the sequential activation of the classical pathway, and this process was probably operative in promoting phagocytosis and killing of the microorganisms which required immunoglobulin and classical pathway activity. The role of immunoglobulin in phagocytosis and intracellular killing of those microorganisms which utilized the alternative as well as the classical complement pathway is uncertain. Available evidence indicates that activation of the alternative pathway by inulin or zymosan occurs in the absence of immunoglobulin (28). Lysis of *Trypanosoma cyclops* (22) and rabbit erythrocytes (31) has also been shown to occur via the alternative pathway without a requirement for antibodies. Recently, however, immunoglobulin was demonstrated to exert a rate-limiting effect on alternative pathway activity in lysis of rabbit erythrocytes (32). In addition, IgG has been shown to participate in lysis of measles virus-infected cells via the alternative pathway (8, 20). Immuno-

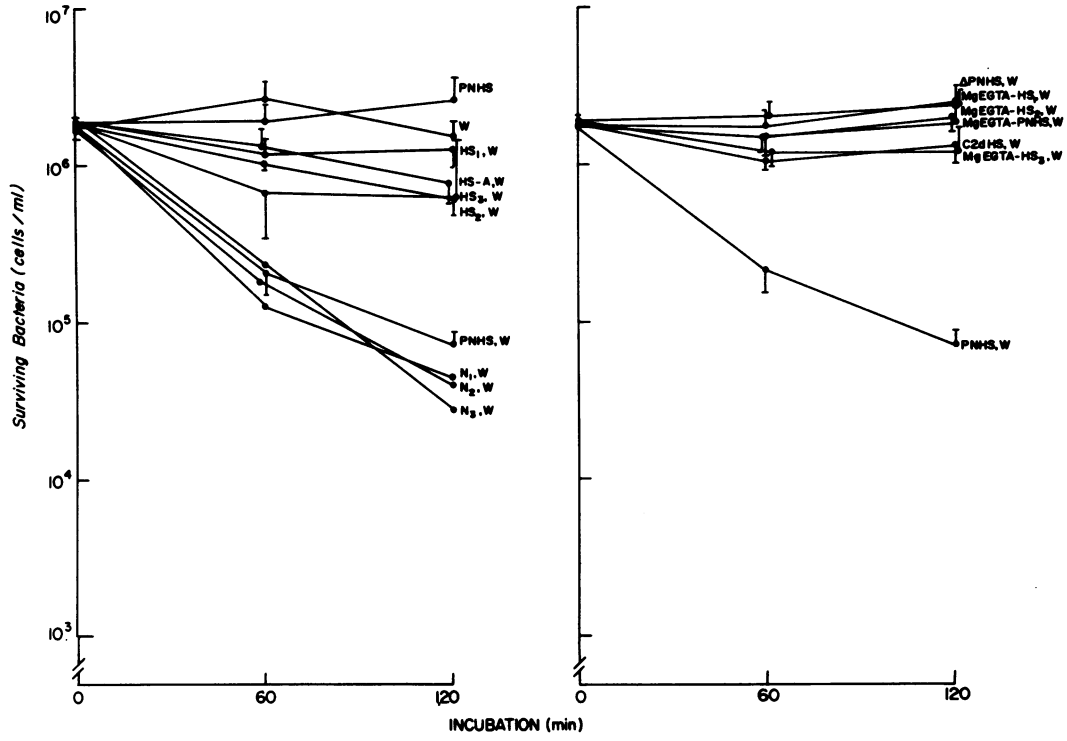

FIG. 7. *Comparison of the abilities of normal human sera and sera deficient in immunoglobulin and/or classical pathway activity to promote phagocytosis and intracellular killing of S. marcescens (W). Abbreviations not identified in the text are as follows: W, polymorphonuclear leukocytes; N_{1-3}, individual normal sera. The concentration of sera used in the reaction mixtures was 5%. The points represent mean values of one to four determinations, and each vertical bar represents the standard error of the mean.*

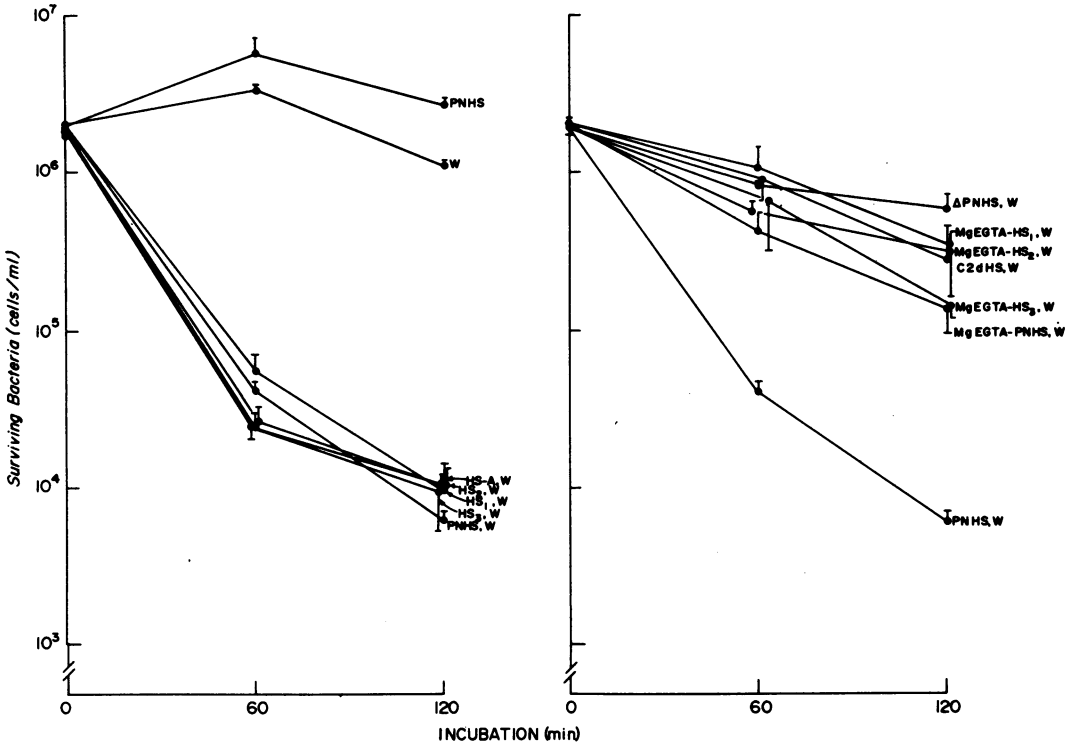

FIG. 8. *Comparison of the abilities of normal human serum and sera deficient in immunoglobulin and/or classical pathway activity to promote phagocytosis and intracellular killing of S. marcescens (S). Abbreviation not identified in the text is as follows: W, polymorphonuclear leukocytes. The concentration of sera used in the reaction mixtures was 5%. The points represent mean values of two to four determinations, and each vertical bar represents the standard error of the mean.*

TABLE 1. *Summary of the requirements for immunoglobulin and the alternative and classical complement pathways for phagocytosis and intracellular killing of clinical isolates of E. coli, P. mirabilis, K. pneumoniae, and S. marcescens by human PMNs*

Microorganism	IgG[a]	Classical pathway	Alternative pathway
E. coli (A)[b]	+	+	−
E. coli (F)[b]	+	+	−
E. coli (P)[b]	−	+	−
E. coli (H)[c]	−	+	−
P. mirabilis (H)[b]	+	+	−
P. mirabilis (A)[b]	+	+	+
P. mirabilis (C)[c]	+	+	+
K. pneumoniae (Wo)[b]	+	+	+
K. pneumoniae (W)[c]	+	+	−
K. pneumoniae (B)[b]	+	+	−
K. pneumoniae (H)[b]	+	+	−
S. marcescens (W)[c]	+	+	−
S. marcescens (S)[c]	−	+	+

[a] Minus sign indicates a minimal requirement for immunoglobulin.

[b] Microorganisms were isolated from burned patients.

[c] Microorganisms were isolated from nonburned patients.

globulin has also been shown to be required for bacteriolysis of an *E. coli* strain in bovine serum (15) and for serum bactericidal activity for *Neisseria gonorrhoeae* (17), both mediated via the alternative complement pathway. It is not known whether immunoglobulin is required for alternative pathway activation or for other steps in the lytic process.

It has been hypothesized that the alternative complement pathway may represent a mechanism for the activation of immune defenses when sufficient quantities of antibody are not available for activation of the classical pathway (27). It is interesting that none of the microorganisms tested in our study utilized only the alternative pathway in the presence of minimal immunoglobulin or could be forced to utilize the alternative pathway during phagocytosis and intracellular killing in sera deficient in both immunoglobulin and classical pathway activity. The inability of the microorganisms to efficiently utilize the alternative pathway in sera treated with MgEGTA could be due to the effects of MgEGTA or a lack of calcium ions on the ingestion or intracellular killing processes. However, these possibilities are considered unlikely for the following reasons: (i) the results obtained using MgEGTA-sera were similar to those obtained using C2dHS as the serum source; and (ii) identical results to those presented in the manuscript were obtained from experiments using bacteria preopsonized with MgEGTA-sera, washed, and then incubated with PMNs in HBSS containing both calcium and magnesium ions.

The observation that classical pathway utilization could occur in the presence of minimal immunoglobulin during phagocytosis and intracellular killing has not been previously reported. Several investigators have provided evidence to suggest that the lipid A moiety of lipopolysaccharides can interact directly with the C1q subunit of C1 to initiate classical pathway activation in the presence of minimal immunoglobulin (6, 24, 29). The results of our study suggest that this mechanism may be operative during phagocytosis and intracellular killing of certain aerobic microorganisms.

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