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Roles of Antibodies and Complement in Phagocytic Killing of Enterococci

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The contributions of complement and antibodies to polymorphonuclear leukocyte (PMN)-mediated killing of enterococci were investigated with pooled normal human serum (PNHS) or immune human sera (IHS) from patients with serious enterococcal infections. Each IHS containing antienterococcal antibodies demonstrated by enzyme-linked immunosorbent assay and Western blotting (immunoblotting) was examined with the enterococcus strain isolated from the same patient. PNHS promoted PMN-mediated killing of enterococci similar to that for IHS. PMN-mediated killing was consistently abrogated after preopsonization with heat-inactivated PNHS, but some heat-inactivated IHS supported neutrophil bactericidal activity. Inhibition of the classical pathway of complement by chelation of either PNHS or IHS with Mg-EGTA [Mg–ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,N′,N′-tetraacetic acid] did not alter PMN-mediated killing, suggesting that activation of the alternative pathway of complement is sufficient to promote killing of enterococci by PMNs. PMN-mediated killing assays were also performed with normal rabbit serum and immune rabbit serum against enterococci. Preopsonization with heat-inactivated immune rabbit serum resulted in PMN-mediated killing of enterococci, which was ablated after adsorption of the serum with the same isolate used for immunization. The influence of different phenotypic enterococcal traits on neutrophil-mediated killing was also investigated. Similar kinetics of killing were observed for derivatives of *Enterococcus faecalis* strains regardless of resistance to antimicrobial agents or production of β-lactamase, hemolysin, gelatinase, or surface proteins involved in the aggregative response to pheromones. In summary, PMN-mediated killing of enterococci appears to depend primarily on complement activation by either the classical or the alternative pathway. Human antienterococcal antibodies generated during infection variably promoted neutrophil bactericidal activity, while antibody raised in a rabbit supported PMN-mediated killing of the organism examined. Finally, the different phenotypic properties of *E. faecalis* examined did not influence the neutrophil-mediated killing of these organisms.

The most recent National Nosocomial Infection Surveillance system report indicated that enterococci were the second most common organism recovered from patients with nosocomial infections in the United States from 1986 to 1989, accounting for 12% of all infections (25). In that report, enterococci were recovered from 16% of urinary tract infections, 13% of surgical wound infections, and 8% of bloodstream infections. Not only have enterococci become a frequent cause of nosocomial infection, they have become increasingly resistant to antimicrobial agents as well (15). The emergence of high-level resistance to aminoglycosides, which abolishes the synergistic bactericidal effect of aminoglycosides when they are used with cell wall-active agents such as penicillin or vancomycin (13), along with β-lactamase production (17) and the recent development of glycopeptide resistance (14), have seriously complicated the treatment of these organisms (16). As antibiotic resistance increases, a better understanding of the pathogenesis of these infections and the interaction of these organisms with the human host defense system becomes even more important. Until recently, the interaction of enterococci with neutrophils and serum was poorly defined. In a recent study, complement promoted polymorphonuclear leukocyte (PMN)-mediated killing of these organisms very efficiently, while antibodies appeared to play a less important role (8).

To date, two properties, aggregative substance and hemolysin, have been suggested as virulence factors of enterococci. Aggregative substance, a plasmid-encoded surface protein synthesized by certain *Enterococcus faecalis* strains in response to pheromones (3, 18), has been postulated to mediate adhesion of these organisms to eukaryotic cells as well as to other *E. faecalis* organisms (12). While pheromones themselves have been demonstrated to stimulate human neutrophil responses (5), the influence of the pheromone-induced aggregative substance on PMN-mediated killing has not been reported. Hemolytic strains of enterococci have been found to be more virulent than nonhemolytic strains in an experimental mouse peritonitis model (10) and an endophthalmitis model (11). However, little is known about the bactericidal activity of neutrophils against these strains; recently, it has been reported that hemolysin production did not affect the PMN-mediated killing of enterococci (22).

The current study was undertaken to further define the interaction between enterococci and the human host defense system. We identified sera from patients with serious enterococcal infections containing antienterococcal antibodies and examined the capacity of human complement and antibodies to promote PMN-mediated killing. We also explored the contribution of antienterococcal antibodies raised in rabbits and determined the influence of different bacterial phenotypic traits on the susceptibility of enterococci to PMN-mediated killing.

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TABLE 1. Laboratory enterococcal isolates used in this study

Strain	Phenotype[a]	Plasmid(s)[b]	Reference
HH22	Sm^r Tc^r Gm^r Bla^+ Em^r, cryptic	pBEM10, pAM323, pAM324	19
HH22-Pen^s	Sm^r Tc^r Gm^r Bla^- Em^r, cryptic	pBEM10Δ, pAM323, pAM324	20
OG1RF	Gel^+ Rif^r Fa^r	None	18
OG1RF(pBEM10)	Gel^+ Rif^r Fa^r Gm^r Bla^+	pBEM10	18
OG1RF(pAM323)	Gel^+ Rif^r Fa^r Em^r	pAM323	18
OG1X	Gel^- Sm^r	None	18
OG1X(pAD1)	Gel^- Sm^r Hly^+ Bac^+	pAD1	18
STG3[c]	Sm^r Sp^r, Agg constitutive Tc^r Gm^r	pCF10::Tn*5281*	9
INY3000[d]	Sm^r Sp^r Tc^r Con^-	None	27
INY3039[e]	Sm^r Sp^r Tc^r Con^+	None	27
INY3040[e]	Sm^r Sp^r Tc^r Con^+	None	27
INY3044[e]	Sm^r Sp^r Tc^r Con^+	None	27
INY3048[e]	Sm^r Sp^r Tc^r Con^+	None	27

[a] Agg, aggregative; Bac, bacteriocin; Bla, β-lactamase; Con^-, conjugation defective; Em, erythromycin; Fa, fusidic acid; Gel, gelatinase; Gm, gentamicin; Hly, hemolysin; Pen, penicillin; Rif, rifampin; Sm, streptomycin; Sp, spectinomycin; Tc, tetracycline.

[b] All plasmids are pheromone inducible, except pCF10::Tn*5281*.

[c] OG1SSp with a derivative of pCF10 that directs constitutive production of the aggregation substance.

[d] OG1SSp containing four Tn*916* insertions in the chromosome.

[e] OG1SSp containing a single Tn*916* insertion in the chromosome (one of the four present in INY3000).

MATERIALS AND METHODS

Special reagents. Chemicals were obtained from Sigma Chemical Co. (St. Louis, Mo.), except for heat-inactivated horse serum from GIBCO BRL Life Technologies, Inc. (Gaithersburg, Md.), brain heart infusion (BHI) broth and skim milk from Difco Laboratories (Detroit, Mich.), and standard molecular weight proteins from Bio-Rad Laboratories (Richmond, Calif.).

Bacterial strains. All organisms used in this study were presumptively identified as enterococci by their ability to hydrolyze esculin and to grow in BHI broth containing 6.5% NaCl. Subsequent identification to the species level was performed in our laboratory according to the criteria of Facklam and Collins (6). Eight of the enterococcal strains used in this study were isolated from clinical specimens submitted to the microbiology laboratories of Hermann Hospital, Methodist Hospital, or St. Luke's Episcopal Hospital, Houston, Tex., between June 1991 and September 1992. Seven of these strains (TEX11 through TEX17) were isolated from the bloodstreams of patients who had a diagnosis of infective endocarditis, and one strain (TEX18) was isolated from the bone biopsy of a patient with a diagnosis of chronic osteomyelitis. TEX15 and TEX16 were identified as *Enterococcus faecium*, and the other six strains were identified as *E. faecalis.*

Table 1 shows the laboratory enterococcal isolates used in this study. *E. faecalis* strain HH22 was isolated from a urine specimen at Hermann Hospital, Houston, Tex., in 1981 (19). This strain is highly resistant to gentamicin (MIC, >2,000 μg/ml), produces β-lactamase (Bla^+), and contains three pheromone-responsive plasmids, pBEM10, pAM323, and pAM324, which respond to pheromones cAD1, cAM323, and cAM324, respectively (4, 17, 18). *E. faecalis* HH22-Pen^s is a novobiocin-cured derivative of HH22 which no longer produces penicillinase (Bla^-) (20). *E. faecalis* OG1RF, OG1X, and OG1SSp are derivatives of OG1 (18, 27). Several derivatives of OG1SSp were used, including INY3000, containing four random chromosomal copies of the tetracycline resistance transposon Tn*916*, and INY3039, INY3040, INY3044, INY3048, each containing one of the four chromosomal copies of Tn*916* (27). INY3000 is a conjugation-defective (Con^-) mutant whose phenotype is manifested by the inability to conjugate in broth with donor cells carrying a pheromone-inducible plasmid as a result of the failure to express binding substance. Although INY3039, INY3040, INY3044, and INY3048 each carry one of the individual Tn*916* inserts of INY3000, they are Con^+ (9). A constitutive clumping (self-aggregation in liquid media) derivative of OG1SSp harboring plasmid pCF10::Tn*5281*, termed STG3, was also evaluated. This derivative was generated by the insertion of Tn*5281* into pCF10, which resulted in hyperexpression of aggregative substance and consequent constitutive clumping (9). All clinical and laboratory enterococci were stored at −70°C.

Sera. (i) Immune human serum (IHS). Serum was obtained from the seven patients reported to have enterococcal infective endocarditis and one patient with chronic osteomyelitis from whom the clinical enterococcal strains were isolated. The sera were investigated for the presence of specific antibodies against enterococci and then used for the PMN-mediated killing assays. Pooled normal human serum (PNHS) was obtained by venipuncture from 10 healthy adult volunteers. Whole blood was allowed to clot at room temperature and centrifuged at 4°C, and sera were stored in aliquots at −70°C.

(ii) Immune rabbit serum (IRS). *E. faecalis* HH22 was grown in 20 ml of 50% heat-inactivated horse serum in BHI for 18 h. Whole cells were harvested by centrifugation at 6,000 × *g* for 10 min and suspended in 20 ml of 0.6% formalin in 0.9% NaCl. Cells were incubated overnight at 4°C, centrifuged at 6,000 × *g* for 10 min, washed twice, and resuspended in 2 ml of 0.9% NaCl. A New Zealand White rabbit was injected intravenously once every 3 days for three injections and then every 2 weeks for four injections with 0.5 ml of the formalin-killed whole-cell suspension diluted 1:10 in normal saline, resulting in approximately 2×10^9 bacteria per injection. Blood was obtained before (preimmune rabbit serum [PRS]) and after (IRS) immunization. Serum was aliquoted and stored at −70°C.

(iii) Heat-inactivation and Mg-EGTA treatment. To inactivate complement, serum was heated at 56°C for 30 min. The classic complement pathway was selectively inhibited by chelation with 10 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N,N,N′,N′*,-tetraacetic acid] and 10 mM $MgCl_2$ (7).

(iv) Adsorption of sera. Antibodies were adsorbed from IRS by incubation with heat-killed *E. faecalis* HH22 (the same strain as that used for immunization) or *Escherichia coli* ATCC 11775 as a control. Bacteria were grown in BHI broth at 37°C overnight with end-over-end rotation, centrifuged, resuspended in distilled water, and boiled for 10 min. After incubation with $\sim 6.4 \times 10^{10}$ heat-killed bacteria per ml of serum at 4°C for 2 h, the adsorbed serum was centrifuged at 10,000 × *g*, 4°C, for 10 min and used for experiments.

ELISA. An enzyme-linked immunosorbent assay (ELISA) was performed by a modification of a previously described method (26). A whole-cell antigen preparation was utilized, because when we attempted to use the putatively more specific antigen preparation reported by Shorrock et al. (26) we were unable to distinguish patients with endocarditis from normal controls (data not shown). The whole-cell antigen was prepared from *E. faecalis* HH22 grown in 100 ml of BHI broth in shaking flasks at 37°C overnight, diluted 1:10 in 0.05 M sodium carbonate buffer (pH 9.6), and allowed to adhere to 96-well polystyrene ELISA plates (flexible round bottom; Dynatech Laboratories Inc., Alexandria, Va.) overnight at 4°C. After

washing, wells were blocked with 1% skim milk in phosphate-buffered saline containing 0.05% Tween 20 (PBST). Each serum was assayed in duplicate in a dilution of 1:100 to 1:12,800 in 1% skim milk in PBST. Goat anti-human immunoglobulin G (IgG) or goat anti-rabbit IgG peroxidase conjugates were used for the detection of human or rabbit antibodies to enterococci, respectively. Titers were expressed as the highest dilution of the serum tested that gave an A_{450} of 0.2 optical density units above background (10-fold higher than the absorbance produced by controls with no serum present).

Western blotting. The antigen for Western blotting (immunoblotting) was prepared from HH22 grown in 1% heat-inactivated horse serum in BHI as described previously (1, 26) by sarcosyl extraction and sequential ammonium sulfate precipitation. The 90% ammonium sulfate pellet was suspended in distilled water, dialyzed, and stored at $-20°C$.

Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electrophoretically transferred onto nitrocellulose membranes. The blots were incubated with sera, and human and rabbit antienterococcal antibodies were subsequently detected by using either goat anti-human IgG or goat anti-rabbit IgG peroxidase conjugates, respectively.

Neutrophil isolation. PMNs were isolated from normal volunteers from EDTA-anticoagulated blood by dextran sedimentation, Ficoll-Hypaque centrifugation, and hypotonic lysis of residual erythrocytes, as previously described (23). Cells were suspended in Hanks' balanced salt solution without Ca^{2+} and Mg^{2+} (HBSS-CM) at approximately 2×10^7 PMNs per ml. Concentrated cells were >95% neutrophils by Diff-Quick staining, and viability was >96% by trypan blue exclusion.

PMN-mediated killing assay. For each killing experiment, bacteria from BHI agar plates were inoculated into BHI broth, incubated overnight at 37°C, subsequently inoculated into fresh BHI broth, and harvested during the mid-log phase of growth. After washing three times with HBSS-CM, the bacterial density was adjusted spectrophotometrically. Aggregates of the constitutively clumping STG3 strain were disrupted by sonication before the bacterial density was adjusted; this procedure did not affect their viability. To examine the effect of PNHS, enterococci were preopsonized by incubation in 10% (vol/vol) PNHS in HBSS-CM at 37°C for 15 min with end-over-end rotation. To examine the effect of IHS or rabbit sera, 5% (vol/vol) PNHS was added to 5% (vol/vol) IHS, PRS, or IRS in HBSS-CM as a source of complement, since delays in processing and prolonged storage of IHS and rabbit sera likely resulted in deterioration of complement activity. While the functional activity of untreated serum was not diminished at 5% serum concentrations (data not shown), the activity of the alternative pathway declines at serum concentrations less than 10%, so we used only 10% PNHS or 5% PNHS plus 5% serum from a patient infected with TEX12 (sTEX12) (which was processed immediately) for examination of the effects of Mg-EGTA treatment. After opsonization, cells were pelleted at $10,000 \times g$, 4°C, for 15 min, washed, and suspended in HBSS-CM to a final concentration of approximately 10^8 CFU/ml.

Each killing mixture contained approximately 2×10^7 to 4×10^7 preopsonized enterococci per ml and 10^7 PMN per ml in a final volume of 200 μl in microtiter plate wells. Calcium and Mg^{2+} were added to the reaction mixture to a final concentration of 1 mM. The mixtures were incubated at 37°C on a shaker. At the times indicated in the figures, 20-μl aliquots were diluted with 180 μl of distilled water for 10 min to lyse the PMNs and release the viable intracellular bacteria. Subsequently, the samples were serially diluted in 0.1 M Na_2SO_4, and colony counts were determined by the pour plate method using BHI agar. Controls lacking PMNs were used to examine the possibility of extracellular killing by serum or a bactericidal effect of the distilled water. Results represent the percentages of initial viable CFU expressed as the means of three experiments, and data were analyzed by Student's t test (two tailed).

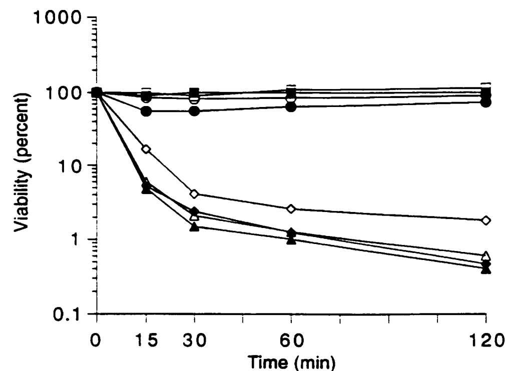

FIG. 1. PMN-mediated killing of a clinical isolate of *E. faecalis* TEX12. TEX12 was preopsonized with PNHS (△), IHS from a patient infected with TEX12 (sTEX12) (▲), heat-inactivated PNHS (○), heat-inactivated IHS (●), Mg-EGTA-treated PNHS (◇), or MgEGTA-treated IHS (◆). Controls lacking preopsonization (□) or lacking neutrophils (■) were included. The data are means of three experiments performed in duplicate.

RESULTS

Kinetics of PMN-mediated killing of enterococci. Initial experiments were carried out to determine the interaction of enterococci with human PMNs and the role of the classical or alternative pathway of complement in the PMN-mediated killing of these organisms. Preopsonization of *E. faecalis* TEX12 with PNHS promoted more than 90% killing after 30 min and 99% killing after 120 min of incubation with PMNs (Fig. 1). Heat inactivation of complement in PNHS, however, abrogated the neutrophil bactericidal activity. When preopsonized with PNHS, all other clinical isolates of *E. faecalis* were also efficiently killed by PMNs in a similar manner as *E. faecalis* TEX12 (Fig. 2), but TEX15 and TEX16, which are both strains of *E. faecium*, were resistant to PMN-mediated killing.

The efficiency of the alternative pathway of complement to promote PMN-mediated killing was examined by selectively inhibiting the classical pathway with 10 mM EGTA–10 mM $MgCl_2$. PNHS chelated with Mg-EGTA was no different than nonchelated serum in promoting neutrophil bactericidal activity of TEX12 (Fig. 1). This result suggests that the alternative pathway of complement activation is sufficient for neutrophil killing of enterococci. Absence of serum preopsonization resulted in no killing by PMNs, and serum alone was not bactericidal for enterococci (Fig. 1).

Determination of specific antibodies against *E. faecalis* HH22 by ELISA and Western blotting. In order to verify the presence of antibodies in each serum used for PMN-mediated killing assays, we performed ELISA and Western blotting using sera from eight patients with severe enterococcal infection (seven patients reported to have infective endocarditis and one reported to have chronic osteomyelitis) and from a rabbit before (PRS) and after (IRS) immunization with *E. faecalis* HH22. PNHS, used as a negative control, had a titer of 1:200; some of the donors for the PNHS were laboratory workers who had worked with enterococci for several years, and this may

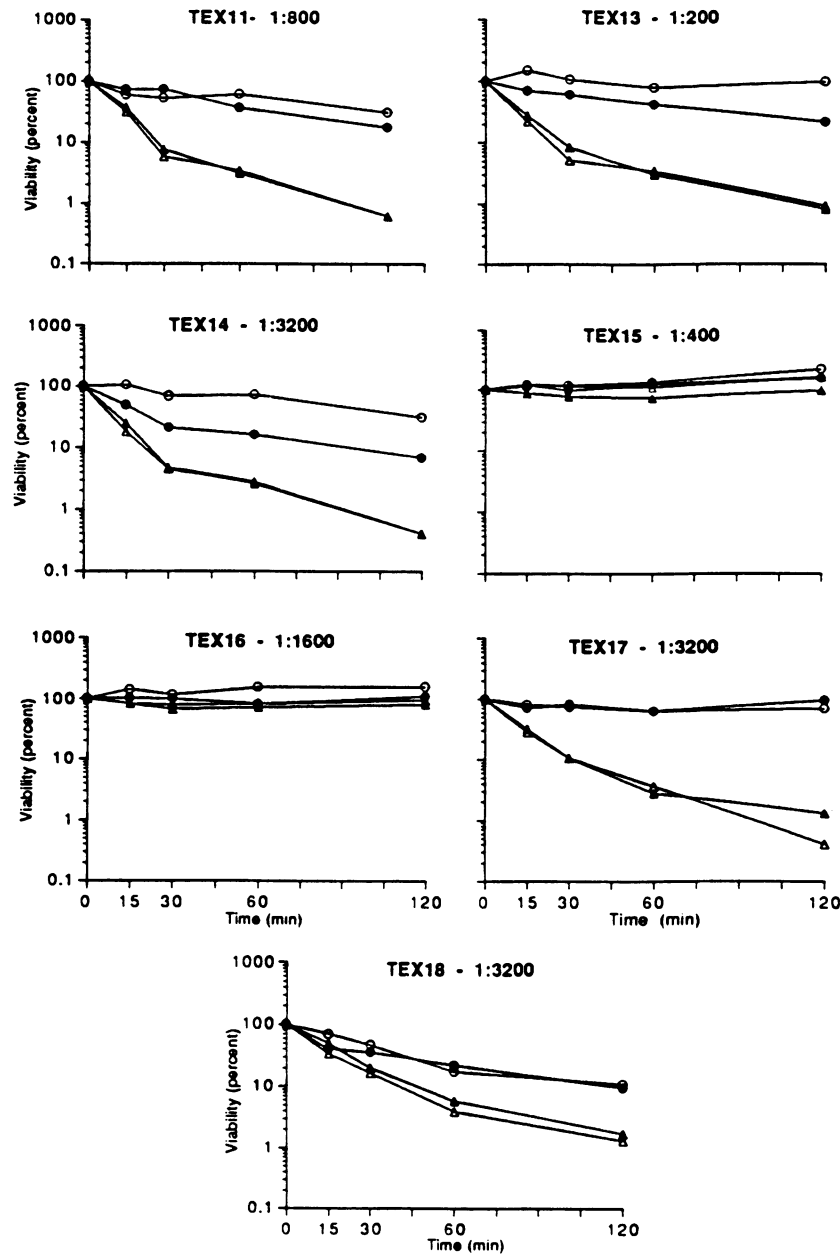

FIG. 2. PMN-mediated killing of clinical enterococcal isolates. ELISA titers of the homologous IHSs used are shown. The organisms were preopsonized with PNHS (△), homologous IHS (▲), heat-inactivated PNHS (○), or heat-inactivated homologous IHS (●). The data are means of three experiments performed in duplicate.

explain the presence of low-level antibodies. PRS had a titer of <1:100 to HH22 whole-cell antigen. Except for serum from one patient whose titer was 1:200 (sTEX13), serum antibody titers from the other six patients reported to have enterococcal infective endocarditis were higher than that for PNHS, ranging from 1:400 to 1:3,200 (1:800 for sTEX11, 1:3,200 for sTEX12, 1:3,200 for sTEX14, 1:400 for sTEX15, 1:1,600 for sTEX16, and 1:3,200 for sTEX17). Serum TEX13 was obtained after the patient had undergone plasmapheresis, which could have lowered the level of antibodies present. Serum TEX18 from the patient with chronic osteomyelitis was positive at 1:3,200. IRS had a titer of 1:1,600.

Figure 3 shows the immunoblot probed with sera from

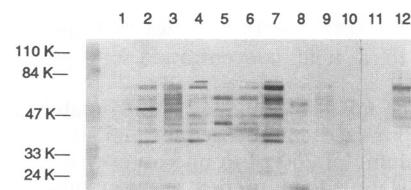

FIG. 3. Western blotting of *E. faecalis* HH22 probed with different sera. Lanes 1 to 7, patients with infective endocarditis (TEX11 to TEX17); lane 8, patient with chronic osteomyelitis (TEX18); lane 9, PNHS; lane 10, patient with possible infective endocarditis not included in the study; lane 11, PRS; lane 12, IRS. Molecular weights in thousands (K) are indicated on the left.

patients and the immunized rabbit. Sera from the seven patients with infective endocarditis (lanes 1 to 7), and serum from the patient with chronic osteomyelitis (lane 8) recognized one or more of the antigens from *E. faecalis* HH22. Lane 9 shows the PNHS, and lane 10 shows the serum from a patient with possible infective endocarditis, not included in this study. PRS was not reactive (lane 11), and IRS reacted strongly against *E. faecalis* HH22 antigens (lane 12). While certain bands were frequently seen among the different serum samples (e.g., 36, 43, 58, and 69 kDa), no particular band or pattern was obviously present in all of the immune sera tested.

Opsonic role of antienterococcal antibodies. To assess the role of antienterococcal antibodies alone in the PMN-mediated killing of enterococci, isolates from patients with severe enterococcal infection were preopsonized with heat-inactivated serum from the same patient from whom the organism had been isolated (homologous serum), and PMN-killing activity was compared with that following preopsonization with heat-inactivated PNHS (Fig. 2). A trend towards greater killing was observed with heat-inactivated homologous IHS versus heat-inactivated PNHS. However, only three of the six *E. faecalis* strain-serum pairs tested reached statistically significant differences by either 60 or 120 min of incubation. At 60 min of incubation, the percentages of initial viable CFU of these isolates preopsonized with heat-inactivated PNHS versus homologous IHS were 95% ± 5% versus 64% ± 3% for TEX12, 78% ± 15% versus 42% ± 8% for TEX13, and 73% ± 23% versus 16% ± 11% for TEX14 (mean ± standard deviation; $P \leq 0.05$ for each pair). Thus, certain human sera containing human antibodies against enterococci promoted PMN-mediated killing in the absence of intact complement, while others did not. Immune serum in the absence of PMNs showed no bactericidal activity against enterococci. PMN-mediated killing of the two *E. faecium* isolates resistant to killing (TEX15 and TEX16) was not enhanced by the presence of antienterococcal antibodies (Fig. 2). Interestingly, enterococcal strains TEX11, TEX14, and TEX18 preopsonized with heat-inactivated PNHS lost 70 to 90% of their viability after 120 min of neutrophil exposure (Fig. 2). However, there was no PMN-mediated killing of these isolates in the absence of serum (data not shown). Since certain sera containing antienterococcal antibodies promoted neutrophil bactericidal activity, the presence of low levels of antienterococcal antibodies in the PNHS (the ELISA titer of PNHS was 1:200, as mentioned above) might explain, in part, this finding.

To determine whether antienterococcal antibodies could promote additional neutrophil bactericidal activity when active complement was present, enterococci were preopsonized with homologous IHS or PNHS (Fig. 1 and 2). IHS did not promote more PMN-mediated killing than PNHS for any of the strains tested, independently of the ELISA titer.

Contribution of antibodies raised in rabbits to PMN-mediated killing. Since only some human antibodies from patients with serious enterococcal disease effectively promoted neutrophil bactericidal activity, we wondered whether immune serum raised in rabbits against killed enterococci might be effective. When complement was intact, preopsonization with IRS promoted the same PMN-mediated killing as PRS at all time points (Fig. 4); preopsonization with PRS without the addition of PNHS as a complement source gave the same results as those obtained when PNHS was added (data not shown). When complement was heat inactivated, IRS was significantly less effective than nonheated PRS or IRS at all time points ($P < 0.05$), but it was significantly better than heat-inactivated PRS ($P < 0.05$), whose neutrophil bactericidal activity was completely abrogated by heat inactivation. IRS in the absence of PMNs was not bactericidal for enterococci.

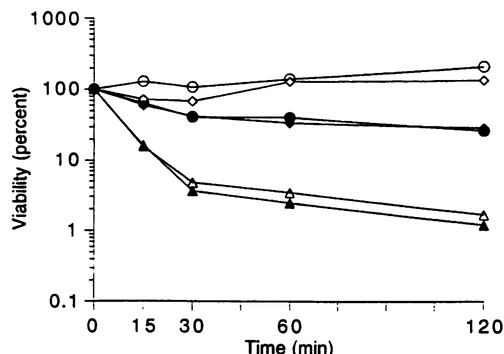

FIG. 4. Role of IRS in PMN-mediated killing of *E. faecalis*. *E. faecalis* HH22 was preopsonized with PRS (△); IRS (▲); heat-inactivated PRS (○); heat-inactivated IRS (●); heat-inactivated IRS adsorbed with *E. faecalis* HH22 (◇); or heat-inactivated IRS adsorbed with *E. coli* (◆). The data are means of three experiments performed in duplicate.

Since either antienterococcal antibodies or another heat-stable component present in rabbit serum could have been responsible for the effect described above, we adsorbed IRS using heat-killed *E. faecalis* HH22 (the same strain used for immunization) to remove the antienterococcal antibodies produced by the rabbit. Adsorption with *E. coli* ATCC 11775 was performed as a control. Heat-inactivated IRS that had been adsorbed with HH22 lost all capacity for promoting PMN-mediated killing, while heat-inactivated IRS adsorbed with *E. coli* ATCC 11775 was unchanged (Fig. 4). Thus, although intact complement in both human and rabbit sera is the predominant contributor to PMN-mediated killing of enterococci, antienterococcal antibodies raised in rabbits do promote neutrophil killing in the absence of active complement.

Influence of enterococcal phenotypic traits on neutrophil bactericidal activity. We next examined the effects of various enterococcal phenotypic traits on PMN-mediated killing. When preopsonized with 10% PNHS, *E. faecalis* HH22 and OG1 and their derivatives listed in Table 1 were all susceptible to PMN-mediated killing, demonstrating more than 90% reduction of initial inoculum at 60 and 120 min. There were no significant differences in the PMN-mediated bactericidal rate among the derivatives (data not shown). These results indicate that neither the presence of pheromone-responsive plasmids; nor the production of β-lactamase, hemolysin, or gelatinase; nor antimicrobial susceptibility affects the susceptibility of enterococci to PMN-mediated killing.

In addition, six derivatives of *E. faecalis* OG1SSp which are either conjugation defective (INY3000), Con^+ (INY3039, INY3040, INY3044, and INY3048), or self-aggregative in liquid media (STG3) were examined for susceptibility to PMN-mediated killing after preopsonization with PNHS. Although STG3 showed a trend towards lower neutrophil-mediated killing compared with INY3000, with an average difference of 0.9 $\log_{10}$ CFU at 120 min, this difference did not reach statistical significance ($P > 0.05$ between both groups at each time point). The other four Con^+ and non self-aggregative derivatives (INY3039, INY3040, INY3044, and INY3048) showed similar neutrophil-mediated killing rates.

DISCUSSION

This study describes the ability of human neutrophils to kill enterococci in vitro and the role of antibodies and complement in this process. Since heat inactivation of normal serum consistently decreases or abrogates PMN-mediated killing, complement appears to be the major component of the serum that promotes neutrophil killing of enterococci. Experiments using Mg-EGTA chelation to inhibit the classical pathway suggest that the alternative pathway of complement alone is sufficient in promoting PMN-mediated killing. The convergence point for the alternative and classical complement pathways is C3, which is cleaved to C3b and subsequently to iC3b. These have been recognized as the major bacterial opsonins (21, 24) and most likely represent the common opsonins which promote the ingestion and killing of enterococci by PMNs.

Since patients with infective endocarditis are able to mount an antibody response to *E. faecalis* (1, 2, 26), we wondered whether serum containing human antienterococcal antibodies promotes PMN-mediated killing of enterococci. Although there was a trend towards enhanced neutrophil bactericidal activity when antibodies were present without active complement, these differences reached statistical significance for only three of the six *E. faecalis* strain-serum pairs examined. In addition, no relationship between the titer of antienterococcal antibody by our ELISA and PMN-mediated killing was apparent, suggesting that a higher level of antibody was not more helpful. Lower concentrations of immune serum were not more effective in promoting PMN-mediated killing (data not shown), suggesting that a prozone phenomenon, whereby serum containing large amounts of specific antibodies may appear ineffective, was not present. Although patients with infective endocarditis have specific IgG directed against *E. faecalis*, the nature of the antigens recognized is unknown. The use of a whole-cell antigen preparation in our ELISA may have reduced the specificity of this test and could have accounted for some of the variability observed. Therefore, the antibodies that we detected by ELISA or Western blotting may not be ones that particularly enhance the neutrophil bactericidal activity of enterococci.

The contribution of antienterococcal antibodies raised in a rabbit to the neutrophil killing of enterococci was also investigated. Rabbit antienterococcal antibodies effectively promoted PMN-mediated killing of enterococci. However, complement still appeared to be more important, since rabbit serum lacking antibodies but containing active complement promoted greater killing than that containing antibodies alone. Adsorption of immune serum with heat-killed *E. faecalis* HH22 abrogated the neutrophil bactericidal activity, confirming that antibodies promoted this activity. Comparison of results using rabbit serum versus human serum must take into account that antibodies produced against antigens on killed organisms used for rabbit immunization may promote a neutrophil bactericidal activity different from those stimulated by live organisms causing enterococcal infections in humans.

Harvey et al. (8) examined the contributions of complement and antibody in the PMN-mediated killing of enterococci. Our findings concur with their study that complement was of primary importance in the neutrophil killing of enterococci. Unlike our results, however, when these investigators explored the role of antibodies using normal rabbit serum and IRS, they found that IRS promoted greater neutrophil killing than PRS only in the presence of active complement. In addition, PRS was markedly deficient in promoting PMN-mediated killing of enterococci compared with NHS. In contrast, we found that IRS was not better than PRS when complement was active and that heat-inactivated IRS still promoted some neutrophil bactericidal activity.

Although only eight *E. faecalis* and two *E. faecium* strains were studied, it is interesting that PMN-mediated killing was different among these two species (Fig. 2). The two *E. faecium* isolates were more resistant to PMN-mediated killing than the *E. faecalis* isolates, and their killing was not enhanced by preopsonization with homologous serum containing both active complement and antienterococcal antibodies. In the study of Harvey et al. (8), the *E. faecium* isolate described showed a reduction in CFU of more than 1 $\log_{10}$ unit by 60 min of incubation, no different than that seen with *E. faecalis*. Only one strain was reported to be more resistant to killing in that study, but the species was not identified.

Some of the pheromones produced and released by *E. faecalis* promote a chemotactic cell response and activate the respiratory burst in human neutrophils (5). *E. faecalis* HH22, which is known to produce pheromones in vitro (19), was killed in the same fashion as other enterococcal strains. Enterococci used for the PMN-killing assays, however, were washed before preopsonization, which might have removed these extracellular bacterial oligopeptides. We also examined PMN-mediated killing of enterococcal strains OG1 displaying different phenotypes. Antimicrobial resistance and gelatinase production did not influence the neutrophil killing rate of the derivatives examined. In accordance with a recent report (22), our study also failed to show any difference in susceptibility to PMN-mediated killing conferred by the presence of the hemolysin-encoding plasmid pAD1. All *E. faecalis* OG1SSp derivative strains showed similar susceptibilities to PMN-mediated killing independently of the production of binding substance. Although there was a trend towards lower neutrophil killing for strain STG3, which is a constitutive producer of aggregative substance and self-aggregative in liquid media, this difference was not statistically significant. Therefore, neither binding substance nor aggregative substance appeared to play a major role in the interaction of enterococci with neutrophils in vitro.

We conclude that PMN-mediated killing of *E. faecalis* is dependent primarily on complement and that some human and rabbit antienterococcal antibodies promote PMN-mediated killing activity, though to a lesser degree. Different phenotypic traits of *E. faecalis*, including antimicrobial susceptibility and production of gelatinase, hemolysin or aggregative substance, did not influence the susceptibility to killing by neutrophils. There was a difference in susceptibility to killing between the two species of enterococci studied, with the two strains of *E. faecium* being resistant to PMN killing. Factors associated with complement activation, binding to PMNs, or intraphagosomal microbicidal systems may be responsible for this species-specific resistance, and further studies exploring these speculations are being pursued.

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