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Effects of Human Neutrophil Granule Extracts on Macromolecular Synthesis in *Neisseria gonorrhoeae*

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Neisseria gonorrhoeae were exponentially killed for 120 min (i.e., they were prevented from forming colonies on agar) by extracts of human neutrophil granules; however, macromolecular synthesis, indicated by incorporation of radiolabeled precursors in trichloroacetic acid-precipitable material, continued at or above zero time control values for 45 min. Protein, deoxyribonucleic acid, and ribonucleic acid synthesis appeared to decrease simultaneously after 45 min. Little or no lysis of gonococci occurred during the first 60 min of incubation. The ions K^+, Na^+, Ca^{2+}, Cl^{1-}, SO_4^{2-} and PO_4^{3-} at concentrations of $\leq$100 mM did not affect granule extract bactericidal activity. On the other hand, 20 mM Mg^{2+} completely inhibited killing when initially present along with granule extract or when added within 2 to 5 min after granule extract was added to a suspension of gonococci. Gonococci treated with granule extract, washed, and then incubated in the absence of extract died as if extract were still present. The ability of subinhibitory concentrations of actinomycin D or erythromycin to inhibit growth and protein and nucleic acid synthesis was synergistically increased in the presence of granule extract. The above information suggests that a bactericidal component(s) of human neutrophil granules sticks to gonococci, altering their outer membrane permeability and their ability to divide.

In vitro, most nonpiliated *Neisseria gonorrhoeae* obtained from uncomplicated gonococcal infections are phagocytized and killed by human polymorphonuclear neutrophils, whereas piliated gonococci are usually not internalized and, therefore, not killed (2, 4, 5, 18, 19). For bacterial death to occur, the gonococci must be intraphagolysosomal (1). Neutrophil microbicidal mechanisms responsible for the death of phagocytized gonococci and other gram-negative bacteria are not fully understood. Oxygen-dependent systems, including the myeloperoxidase-Cl-H_2O_2 system (13) and superoxide- (3) and oxygen-independent systems (13) have been implicated in neutrophil gonococcicidal activity.

Recently, a number of investigations have more completely characterized human neutrophil oxygen-independent bactericidal systems. Odeberg and Olsson (8, 9) studied the mechanisms of microbicidal activity of chymotrypsin-like cationic proteins (CCP) first described by Welsh and Spitznagel (23). These proteins possess heat-stable microbicidal activity, are more active against gram-positive bacteria, and appear to act due to their highly charged nature. More recently, Weiss et al. purified and characterized an apparently nonenzymatic, cationic "bactericidal/permeability increasing" (B/PI) protein of 60,000 daltons that is active against some gram-negative bacteria. This protein has the novel mechanism of rapidly increasing outer membrane permeability and inhibiting bacterial multiplication, while minimally affecting macromolecular synthesis or ultrastructure (21, 22).

The question can be asked, however, whether the bactericidal activity of the individually identified lysosomal components may be greater than or less than the total of the granule enzymes. Odeberg and Olsson have shown synergistic bactericidal effects between lysosomal proteins such as elastase or myeloperoxidase and the chymotrypsin-like cationic proteins (10). Certainly other synergies and possibly inhibitory processes occur. By using unfractionated extracts of purified neutrophil granules (lysosomes), in addition to purified bactericidal agents, we can better understand the total oxygen-independent bactericidal capacity of the neutrophil.

In this communication we extend previous observations (13) on the oxygen-independent bactericidal mechanisms of human neutrophil lysosomal contents against *N. gonorrhoeae*.

MATERIALS AND METHODS

Bacteria. *N. gonorrhoeae* strain F62 was obtained

from P. Fred Sparling, University of North Carolina, Chapel Hill, and was identified to species as previously described (13–15). Cultures were selectively passaged daily on GC medium base agar (GC agar; Difco Laboratories, Detroit, Mich.) with supplements as described previously (13–15). Colony type 4 was chosen by morphology as described by Kellogg et al. (4, 5) and by transparency as described by Swanson (17). Log-phase gonococci were obtained by inoculating 20 ml of warm GC broth containing supplements and 400 μg of $NaHCO_3$ per ml with 5 ml of a suspension of 16- to 20-h plate-grown gonococci (optical density at 550 nm, 0.4; Bausch & Lomb Spectronic 20, 1-cm light path) and incubated in a rotary shaker water bath at 36°C for approximately 3 h to a Klett reading (green filter) of approximately 120 (4×10^8 to 5×10^8 cells per ml).

Neutrophils, granules, and granule extract. Preparation of neutrophils, granules, and granule extract was as described elsewhere (14, 15). Briefly, neutrophils were obtained from healthy adult human volunteers by leukapheresis and were sedimented first through Hydroxyethyl starch or Plasmagel and then through Ficoll-Hypaque, yielding approximately 10^{10} purified neutrophils from each donor. Final cell suspensions were ≥93% neutrophils, with eosinophils the major contaminating cell population.

Granules were pelleted (20,000 × *g*, 20 min) from postnuclear supernatants (250 × *g*, 15 min) of neutrophil homogenates (90% breakage in 0.35 M sucrose). Granule pellets containing both azurophil and specific granule populations were twice extracted overnight at 3°C with 0.2 M sodium acetate buffer (pH 4.0) containing 10 mM $CaCl_2$. Extracts were clarified by centrifugation (20,000 × *g*, 20 min) and dialyzed against phosphate-buffered saline (PBS; pH 7.4; containing, per liter of distilled, deionized water: NaCl, 8 g; KCl, 0.2 g; Na_2HPO_4, 1.15 g; and KH_2PO_4, 0.2 g) with dialysis tubing with an average molecular weight cutoff of 3,500. This dialyzed preparation of acetate-extracted neutrophils is referred to as "granule extract" in this manuscript. Extract of granules from 10^{10} neutrophils yielded approximately 90 mg of protein.

Bactericidal and incorporation assays. Experimental mixtures containing 1×10^8 to 2×10^8 gonococci per ml, granule extract to the desired concentration, and GC broth in sterile borosilicate glass tubes (13 by 100 mm) were incubated with shaking at 36°C. Assay volumes varied with the number of data points required. Control mixtures contained PBS instead of extract. Bacterial viability was determined by spreading appropriate dilutions (made in GC broth without supplements) on GC agar plates which were incubated for 24 h at 36°C in ~6% CO_2 in air. For all points, viability is defined as the ability of gonococci to form colonies on GC agar under the above described conditions and is calculated as: percent viability = (test viability/initial (zero time) viability) × 100.

Macromolecular synthetic capability was measured by following incorporation of radiolabeled precursors into trichloroacetic acid-precipitable material. To study nucleic acid synthesis, [5-^{3}H]uridine, [6-^{3}H]-uridine (6 mM, 1.67 μCi/μmol), [6-^{3}H]uracil (2 mM, 10 μCi/μmol), or deoxy[G-^{3}H]adenosine (5 mM, 10 μCi/μmol) was used. Protein synthesis was followed by incorporation of ^{3}H-amino acid mix or L-[4,5-^{3}H]leucine (2 mM, 10 μCi/μmol). Radiolabels were obtained from Amersham Corp., Arlington Heights, Ill. At the times indicated below, 100 μl of assay mixture was removed (in triplicate) and placed in 2 ml of ice-cold 10% (wt/vol) trichloroacetic acid in borosilicate glass tubes (13 by 100 mm) and blended in a Vortex mixer and kept on ice for 1 h. The trichloroacetic acid solutions were then filtered through 25-mm cellulose filters (0.45-μm pore size; Amicon Corp., Lexington, Mass.). The tubes and filters were rinsed with 10 ml of 5% (wt/vol) ice-cold trichloroacetic acid and 4 ml of ice-cold 95% ethanol. Filters were dried with an infrared lamp, placed in 4 ml of Formula 963 scintillation fluid (New England Nuclear Corp., Boston, Mass.) in minivials (West Chem Products, San Diego, Calif.) and counted in a Tracor Analytic Liquid Scintillation System, model G892. Only counts of triplicate samples that had channel ratios within 5% of each other were used in the results. Controls were performed by placing complete incubation mixtures on ice or at 60°C for the duration of the experiment. The method of Schmidt and Tannhauser was used to determine whether radiolabeled nucleotides were incorporated into deoxyribonucleic acid or ribonucleic acid (16).

Permeability. Sensitivity of gonococci to various antibiotics was used to detect possible outer membrane permeability changes caused by granule extract. These antibiotics were added to the reaction mixtures described above, and gonococcal viability and incorporation were measured. In addition, the uptake of [^{3}H]actinomycin D (ActD; 0.5 μCi/ml, 0.2 nM) during a 10-min pulse in the absence or presence of extract was used as an indication of permeability. [^{3}H]ActD was added to the reaction mixture described above, and at the indicated times, 100-μl samples were removed and filtered on 25-mm, cellulose filters (0.45-μm pore size), and the filters were rinsed with 5 ml of room-temperature PBS, dried, and counted as described above.

Reagents. All reagents, unless otherwise indicated, were of the highest quality commercially available.

RESULTS

Bactericidal system. The bactericidal system described in this publication is similar to one described previously (13); however, a higher concentration of gonococci was used (2×10^8 cells per ml instead of 6×10^3 cells per ml) so that incorporation of radiolabeled precursors into macromolecules could be accurately measured. Due to the higher numbers of gonococci used, a higher concentration of granule extract was needed to kill 95 to 99% of the gonococci within 1 h (750 μg/ml as opposed to 300 μg/ml) (Fig. 1, lower curve). Gonococci incubated in control mixtures (containing a volume of PBS equivalent to the granule extract added to the test mixtures) doubled their number of colony-forming units every 50 to 60 min. Loss of viability in the presence of extract is defined as a loss of ability to form colonies on agar. Extracts from

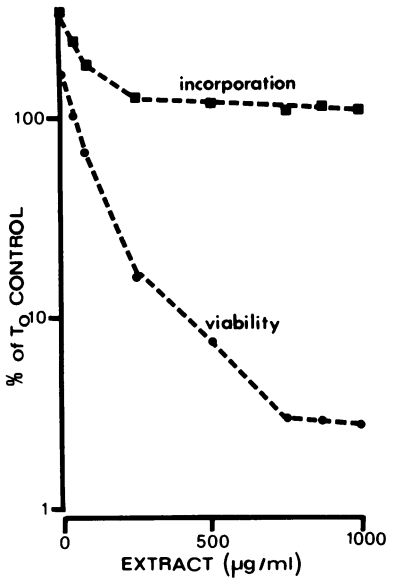

FIG. 1. *Effect of granule extract on viability and ability to incorporate labeled precursors into macromolecules. Gonococci were incubated at 37°C in the presence of increasing concentrations of granule extract. At 1 h, gonococcal viability and incorporation of a 10-min pulse of [^{3}H]uridine into trichloroacetic acid-precipitable material were assessed.*

seven different donors were used for the experiments described in this publication, with no significant variations observed in their effects. Results expressed in the figures are representative experiments. All experiments were repeated at least three times on different days.

Effects of granule extract on macromolecular synthesis. Incorporation of radiolabeled nucleotides or amino acids by 2×10^8 gonococci per ml into trichloroacetic acid-precipitable material was followed over time in the presence or absence of 750 μg of extract per ml. Incorporation by gonococci of [6-^{3}H]uracil, [4,5-^{3}H]leucine, [2,8-^{3}H]adenosine, [5-^{3}H]uridine, or ^{3}H-labeled mixed amino acids into trichloroacetic acid-precipitable material was inhibited by extract. The appropriate labeled precursor was added to a suspension of gonococci, and at 0, 30, 60, 90, and 120 min, samples were removed and precipitated with trichloroacetic acid. Incorporation by gonococci of labeled precursors continued for 60 min at 50 to 70% of the control rate. However, no incorporation was seen after 60 min.

For further characterization of the kinetics of the observed inhibition of macromolecular synthesis by granule extract, gonococci were incubated at zero time with extract, and samples were then pulsed with radiolabeled precursors for 10 min at zero time and at 15-min intervals. Results are expressed as percentage of zero time controls: (trichloroacetic acid-precipitable counts per minute at test time/trichloroacetic acid-precipitable counts per minute at zero time) × 100. Control (zero time) trichloroacetic acid precipitates averaged between 1,200 and 2,500 cpm depending on the labeled precursor. Both nucleic acid and protein synthesis, as indicated by incorporation of [5-^{3}H]uridine and [4,5-^{3}H]leucine, respectively, into trichloroacetic acid precipitates, appeared to remain ≥100% functional after a ≥80% loss of gonococcal viability (Fig. 2). As viability continued to decrease below 20% (i.e., after about 60 min) due to the action of granule extract, macromolecular synthesis decreased sharply, until at 120 min viability was <0.1%, and incorporation of radiolabel was <10%.

In dose-response experiments, in which gonococci were incubated with granule extract for 60 min and then pulsed with [5-^{3}H]uridine or [4,5-^{3}H]leucine for 10 min, incorporation of label into trichloroacetic acid precipitates steadily decreased in the presence of 0 to 100 μg/ml of granule extract but was then little affected by concentrations between 100 and 1,000 μg/ml (Fig. 1, top curve).

To measure whether [5-^{3}H]uridine was entering deoxyribonucleic acid or ribonucleic acid, trichloroacetic acid precipitates from control gonococci that had been pulsed with label for 10 min were subjected to further acid and base treatment. Eighteen percent of the trichloroacetic acid-precipitable material remained insoluble to acid treatment (pH 1.5, 100°C, 30 min), whereas no label remained insoluble when treated with alkali (pH 12, 37°C, 20 h), indicating that approximately 18% of [5-^{3}H]uridine was being incorporated into large molecules of ribonucleic acid. None of the [5-^{3}H]uridine was being incorporated into large molecules of deoxyribonucleic acid. The approximately 80% of [5-^{3}H]uridine label that was solubilized by both acid and base was assumed to be in small oligonucleotides. The above experiment was also performed with [6-^{3}H]uridine and gave similar results, with 35% of the label remaining acid insoluble and no label remaining alkali insoluble.

Since the amount of label incorporated into trichloroacetic acid precipitates by granule extract-treated gonococci, at times up to 45 min, was actually greater than incorporation by the same numbers of untreated, control gonococci, the possibility of increased transport or permeability and thus increased intracellular pools was investigated. Extract-treated or control gonococci were pulsed at 30 min for 10 min with [5-^{3}H]uridine and pelleted by centrifugation, the

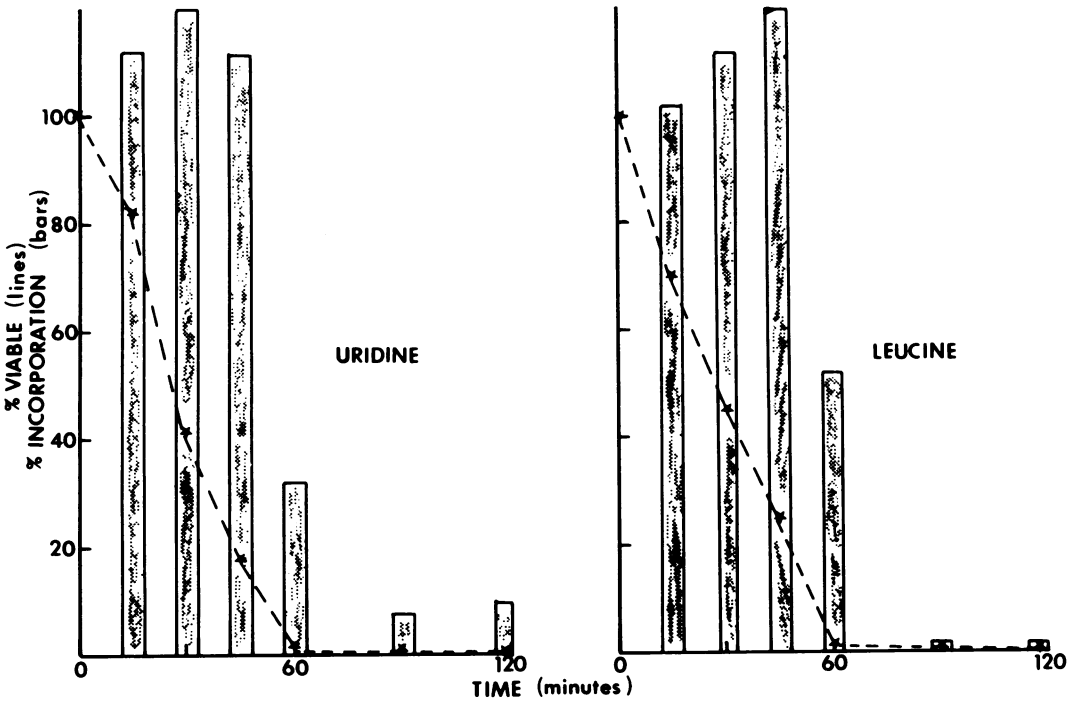

FIG. 2. *Effect of granule extract on viability and on nucleic acid and protein synthesis. Gonococci and granule extract were added at time zero. At the indicated times, viability and incorporation of a 10-min pulse of [^{3}H]uridine or [^{3}H]leucine into trichloroacetic acid-precipitable material were measured.*

pellet was resuspended, and cells were counted. In a representative experiment, extract-treated and control gonococci had 1.6×10^5 and 2.0×10^5 cpm associated with them, respectively. These similar total cell-associated counts suggested similar intracellular accumulation of radiolabeled uridine by control and extract-treated gonococci.

Effects of ions on killing by extract. The ions K^+, Na^+, Ca^{2+}, Mg^{2+}, Cl^-, SO_4^{2-}, and PO_4^{3-} were tested for their effects on the bactericidal activity observed in our system, since the activity of some cationic bactericidal proteins is inhibited or reversed by various types and concentrations of ions. Of the above ions tested at concentrations up to 0.1 M, only Mg^{2+} inhibited extract bactericidal activity. None of the ions stimulated killing. Magnesium had an optimum inhibitory concentration of 40 mM, with high concentrations of Mg^{2+} being lethal for gonococci (Fig. 3). Next, experiments were performed to see whether Mg^{2+} needed to be present initially in order to inhibit extract bactericidal activity, or whether Mg^{2+} could be added at times after the addition of extract. Individual tubes were set up, each containing gonococci and granule extract at zero time. Magnesium was added to a final concentration of 40 mM at 2-min intervals, and the gonococci were incubated until 60 min and plated for viability. Magnesium inhibited killing by extract only when added within the first 2 to 5 min (data not shown). It did not inhibit or reverse killing that had already been initiated or completed.

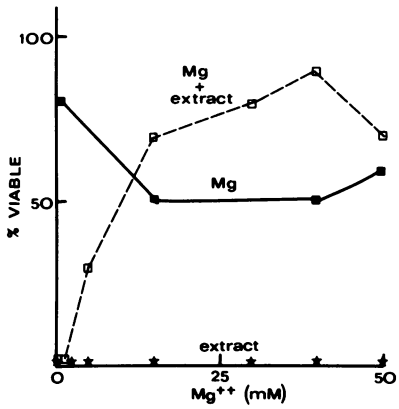

FIG. 3. *Effect of Mg^{2+} on granule extract bactericidal activity. Gonococci were incubated with increasing concentrations of Mg^{2+} with or without granule extract. At 60 min, viability was assessed. Stars represent gonococci incubated with extract in the absence of Mg^{2+}.*

In similar experiments, gonococci were incubated in GC broth with or without the addition of 40 mM Mg^{2+} for 10 min, centrifuged (8,000 × *g*, 5 min), and resuspended in the normal bactericidal mixture containing granule extract, and viability was assessed over time. Gonococci preincubated with Mg^{2+} were killed by extract to the same degree as control gonococci not preincubated with Mg^{2+}.

Adherence of bactericidal components to gonococci. The results of the above experiment suggested a number of alternatives, one being that Mg^{2+} was eluting a bactericidal component from the gonococcal surface. Therefore, experiments were done to see whether extract constituents were adhering to the gonococci. Gonococci (6×10^3 colony-forming units per ml) were incubated with 500 μg of extract per ml at 37°C, and at 0, 10, 20, 30, and 60 min samples were removed and centrifuged (8,000 × *g*, 3 min). The pelleted gonococci were then suspended in GC broth without extract and incubated with shaking at 37°C, and gonococcal viability was assessed at 0, 30, and 60 min (Fig. 4). The data show that gonococci continued to die in the absence of granule extract. The kinetics of gonococcal death after only a few seconds in contact with extract (i.e., 0 min) is slower than death after exposure to extract for 10 or 20 min, which can be seen by the increasing initial slopes (given in parentheses) of the solid lines in Fig. 4. In experiments where less extract (e.g., 300 μg/ml) was used in the initial incubation, results similar to those seen in Fig. 4 were obtained, except that less killing was observed (data not shown). These results indicated that there was a bactericidal component(s) sticking to the gonococci or that an irreversible lethal event(s) had occurred (or both). In addition, they supported the above data with Mg^{2+}, indicating that initiation of lethal events occurs very rapidly.

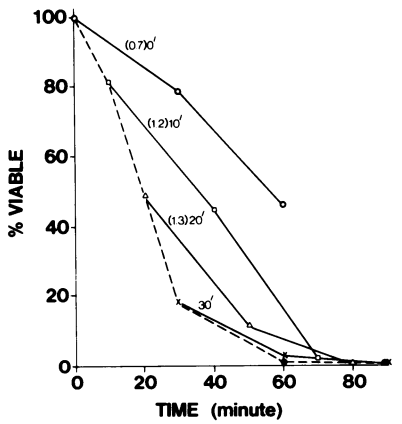

FIG. 4. *Gonococcal viability after removal of granule extract. Gonococci were incubated with extract, and at 0 (○), 10 (□), 20 (△), 30 (×), and 60 (●) min samples of the suspension were centrifuged. The pelleted gonococci were resuspended without extract, and viability was assessed at 0, 30, and 60 min. The solid lines connect data points at 0, 30, and 60 min after removal of extract at the various times. The dotted line connects the data points of all the various times at 0 min, i.e., immediately after removal of extract and resuspension of the gonococci. Numbers within parentheses are initial slopes of the solid lines.*

If a bactericidal component(s) were sticking to gonococci, then that component(s) should be depleted from incubation supernatants. The bactericidal activity of supernatants from incubation mixtures, originally containing 600 μg of extract per ml (now containing "used" extract), was decreased by 95 to 99% (Table 1) when it was reused in our bactericidal system, indicating loss of bactericidal component(s). Supernatants from control 1-h incubations (containing "used" GC broth with no extract) did not affect gonococcal viability, nor did these control supernatants inhibit or enhance the action of fresh extract when used in bactericidal assays.

Indications of extract-induced membrane permeability changes. To study altered outer membrane permeability, we observed for increased sensitivity of gonococci to various antimicrobial agents. ActD (200 ng/ml), erythromycin (500 ng/ml), chloramphenicol (100 ng/ml), and acridine orange (50 μg/ml) were tested in our bactericidal or incorporation assays in the absence or presence of extract. The subinhibitory concentrations of antimicrobial agents used were determined by dose-response experiments with our bactericidal system (data not shown). When the concentrations of the antibiotics listed above were doubled, they all showed degrees of bactericidal activity.

TABLE 1. *Effect of used granule extract (GE) on gonococcal viability*[a]

Time (min)	% Viability			
	Expt 1		Expt 2	
	−GE	+GE	−GE	+GE
0[b]	100	100	100	100
60	205	2	185	0.1
0'[c]	100	100	100	100
60'	286	40	231	8

[a] Figures are from two representative experiments done on separate days.

[b] At 0 min, experimental reaction mixtures contained 600 μg/ml of fresh GE.

[c] At 0 min, the supernatant (6,000 × *g*, 5 min) from the first 60-min incubation (with or without granule extract) was used to resuspend a fresh inoculum of gonococci. Viability was measured 60 min later.

In the following experiments, gonococci were incubated with 750 μg of extract per ml for 30 min, and viability was determined. Gonococci were then pulse-labeled with [5-^{3}H]uridine or ^{3}H-labeled mixed amino acids in the presence of antibiotic for 10 min, and viability and incorporation were determined. Neither chloramphenicol nor acridine orange exhibited increased bactericidal activity in the presence of extract. ActD, at a subinhibitory concentration, caused both increased bactericidal activity and increased inhibition of protein and nucleic acid synthesis in the presence of lethal concentrations of extract (Table 2). Erythromycin also caused increased bactericidal activity in the presence of extract, with results similar to those shown for ActD. Reciprocal experiments in which gonococci were preincubated with subinhibitory concentrations of ActD or erythromycin and then exposed to extract and labeled precursors gave almost identical results.

If extract-treated gonococci are indeed more permeable to these antibiotics, then more should be associated with gonococci in the presence of extract than in its absence. To see whether this was the case, gonococci were preincubated with or without extract for 30 min, at which time [^{3}H]ActD was added. At various time intervals, samples were removed, filtered, rinsed, and counted. Extract-treated gonococci took up substantially more [^{3}H]ActD than did untreated controls in 10 min (Fig. 5). Uptake of ActD by gonococci treated with 0.1% Triton X-100 is shown as a positive control.

Although direct measurements have not yet been made, the effects of granule extract on osmotic stability of gonococci seem to be minimal. Bactericidal assays done in the presence of 0.15 M NaCl or 8.6% (0.25 M) sucrose yield the same data as assays done in their absence, indicating that these stabilizing agents do not prevent killing (data not shown). In addition, lysis of gonococci by extract was not observed, as measured spectrophometrically (Table 3). Actually, the optical density of gonococcal suspensions increased during incubation with extract, possibly due to swelling of the gonococci, or the interaction of extract components with incubation reagents, causing cloudiness.

DISCUSSION

In the experiments described in this publication we used nonpiliated, transparent *N. gonorrhoeae* as a probe to investigate the mechanisms of bactericidal activity of human neutrophil granule contents. Nonpiliated gonococci were used since we have shown previously that both piliated and nonpiliated gonococci are killed similarly by granule extract in regard to time, concentration, pH, and extract heat lability (13). In addition, both types are killed to a similar degree by the myeloperoxidase–Cl–H_2O_2 system (13). In this regard, our in vitro bactericidal system probably measures oxygen-independent bactericidal components for a number of reasons (13). It is unlikely that an oxygen-dependent system is involved since no H_2O_2 can be measured in the reaction mixtures (unpublished results), gonococci are strongly catalase positive, exogenous catalase does not abrogate killing, and the bacteriological growth medium (GC broth) used in the bactericidal assays inhibits the myeloperoxidase–Cl–H_2O_2 bactericidal system (13).

If, then, the observed killing by granule extracts is oxygen independent, are the characteristics of the bactericidal activity that we observe similar to the killing characteristics of the recently described human neutrophil cationic bactericidal proteins (6, 8, 22)? Indeed, is the bactericidal activity due solely to the cationic proteins? In our system, the gonococci are apparently not killed solely by the CCP described by

TABLE 2. *Effect of ActD and granule extract on protein and ribonucleic acid synthesis and gonococcal viability*[a]

Prepn	Expt 1			Expt 2		
	% Leucine incorporation[b]	% Viable at 30 min (pre-ActD)[c]	% Viable at 40 min (post-ActD)	% Uridine incorporation[b]	% Viable at 30 min (pre-ActD)	% Viable at 40 min (post-ActD)
PBS	100	165.0 ± 9.2	141.0 ± 3.5	100	153.0 ± 10.3	131.0 ± 9.0
PBS plus ActD	96.0 ± 3.0		180.0 ± 21.0	90.5 ± 0.5		142.0 ± 31.0
GE	47.0 ± 6.0	26.8 ± 6.8	4.5 ± 1.5	49.5 ± 16.5	16.8 ± 2.4	2.44 ± 0.35
GE plus ActD	14.0 ± 2.0		0.26 ± 0.01	0		0.12 ± 0.03

[a] Results are expressed as the percentage of the mean ± the standard error of the mean. ActD, ActD at 200 ng/ml; GE, granule extract at 750 μg/ml.

[b] At 30 min, gonococci were pulsed for 10 min with L-[4,5-^{3}H]leucine or [5-^{3}H]uridine, and radioactivity was measured in trichloroacetic acid precipitates.

[c] Viability at 30 or 40 min was compared with viability at zero time, which was considered 100%.

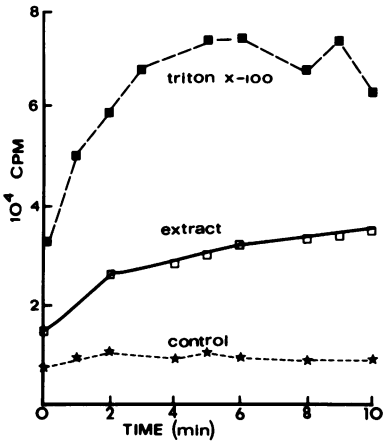

FIG. 5. *Uptake of [^{3}H]ActD by granule extract treated-gonococci. Gonococci, granule extract, and [^{3}H]ActD were incubated together, and ActD uptake was measured at the indicated times. Average of two experiments.*

Odeberg and Olsson (8, 9) for a number of reasons. (i) The bactericidal activity of CCP is more active against, although not limited to, gram-positive bacteria. The granule extract concentrations used in this manuscript and in a previous one (13) appear to contain insufficient amounts of CCP to be responsible for the observed killing. (ii) The bactericidal activity of CCP is inhibited by the concentration of NaCl (0.15 M) present in our assay mixtures. (iii) Inhibition by CCP of macromolecular synthesis in *Escherichia coli* parallels loss of *E. coli* viability, whereas loss of macromolecular synthetic capability in gonococci caused by granule extract occurs only after 45 min, at which time a 10-fold decrease in gonococcal viability has occurred. (iv) The bactericidal activity of CCP is activated by heating at 100°C for 10 min, whereas the gonococcicidal activity of granule extract is slightly inhibited by the same conditions (13). Fractions (dialyzed against PBS, pH 7.4) containing CCP, obtained by passing granule extract through a Sephadex G-100 column by the method of Odeberg and Olsson (8, 11), were not bactericidal against transparent or opaque gonococci at protein concentrations as high as 300 μg/ml (manuscript in preparation).

It appears that granule extract bactericidal activity is more likely due to proteins such as the B/PI protein described by Weiss et al. (21, 22). The most striking similarity between the killing mechanisms of the two (granule extract and B/PI) is the ability to inhibit bacterial multiplication (i.e., to inhibit colony-forming ability) without inhibiting deoxyribonucleic acid, ribonucleic acid, or protein synthesis. The initial increase in macromolecular synthesis by extract-treated gonococci (Fig. 2) was not expected. A reasonable explanation of the data is that the gonococci continue to increase their mass, but are unable to reproduce, i.e., form colonies. After 45 to 60 min, in spite of their increased mass, the gonococci lose all synthetic capabilities. Work is in progress using enzymatic, physical, and morphological criteria to measure this expected increase in mass. In addition, the gonococci were not lysed by incubation in isotonic medium with granule extract for 60 min, which correlates with the response of *E. coli* to B/PI. Interestingly, *E. coli* treated with B/PI retain their ability to be induced for β-galactosidase activity. Since we are not aware of such an easily inducible enzyme system in gonococci, this type of experiment is not feasible at the present time.

The bactericidal activity of granule extracts might also be due to any one, or to a combination, of a number of other proteins from human neutrophils, none of which have as yet been as extensively characterized as the cationic proteins (CCP and B/PI) described above. Modrzakowski et al. found numerous bactericidal fractions from chromatographically fractionated granule contents and went on to more completely describe a fraction (valley AB from a Sephadex G-100 column) that contained a potent bactericidal cationic protein with an apparent molecular weight of approximately 36,500 (6, 7). Gram-negative bacteria, possessing lipopolysaccharide with the Ra chemotype, appeared to have similar sensitivities to Modrzakowski's valley AB protein and Weiss's B/PI. The gonococci used in this report apparently have the Ra chemotype lipopolysaccharide (12, 24) and appear to react to partially purified B/PI protein with the same sensitivity as Ra mutants of *E. coli* (manuscript in preparation).

Discrepancies exist, however, between the characteristics of our bactericidal system and

TABLE 3. *Effect of granule extract on absorbance of gonococcal suspensions*[a]

Time (min)	Absorbance		
	PBS control	Triton X-100 (0.1%)	Granule extract (750 μg/ml)
0	0.000 (100)[b]	0.000 (100)	0.375 (100)
30	0.146 (174)	−0.174 (0.44)	0.476 (16.1)
60	0.340 (242)	−0.179 (0.05)	0.580 (0.02)

[a] At 0 min, the spectrophotometer was zeroed at 550 nm to the PBS control containing gonococci and was used as the blank for the other assays. At indicated times the absorbance and viability were determined.

[b] Numbers within parentheses indicate percent viability compared to zero time controls.

those described by others for human neutrophil granule contents. The question arises whether the individual bactericidal proteins described by various investigators may work in concert with or antagonistically toward each other. Odeberg and Olsson have shown such interactions with human neutrophil elastase or chymotrypsin and the myeloperoxidase-Cl-H_2O_2 bactericidal system (10) and have suggested the presence of a protease inhibitor within neutrophils. Thorne et al. showed that the gram-negative *Acinetobacter* 199A was rendered sensitive to lysozyme by preincubation with human lysosomal proteinases, including cathepsin G and elastase (20).

Work continues in our laboratory on the interaction of the many lysosomal constituents and their role in bactericidal activity.

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LITERATURE CITED

1. **Densen, P., and G. L. Mandell.** 1978. Gonococcal interactions with polymorphonuclear neutrophils. Importance of the phagosome for bactericidal activity. J. Clin. Invest. **62**:1161-1171.
2. **Dilworth, J. A., J. O. Hendley, and G. L. Mandell.** 1975. Attachment and ingestion of gonococci by human neutrophils. Infect. Immun. **11**:512-516.
3. **Ismail, G., W. D. Sawyer, and W. S. Wegener.** 1977. Effect of hydrogen peroxide and superoxide radical on viability of *Neisseria gonorrhoeae* and related bacteria. Proc. Soc. Exp. Biol. Med. **155**:264-269.
4. **Kellogg, D. S., Jr., I. R. Cohen, L. C. Norins, A. C. Schroeter, and G. Ressig.** 1968. *Neisseria gonorrhoeae*. II. Colonial variation and pathogenicity during 35 months in vitro. J. Bacteriol. **96**:596-605.
5. **Kellogg, D. S., Jr., W. L. Peacock, Jr., W. E. Deacon, C. Brown, and C. I. Pirkle.** 1963. *Neisseria gonorrhoeae*. I. Virulence genetically linked to clonal variation. J. Bacteriol. **85**:1274-1279.
6. **Modrzakowski, M. C., M. H. Cooney, L. E. Martin, and J. K. Spitznagel.** 1979. Bactericidal activity of fractionated granule contents from human polymorphonuclear leukocytes. Infect. Immun. **23**:587-591.
7. **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.
8. **Odeberg, H., and I. Olsson.** 1975. Antibacterial activity of cationic proteins from human granulocytes. J. Clin. Invest. **56**:1118-1124.
9. **Odeberg, H., and I. Olsson.** 1976. Mechanisms for the microbicidal activity of cationic proteins of human granulocytes. Infect. Immun. **14**:1269-1275.
10. **Odeberg, H., and I. Olsson.** 1976. Microbicidal mechanisms of human granulocytes: synergistic effects of granulocyte elastase and myeloperoxidase or chymotrypsin-like cationic protein. Infect. Immun. **14**:1276-1283.
11. **Odeberg, H., I. Olsson, and P. Venge.** 1975. Cation proteins of human granulocytes. IV. Esterase activity. Lab. Invest. **32**:86-90.
12. **Perry, M. B., V. Daoust, B. B. Diena, F. E. Ashton, and R. Wallace.** 1975. The lipopolysaccharides of *Neisseria gonorrhoeae* colony types 1 and 4. Can. J. Biochem. **53**:623-629.
13. **Rest, R. F.** 1979. Killing of *Neisseria gonorrhoeae* by human polymorphonuclear neutrophil granule extracts. Infect. Immun. **25**:574-579.
14. **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.
15. **Rest, R. F., M. H. Cooney, and J. K. Spitznagel.** 1978. Bactericidal activity of specific and azurophil granules from human neutrophils: studies with outer membrane mutants of *Salmonella typhimurium* LT-2. Infect. Immun. **19**:131-137.
16. **Schmidt, G., and S. J. Tannhauser.** 1945. A method for the determination of DNA, RNA, and phosphoproteins in animal tissues. J. Biol. Chem. **161**:83-89.
17. **Swanson, J.** 1978. Studies on gonococcus infection. XII. Colony color and opacity variants of gonococci. Infect. Immun. **19**:320-331.
18. **Thomas, D. W., J. C. Hill, and F. J. Tyeryar, Jr.** 1973. Interaction of gonococci with phagocytic leukocytes from man and mice. Infect. Immun. **8**:98-104.
19. **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.
20. **Thorne, K. J. I., R. C. Oliver, and A. J. Barrett.** 1976. Lysis and killing of bacteria by lysosomal proteinases. Infect. Immun. **14**:555-563.
21. **Weiss, J., S. Beckerdite-Quaghata, and P. Elsbach.** 1980. Resistance of gram-negative bacteria to purified bactericidal leukocyte proteins. Relation to binding and bacterial lipopolysaccharide structure. J. Clin. Invest. **65**:619-628.
22. **Weiss, J., P. Elsbach, I. Olsson, and H. Odeberg.** 1978. Purification and characterization of a potent bactericidal and membrane active protein from the granules of human polymorphonuclear leukocytes. J. Biol. Chem. **253**:2664-2672.
23. **Welsh, I. R. H., and J. K. Spitznagel.** 1971. Distribution of lysosomal enzymes, cationic proteins, and bactericidal substances in subcellular fractions of human polymorphonuclear leukocytes. Infect. Immun. **4**:97-106.
24. **Wiseman, G. M., and J. D. Caird.** 1977. Composition of the lipopolysaccharide of *Neisseria gonorrhoeae*. Infect. Immun. **16**:550-556.