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# Contributions of Complement and Immunoglobulin to Neutrophil-Mediated Killing of Enterococci

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**Enterococci have become a frequent causative agent in neonatal sepsis. The relative contributions of antibody and complement and their interactions in the neutrophil-mediated bacterial killing of 11 *Enterococcus* strains from neonates were investigated. Polymorphonuclear leukocytes (PMNL) from adult and term newborn infants were tested with normal human serum, adult hypogammaglobulinemic serum, and normal newborn serum in a neutrophil bactericidal assay. Neutrophil bactericidal activity for enterococci was not influenced by the serum source but was essentially ablated after heat inactivation of complement in all sera. No differences were observed in the killing capacity of healthy newborn versus adult PMNL regardless of serum source. Representative *Enterococcus* strains were then tested with agammaglobulinemic serum or C4-deficient serum, resulting in neutrophil bactericidal activities consistently exceeding 90%. A neutrophil bactericidal assay performed with normal rabbit serum and hyperimmune rabbit serum against enterococci showed that antibodies to enterococci enhanced neutrophil-mediated killing of this organism. Thus, neutrophil killing of enterococci appears to be mediated primarily by complement, with antibody playing a less essential but potentially important role. PMNL from adult and healthy term infants functioned with equal efficiency in the neutrophil killing of enterococci.**

In recent years, enterococci have become a frequent causative agent in both early- and late-onset neonatal sepsis. Previously placed in the genus *Streptococcus* and designated group D by Lancefield's classification (21), enterococci were first recognized as a cause of neonatal sepsis in the late 1950s, when McCracken and Shinefield (20) reported two cases over the 11-year interval from 1954 to 1964. Buchino et al. (7) reported 13 cases of group D streptococcal sepsis and/or meningitis at the Cincinnati Children's Hospital from 1970 to 1976. The incidence of enterococcal sepsis increased in metropolitan Houston from 0.12 to 0.7 cases per 1,000 live births between 1982 and 1986 (10). Enterococci also have been implicated in nursery outbreaks, implying a nosocomial origin. An outbreak of *Enterococcus faecalis* in Denver accounted for 42% of neonatal sepsis cases during one 6-month interval (17). A report by Coudron and associates (9) of invasive *E. faecium* infections among premature infants with intravascular devices suggested that hand-to-hand spread was the mode of transmission. In a recent multicenter trial, *Enterococcus* spp. were the third most frequent cause of late-onset gram-positive sepsis among low-birth-weight infants, accounting for 15% of these cases (5).

The susceptibility of neonates to invasive infections has prompted delineation of the pathogenesis of these infections, with particular emphasis on group B streptococci (4, 6, 11, 12). The opsonic requirements of enterococci, however, have not been fully elucidated. In 1984, Willemien et al. (24) examined the killing capacity of human polymorphonuclear leukocytes (PMNL) in aerobic and anaerobic conditions. They found that in vitro, killing of *Streptococcus faecalis* was unaffected by changes in oxygen tension. Akriotis and Biggar (1) reported in 1985 that PMNL-mediated killing of *S. faecalis* was significantly reduced by hypothermia. Chirico et al. (8) in 1985 investigated bactericidal activity against *S. faecalis* by PMNL from term and preterm infants, finding a transient but significant impairment in both groups.

The purpose of our experiments was to investigate the host factors predicting susceptibility to enterococcal infection in neonates. Using a number of serum and PMNL sources in a neutrophil bactericidal assay, we characterized the relative contributions of antibody and complement as well as their interactions in the neutrophil killing of enterococci.

## MATERIALS AND METHODS

**Collection and preparation of sera.** An adult normal human serum (NHS) pool obtained from five healthy adults had a normal level of immunoglobulin G (IgG) (1,080 mg/dl) and a total hemolytic complement ($CH_{50}$) of 500 U/ml (normal range, 300 to 500 U/ml). Hypogammaglobulinemic serum (AHG) was obtained from an adult with common variable hypogammaglobulinemia who was not receiving antimicrobial agents or immunoglobulin replacement therapy. The IgG level in this patient's serum was 48 mg/dl, and the $CH_{50}$ was 532 U/ml. A normal newborn serum (NNS) pool was collected by venipuncture from healthy term neonates within the first 48 h of life and contained 1,140 mg of IgG per dl and a normal $CH_{50}$ (385 U/ml). C4-deficient serum (C4D) was collected from a child with congenital C4 deficiency (IgG, 1,740 mg/dl; C4, 0 mg/dl; $CH_{50}$, 0 U/ml). Agammaglobulinemic serum (AGS) was obtained from a child with severe combined immunodeficiency (IgG, 10 mg/dl; IgA and IgM, undetectable) and a $CH_{50}$ level of 352 U/ml. All sera were allowed to clot at room temperature, centrifuged at 4°C, aliquoted, and stored at −70°C within 1 h of collection. Complement in selected sera was inactivated by heating to 56°C for 30 min.

**Preparation of rabbit antisera.** New Zealand White rabbits were immunized with a representative strain of Formalin-

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killed enterococci in Todd-Hewitt broth (THB) (Difco Laboratories, Detroit, Mich.) by the method of McCarty and Lancefield (19). Blood was collected pre- and postimmunization, centrifuged at 4°C, and aliquoted, and serum was stored at −70°C within 1 h of collection.

**Isolation of neutrophils.** Peripheral venous blood from a laboratory volunteer was collected in a syringe containing citrate phosphate dextrose solution (Abbott Laboratories, North Chicago, Ill.) for anticoagulation and 6% dextran (Spectrum Chemical Mfg. Corp., Gardena, Calif.) for sedimentation before purification over Ficoll-Hypaque gradients. For assays requiring neonatal PMNL, blood was collected from the placentas of term healthy neonates born via vaginal delivery. The isolated PMNL were resuspended in Dulbecco's phosphate-buffered saline (GIBCO, Grand Island, N.Y.) to a concentration of $\sim 2 \times 10^7$/ml. Leukocytes were counted manually with a hemacytometer, and purity and viability of ≥95% were verified by using Wright's stain and trypan blue dye exclusion, respectively.

**Bacteria.** The *Enterococcus* strains included all blood or cerebrospinal fluid isolates from neonates hospitalized in the nurseries of the Harris County Hospital District between January 1989 and July 1990. At least one enterococcal isolate was obtained from 14 infants. Thirteen were considered clinically significant upon review of the medical record. Of these, eight were from neonates with early-onset infection, defined as that occurring within the first 5 days of life. Seven of the eight early-onset infections occurred in term infants, and the mean birth weight was 3,600 g (range, 2,030 to 4,260 g). Four were evaluated for symptoms suggesting sepsis, and four were evaluated because of maternal risk factors.

Four of the five late-onset infections occurred in preterm infants at a mean of 42 days. The original isolates were stored in aliquots of THB at −70°C. For each experiment, an aliquot was thawed, inoculated onto a blood agar plate, incubated overnight at 37°C, subsequently inoculated into THB, and harvested during the mid-log phase of growth.

Identification to species level and susceptibility testing by the Vitek Identification System (Vitek Systems, Inc., St. Louis, Mo.) were done for the 13 clinical isolates obtained from neonates. Of the 13 isolates, 11 were *E. faecalis* and 2 were *E. faecium*. All isolates were susceptible to ampicillin and vancomycin. All strains were tested for β-lactamase activity with nitrocefin disks, and none was positive. All isolates were tested for high-level resistance to gentamicin at concentrations of 1,000 and 2,000 μg/ml in Mueller-Hinton agar (Baltimore Biologic Laboratories, Cockeysville, Md.) (23). None of the isolates showed high-level resistance with this method. Chromosomal restriction endonuclease digestion patterns were obtained by pulsed-field electrophoresis of large chromosomal fragments for all of the late-onset isolates and three of the early-onset isolates by Barbara E. Murray (22). Three of the late-onset isolates appeared to represent a single strain, while the other two late-onset and all early-onset isolates tested clearly represented distinct strains. Data from assays with the three identical strains were subsequently averaged and used as a single value for the purpose of statistical analysis.

**Neutrophil bactericidal assay.** A neutrophil bactericidal assay described previously (11) was modified to obtain a bacteria-to-PMNL ratio of ~3:1 in the reaction mixture. The incubation mixture contained 50 μl of freshly isolated PMNL ($\sim 10^6$ cells), 50 μl of a bacterial suspension ($\sim 3 \times 10^6$ CFU), 100 μl of a serum source, and 100 μl of Dulbecco's phosphate-buffered saline or minimal essential medium (Whittaker Bioproducts, Walkersville, Md.) with 1% bovine serum albumin. Where indicated, either the bacteria or the PMNL were first exposed to NHS (30 min at 37°C), and then the serum was removed by centrifugation before incubation of bacteria with PMNL. Control tubes lacking PMNL or serum were included in each assay. Results were expressed as bacterial killing or percent reduction in the initial inoculum at 30 and 60 min. All neutrophil bactericidal assays were performed in duplicate.

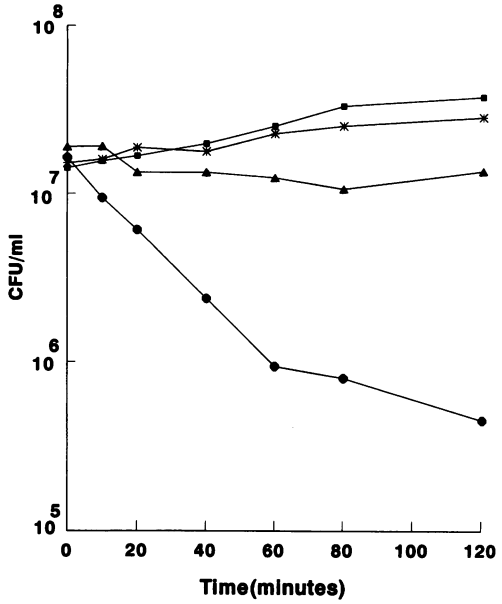


FIG. 1. Kinetic study with a representative strain of *E. faecalis* and adult PMNL. NHS (●) promoted greater than 1 $\log_{10}$ reduction in CFU by 60 min. Heat-inactivated NHS (▲) effected a minimal reduction in CFU, and controls lacking serum (■) or PMNL (*) promoted bacterial growth. Results represent the means of duplicate experiments.

**Statistical analysis.** Data were analyzed by Student's unpaired *t* test (two tailed) (13).

## RESULTS

Preliminary experiments were performed to determine the kinetics of neutrophil-mediated killing of enterococci. This was examined by using NHS, adult PMNL, and a representative strain of *E. faecalis*. Aliquots were plated at 10, 20, 40, 60, 80, and 120 min. At 60 min, NHS promoted a greater than 1 $\log_{10}$ (>90% of initial inoculum) reduction in CFU (Fig. 1). Heat-inactivated serum effected a 27% reduction in CFU at 120 min. Reaction mixtures lacking serum or PMNL promoted bacterial growth.

A similar experiment was performed with a representative strain of *E. faecium* (Fig. 2). Aliquots were plated at 0, 30, and 60 min. Again, and in other experiments with additional strains, killing by NHS was more pronounced at 60 than at 30 min. These intervals were chosen to examine neutrophil bactericidal activity with different serum and PMNL sources.

An experiment was performed to examine the effects of preopsonization on neutrophil bactericidal activity. A strain previously found to be more resistant to killing was used to emphasize possible differences in neutrophil bactericidal activity. At 30 and 60 min of incubation, mixtures containing preopsonized bacteria revealed markedly greater neutrophil

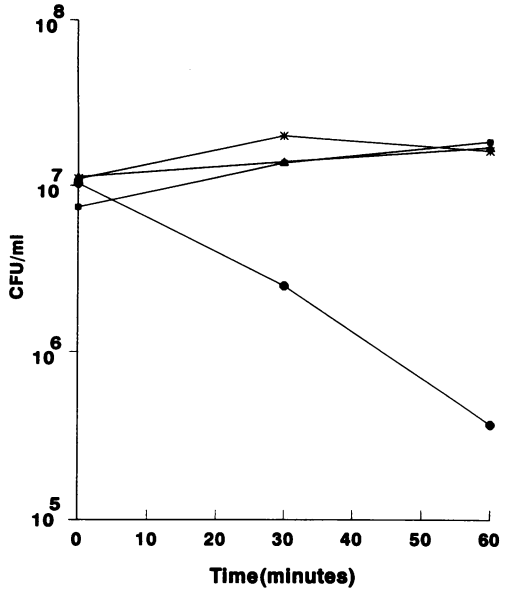


FIG. 2. Kinetic study with a representative strain of *E. faecium* and adult PMNL. NHS (●) promoted greater than 1 $\log_{10}$ reduction in CFU by 60 min. Heat-inactivated NHS (▲) and controls lacking serum (■) or PMNL (*) promoted bacterial growth. Results represent the means of duplicate experiments.

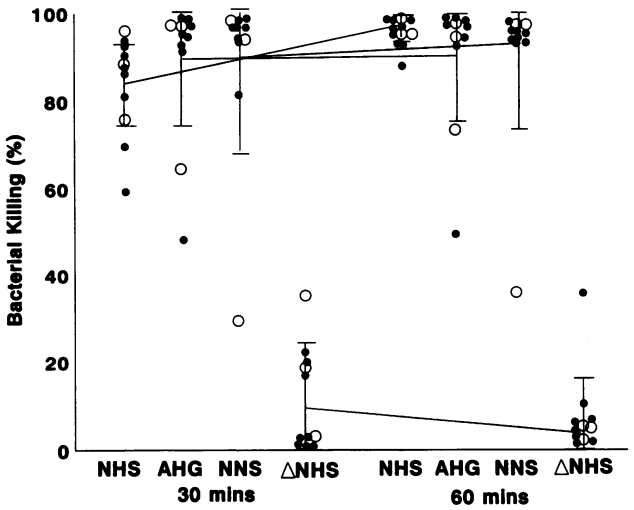


FIG. 3. Neutrophil killing of all strains at 30 and 60 min of incubation with NHS, AHG, NNS, and heat-inactivated NHS (ΔNHS). Early-onset (●) and late-onset (○) strains demonstrated no significant differences regardless of serum source. Heat-inactivated NHS allowed only minimal neutrophil killing. All sera supported a mean of at least 90% reduction in CFU by 60 min. Data represent means ± standard deviations (bars) for at least two experiments.

bactericidal activity than those containing NHS and untreated bacteria (Table 1). Incubation mixtures containing PMNL pretreated with NHS failed to demonstrate bacterial killing. Thus, preopsonization of bacteria but not PMNL enhanced killing of enterococci.

**Influence of serum source on neutrophil bactericidal activity.** Experiments were next performed to compare the effects of different serum sources on neutrophil bactericidal activity (Fig. 3). Each of the 11 strains was tested with adult PMNL and NHS, AHG, and NNS. At 30 min, there was no significant difference when strains causing early- and late-onset disease were compared, regardless of the serum source. Testing with NNS showed a trend towards lower neutrophil killing of the late-onset strains, 74% ± 39% versus 96% ± 6%, but this was not a statistically significant difference ($P > 0.20$). When all 11 strains were compared, there was no significant difference between the neutrophil bactericidal activities of the serum sources (Fig. 3). Heated NHS supported only very low-level neutrophil bactericidal activity (10%), whereas heated NNS and AHG showed none (data not shown).

At 60 min of incubation, the mean bacterial kill was 97% ± 1% for NHS, 91% ± 16% for AHG, and 93% ± 19% for NNS (Fig. 3). There were no significant differences in neutrophil bactericidal activity when strains causing early- versus late-onset disease were compared in NHS, AHG, or NNS. Late-onset strains tested with NNS continued to show a trend towards decreased neutrophil bactericidal activity, 77% ± 37% compared with 99% ± 1% in early-onset strains, as observed at 30 min of incubation. This result, however, was not statistically significant ($P > 0.20$). One of the late-onset strains was consistently more resistant to killing than the others, accounting for the wide standard deviation. When all 11 strains were compared, each serum source supported more than 1 $\log_{10}$ reduction in CFU, or a bacterial kill exceeding 90% by 60 min (Fig. 3). The neutrophil bactericidal activity mediated by heated NHS declined to 4% ± 12% by 60 min, and again, heat inactivation of AHG and NNS ablated all neutrophil bactericidal activity (data not shown).

These results indicate that complement is important in the opsonization and phagocytosis of enterococci, as evidenced by efficient killing by all three serum sources. Intact adult, neonatal, and hypogammaglobulinemic sera efficiently promoted killing by adult neutrophils, whereas complement inactivation by heating essentially ablated it.

**Influence of PMNL source.** The influence of PMNL source on neutrophil killing of enterococci was then determined (Fig. 4). All 11 strains were tested with healthy term-newborn PMNL together with NHS, AHG, and NNS. At 30 min of incubation, newborn PMNL tested with NHS showed significantly increased neutrophil bactericidal activity compared with adult PMNL, 95% versus 84% ($P < 0.005$). Newborn PMNL tested with AHG and NNS showed no significant difference compared with adult PMNL. By 60 min, no difference was observed in the neutrophil bactericidal activity with newborn versus adult PMNL regardless of the serum source. These results suggested that healthy term-newborn PMNL function at least as efficiently as adult PMNL in the neutrophil-mediated killing of enterococci.

**Role of complement.** To assess the role of complement alone in the neutrophil bactericidal activity of enterococci, an assay was performed with AGS. With a representative strain of *E. faecalis*, AGS in combination with adult PMNL

TABLE 1. Effect of opsonization with NHS

| Mixture | Bacterial killing (%) | |
|---|---|---|
| | 30 min | 60 min |
| Enterococci + PMNL + NHS | 43 | 56 |
| NHS-pretreated enterococci + PMNL | 81 | 76 |
| Enterococci + NHS-pretreated PMNL | 0 | 0 |

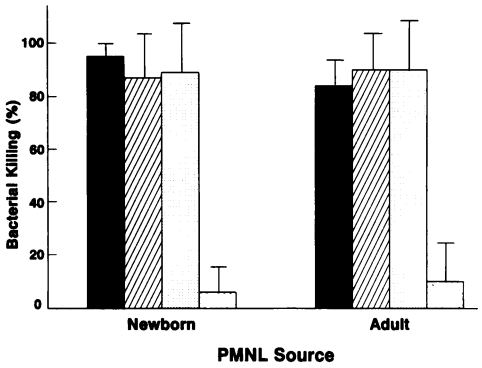


FIG. 4. Neutrophil killing of enterococci, expressed as percent bacterial killing by PMNL from adults and term infants with NHS (solid bars), AHG (hatched bars), NNS (stippled bars), and heat-inactivated NHS (open bars). Data represent means ± standard deviations (bars) for duplicate experiments for all strains at 30 min of incubation. The percent bacterial kill by newborn PMNL with NHS significantly exceeded that by adult PMNL with NHS ($t = 3.50$, $P < 0.005$).

promoted a neutrophil bactericidal activity of 96 and 99% at 30 and 60 min, respectively (Table 2). Thus, complement in the absence of antibody appeared to efficiently promote killing by adult PMNL. This again supports the relative importance of complement in the neutrophil bactericidal activity of enterococci and suggests that antibody directed at specific epitopes of this microorganism does not appear to be an absolute requirement for these interactions.

In view of the essential role of complement in neutrophil bactericidal activity, an experiment was carried out to assess the roles of the classical and alternative complement pathways. Four strains of *E. faecalis* were tested with C4D serum and adult PMNL (Table 2). Neutrophil bactericidal activity was 96% ± 2% at 30 min and 99% ± 1% at 60 min. These results suggest that complement-mediated neutrophil bactericidal activity of enterococci may proceed efficiently via the alternative pathway.

**Role of antibody.** The role of antibody in neutrophil killing of enterococci was investigated next. A neutrophil bactericidal assay was performed with normal rabbit serum before and after (hyperimmune rabbit serum) immunization. Aliquots were plated at 0, 15, 30, and 60 min. As shown in Fig. 5, at 15 and 60 min, hyperimmune rabbit serum promoted a significantly greater reduction in CFU than normal rabbit serum ($P < 0.05$). Killing by hyperimmune rabbit serum was also more pronounced at 30 min, although this did not reach statistical significance. These results suggest that enterococcus-specific antibodies may serve an important role in the neutrophil killing of enterococci by enhancing and accelerating the process.

TABLE 2. Comparative neutrophil bactericidal activity

| Serum | Mean % bacterial killing[a] (SD) | |
|---|---|---|
| | 30 min | 60 min |
| NHS | 82 (12) | 95 (5) |
| AGS | 96 (1) | 99 (1) |
| C4D | 96 (2) | 99 (1) |

[a] Results represent means of at least two determinations.

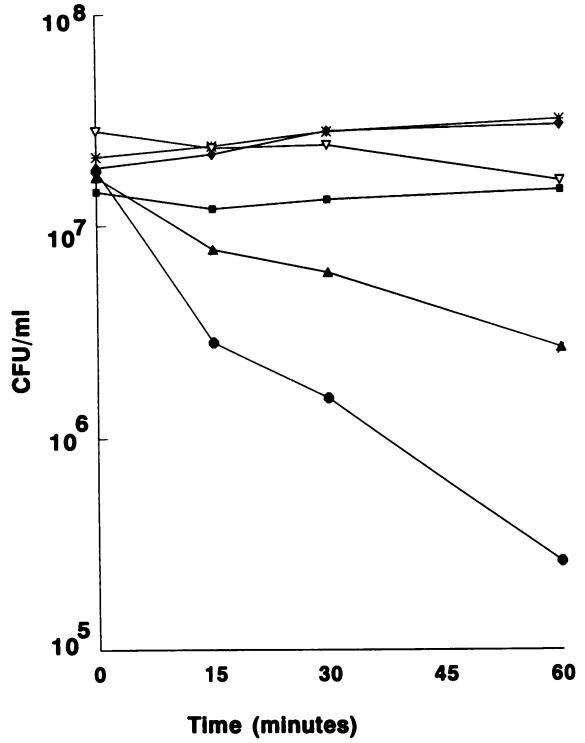


FIG. 5. Neutrophil bactericidal assay with normal rabbit serum (▲), heat-inactivated normal rabbit serum (▽), hyperimmune rabbit serum (●), and heat-inactivated hyperimmune rabbit serum (◆) at 0, 15, 30, and 60 min of incubation. Hyperimmune rabbit serum showed significantly increased killing versus normal rabbit serum at 15 and 60 min ($P < 0.05$). Controls lacking serum (*) or PMNL (■) promoted bacterial growth. Data represent the means of duplicate determinations.

## DISCUSSION

The host factors predicting susceptibility to neonatal *Enterococcus* infection have not previously been elucidated fully. The aim of the preceding experiments was to examine the requirements for neutrophil killing of enterococci by serum and PMNL from adults and neonates and to define the relative contributions and interactions of antibody and complement to this process. Several conclusions may be drawn. First, complement appears to play an important role in the neutrophil bactericidal activity toward enterococci. This conclusion is based on several findings. Regardless of serum source, sera with intact complement routinely exhibited neutrophil bactericidal activity exceeding 90% by 60 min of incubation. When complement was inactivated by heating, however, neutrophil bacterial killing was consistently reduced to minimal or undetectable levels. The results of experiments with AHG showed efficient neutrophil killing of enterococci in the presence of decreased antibody levels. Additionally, AGS promoted greater than 90% neutrophil bactericidal activity in the absence of antibody. As seen previously with *Escherichia coli* (18), experiments with preopsonized bacteria demonstrated not only efficient but enhanced neutrophil killing. These experiments imply that complement in the presence of PMNL from healthy neonates is not only adequate but very efficient in the killing of enterococci in vitro. Thus, it appears that complement plays an essential role in the neutrophil killing of enterococci in both neonates and adults.

The second conclusion that may be reached from these studies is that sera from healthy, term neonates are as efficient in the neutrophil killing of enterococci as adult sera. NNS overall exhibited neutrophil bactericidal activity exceeding 90%. When strains were compared by age or onset of sepsis (early versus late onset), there was a trend towards decreased neutrophil bactericidal activity for the strains from infants with late-onset sepsis when NNS was used. This result, however, did not reach statistical significance. Although the $CH_{50}$ of NNS was lower than normal adult $CH_{50}$ values, complement levels apparently were adequate to provide the necessary activity for neutrophil killing to proceed efficiently. A previous study found that the activity of the alternative pathway appears to be more frequently subnormal in newborn sera than the activity of the classical pathway and that gestational age appears to correlate with alternative-pathway hemolytic activity and properdin concentrations (14). Our experiment with C4D serum showed that complement activity may proceed via the alternative pathway alone, resulting in bactericidal activity equal to that of systems in which both pathways are intact. In light of this, it is tempting to speculate that premature infants' complement levels would be particularly diminished, especially those of the alternative pathway, rendering them more susceptible to infection with enterococci. This speculation, however, requires further study.

Third, specific antibody appears to have an enhancing role in the neutrophil killing of enterococci. In the presence of heat-inactivated sera and PMNL, antibody alone supported only minimal neutrophil bactericidal activity with NHS and essentially none with AHG and NNS. As noted above, AHG and AGS both demonstrated efficient neutrophil killing, with bacterial kill exceeding 90%. Thus, antibody does not appear to be absolutely essential to the efficient neutrophil killing of enterococci. However, studies of group B streptococci have shown the importance of specific antibody in enhancing alternative pathway activation (12). Our findings with hyperimmune rabbit serum support the conclusion that specific antibody, though not essential, may accelerate the neutrophil killing of enterococci. Consequently, under conditions in which premature infants are complement restricted, low concentrations of specific antibody may play an important role in defining susceptibility to neonatal *Enterococcus* infection.

Last, no impairment was found for PMNL from healthy term neonates in the neutrophil killing of enterococci. Neonatal PMNL consistently achieved more than 90% bacterial kill regardless of serum source. It is well documented that neonatal PMNL demonstrate impaired mobility and adherence (2, 16). Chirico et al. (8) examined bactericidal activity against *S. faecalis* with PMNL from term and preterm infants. Their results showed that in term infants, neutrophil bactericidal activity was normal during the first 48 h of life, decreased significantly to defective values between days 4 and 10, and returned to normal values toward days 13 to 16 of life. Preterm infants, however, showed defective neutrophil bactericidal activity in the first 48 h of life that persisted through the 32nd day of life. Our results concur with this study in that PMNL obtained from placentas of term infants at delivery showed normal neutrophil bactericidal activity compared with adult PMNL. Additionally, PMNL from stressed neonates have been shown to exhibit even greater impairment of adherence and chemotaxis compared with PMNL from healthy neonates (3, 15). It is interesting that six of the eight infants with early-onset sepsis had meconium staining at birth, suggesting the possibility of some form of intrauterine stress. The adverse effect of hypothermia on neutrophil function (1) may also be important in that hypothermia is commonly seen in the stressed neonate. We may speculate that in term healthy infants, PMNL function may be diminished but adequate to promote efficient neutrophil killing of enterococci. In a stressed infant, however, further impairment of PMNL function could potentially predispose these infants to infection with enterococci.

## ACKNOWLEDGMENTS

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