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Anaerobiosis Increases Resistance of *Neisseria gonorrhoeae* to O_2-Independent Antimicrobial Proteins from Human Polymorphonuclear Granulocytes

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We investigated the in vitro resistance of *Neisseria gonorrrhoeae* FA19 to the O_2-independent antimicrobial systems of human polymorphonuclear leukocytes. Acid extracts of polymorphonuclear leukocyte granules (crude granule extracts) and a purified granule protein (57 kilodaltons) were, at low concentrations, bactericidal for gonococci under aerobic conditions that permitted growth. However, they were less effective under anaerobic conditions that imposed bacteriostasis. We found that adding sodium nitrite to reduced growth media permitted the growth of strain FA19 in an anaerobic environment. Under these conditions with nitrite, anaerobic cultures of strain FA19 were no more resistant to the crude granule extract and the 57-kilodalton protein than aerobic cultures. In contrast, *Salmonella typhimurium* SL-1004, a facultative anaerobe, was readily killed by both the crude granule extract and the 57-kilodalton antimicrobial protein regardless of the presence or absence of free molecular oxygen. This is the first demonstration that an isolated antimicrobial protein from polymorphonuclear leukocyte granules is active against bacteria under anaerobic conditions. Our results also indicated that the efficacy of human polymorphonuclear leukocyte O_2-independent killing of *N. gonorrhoeae* may, in part, be inhibited by bacteriostatic conditions imposed by hypoxia.

Among the antimicrobial systems of polymorphonuclear leukocytes (PMN) are those dependent on the reduction of molecular O_2 and those independent of O_2 (15, 16). O_2-independent antimicrobial systems have attracted renewed attention, perhaps because of the observation that PMN obtained from patients with chronic granulomatous disease readily ingest and kill certain bacteria (24). These PMN are unable to reduce molecular O_2 and must therefore utilize O_2-independent antimicrobial mechanisms for intraleukocytic killing of bacteria (15, 16). In normal individuals, O_2-independent antimicrobial mechanisms might represent a significant cellular response against infection, particularly at sites where hypoxia occurs.

Piliated (T1 and T2) gonococci readily attach to PMN but apparently resist phagocytosis (6, 11) to a greater extent than nonpiliated (T3 and T4) gonococci (20, 21, 29–31). The fate of such ingested gonococci has been the subject of controversy. Although most studies indicate little if any intracellular survival of gonococci (6, 7, 11, 21, 29, 33, 36, 37), other investigators have proposed that some intracellular gonococci survive and even replicate within human phagocytic cells (2, 3, 32). The mechanisms by which such intracellular gonococci might survive the toxic environment of the phagolysosome are unknown.

Studies by Rest (22) and Daley et al. (4) documented the in vitro sensitivity of gonococci to the granule proteins which contribute significantly to the O_2-independent antimicrobial activity of human PMN. This PMN antimicrobial system is relevant because gonococci are often isolated in conjunction with strict anaerobes (8). Thus, in vivo, gonococci might find that hypoxic conditions are bacteriostatic and therefore protective against O_2-independent killing due to PMN. At such hypoxic sites, the O_2-independent antimicrobial systems would presumably represent the major antigonococcal mechanism available to PMN. Accordingly, we studied the antigonococcal activity of human PMN granule proteins under both aerobic and anaerobic conditions.

We report here that anaerobic conditions are bacteriostatic for gonococci and increase their resistance to both crude granule extracts (CGE) and to an isolated granule antimicrobial protein. However, gonococci cultured anaerobically in reduced media containing sodium nitrite grew and were as sensitive as bacteria grown aerobically. In contrast, *Salmonella typhimurium*, a facultative anaerobe, was equally sensitive to the antimicrobial proteins whether it was exposed to aerobic or anaerobic conditions. The data indicate that although the exposure of gonococci to anaerobic conditions increased their resistance to O_2-independent killing by PMN, this effect could be reversed when gonococci were permitted to grow in an anaerobic environment supplemented with sodium nitrite.

MATERIALS AND METHODS

Bacteria. *Neisseria gonorrhoeae* FA19 (provided by P. F. Sparling, University of North Carolina) was maintained as T1 transparent colonies on Gonococcal Base (GCB) agar (Difco Laboratories, Detroit, Mich.) *S. typhimurium* SL-1004, an RD_1 lipopolysaccharide mutant of strain LT2 (18), was also used in this study.

Media. *N. gonorrhoeae* was maintained and the number of CFU from bactericidal assays was determined on GCB agar base with defined supplements (4). Broth cultures of gonococci were prepared in GCB broth with defined supplements and sodium bicarbonate (0.5 g/liter; Mallinckrodt Inc.). *S. typhimurium* was maintained and the number of CFU from bactericidal assays was assessed on Trypticase soy agar (BBL Microbiology Systems, Cockeysville, Md.). Broth cultures were prepared in Trypticase soy broth (BBL). All broths were adjusted to pH 7.0 before use.

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Anaerobic conditions. All anaerobic procedures were conducted in a VPI-type anaerobic chamber (Virginia Polytechnic Institute and State University, Blacksburg, Va.), which was filled and purged with an anaerobic gas mixture (5% CO_2, 10% H_2, and 85% N_2; Union Carbide Corp. Atlanta, Ga.). The chamber contained a palladium-coated catalyst to remove residual oxygen. Strict anaerobic conditions were maintained in the chamber and were checked with methylene blue anaerobic indicator strips (BBL). All materials were reduced before use by placing them in the anaerobic chamber overnight. To reduce broth, 100-ml samples were placed in the chamber and gently aerated overnight with the anaerobic gas mixture from a Pasteur pipette that was connected to a gas regulator tap and a diaphragm pump with in-line membrane filters (sterile disposable Millex-GV 0.22-μm-pore-size filter unit; Millipore Corp., Bedford, Mass). This overnight gassing reduced the percentage of free-O_2 saturation from 5.15 to 0.03 ppm in GCB broth in the absence or presence of 0.01% (wt/vol) sodium nitrite. The free O_2 was measured with an oxygen meter, (model 24-5513-50; Cole-Parmer Instrument Co., Chicago, Ill.). The slow addition of grains of sodium dithionite (J. T. Baker Chemical Co., Phillipsburg, N.J.) to reduced broth in the absence or presence of sodium nitrite did not cause further detectable reduction of the broth. After reduction, the broth was adjusted to pH 7.0.

Cultivation of gonococci in an anaerobic environment. A 5-mm-diameter paper disk (Whatman no. 1; Whatman Inc., Clifton, N.J.) was soaked in a filter-sterilized 10% (wt/vol) sodium nitrite (Mallinckrodt) solution, placed on a freshly prepared lawn of gonococci (with bacteria taken directly from a frozen stock culture), and incubated for 24 h at 37°C in the anaerobic chamber. The GCB agar was further moistened with 1 ml of reduced GCB broth (containing supplements, sodium bicarbonate, and 0.01% [wt/vol] sodium nitrite) and was incubated for an additional 12 h in the anaerobic chamber.

Gonococci grown in the anaerobic chamber were harvested by removing the GCB agar plates from the anaerobic chamber, flooding the plates with 2 ml of reduced GCB broth, scraping the agar surface with a sterile glass Pasteur pipette, and removing the broth. The gonococci were then centrifuged (1,200 × *g*, 10 min), and the supernatant was discarded. The pellet was gently resuspended in reduced GCB broth containing 0.01% (wt/vol) sodium nitrite. This suspension was transferred to the anaerobic chamber, gently aerated with the anaerobic gas mixture for 15 min, and incubated for another 6 h at 37°C. Appropriate dilutions of the bacterial suspensions in fresh reduced GCB broth were then prepared for incorporation into the bactericidal assay.

Monitoring of gonococcal metabolism and growth during exposure to anaerobic conditions. Gonococci were grown in the anaerobic chamber as outlined above with one exception: after centrifugation, the bacterial pellet was gently resuspended in 1 ml of reduced GCB broth in the absence of sodium nitrite. A 300-μl portion of this bacterial suspension was used to inoculate each of six 14-ml samples of reduced GCB broth. These samples contained defined supplements, sodium bicarbonate, 1 μCi of either uniformly ^{14}C-labeled L-amino acid mixture (specific activity, 310 mCi/mmol) or adenine (specific activity, 1.99 mCi/mg) (Amersham Corp., Arlington Heights, Ill.), and 0.005, 0.01, or 0.05% (wt/vol) sodium nitrite. Gonococci grown aerobically (in 5% CO_2–air at 37°C for 18 h) on GCB agar were suspended in reduced GCB broth, centrifuged (1,200 × *g*, 10 min) and resuspended in reduced GCB broth in the absence of sodium nitrite. These bacteria were further diluted in reduced GCB broth and used to inoculate two 14-ml portions of reduced GCB broth containing defined supplements, sodium bicarbonate, and 1 μCi of either [U-^{14}C]adenine or uniformly ^{14}C-labeled L-amino acids but without sodium nitrite. These eight 14-ml samples of gonococcal suspensions were transferred to the anaerobic chamber and gently aerated with the anaerobic gas mixture for 15 min. At hourly intervals for up to 9 h, small samples of each suspension were removed, and the incorporation of uniformly ^{14}C-labeled L-amino acids or [U-^{14}C]adenine was determined. In addition, the number of viable CFU was determined by plating appropriate dilutions (0.1 ml) onto GCB agar. All plates were incubated aerobically at 37°C under 5% CO_2 for 48 h before determining viability. To determine the uptake of uniformly ^{14}C-labeled L-amino acids or adenine, a 200-μl sample was pipetted onto a glass microfiber filter disk (2.4 cm; GF/C; Whatman). The protein was precipitated onto the fiber disk with 10% (wt/vol) trichloroacetic acid. The filter was washed four times with 1 ml of 10% trichloroacetic acid each time and air dried at 37°C overnight before being solubilized in 800 μl of Protosol (New England Nuclear Corp., Boston, Mass.) for 15 min. After this treatment, 4.2 ml of scintillation fluid was added (Scinti-Verse I; Fisher Scientific Co., Pittsburgh, Pa.), and the samples were stored in the dark overnight before being read on a Beckman LS8000 series liquid scintillation system (Beckman Instruments, Inc., Fullerton, Calif.). Each sample was assessed in duplicate.

Preparation of CGE and purification of the 57-kD antimicrobial protein. A granulocyte concentrate (95% PMN) was obtained by leukophoresis from a patient with chronic myelocytic leukemia undergoing treatment at Emory University Hospital (informed consent was obtained). The preparation and disruption of PMN have been described previously (18, 23). Granules were obtained by high-speed centrifugation and extracted with 0.2 M sodium acetate (pH 4.0). The CGE (525 mg of total protein) was dialyzed overnight against 0.05 M sodium acetate (pH 5.0)–0.15 M sodium chloride and then chromatographed on carboxymethyl Sephadex (Pharmacia Fine Chemicals, Piscataway, N.J.). Protein was eluted from the column with a two-step linear sodium chloride gradient (0.15 to 0.5 and 0.5 to 1.0 M sodium chloride) in 0.05 M sodium acetate (pH 5.0). The antigonococcal activity (in 2.7% of the total protein applied to the column) was eluted from the column at sodium chloride concentrations ranging from 0.6 to 0.7 M. This sample was dialyzed overnight at 4°C against 0.2 M sodium acetate, concentrated by ultrafiltration with a YM-5 membrane filter, and chromatographed on Sephadex G-75-SF (Pharmacia). Antimicrobial activity against *N. gonorrhoeae* FA19 was recovered, and the protein portion of the recovered material migrated as a single entity of approximately 57 kilodaltons (kD) as determined by silver staining of sodium dodecyl sulfate-polyacrylamide gel electrophoresis profiles (Fig. 1). The 57-kD protein was stored at 4°C in 0.2 M sodium acetate (pH 4.0) before use. Before the incorporation of these preparations in bactericidal assays, both the CGE and the 57-kD protein were dialyzed overnight against glass-distilled water with dialysis tubing that retained proteins of greater than 3,500 molecular weight. Twofold dilutions of the dialyzed preparations were made in Linbro 96-well microtiter plates (Flow Laboratories, Inc., McLean, Va.) with the appropriate reduced broth and then sterilized with UV irradiation (10 cm from the source for 5 min with two bactericidal lamps, each producing 8×10^{-4} W cm^{-2} at 30 cm from the source, with 90% of the radiation in the

region of 253 nm) (Germicidal G15T8; Sylvania). Protein concentrations were determined with chicken egg-white lysozyme as a standard, as described by Bradford (1).

Bactericidal assay. CGE (10 μl) or 57-kD antimicrobial protein (50 μl) and the same volumes of twofold serial dilutions of these preparations (in the appropriate reduced broth) were added to 100 μl of bacterial suspension and then adjusted to a final volume of 220 μl (by the addition of appropriate broth) in Linbro 96-well microtiter plates. When *N. gonorrhoeae* was incorporated in bactericidal assay, the bacteria were harvested from GCB agar into GCB broth and incubated at 37°C to midlogarithmic phase. These cultures were serially diluted to provide 10^3 to 10^4 CFU/ml in GCB broth. In all of the anaerobic bactericidal assays, the final bacterial suspension was prepared in prereduced media and further gassed with the anaerobic gas mixture (15 min) before being added to the wells of the microtiter plates. When *S. typhimurium* was incorporated in the bactericidal assays, the bacterial suspension was prepared from an overnight culture of bacteria in Trypticase soy broth. This culture was diluted 100-fold in 10 ml of fresh medium and incubated at 37°C to midlogarithmic phase (optical density at 650 nm of 0.5 per cm). These cultures were washed twice with tryptone saline (23) and serially diluted to provide 10^3 to 10^4 CFU/ml. These final dilutions of *S. typhimurium* were prepared in reduced broth. When the bactericidal assays were conducted under anaerobic conditions, the samples were added to wells containing the bactericidal preparations in reduced broth. Control samples contained 100 μl of bacterial suspension and 120 μl of broth. After a 1-h incubation period at 37°C, two 100-μl portions of each sample were plated onto the appropriate agar and incubated (5% CO_2–air at 37°C for 12 to 48 h) to permit the determination of recovered CFU. The number of recovered CFU of bacteria incubated with the bactericidal preparations was calculated as a percentage of the number of CFU recovered from control samples.

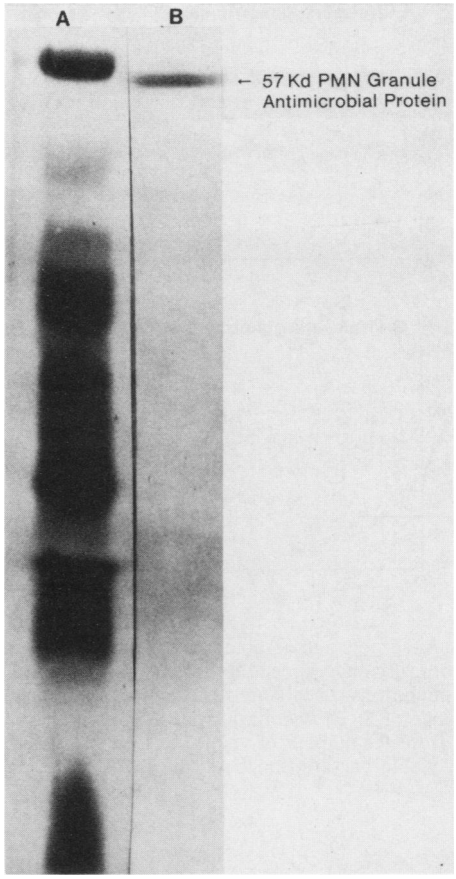

FIG. 1. Silver-stained sodium dodecyl sulfate-polyacrylamide gel electrophoresis profile of CGE and 57-kD antimicrobial protein. Proteins were solubilized and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and separated proteins were visualized by silver staining. Lanes: A, 15 μg of CGE; B, 0.5 μg of purified 57-kD antimicrobial protein.

RESULTS

Anaerobic and aerobic sensitivity of *N. gonorrhoeae* and *S. typhimurium* to CGE and 57-kD granule protein. The antigonococcal activities of both the CGE and the 57-kD granule protein were assessed under aerobic and anaerobic conditions. In a parallel experiment, the same granule proteins were examined for antimicrobial activity against *S. typhimurium* SL-1004. In these experiments, bacteria were grown aerobically to midlogarithmic phase and diluted to 10^3 to 10^4 CFU/ml. They were then transferred to the anaerobic chamber and bubbled with the anaerobic gas mixture for 15 min before exposure to the granule protein preparations. The results (Fig. 2) showed that *S. typhimurium* SL-1004 was readily killed by both the CGE and the 57-kD protein under both aerobic and anaerobic conditions. However, under anaerobic conditions, *N. gonorrhoeae* FA19 exhibited increased resistance to both the CGE and the 57-kD protein. The results (Fig. 2) indicated that the 50% lethal dose (LD_{50}) of the CGE against strain FA19 was approximately 20 μg/ml of protein under aerobic conditions, whereas under anaerobic conditions it was greater than 310 μg/ml. Similarly, the LD_{50} of the 57-kD protein against strain FA19 was increased under anaerobic conditions to greater than 1.2 μg/ml, whereas under aerobic conditions it was 0.2 μg/ml. Anaerobic conditions per se were not responsible for the observed changes in the LD_{50} for both the CGE and the 57-kD protein, because both preparations killed *S. typhimurium* in approximately the same fashion under both aerobic and anaerobic conditions.

Growth of *N. gonorrhoeae* in an anaerobic environment. We sought to determine whether gonococci grown under anaerobic conditions exhibited increased resistance to granule proteins under anaerobic conditions as described above for aerobically grown gonococci. The incorporation of sodium nitrite into reduced gonococcal growth media permitted replication of the bacteria in the anaerobic chamber. *N. gonorrhoeae* was successfully grown anaerobically on GCB agar plates that were impregnated with sterile disks soaked with a 10% (wt/vol) sodium nitrite solution. After 48 h of incubation in the anaerobic chamber, the plates were removed and inspected for growth with a dissecting microscope. Plates containing sodium nitrite revealed pinpoint transparent colonies that grew as a halo around the disk. However, growth was more obvious when the plates were flooded with oxidase reagent (*p*-aminodimethylaniline oxalate) (Fig. 3). The apparent toxicity of high concentrations of sodium nitrite was suggested by the absence of growth immediately around the disk. Diffusion of the nitrite from the disk apparently permitted it to achieve an optimum concentration supporting gonococcal growth.

The metabolic activity and growth of gonococci in reduced broth containing various concentrations of sodium

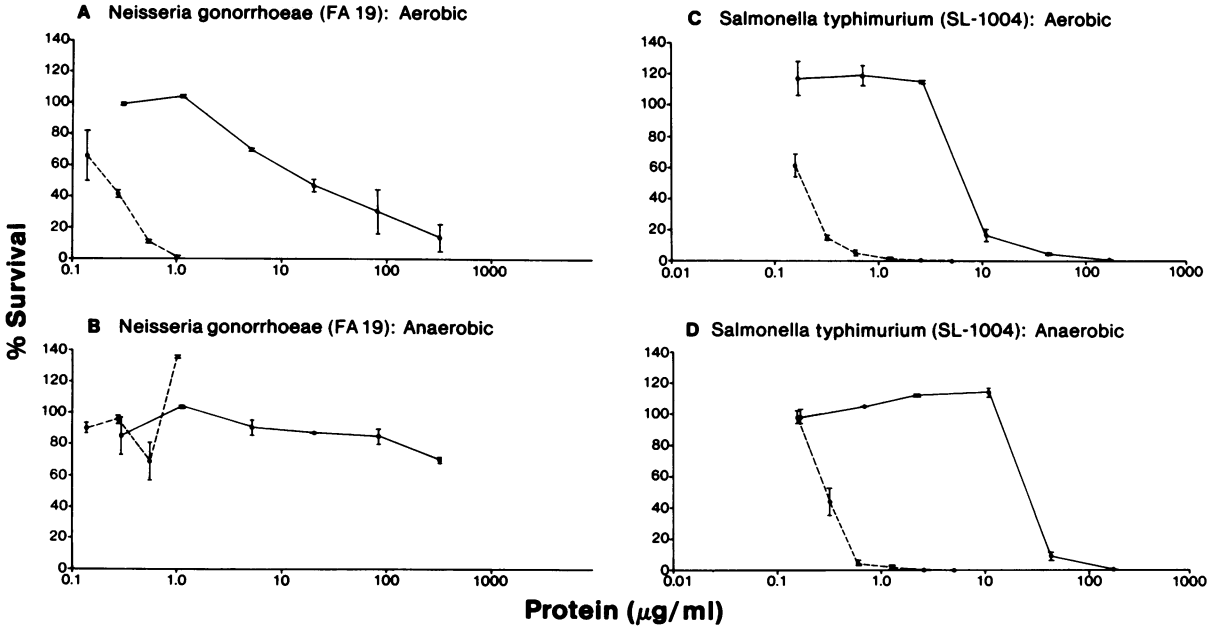

FIG. 2. Anaerobic and aerobic resistance of *N. gonorrhoeae* and *S. typhimurium* to CGE and 57-kD antimicrobial protein. Bacteria were grown aerobically and exposed to various concentrations of CGE (solid line) or 57-kD antimicrobial protein (dashed line) under aerobic or anaerobic conditions. Shown are resistance of *N. gonorrhoeae* FA19 to antimicrobial proteins under aerobic (A) and anaerobic (B) conditions and resistance of *S. typhimurium* SL-1004 to antimicrobial proteins under aerobic (C) and anaerobic (D) conditions. Percent survival was calculated from the number of viable bacteria recovered after incubation with the antimicrobial proteins and equated as a percentage of the number of viable bacteria incubated in media alone. Results are the average of two experiments; the vertical lines indicate variance of duplicates.

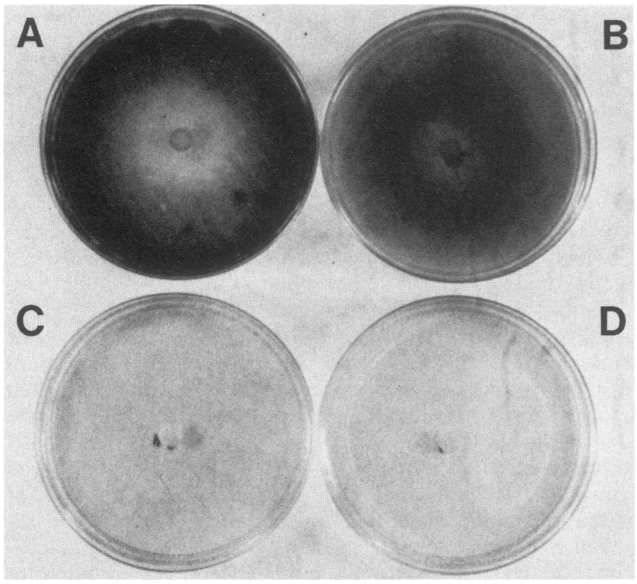

FIG. 3. Sodium nitrite promotes anaerobic growth of *N. gonorrhoeae* FA19 in an anaerobic environment. (A) Aerobically grown *N. gonorrhoeae* FA19 (5% CO_2–air, 37°C, 20 h) stained with oxidase reagent (*p*-aminodimethylaniline oxalate). (B) Anaerobic incubation of FA19 (5% CO_2, 10% H_2, 85% N_2; 37°C, 48 h; with paper disk soaked in 10% [wt/vol] sodium nitrite) stained with oxidase reagent. (C) Anaerobic incubation of FA19 (5% CO_2, 10% H_2, 85% N_2; 37°C, 48 h; with paper disk soaked in distilled water) stained with oxidase reagent. (D) Anaerobic incubation of GCB agar alone (5% CO_2, 10% H_2, 85% N_2, 37°C 48 h; with paper disk soaked in 10% sodium nitrite) stained with oxidase reagent.

nitrite were monitored during incubation in the anaerobic chamber. Incorporation of ^{14}C-labeled adenine or amino acid mixture permitted the monitoring of nucleic acid or protein synthesis during a 9-h incubation period. The results (Fig. 4) indicated that optimum nucleic acid and protein synthesis by anaerobic cultures of FA19 occurred when 0.01% (wt/vol) sodium nitrite was present. The metabolic activity of gonococci in the presence of 0.005% (wt/vol) sodium nitrite appeared to reach a plateau after 6 h, whereas 0.05% sodium nitrite permitted only minimal nucleic acid and protien synthesis. This high concentration of nitrite was also apparently toxic, since viability was decreased by three logs. In contrast, 0.01% sodium nitrite supported the growth of FA19 in reduced GCB broth. During the 9-h incubation period, the cell density of this culture increased from 5×10^6 to 1.5×10^8 CFU/ml. Without nitrite there was negligible growth and ^{14}C-labeled adenine or amino acid incorporation.

Sensitivity of gonococci grown under anaerobic conditions to CGE and the 57-kD antimicrobial protein. To assess whether anaerobically cultivated gonococci resisted the antimicrobial activity of the granule proteins in a manner similar to that of gonococci grown aerobically but exposed to granule protein under anaerobic conditions, strain FA19 was grown under anaerobic conditions on agar plates containing sodium nitrite. The gonococcal suspension was transferred to reduced GCB broth containing 0.01% (wt/vol) sodium nitrite and incubated in the anaerobic hood for 6 h. Gonococci grown in this manner were then treated with various concentrations of the CGE or the 57-kD granule protein. This mixture was incubated in the anaerobic chamber for 45 min. The results (Fig. 5) showed that gonococci grown in the anaerobic chamber exhibited decreased resistance to the granule proteins and, in this respect, resembled aerobically grown gonococci in aerobic bactericidal assays

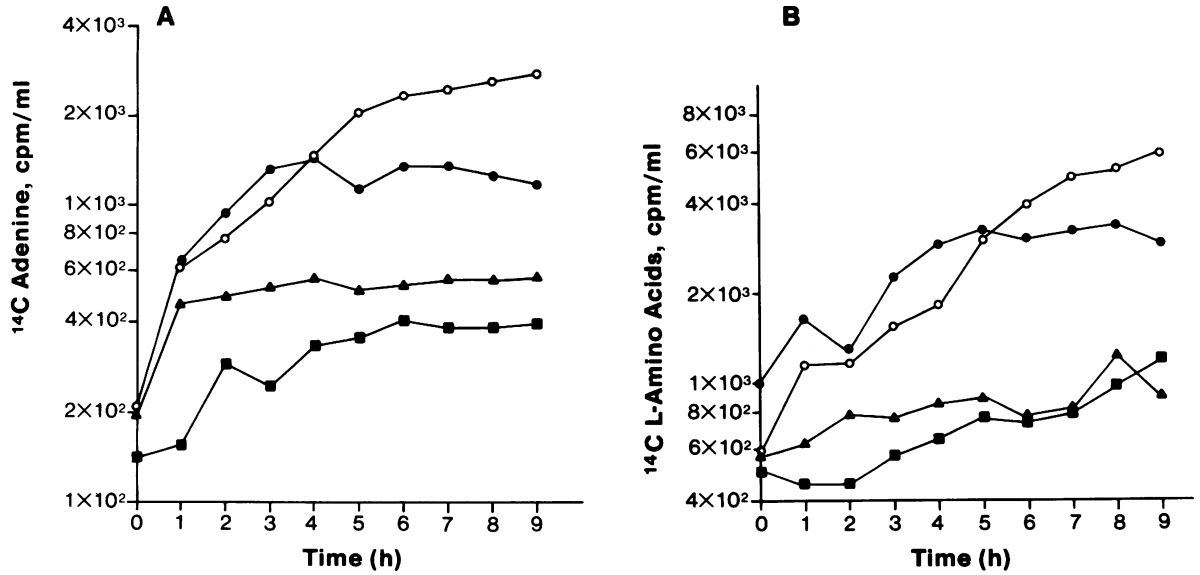

FIG. 4. Demonstration of nucleic acid and protein synthesis by *N. gonorrhoeae* FA19 under anaerobic conditions. Strain FA19 was grown on GCB agar in the anaerobic chamber and transferred to GCB broth containing uniformly ^{14}C-labeled adenine (A) or amino acid mixture (B) with or without sodium nitrite. Each data point represents the mean of duplicate samples of trichloroacetic acid-precipitable counts per minute per milliliter. Symbols: ●, 0.005% (wt/vol) sodium nitrite; ○, 0.01% sodium nitrite; ■, 0.05% sodium nitrite; ▲, no added sodium nitrite.

(Fig. 2). The approximate LD_{50} values of the CGE and the 57-kD antimicrobial proteins for strain FA19 grown aerobically or anaerobically and tested in aerobic or anaerobic bactericidal assays are summarized in Table 1. This table permits a direct comparison of the differences in *N. gonorrhoeae* resistance to the antimicrobial proteins of PMN.

DISCUSSION

Densen and Mandell (5) demonstrated that the killing of *N. gonorrhoeae* with PMN required the intraphagosomal sequestration of gonococci. Such intraphagosomal killing of gonococci could occur via an O_2-independent system, since PMN obtained from patients with chronic granulomatous disease killed ingested gonococci to the same extent as normal PMN (24). In addition, earlier work by Daly et al. (4) and Rest (22) demonstrated that proteins prepared from azurophil granules kill gonococci in vitro. However, the biochemical nature of such proteins and whether they functioned in the absence of oxygen was not reported.

We examined the sensitivity of *N. gonorrhoeae* to CGE and a purified antimicrobial granule protein under anaerobic conditions to assess whether such proteins could exert antigonococcal activity in hypoxic environment. This is relevant because gonococci are often isolated from infected urogenital tracts in the presence of a milieu of strict anaerobes (8). At such infectious sites, the decreased pO_2 levels would presumably diminish the antimicrobial activity of the O_2-dependent systems, and the intraphagosomal killing of gonococci would depend on O_2-independent means.

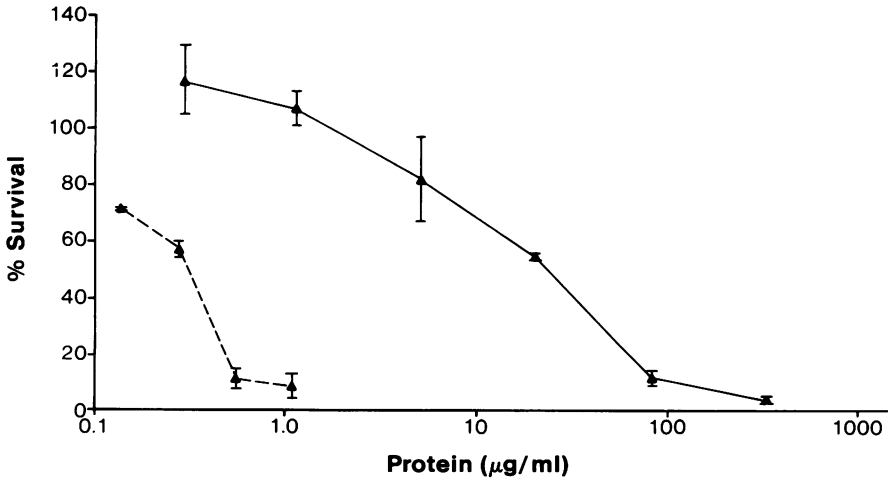

FIG. 5. Antimicrobial activity of CGE and 57-kD antimicrobial protein against gonococci grown in the anaerobic chamber. Strain FA19 in reduced GCB broth containing defined supplements and 0.01% (wt/vol) sodium nitrite was grown in the anaerobic chamber and exposed to various concentrations of CGE (solid line) or 57-kD antimicrobial protein (dashed line) under anaerobic conditions. The percent survival at each protein concentration tested was calculated as described in the legend to Fig. 2. Results are the average of two experiments.

TABLE 1. Antigonococcal activity of CGE and 57kD antimicrobial protein in aerobic and anaerobic bactericidal assays

Atmosphere during gonococcal cultivation[a]	Atmosphere during bactericidal assay	Presence (+) or absence (−) of 0.01% (wt/vol) sodium nitrite in bactericidal assay	Approximate LD_{50} (μg/ml)	
			CGE	57 kD
Aerobic	Aerobic	−	20	0.2
Aerobic	Aerobic	+	50	0.2
Aerobic	Anaerobic	−	>310	>1.2
Anaerobic	Anarobic	+	25	0.3

[a] Aerobic conditions, 5% CO_2–air; anaerobic conditions, 5% CO_2–10% H_2–85% N_2.

[b] The LD_{50} was the protein concentration of CGE or the 57 kD protein which killed 50% of the bacteria that survived in controls in the absence of the antimicrobial protein and was estimated from the graph of percent survival versus protein concentration (see Fig. 2 and 5).

The isolation of gonococci with strict anaerobes suggests that despite being considered aerobic (19, 25), gonococci could survive hypoxic conditions in vivo. Accordingly, we examined the antigonococcal efficacy of both CGE and a purified antimicrobial granule protein (57kD). We used the 57-kDa protein as described above because on a weight basis it is at least 15-fold more active against *N. gonorrhoeae* FA19 than any other granule protein examined thus far (Shafer et al., unpublished data). This protein probably contributes significantly to O_2-independent killing of gonococci in vitro. However, this in vitro behavior cannot be directly extrapolated to in vivo conditions.

The significant observation of our study is that anaerobic conditions substantially diminished the in vitro potency of granule proteins against *N. gonorrhoeae* FA19 but not against the facultative anaerobe *S. typhimurium* SL-1004 (Fig. 2). Furthermore, this anaerobic resistance of gonococci decreased when bacteria were grown under anaerobic conditions in reduced media containing sodium nitrite. Under such conditions, sodium nitrite relieved the bacteriostatic effect of the anaerobic environment. It apparently served as a surrogate electron acceptor (17). The simplest explanation for this decreased resistance of gonococci to both the CGE and the 57-kD protein is that the bactericidal activity of these preparations depends upon bacterial growth. Thus, under the anaerobic conditions used, i.e., the exposure of gonococci to anaerobic media without sodium nitrite (Fig. 2), the bactericidal activity of both the CGE and the 57-kD protein was inhibited because the target bacteria were in a state of bacteriostasis. When bacteriostasis was relieved by adding sodium nitrite to the growth media, gonococcal resistance to the protein decreased (Fig. 5). Gladstone et al. (12) also observed that culture conditions influence the susceptibility of staphylococci to rabbit cationic granule proteins. That bacteriostatic cultures of gonococci are more resistant to neutrophil proteins is consistent with the early observations of Smith and Wood (27, 28). They demonstrated that penicillin failed to kill pneumococci in the stationary phase of growth or in anaerobic subcutaneous abscesses. Thus, the increased resistance of gonococci to neutrophil proteins when subjected to an anaerobically induced state of bacteriostasis may be similar to the well-established observation that certain antibiotics, such as penicillin (9, 10), kill only replicating bacteria.

Results from several independent studies (4, 18, 22–25, 34, 35) suggest that O_2-independent antimicrobial systems of human PMN contribute significantly to host defense against infection and that cationic proteins derived from the membrane-bound azurophil granules are mediators of such behavior (4, 23). Wetherall et al. (35) recently demonstrated that under strict anaerobic conditions, the CGE of human PMN readily kill *Bacteroides fragilis*. Our results support and extend the contention that granule proteins are capable of O_2-independent antimicrobial activity. We demonstrated that the 57-kD protein prepared from human PMN granules readily killed *S. typhimurium* SL-1004 under both aerobic and anaerobic conditions (Fig. 2). To our knowledge this is the first study directly addressing the question of whether a granule protein has primary antimicrobial activity under strict anaerobic conditions.

Gonococci are considered to be aerobes (19, 25). However, the maintenance of gonococcal viability in clinical isolates under anaerobic conditions (26) and the cultivation of laboratory strains of gonococci on solid media under low oxygen tension (13, 14) or under strict anaerobic conditions (17; this study) demonstrate that gonococci remain viable in an anaerobic environment. We are currently examining whether other biologic activities and outer membrane chemistry of gonococci are altered under anaerobic conditions. In this regard, we have also observed that the level of resistance of strain FA19 to spectinomycin under anaerobic conditions increased almost 10-fold (Casey et al., unpublished data). An anaerobic environment might, therefore, augment the resistance of gonococci not only to PMN granule proteins but also to antibiotics routinely used in therapy.

The increased resistance of anaerobically exposed gonococci not only to the 57-kD protein but also to CGE indicates that in hypoxic environments and in the absence of a surrogate terminal electron acceptor gonococci could survive O_2-independent antimicrobial activity in the phagolysosome. Consequently, anaerobiosis may be important in in vitro studies attempting to elucidate some of the pathogenic mechanisms of this bacterium. We are presently examining the intraphagosomal fate of strain FA19 under aerobic and anaerobic conditions.

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LITERATURE CITED

1. **Bradford, H. M.** 1976. A rapid sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**:248–254.
2. **Casey, S. G., D. R. Veale, and H. Smith.** 1979. Demonstration of intracellular growth of gonococci in human phagocytes using spectinomycin to kill extracellular organisms. J. Gen. Microbiol. **113**:395–398.
3. **Casey, S. G., D. R. Veale, and H. Smith.** 1980. Intracellular survival of *Neisseria gonorrhoeae* in human urethral exudate. FEMS Microbiol. Lett. **8**:97–100.
4. **Daly, J. A., T. J. Lee, J. K. Spitznagel, and P. F. Sparling.** 1982. Gonococci with mutations to low level penicillin resistance exhibit increased sensitivity to the oxygen-independent bactericidal activity of human polymorphonuclear leukocyte granule extracts. Infect. Immun. **35**:826–833.

5. **Densen, P., and G. L. Mandell.** 1978. Gonococcal interaction with polymorphonuclear neutrophils: importance of the phagosome for bactericidal activity. J. Clin. Invest. **62**:1161–1171.
6. **Dilworth, J. A., J. O. Hendley, and G. L. Mandell.** 1975. Attachment and ingestion of gonococci by human neutrophils. Infect. Immun. **11**:512–516.
7. **Drutz, D. J.** 1978. Intracellular fate of *Neisseria gonorrhoeae*, p. 232–235. *In* G. Brooks, E. C. Gotschlich, K. K. Holmes, W. D. Sawyer, and F. E. Young (ed.), Immunobiology of *Neisseria gonorrhoeae*. American Society for Microbiology, Washington, D.C.
8. **Fontaine, E. A., D. Taylor-Robinson, N. F. Hanna, and E. D. Coufalik.** 1982. Anaerobes in men with urethritis. Br. J. Vener. Dis. **58**:321–326.
9. **Frere, J. M.** 1977. Mechanisms of action of β-lactam antibiotics at the molecular level. Biochem. Pharmacol. **26**:2203–2210.
10. **Ghuysen, J. M.** 1977. A new insight into the action of penicillin, p. 32. *In* W. E. Brown (ed.), The bacterial DD-carboxypeptidase-transpeptidase system. University of Tokyo Press, Tokyo.
11. **Gibbs, D. L., and R. B. Roberts.** 1975. The interaction *in vitro* between human polymorphonuclear leukocytes and *Neisseria gonorrhoeae* cultivated in the chick embryo. J. Exp. Med. **141**:155–171.
12. **Gladstone, G. P., E. Walton, and U. Kay.** 1974. The effect of cultural conditions on the susceptibility of staphylococci to killing by the cationic proteins from rabbit polymorphonuclear leukocytes. Br. J. Exp. Pathol. **55**:427–447.
13. **James-Holmquest, A. N., R. D. Wende, R. L. Mudd, and R. P. Williams.** 1973. Comparison of atmospheric conditions for culture of clinical specimens of *Neisseria gonorrhoeae*. Appl. Microbiol. **26**:466–469.
14. **Kellogg, D. S., J. A. Crawford, and C. S. Callaway.** 1983. Cultivation of *Neisseria gonorrhoeae* under low-oxygen conditions. J. Clin. Microbiol. **18**:178–184.
15. **Klebanoff, S. J.** 1975. Antimicrobial systems of the polymorphonuclear leukocyte, p. 45. *In* J. A. Bellanti and D. H. Dayton (ed.), The phagocytic cell in host resistance. Raven Press, New York.
16. **Klebanoff, S. J.** 1975. Antimicrobial mechanisms in neutrophilic polymorphonuclear leukocytes. Semin. Hematol. **12**:117–142.
17. **Knapp, J. S., and V. L. Clark.** 1983. Anaerobic growth of *Neisseria gonorrhoeae* coupled to nitrite reduction. Infect. Immun. **46**:176–181.
18. **Modrzakowski, M. C., and J. K. Spitznagel.** 1979. Bactericidal activity of fractionated granule contents from human polymorphonuclear leukocytes: antagonism of granule cationic proteins by lipopolysaccharide. Infect. Immun. **25**:597–602.
19. **Morse, S. A., R. D. Miller, and B. H. Hebeler.** 1977. Physiology and metabolism of *Neisseria gonorrhoeae*, p. 236–253. *In* R. B. Roberts (ed.), The gonococcus. John Wiley & Sons, Inc., New York.
20. **Ofek, I., E. H. Beachey, and A. L. Bisno.** 1974. Resistance of *Neisseria gonorrhoeae* to phagocytosis: relationship to colonial morphology and surface pili. J. Infect. Dis. **129**:310–316.
21. **Punsalang, A. P., Jr., and W. D. Sawyer.** 1973. Role of pili in the virulence of *Neisseria gonorrhoeae*. Infect. Immun. **8**:255–263.
22. **Rest, R. F.** 1979. Killing of *Neisseria gonorrhoeae* by human polymorphonuclear neutrophil granule extracts. Infect. Immun. **25**:574–579.
23. **Rest, R. F., M. H. Cooney, and J. K. Spitznagel.** 1977. Susceptibility of lipopolysaccharide mutants to the bactericidal action of human neutrophil lysosomal fractions. Infect. Immun. **16**:145–151.
24. **Rest, R. F., S. H. Fischer, Z. Z. Inghamz, and J. F. Jones.** 1982. Interactions of *Neisseria gonorrhoeae* with human neutrophils: effects of serum and gonococcal opacity on phagocyte killing and chemiluminescence. Infect. Immun. **36**:737–744.
25. **Reyn, A.** 1974. Gram-negative cocci and coccibacilli, p. 427–444. *In* R. E. Buchanan and N. E. Gibbons (ed.), Bergey's manual of determinative bacteriology, 8th ed. The Williams & Wilkins Co., Baltimore.
26. **Short, H. B., V. L. Clark, D. S. Kellogg, Jr., and F. E. Young.** 1982. Anaerobic survival of clinical isolates and laboratory strains of *Neisseria gonorrhoeae*: use in transfer and storage. J. Clin. Microbiol. **15**:915–919.
27. **Smith, M. R., and W. B. Wood, Jr.** 1956. An experimental analysis of the curative action of penicillin in acute bacterial infections. I. The relationship of the antimicrobial effect of penicillin. J. Exp. Med. **103**:487–498.
28. **Smith, M. R., and W. B. Wood, Jr.** 1956. An experimental analysis of the curative effect of penicillin in acute bacterial infections. III. The effect of suppuration upon the antibacterial action of the drug. J. Exp. Med. **103**:509–519.
29. **Swanson, J., E. Sparks, B. Zeligs, M. A. Siam, and C. Parrott.** 1974. Studies on gonococcus infection. V. Observations on in vitro interactions of gonococci and human neutrophils. Infect. Immun. **10**:633–644.
30. **Swanson, J. S., J. Kraus, and E. C. Gotschlich.** 1971. Studies on gonococcus infection. I. Pili and zones of adhesion: their relation to gonococcal growth patterns. J. Exp. Med. **123**:886–906.
31. **Thongthai, C., and W. D. Sawyer.** 1973. Studies on the virulence of *Neisseria gonorrhoeae*. I. Relation of colonial morphology and resistance to phagocytosis by polymorphonuclear leukocytes. Infect. Immun. **7**:373–379.
32. **Veale, D. R., M. Goldner, C. W. Penn, J. Ward, and H. Smith.** 1979. Intracellular survival and growth of gonococci in human phagocytes. J. Gen. Microbiol. **113**:383–393.
33. **Watt, P. J.** 1970. The fate of gonococci in polymorphonuclear leukocytes. J. Med. Microbiol. **3**:501–509.
34. **Weiss, J., M. Victor, O. Stendhal, and P. Elsbach.** 1982. Killing of gram-negative bacteria by polymorphonuclear leukocytes. Role of an O_2-independent bactericidal system. J. Clin. Invest. **69**:959–970.
35. **Wetherall, B. L., H. Pruul, and P. J. MacDonald.** 1984. Oxygen-independent killing of *Bacteroides fragilis* by granule extracts from human polymorphonuclear leukocytes. Infect. Immun. **43**:1080–1084.
36. **Witt, K., D. R. Veale, H. Finch, C. W. Penn, D. Sen, and H. Smith.** 1976. Resistance of *Neisseria gonorrhoeae* grown *in vivo* to ingestion and digestion by phagocytes of human blood. J. Gen. Microbiol. **96**:341–350.
37. **Witt, K., D. R. Veale, and H. Smith.** 1976. Resistance of *Neisseria gonorrhoeae* to ingestion and digestion by phagocytes of human buffy coat. J. Med. Microbiol. **9**:1–12.