MOLECULAR BIOLOGY OF GONOCOCCAL DEATH FOLLOWING EXPOSURE TO THE GRANULE EXTRACTS OF HUMAN NEUTROPHILS

by

John Patrick Rock

A Dissertation Submitted to the Faculty of the

DEPARTMENT OF MOLECULAR AND CELLULAR BIOLOGY

In Partial Fulfillment of the Requirements
For the Degree of

DOCTOR OF PHILOSOPHY
WITH A MAJOR IN MOLECULAR BIOLOGY

In the Graduate College

THE UNIVERSITY OF ARIZONA

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The University of Arizona PH.D. 1986

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As members of the Final Examination Committee, we certify that we have read the dissertation prepared by John P. Rock

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and recommend that it be accepted as fulfilling the dissertation requirement for the Degree of Doctor of Philosophy.

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I hereby certify that I have read this dissertation prepared under my direction and recommend that it be accepted as fulfilling the dissertation requirement.

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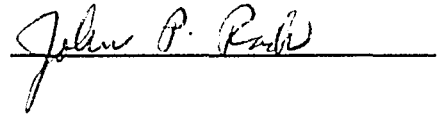
SIGNED: 

DEDICATION

This dissertation is dedicated to the members of my family, who have given their unquestioning and unwavering support throughout the long ordeal of graduate school. It is only because of their love and understanding that I am able to complete this degree, and to all of them I am humbly, eternally grateful.

PREFACE

The format of this dissertation is designed around journal articles, written from the results of my research, which have either been published or submitted for publication. As such, Chapters 2, 3, and 4 are organized into sections appropriate for publication in the journals of the American Society for Microbiology. Therefore, there is some redundancy of information among the chapters, particularly within the Materials and Methods sections. It was felt, however, that the individual chapters would each be more complete and coherent if they contained the repetitive sections.

I wish to acknowledge the assistance of Linda Eaton, Christopher Elkins, Stephen Fischer, and Joseph Mezzatesta for their valuable contributions, including suggestions, technical help, and brainstorming, at various points during this research. Lastly, I wish to thank Richard Rest for his patience, wisdom, and unfailing excitement about research, and for instilling a bit of those qualities in me.

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ABSTRACT

Gonococci that have been phagocytized by human neutrophils are killed very effectively. While much research has focused on defining the microbicidal mechanisms of the neutrophil arsenal, substantially less is known regarding why phagocytized bacteria die. Gonococci were examined, at the molecular level, following exposure to human neutrophil granule extracts (GE) in an effort to discover the "lethal lesion", that injury to the bacterial cell which results in its death. The cytoplasm-based metabolism of GE-treated gonococci continues to function normally for at least 30 minutes, although these same cells have lost the ability to divide and are reproductively dead. GE-treated gonococci were found to utilize less oxygen than control cells, indicative of damage to the cytoplasmic membrane. Visual examination of GE-treated gonococci by light microscopy revealed that the cells undergo very minimal division once exposed to GE. GE-treated gonococci visualized by transmission electron microscopy had outer membranes which suffered time-related disorganization and disruption; the effects began immediately upon contact with GE. GE-treatment was also observed to cause aberrant structure and orientation of forming bacterial septa. Investigation of gonococcal peptidoglycan, the structural

component of the bacterial membrane, yielded interesting results when the effects of GE were scrutinized. GE caused subnormal incorporation of peptidoglycan precursors, and also induced a twofold higher rate of release of peptidoglycan turnover fragments than was seen from control cells. After analysis of peptidoglycan fragments released into culture supernatants by thin-layer chromatography and high-pressure liquid chromatography, it was found that the small amount of high-molecular-weight fragments exhausted by control cells was not present with treated cells. Investigation of the cell-associated peptidoglycan, by the above methods, after exposure to GE revealed differences in the digestion products. There was a distinct reduction in the amount of penicillin bound by the penicillin-binding proteins of GE-treated cells. There was, however, no observed change in the electrophoretic mobility between the PBPs of control and treated cells.

CHAPTER 1

INTRODUCTION

Gonorrhea is a disease that has plagued man since ancient times; its symptoms were referred to by the Chinese emperor Huang Ti over 4,600 years ago. Currently, gonorrhea is a disease of epidemic proportions. The present venereal disease crises of herpes and AIDS have contributed to a loss of awareness of the problems of gonorrhea. However, complications of gonorrhea, which include pelvic inflammatory disease, arthritis, dermatitis, and sterility, are significant in terms of the impact they have on the human population.

Man is the only known host and reservoir for Neisseria gonorrhoeae. A gonococcal infection frequently manifests itself as a urethral discharge, classically known as "The Drips" in street slang. This discharge results from a massive influx of neutrophils to the site of infection, and gram-negative diplococci are clearly visible within these neutrophils. It follows, then, that understanding the interaction of gonococci with human neutrophils is critical to an understanding of the pathogenics of the bacteria and the defense mechanisms of the host.

Neutrophils are one of the major, secondary defense mechanisms for dealing with bacteria that have penetrated the primary, passive barriers of the host. Neutrophils attempt to deal with pathogenic bacteria by phagocytizing and killing them. Densen and Mandell (1978) reported that gonococci must be phagocytized to be killed by neutrophils. Internalized bacteria are held within a membrane-bound vacuole termed the phagosome. The components of the neutrophil microbicidal systems, also within membrane-bound vesicles called granules, are introduced to the bacteria when granules fuse with phagosomes. Intracellular pathogens are able to avoid being killed by selectively inhibiting various parts of these microbicidal mechanisms (Filice 1983, Quie 1983). There have been reports that gonococci can also survive within neutrophils (Parsons et al. 1981, 1982), but these results have not been confirmed by others.

The microbicidal mechanisms of the neutrophil have been reviewed by Root and Cohen (1981). Basically, these mechanisms can be divided into two categories: (i) oxidative, and (ii) non-oxidative systems. The oxidative, or oxygen-dependent, systems are mediated by the neutrophil oxidative burst which occurs concurrently with phagocytosis. McPhail and Snyderman (1983) have reported on the mechanism of activation of the respiratory burst. Neutrophils create a variety of toxic oxygen species such as superoxide anion, hydroxyl radicals, singlet oxygen, and hydrogen peroxide

during the respiratory burst. Although hydrogen peroxide alone can kill bacteria (Clifford and Repine 1982), enzymes such as myeloperoxidase can convert these oxygen species into extremely bactericidal products such as hypochlorous acid. However, not all organisms are susceptible to oxidative killing; resistance to oxygen-dependent neutrophil killing by Nocardia asteroides has been noted (Filice 1983).

The non-oxidative (oxygen-independent) microbicidal mechanisms, recently summarized by Spitznagel and Shafer (1985) are very important to host defense. Anaerobic bacteria from oxygen-free sites of the body are killed by neutrophils (Klempner 1984), which precludes involvement of the oxygen-dependent mechanisms in this instance. Thore et al. (1985) found that neutrophils can kill Streptococcus pneumoniae anaerobically, while Wetherall, Pruul, and McDonald (1984) described the oxygen-independent killing of Bacteroides fragilis by neutrophil granule extract (GE). Rest et al. (1982) found that neutrophils obtained from donors with chronic granulomatous disease, which is characterized by the absence of a neutrophil respiratory burst, kill gonococci as well as normal neutrophils do.

Various components of the non-oxidative neutrophil arsenal have been investigated. Some of these are non-specific, such as the drop in pH that occurs within the forming phagocytic vacuole (Styrt and Klempner 1982). The mechanism of the pH drop has been studied (Mollinedo,

Manara, and Schneider 1986), and the importance of acidification of the vacuole for killing of bacteria has been established (Styrt and Klempner 1985). Low-molecular-weight polypeptides with a very broad spectrum of microbicidal activity have recently been discovered in neutrophils (Selsted, Szklarek, and Lehrer 1984).

Other parts of the microbicidal system have much more specific effects on ingested bacteria. A bactericidal, permeability-increasing protein found in rabbit and human neutrophils has been found to be very bactericidal in purified form (Elsbach et al. 1979; Forst, Weiss, and Elsbach 1982; van Houte et al. 1977; Weiss et al. 1976, 1978, 1984; Weiss, Victor, and Elsbach 1983). Odeberg and Olsson (1975, 1976a, 1976b) and Modrzakowski and Spitznagel (1979) have investigated the microbicidal mechanisms of cationic proteins isolated from human neutrophils. Degradation of gonococcal outer membrane proteins by the neutrophil components elastase and cathepsin G was determined by Rest and Pretzer (1981). The bactericidal activity of GE has been demonstrated against several species of bacteria (Buck and Rest 1981; Rest 1979; Rest, Cooney, and Spitznagel 1977, 1978; Wetherall et al. 1984). Degradation of internalized bacteria, reported by Eaton and Rest (1983), Ginsburg and Lahav (1983), and Ginsburg, Lahav, and Giesbrecht (1982), is

as important to disposal of killed bacteria as it is to the actual killing.

Gonococcal ultrastructure has been determined by electron microscopy of cells from both in vivo and in vitro sources (Fitz-James 1964; Hendley et al. 1981; Novotny, Short, and Walker 1975; Ovcinnikov and Delektorskij 1971). This information is useful for comparing normal gonococcal structure with the structural changes observed after bacteria have been either phagocytized (Rozenberg-Arska et al. 1985, Swanson and Zeligs 1974) or exposed to isolated granule constituents (van Houte et al. 1977).

Many other biochemical and morphological aspects of gonococci have been extensively investigated. In addition, these same systems have been studied in other bacteria, which helps in understanding the composition of gonococci. The respiratory chain, an integral component of the inner surface of the cytoplasmic membrane, has been partially characterized in gonococci (Kenimer and Lapp 1978, Winter and Morse 1975). Norrod and Morse (1979) examined gonococcal species for the presence of superoxide dismutase, an enzyme that is potentially protective against neutrophil-generated superoxide anion. Archibald and Duong (1986) examined gonococci recently and found no evidence for a bacterial superoxide dismutase.

Bacterial membranes are the first component of the cell to interact with GE. Therefore, a detailed knowledge

of the structure and composition of the gonococcal membrane is important. Phospholipids, the main structural component of the cytoplasmic membrane and the inner surface of the outer membrane, have been identified by Beebe and Wlodkowski (1976), Sud and Feingold (1975), and Senff et al. (1976). Mintz, Apicella, and Morse, (1984), and Schneider et al. (1984, 1985) looked at the lipopolysaccharide component of gonococci. Permeability of the outer membrane was examined by Lysko and Morse (1981). Mutations causing altered permeability were also identified and investigated (Eisenstein and Sparling 1978, McFarland et al. 1983, Morse et al. 1982), and the implications of these permeability changes to killing by neutrophils (Daly et al. 1982) and antibiotics (Shafer et al. 1984) were examined. The presence of a protein-macromolecular complex has been reported in the outer membrane of gonococci (Hansen and Wilde 1984). Blake, Gotschlich, and Swanson (1981) have examined gonococcal outer membrane proteins with regard to their susceptibility to proteolytic cleavage.

Peptidoglycan is the structural component of the bacterial membrane. It is the rigid, single-molecule network that regulates the size and shape of the bacterial cell, and also protects the cell from the osmotic imbalance across the cell membrane. Peptidoglycan is (retrospectively) the membrane component that has become the focus of this research.

The basic structure of gonococcal peptidoglycan has been elucidated (Blundell and Perkins 1985, Dougherty 1983a, Greenway and Perkins 1985, Hebeler and Young 1976a); the peptidoglycan structure of a nonpathogenic Neisseria sp. has been found to be similar (Martin et al. 1973). Gonococcal peptidoglycan is of chemotype I, which is the same group to which Escherichia coli peptidoglycan belongs.

Mengin-Lecreulx and van Heijenoort (1985) have reported on E. coli peptidoglycan synthesis; presumably the steps would be similar for gonococcal peptidoglycan. Sinha and Rosenthal (1980), and Blundell and Perkins (1985) have found that the muramyl residue, on what would normally be the reducing end of the glycan chain, has a 1,6-anhydro-intramolecular bond. The extent of peptide cross-linking (intermolecular, diaminopimelic acid to D-alanine) in gonococcal peptidoglycan averages roughly 40% (Rosenthal, Wright, and Sinha 1980), somewhat high for gram-negative bacteria. In contrast, the peptidoglycan of Streptococcus pneumoniae has cross-links in less than 10% of its peptide side chains (Fischer and Tomasz 1985). Gonococcal peptidoglycan is O-acetylated at the 6-position of muramic acid (Rosenthal, Gfell, and Folkening 1985), a feature that renders the peptidoglycan relatively insensitive to lysozyme from human sources (Blundell, Smith, and Perkins 1980; Lear and Perkins 1983; Rosenthal, Blundell, and Perkins 1982; Rosenthal et al. 1983; Swim et al. 1983). Early reports by

Hebeler et al. (1978, 1979) presented evidence for a peptidoglycan-associated protein in gonococci; this is analogous to the lipoprotein of E. coli, which anchors the outer membrane to the underlying peptidoglycan. Subsequent examination has not confirmed these results.

Some of the enzymes of peptidoglycan synthesis and turnover have been identified. Rosenthal (1979) discovered hexaminidase and amidase activities in gonococci. Gubish, Chen, and Buchanan (1982) found an endo-N-acetylglucosaminidase, and an exo-N-acetylglucosaminidase was detected by Chapman and Perkins (1983). Gonococcal penicillin-binding proteins are responsible, among other associated enzymatic activities, for formation of the intermolecular peptide cross-links. Dougherty, Koller, and Tomasz (1980, 1981), and Barbour (1981) identified the three known penicillin-binding proteins of gonococci. Comparatively little is known about the three gonococcal enzymes, especially when contrasted with the relative wealth of information available about the penicillin-binding proteins of bacteria such as E. coli (de la Rosa, Pedro, and Vasquez 1985; Ishino and Matsuhashi 1981; Nicholas et al. 1985; Rodriguez-Tebar, Barbas, and Vazquez 1985), Streptococci (Fontana et al. 1983, Handwerger and Tomasz 1986), Staphylococcus aureus (Chambers, Hartman, and Tomasz 1985), and Bacillus subtilis (Buchanan and Strominger 1976).

Lastly, an understanding of the autolytic enzymes of gonococci is important to the comprehension of the effects of GE on gonococci. Gonococci spontaneously undergo autolysis when a broth- or plate-grown culture gets old (typically older than 24 hours). Morse and Bartenstein (1974), Hebeler and Young (1975), and Elmros, Burman, and Bloom (1976) determined the factors affecting autolysis. These include depletion of nutrient source (glucose), determination of optimal pH and temperature, and identification of external agents such as EDTA, divalent cations, etc. Further elaboration of the mechanism of autolysis was reported by Cacciapuoti, Wegener, and Morse (1978). This elaboration involves the interrelationship of pH, presence of divalent cations, and phospholipid hydrolysis. Hebeler and Young (1976b) identified the major autolysin as N-acetylmuramyl-L-alanine amidase. Wegener, Hebeler, and Morse (1977a) examined autolysis in relation to peptidoglycan hydrolysis.

In light of the background information presented, it is therefore relevant to examine the interactions of gonococci with the extracts of human neutrophils. Groundwork to this project, performed by Buck and Rest (1981), points to non-cytoplasm-based effects involved in the early events of this interaction. It is logical to turn towards the bacterial membrane as the structure likely to provide useful results for defining why gonococci die when phagocytized by

neutrophils. Knowledge of some parts of the gonococcal membrane is sketchy and rudimentary; however, it should prove sufficient to identify whether or not the bacterial death-associated lesion is caused by disruption of that particular component.

CHAPTER 2

EFFECT OF HUMAN NEUTROPHIL LYSOSOMAL CONTENTS ON PEPTIDOGLYCAN TURNOVER AND MEMBRANE STRUCTURE IN *NEISSERIA GONORRHOEAE*

This chapter comprises a manuscript that has already been published (Rock 1985). The results presented include the earliest observed changes in gonococci caused by granule extract; these changes occur within the time necessary to be causal for the loss of colony-forming ability, which is the basis of my research project.

Abstract

The effect of human neutrophil lysosomal contents (granule extract) on the peptidoglycan turnover and gross membrane morphology of mid-log-phase gonococci was investigated. Transmission electron microscopy of granule extract-treated gonococci revealed alterations in membrane structure which became apparent immediately upon treatment and increased proportionally over time. These alterations include aberrations in septum structure and orientation, a decrease in the "ruffled" appearance of the outer membrane, and the appearance of amorphous, electron dense material adhering externally to the outer membrane. During the first 20 min of treatment with granule extract, gonococci whose peptidoglycan backbone had been radiolabeled revealed a rate of

release of peptidoglycan breakdown products of 24.9% per h, compared with the rate in controls of 13.7% per h. After the first 20 min of treatment, the rates of release by control and treated gonococci became similar, but the amounts of material released differed. Uptake of peptidoglycan precursors was also affected by treatment with granule extract: control gonococci took up linearly increasing amounts of a 5-min radiolabel pulse over time, but the uptake by treated gonococci was immediately and completely halted. This suggests that attack of the gonococcal membrane by granule extract produces immediate membrane damage. This damage may ultimately be responsible for bacterial death by specifically inhibiting division, but not general metabolism.

Introduction

Gonococci invading the human host have to contend with polymorphonuclear neutrophils as a major host defense mechanism. Neutrophils have been shown to phagocytize and kill bacteria by a variety of bactericidal mechanisms (Root and Cohen 1981). On the other hand, phenotypic variants of Neisseria gonorrhoeae may or may not be phagocytized in vitro, depending on whether gonococcal pili or outer membrane proteins (protein II), or host serum are present (Densen and Mandell 1978, Rest et al. 1982). Once phagocytized, however, gonococci are exposed to the combined

contents of neutrophil azurophil and specific granules, and all variants are killed equally well (Rest 1979). Bacterial death, at least in the initial stages of exposure to granule enzymes, is associated only with the loss of the ability to form colonies on agar. Protein, DNA, and RNA synthesis in granule extract (GE)-treated gonococci continues at or above control levels for at least 30 min (Buck and Rest 1981). My research has shown that oxygen uptake by gonococci, a measure of the function of the terminal oxidase of the electron transport chain, also continues at or above control levels for the first 30 min of GE treatment (unpublished data). When taken together, these data indicate that, at least initially, GE-treated gonococci are reproductively dead but metabolically alive.

Since cell division stops but metabolic processes continue, it is appropriate to investigate the effect of GE on the turnover of gonococcal peptidoglycan. As a major structural component of the gram-negative cell membrane, peptidoglycan must be intimately involved in septum formation and cell division. A great deal of recent research has focused on gonococcal peptidoglycan. Studies have determined the amino acid composition, the degree of cross-linking in the peptide side chain, and the fraction of the glycan backbone residues that are O-acetylated (Blundell, Smith, and Perkins 1980; Dougherty 1983a; Hebeler and Young 1976a; Lear and Perkins 1983; Rosenthal, Blundell, and

Perkins 1982; Rosenthal, Wright, and Sinha 1980; Swim et al. 1983). Hebeler, et al. (1978, 1979) found protein complexed with gonococcal peptidoglycan. Several bacterial enzymes (autolysins) of peptidoglycan breakdown have been identified (Chapman and Perkins 1983; Gubish, Chen, and Buchanan 1982; Hebeler and Young 1976b; Rosenthal 1979), as have the breakdown products themselves (Rosenthal 1979, Sinha and Rosenthal 1980).

In the present paper I report on the quantitative aspects of the effects of human neutrophil granule components on gonococcal peptidoglycan synthesis and breakdown. I also provide visual evidence that granule constituents do in fact cause significant morphological aberrations in gonococcal membrane structure.

Materials and Methods

Bacteria

Neisseria gonorrhoeae strain F62 was maintained on GC medium base supplemented with (per liter): dextrose, 4 g; glutamine, 100 mg; cocarboxylase, 0.2 mg; $Fe(NO_3)_3$, 12.5 mg; and $NaHCO_3$), 400 mg. Cultures were incubated at 36°C in 5 to 10% CO_2 in air and subcultured daily to propogate the specific colonial morphology types as identified by Kellogg et al. (1963, 1968). For experiments, type 4 gonococci were grown to mid-log phase by inoculating 5 ml of prewarmed, supplemented GC broth (containing, per liter, 15 g of

Proteose peptone no. 3, 4 g of K_2HPO_4, 1 g of KH_2PO_4, 5 g of NaCl, and 1 g of soluble starch) from a 14- to 18-hour plate and vortexing with glass beads. This GC broth was then transferred to a side-arm flask containing 15 ml prewarmed, supplemented GC broth. An initial turbidity reading (Klett-Summerson Photoelectric Colorimeter, green filter) of 10 to 12 U was obtained. The flask was incubated in a shaker bath at 36°C for approximately 3 h, until the culture reached a Klett value of 80 to 110, which represented mid-log phase. Gonococci were quantitated for assays by transferring samples of the culture into 5 ml of supplemented GC broth until an optical density at 550 nm (Spectronic 20 colorimeter, Bausch and Lomb, Rochester, N.Y.) of 0.30 was reached, which corresponds to 10^8 to 2×10^8 CFU/ml.

Preparation of Granule Extract

Neutrophils were obtained from healthy adult human volunteers by leukapheresis and sedimented for 1 h. Then, as described by Rest (1979) and Rest, Cooney, and Spitznagel (1977, 1978), the cells were washed in phosphate-buffered saline (PBS) and centrifuged through Ficoll-Hypaque. Erythrocytes were lysed hypotonically. This yielded approximately 10^{10} neutrophils per donor with no more than 8% non neutrophils (mostly eosinophils). The neutrophils were either homogenized in 0.34 M sucrose or disrupted in a Parr Bomb (Parr Instrument Company, Moline, Ill.) (Borregaard et

al. 1983) to 90% breakage, and the nongranule cellular debris pelleted (250 × g, 15 min). Azurophil and specific granules were pelleted (20,200 × g, 20 min) from the homogenization or bomb supernatant and extracted by Dounce homogenization (10 min, 4°C) in 0.2 M sodium acetate buffer (pH 4.0) containing 10 mM $CaCl_2$. Extracts were clarified by centrifugation (20,200 × g, 20 min) and dialyzed against PBS (pH 7.4) in tubing with an average molecular weight cutoff of 3500. Extracts from the combined granules of 10^9 neutrophils yielded approximately 10 mg of protein, as determined by the method of Lowry et al. (1951), using egg white lysozyme as a standard.

For each batch of GE, the protein concentration required to reduce gonococcal viability by two logs after 60 min of exposure was determined. This was the GE concentration used in all experiments.

Peptidoglycan Breakdown and Release

Gonococci were grown to mid-log phase with [^{3}H]glucosamine (0.6 μCi/ml; specific activity, 20 to 40 Ci/mmol) added to the culture for the last 2 h of growth (Wegener, Hebeler, and Morse 1977a). The gonococci were cold chased for 30 min, and after an additional wash were resuspended in fresh, supplemented GC broth and mixed with GE (treated) or PBS (control). At indicated times, samples were pelleted and radiolabel release into the medium was determined.

Samples were also plated at 0 and 60 min to determine gonococcal viability.

Uptake of Glucosamine

Mid-log-phase gonococci in fresh, supplemented GC broth were mixed with GE (treated) or an equal volume of PBS (control) and incubated in a water bath at 36°C. At indicated times, a sample was taken from each tube and mixed with [^{3}H]glucosamine (1.0 μCi/ml; specific activity, 20 to 40 Ci/mmol). After a 5-min pulse at 36°C, the gonococci were pelleted (1 min at 12,800 × g, room temperature) and the amounts of radiolabel associated with pellet and supernatant were determined. Viability was assessed as above.

Transmission Electron Microscopy

Samples of control and treated gonococci (4 ml containing 5 × 10^7 CFU/ml) were taken at various times and fixed overnight in 2.5% glutaraldehyde at 4°C. After three washes in phosphate buffer, the pellets were post-fixed for 30 min in 1% osmium tetroxide and washed twice more. Specimens were dehydrated in a graded series of ethanols and embedded in Spurr embedding medium (Electron Microscopy Sciences, Fort Washington, Pa.). Sections (60 nm thick) were cut on an LKB 8800 Ultramicrotome III (LKB Instruments, Rockville, Md.) with a diamond knife. The sections were stained for 20 min in 5% uranyl acetate and 5 min in lead

citrate. Micrographs were taken on a Zeiss EM-9 electron microscope.

Materials

Tritiated glucosamine was purchased from Amersham Corp., Arlington Heights, Ill. GC medium base, Proteose peptone no. 3, and soluble starch were from Difco Laboratories, Detroit, Mich. Ficoll and egg white lysozyme were purchased from Sigma Chemical Co., St. Louis, Mo. Hypaque (50% sodium) was from Winthrop Laboratories, Menlo Park, Calif. Electron microscopy supplies were kindly donated by Sheila Katz of Hahnemann University.

Results

Killing of Mid-log-phase Gonococci by GE

I compared peptidoglycan turnover between control and GE-treated gonococci to determine whether GE affected synthesis and degradation of this structural component of the gram-negative cell membrane. When gonococcal viability was assayed, controls increased in number exponentially over the course of the experiment, with no appreciable lag. GE-treated gonococci, however, rapidly lost their ability to form colonies on agar, and the decrease began immediately (Fig. 1).

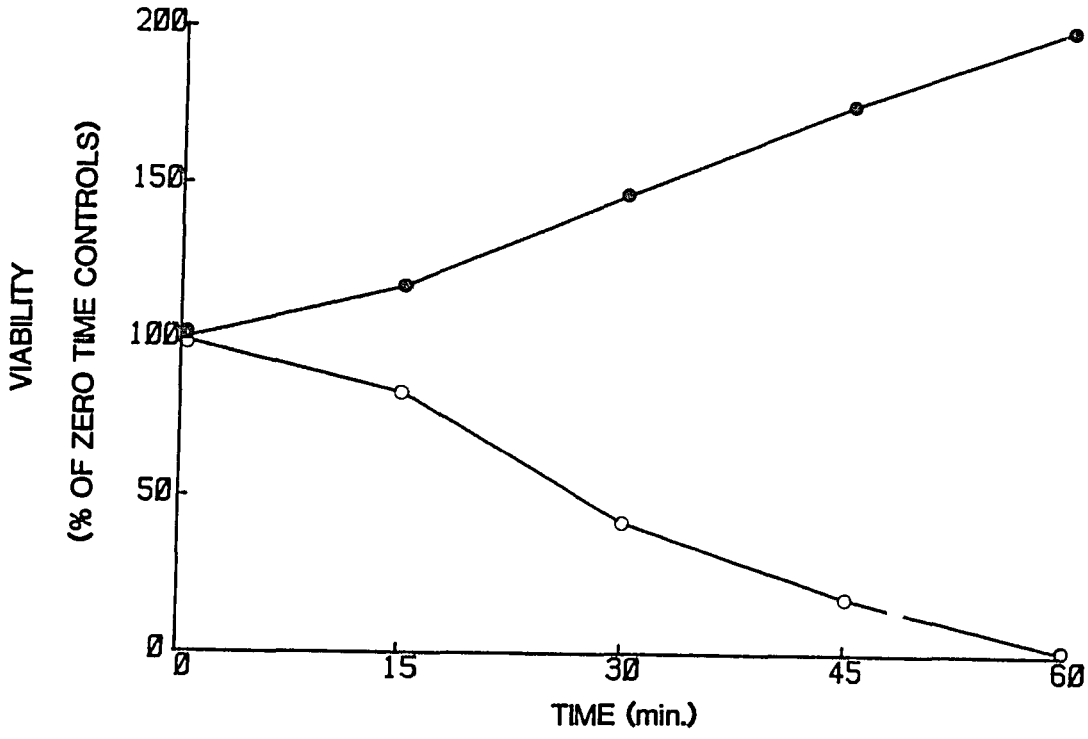

Fig. 1. Effect of Neutrophil Granule Components on Gonococcal Viability

Mid-log-phase gonococci (5×10^7 CFU/ml) were incubated at 36°C. At zero time, GE (500 μg of protein per ml, final concentration) or an equal volume of PBS (control) was added to the cultures. Viability was assessed at indicated times by the ability to form colonies on agar. Symbols: ●, control gonococci; ○, treated gonococci. Data are from a typical experiment.

Release of Soluble Fragments of Prelabeled Peptidoglycan into Culture Supernatants

Gonococci hydrolyze previously synthesized peptidoglycan during normal growth. To determine the effects of GE on peptidoglycan breakdown, I measured the release of hydrolysis products from GE-treated and control cells. I assayed culture supernatants for the presence of peptidoglycan breakdown fragments from prelabeled gonococci. The resulting data show that the amount of radiolabel released by GE-treated gonococci during the first 20 min of the experiment roughly paralleled the amount released by PBS controls. Control gonococci discharged slightly more label initially, but by 20 min the release from treated gonococci had overtaken that of controls during the same time. However, a significant difference existed in the rate of release of radiolabel into the culture supernatant (Fig. 2). Over the initial 20 min, GE-treated gonococci discharged radiolabel at a rate of 24.9% of total incorporated tritium per h while the rate of release in PBS controls was only 13.7% per h. After the first 20 min, the rates of release by treated and control gonococci were similar. However, during this time the amount of label released into the medium showed a slight but reproducible difference; GE-treated gonococci released consistently more label than controls.

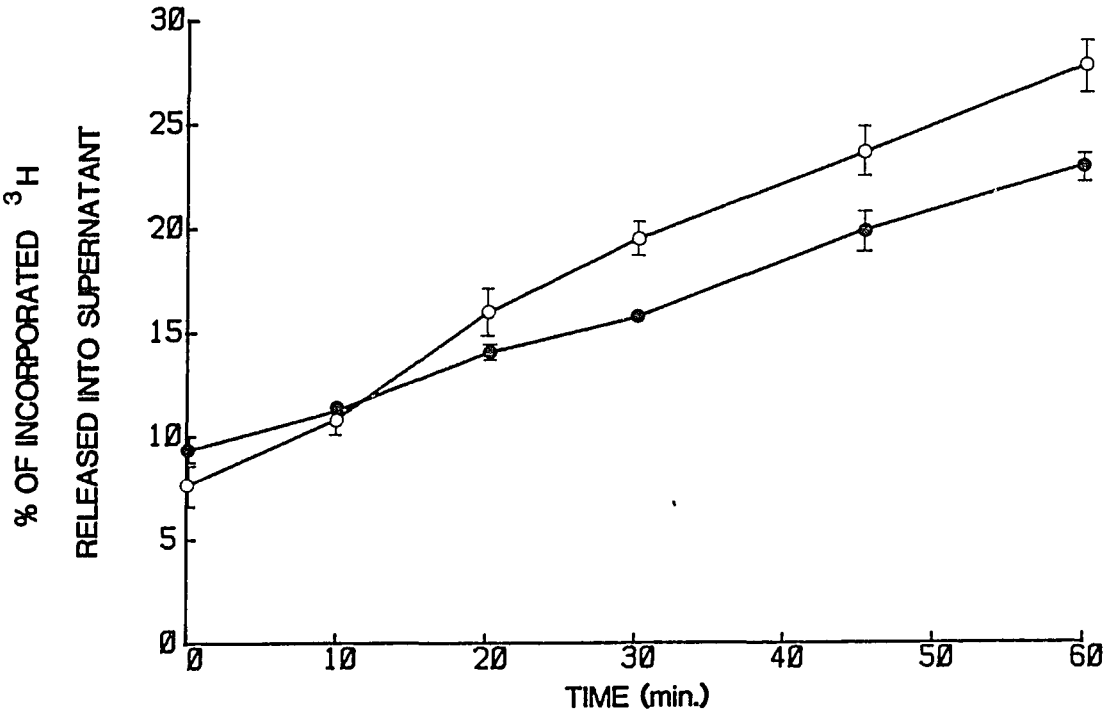

Fig. 2. Release of Peptidoglycan Breakdown Products from Gonococci into Culture Supernatants

Prelabeled (see Materials and Methods in this chapter), mid-log-phase gonococci (5×10^7 CFU/ml) were incubated at 36°C with 500 μg of GE per ml (○) or an equal volume of PBS (●). At indicated times, culture samples were centrifuged (1 min at 12,800 × g, room temperature) to pellet bacteria. Tritium release into supernatants was determined by liquid scintillation counting. Data are the average of three separate experiments. Full error bars represent one standard deviation.

Uptake of Tritiated Glucosamine from Culture Medium

The data above indicate that peptidoglycan breakdown is altered by GE. In an effort to measure peptidoglycan synthesis, I assayed for the uptake of radiolabeled glucosamine during a 5-min pulse. Control gonococci took up linearly increasing amounts of precursor over the course of the experiment. This was expected when related to a concurrent increase in the number of CFU. When I measured the uptake of label by GE-treated gonococci, however, the results were quite different (Fig. 3). Initial uptake was significantly less than that seen in controls. More importantly, treated gonococci exhibited the same basal level of label uptake over time. The absence of any time-related increase in peptidoglycan precursor utilization indicated that peptidoglycan synthesis was immediately and completely halted by exposure to GE.

Membrane Morphology Visualized by Transmission Electron Microscopy

Since peptidoglycan synthesis and degradation were affected by GE, it followed that gross membrane morphology might also be affected. Transmission electron microscopy of GE-treated gonococci revealed obvious morphological aberrations. Whereas control gonococci had a distinctly ruffled outer membrane and the peptidoglycan layer inside the outer membrane was also very wavy (Fig. 4), the outer membranes of GE-treated gonococci seemed to have much less variation in

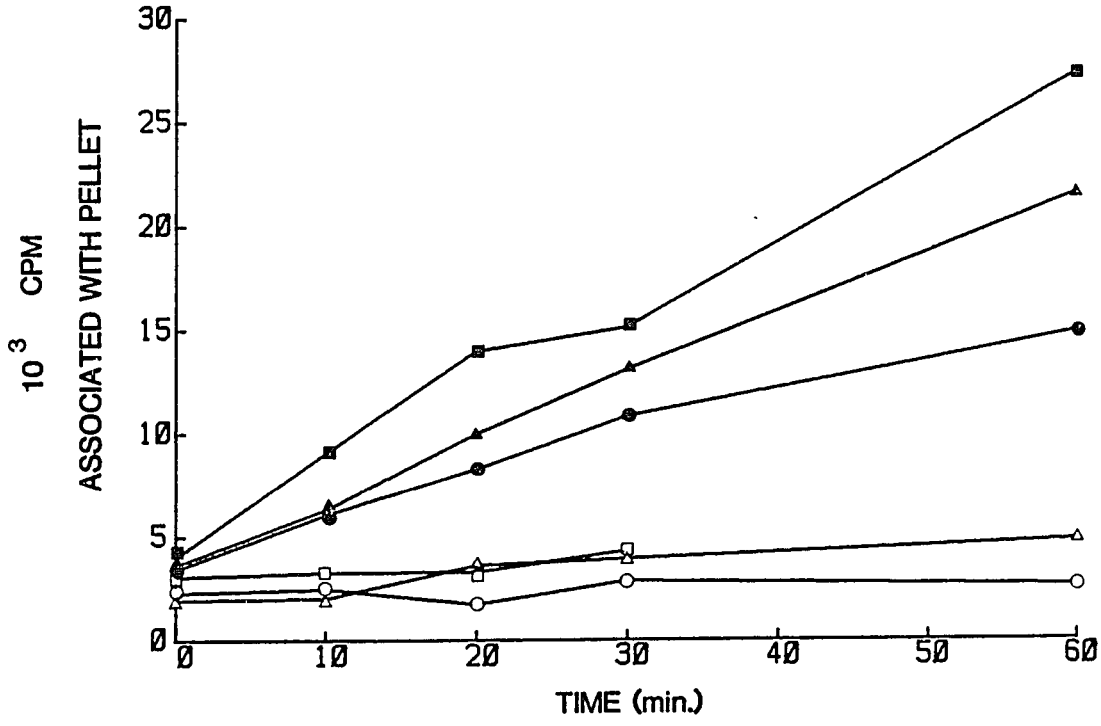

Fig. 3. Uptake of [^{3}H]glucosamine by Gonococci

Mid-log-phase gonococci (5 × 10^7 CFU/ml) were incubated at 36°C with 500 μg of GE per ml (○, □, △) or an equal volume of PBS (●, ■, ▲). At the times indicated, a culture sample was pulsed with [^{3}H]glucosamine for 5 min at 36°C. At the end of the pulse, the samples were centrifuged (1 min at 12,800 × g, room temperature), and the amount of radiolabel associated with the pellets was determined by liquid scintillation counting. The three symbols indicate data from three separate experiments.

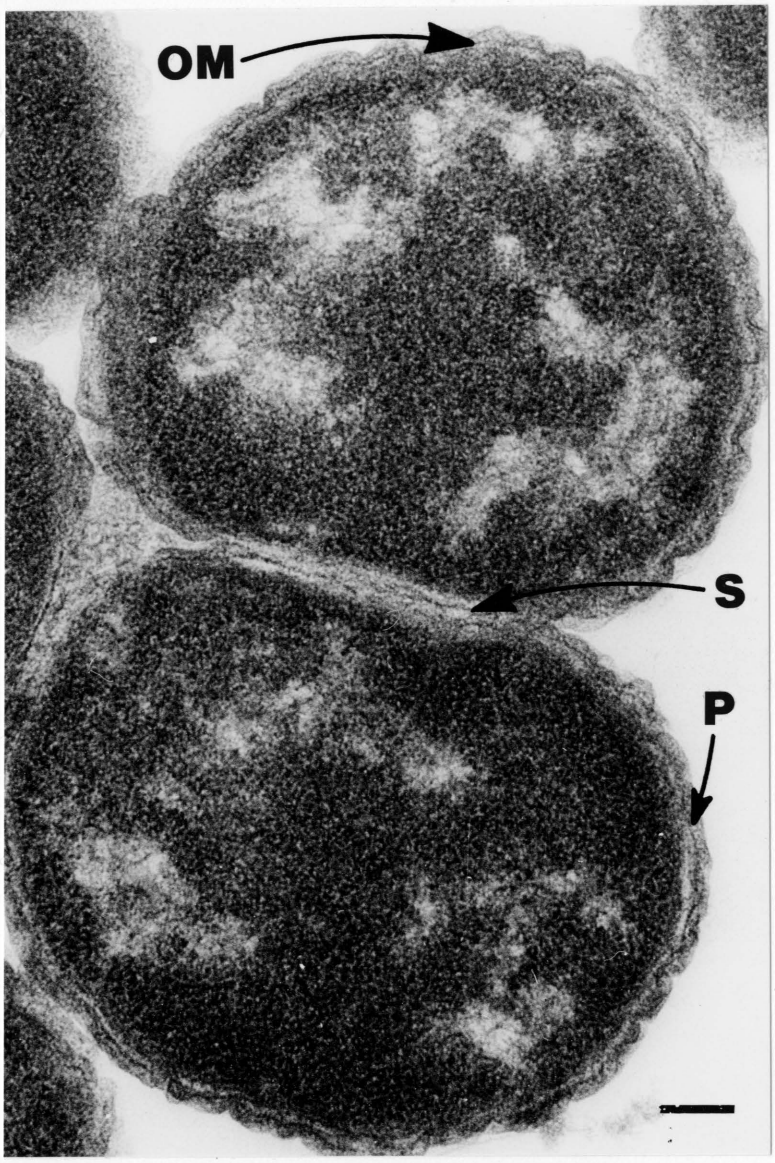

Fig. 4. Transmission Electron Micrograph of Gonococci Exposed to PBS (controls)

Note the ruffled appearance of the outer membrane (OM) and peptidoglycan layer (P). Completed septum (S) contains outer membrane material. Bar, 0.1 μm.

thickness (Fig. 5A), giving them a less ruffled appearance. The peptidoglycan layer on GE-treated gonococci was very straight and more highly visible. These membranes also had an accumulation of amorphous material clinging to them, the amount of which increased over the course of the experiment.

My investigations revealed GE-induced changes in septum formation and orientation in dividing gonococci. Partial septa were seen in both control and GE-treated gonococci. However, the orientation of the partial septa in the treated gonococci was aberrant (Fig. 5B). When extended linearly, the misaligned partial septa of treated gonococci do not appear oriented toward an intersecting point, as would be necessary to form a complete septum. When viewing completely formed septa separating diplococci, I also saw major structural differences between treated and control gonococci. The "normal" septum seen in controls has distinct layers which include apparently intact outer membrane material (Fig. 4), but septa formed during exposure to GE do not have any outer-membrane-type material in the space between the separating cells (Fig. 5A). In Fig. 5A the outer membrane can be seen to penetrate this space only partially, although the underlying peptidoglycan layer appears to be intact.

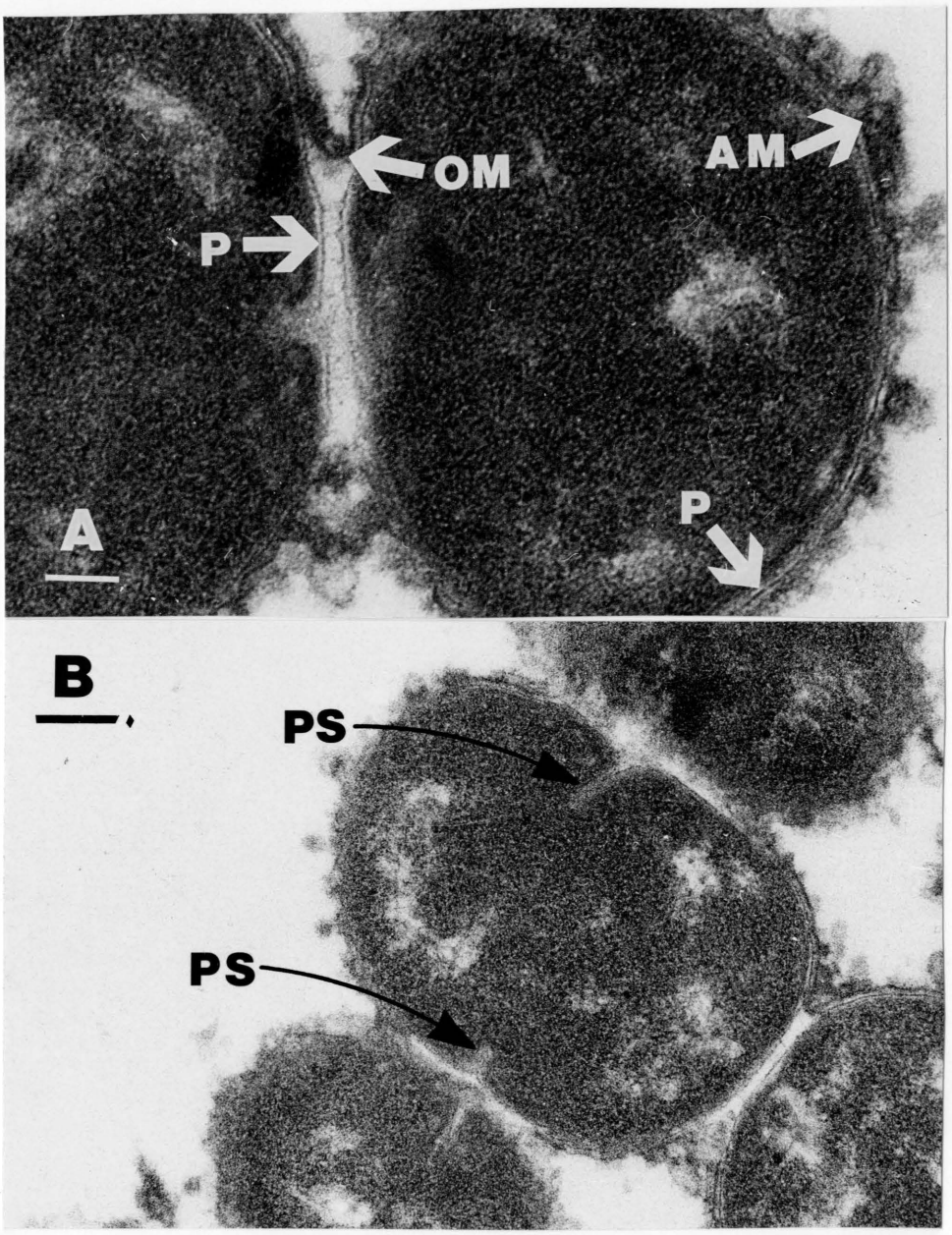

Fig. 5. Transmission Electron Micrographs of Gonococci Exposed to GE (treated)

(A) Note amorphous material (AM) adhering to the outer membrane (OM). Peptidoglycan layer (P) is more distinct and more linear than in control gonococci. Peptidoglycan is visible in the completed septum, but outer membrane material pulls away at the edge of the septum, leaving an electron-translucent gap. Bar, 0.1 μm. (B) Note the misalignment of the partially formed septum (PS). Bar, 0.2 μm.

Discussion

The contents of human neutrophils are responsible for a wide variety of insults to the membranes of gram-negative bacteria. Recent investigations have concentrated on identifying and explaining the perturbations in membrane structure and function caused by individual components of the neutrophil arsenal. Rest and Pretzer (1981) showed that purified lysosomal proteases (elastase and cathepsin G) degrade gonococcal outer membrane proteins. Eaton and Rest (1983) similarly reported that degradation of outer membrane protein occurs within whole neutrophils as well. I have also determined that high concentrations of purified elastase kill gonococci, although elastase is not bactericidal at concentrations present in GE (unpublished observations). Elastase probably contributes to killing within the neutrophil, since its bactericidal activity is synergistic with other granule components that kill gonococci. On the other hand, elastase may be equally important as a degradative enzyme once ingested bacteria are killed. Investigations by Modrzakowski and Spitznagel (1979) revealed a neutrophil granule cationic protein whose bactericidal activity decreases directly with the increase in complexity of the lipopolysaccharide of Salmonella typhimurium. This suggests that interaction of granule constituents with lipopolysaccharide is an important factor in bacterial killing. A bactericidal, permeability-increasing protein was described

in rabbit neutrophils by van Houte et al. (1977), and in human neutrophils by Weiss et al. (1978). This cationic protein is highly bactericidal against Escherichia coli, although it has no protease, lysozyme, myeloperoxidase or phospholipase A activity. Recently, the bactericidal, permeability-increasing protein has been shown to cause a marked and selective stimulation of the synthesis of lipopolysaccharide (Weiss et al. 1984). Therefore, it is apparent that isolated neutrophil granule constituents affect bacterial membranes by a variety of mechanisms.

Penicillin, an antibiotic that inhibits the cross-linking of peptidoglycan side chains, also produces a number of effects on membrane morphology and synthesis. Goodell, Fazio, and Tomasz (1978) have described these changes. Penicillin treatment of gonococci causes outer membrane "blebs" to protrude from the cell surface. The peptidoglycan layer under these protrusions is intact in some of their electron micrographs and nicked in others. My micrographs (Fig. 5B) also show outer membrane material detached from the underlying peptidoglycan at the periphery of the completed septum, although no outer membrane "blebs" were seen. Goodell et al. (1978) showed that addition of penicillin to growing gonococci causes glucosamine incorporation to stop within 5 to 10 min. Dougherty (1983b) reported that penicillin treatment can affect the degrees of peptide cross-linking and O-acetylation of nascent peptidoglycan. I have

found that GE treatment of gonococci immediately and completely inhibits the time-dependent increase in glucosamine uptake seen in controls. At the same time, these gonococci are very metabolically active. The basal level of glucosamine uptake by treated gonococci can be accounted for by the 15% of labeled glucosamine that is incorporated into lipopolysaccharide (Sinha and Rosenthal 1980). This is only indirect evidence that peptidoglycan synthesis stops, since uptake of only basal levels of precursors does not preclude de novo synthesis of peptidoglycan precursors.

The results of my electron microscopy differ from those in a previous report. Van Houte et al. (1977) investigated Escherichia coli membrane ultrastructure by freeze-fracture techniques. They reported that bactericidal, permeability-increasing protein from rabbit neutrophils does not change membrane morphology in E. coli even though rapid killing occurs and membrane permeability increases. I have found that whole GE from human neutrophils causes substantial alterations in gonococcal membrane morphology. These alterations, visualized by transmission electron microscopy, include aberrations in septum structure and changes in outer membrane gross morphology.

Penicillin has been shown to accelerate the rate of peptidoglycan hydrolysis in gonococci (Sinha and Rosenthal 1981; Wegener, Hebeler, and Morse 1977b). I have seen granule components cause almost twice the initial rate of

hydrolysis compared to controls. This accelerated rate may be due to (i) hyperactivity of the native peptidoglycan hydrolases, (ii) a greater rate of release of peptidoglycan breakdown products caused by an increase in outer membrane permeability, or (iii) neutrophil enzymes gaining access to and hydrolyzing gonococcal peptidoglycan. Comparing the peptidoglycan hydrolysis products between control and GE-treated gonococci should help to identify the mechanism of this rate increase.

CHAPTER 3

RAPID DAMAGE TO MEMBRANES OF *NEISSERIA GONORRHOEAE* CAUSED BY HUMAN NEUTROPHIL GRANULE EXTRACTS

This chapter consists of a manuscript that has been submitted for publication in the American Society for Microbiology journal "Infection and Immunity". It contains additional studies of the membranes of gonococci exposed to granule extract; it includes examination of ultrastructure by electron microscopy, assessment of division capacity by light microscopy, and examination of cytoplasmic membrane integrity by measurement of oxygen uptake.

Abstract

Gonococci exposed to acid extracts of human neutrophil granules were compared to untreated gonococci to determine the effects of such extracts on gonococcal membranes. Enumeration of cells by phase contrast microscopy at 0 and 60 min (one generation interval) revealed that treated gonococci undergo very limited cell division once exposed to granule extracts. At 60 min the treated cells tended to clump, and many lost their refractivity under phase, which indicated membrane damage. As measured by the depletion of dissolved oxygen from the growth medium, treated gonococci used oxygen at a lower rate than untreated

cells. This decreased oxygen utilization occurred immediately after contact with granule extract. After 60 min of extract treatment, oxygen uptake by treated cells was dramatically decreased. Membrane ultrastructure of control and treated gonococci was compared in thin section by transmission electron microscopy. Extract treatment resulted in a time-related increase in disruption of the bacterial outer membrane, which became apparent almost immediately after GE treatment. This was accompanied by increasingly aberrant septum structure. Resolution of peptidoglycan by electron microscopy was also affected by extract treatment, as early as 10 min after treatment. These data, together with other studies, suggest that extract treatment of gonococci causes a rapid loss of the ability to form colonies on agar due to alterations of membranes and peptidoglycan.

Introduction

In vivo, localized sites of infection by Neisseria gonorrhoeae are marked by an influx of polymorphonuclear neutrophils. These neutrophils are capable of phagocytizing and killing gonococci. Neutrophil granule extracts (GE) kill gonococci in vitro (Rest 1979), presumably by the same or similar mechanisms as in vivo. In the in vitro context, bacterial death can be defined as the rapid loss of the ability to form colonies on agar. Although these cells are reproductively dead, they still maintain a quantitatively

normal or supernormal metabolic capacity for >=30 min after exposure to GE. Buck and Rest (1981) were able to demonstrate this by measuring the incorporation of radiolabeled precursors into trichloroacetic acid-precipitable material as an indication of macromolecular (DNA, RNA and protein) synthesis. It is unlikely that gonococci lyse during these first 30 min of extract treatment (Buck and Rest 1981).

An obvious question then arises as to the actual molecular mechanism(s) involved in this loss of cell division capability. A bactericidal, permeability-increasing (BPI) granule protein has been isolated from rabbit (Elsbach et al. 1979) and human (Weiss et al. 1978) neutrophils that kills Escherichia coli and several other gram-negative bacteria. Although BPI has no demonstrated enzymatic activity, it increases outer membrane permeability and affects phospholipid biosynthesis very rapidly (Weiss et al. 1984); these effects occur within the time necessary to halt bacterial cell division. However, van Houte et al. (1977) did not find any observable ultrastructural alterations in E. coli killed by BPI, nor has the observed phospholipid alteration been causally associated with death (Weiss et al. 1984).

My studies (Rock 1985) have been devoted to determining the morphologic and biochemical events responsible for GE-induced gonococcal death. I have focused on changes in membrane structure and peptidoglycan biosynthesis which

occur rapidly after GE treatment; i.e. while general metabolism continues but the capacity to divide appears to decrease.

In the present study, I look at gonococcal morphology in detail by light and electron microscopy to determine the point at which bacterial replication stops in GE-treated cells. Oxygen uptake, a measure of the terminal oxidase of the electron transport chain, was also examined to resolve the state of the gonococcal cytoplasmic membrane.

Materials and Methods

Bacteria

Neisseria gonorrhoeae strain F62 was maintained on GC medium base supplemented with (per liter): dextrose, 4 g; glutamine, 100 mg; cocarboxylase, 0.2 mg; $Fe(NO_3)_3$, 12.5 mg; and $NaHCO_3$, 400 mg. Cultures were incubated at 36°C in a humidified atmosphere of 5 to 10% CO_2 in air and were subcultured daily to propogate the specific colonial morphology types as identified by Kellogg et al. (1963, 1968) and Swanson (1978). For experiments, type 4 (nonpiliated, protein II^-) gonococci were grown to mid-log phase in supplemented (as above) GC broth (GCB) containing (per liter): Proteose peptone no. 3, 15 g; K_2HPO_4, 4 g; KH_2PO_4, 1 g; NaCl, 5 g; and soluble starch, 1 g. Five milliliters of prewarmed medium was inoculated from a 14- to 18-h plate and vortexed with glass beads. The inoculum was transferred to a 125 ml

sidearm flask containing 15 ml of prewarmed, supplemented GCB. An initial turbidity reading (Klett-Summerson photoelectric colorimeter, green filter) of 10 to 12 U was obtained. The flask was shaken at 225 rpm in a 36°C shaker bath for 3 to 4 h, until the culture reached a Klett value of 80 to 110, representing mid-log phase. Concentrations of gonococci were determined by diluting the log-phase culture with supplemented GC broth to an optical density at 550 nm (Spectronic 20 colorimeter, Bausch and Lomb Inc., Rochester, N.Y.) of 0.30 ± 0.01. This cell density corresponds to 10^8 to 2×10^8 CFU/ml, as verified by plate count.

Preparation of GE

Neutrophils were obtained from healthy adult human volunteers by leukapheresis. Then, as described by Rest (1979) and Rest, Modrzakowski, and Spitznagel (1977, 1978), the cells were washed in phosphate-buffered saline (PBS) containing (per liter): NaCl, 8 g; KCl, 0.2 g; Na_2HPO_4, 1.15 g; and KH_2PO_4, 0.2 g; pH 7.4. After sufficient washing (150 × g, 10 min) to remove serum components, the cells were centrifuged through Ficoll-Hypaque; erythrocytes were lysed hypotonically. This yielded approximately 10^{10} neutrophils per donor with no more than 8% contaminating cell types (mostly eosinophils). The neutrophils were disrupted to 90% breakage by nitrogen cavitation (Parr Bomb, Parr Instrument Co., Moline, Ill.), as described by Borregaard et al.

(1983). The cellular debris was pelleted (250 × g, 15 min) and discarded. The entire granule population was pelleted (20,200 × g, 20 min) from the bomb supernatant and extracted by douncing (3 × 10 min, 4°C) in 0.2 M sodium acetate buffer (pH 4.0) containing 10 mM $CaCl_2$. Acetate extracts of granules were clarified by centrifugation (20,200 × g, 20 min) and dialyzed against PBS in tubing with an average molecular weight cutoff of 3500. Extracts from the granules of 10^{10} neutrophils yielded approximately 100 mg of protein, as determined by the method of Lowry et al. (1951), with egg white lysozyme as a standard.

The protein concentration required to reduce gonococcal viability by two logs (from 5×10^7 to 5×10^5 CFU/ml) after 60 min of exposure was determined for each batch of GE. This GE concentration (typically, 500 μg/ml) was used in all experiments.

Light Microscopy

Either GE or the same volume of PBS was added to tubes containing 5×10^7 CFU/ml at 36°C. At indicated times an 8 μl sample from each assay tube was spread over the grids of separate Petroff-Hausser counting chambers (Hausser Scientific, Blue Bell, Pa.). After placing the coverslip, each chamber was mounted on the microscope stage and left undisturbed for 5 min to allow the gonococci to settle onto the grid. Gonococci were enumerated under 1000X phase.

Results are presented as the total number of individual cocci (alone or in aggregates), and as the number of aggregates of cocci (which also includes isolated cocci). For each experiment, counts were the totals from four noncontiguous 0.2 × 0.2 mm squares. Duplicate samples were assayed for viability as above.

Oxygen Uptake

Oxygen uptake was determined using a YSI model 53 biological oxygen monitor with probe conversion for small sample volumes (Yellow Springs Instrument Co., Inc., Yellow Springs, Ohio). The water jacket surrounding the probe chambers was maintained at 37°C. Assay tubes, also maintained at 37°C, contained 10^8 CFU/ml to which GE or an equal volume of PBS were added. At the times indicated 1.5 ml of control or GE-treated gonococci were transferred to separate probe chambers and allowed to equilibrate with rapid stirring for approximately 3 min. Probes were inserted and the rate of decrease of oxygen saturation in the medium was measured. In addition, duplicate samples were removed, diluted in GC broth, and plated in duplicate to determine gonococcal viability. Data from individual experiments are expressed as oxygen uptake per 10^8 CFU at 0 min.

Transmission Electron Microscopy

Assay mixtures were exactly the same as for light microscopy (above). At the times indicated 4 ml of control

and treated gonococci (5×10^7 CFU/ml) were pelleted (3400 × g, 10 min, 4°C). Pellets were fixed overnight in 2.5% glutaraldehyde in 0.2 M NaH_2PO_4 and 1% $CaCl_2$ at 4°C. After three washes (12,800 × g, 2 min, 20°C) in 0.1 M Na_2HPO_4, pH 7.4, the pellets were postfixed for 30 min in 1% osmium tetroxide in 0.1 M Na_2HPO_4, pH 7.4, and washed twice more (as above). Specimens were dehydrated in a graded series of ethanols and embedded in Spurr embedding medium (Electron Microscopy Sciences, Fort Washington, Pa.). Sections (60 nm thick) were cut on an LKB 8800 Ultramicrotome III (LKB Instruments, Rockville, Md.) with a diamond knife. Sections were stained for 20 min in 5% uranyl acetate and 5 min in lead citrate (Reynolds 1963). Micrographs were taken on a Zeiss EM-9 electron microscope.

Materials.

GC medium base, Proteose peptone no. 3 and soluble starch were purchased from Difco Laboratories, Detroit, Mich. Ficoll and egg white lysozyme were from Sigma Chemical Co., St. Louis, Mo. Hypaque (50% sodium) was from Winthrop Laboratories, Menlo Park, Calif. Electron microscopy supplies were kindly donated by Sheila Katz of Hahnemann University.

Results

Light Microscopy and Cell Division-Morphologic Observations

Gonococcal viability, measured as the ability to form colonies on agar, begins to decline immediately upon exposure to GE (Rest 1979). Since formation of a visible colony is a process which requires many cell divisions, it was important to know if gonococci undergo even one round of division subsequent to exposure to GE. I observed control and GE-treated gonococci at 0 and 60 min by phase-contrast microscopy. Sixty minutes of GE treatment resulted in aggregation of gonococci (Fig. 6), and resulted in the apparent lysis of a large percentage of the cells. I took the loss of refractivity of the GE-treated gonococci to indicate either spheroplast formation or lysis. Since the nonrefractive cocci did not appear spherical, they were probably lysed. Cells observed immediately after addition of GE were indistinguishable from control cells (photograph not shown).

Light Microscopy and Cell Division-Quantitative Observations

The small increase seen, in the presence of GE, in the total number of cocci indicates that cell division did not stop instantly upon exposure to GE. However, the increase in number of treated cells (34%) was considerably less than the increase seen in controls (84%). The limited amount of cell division during the first one generation

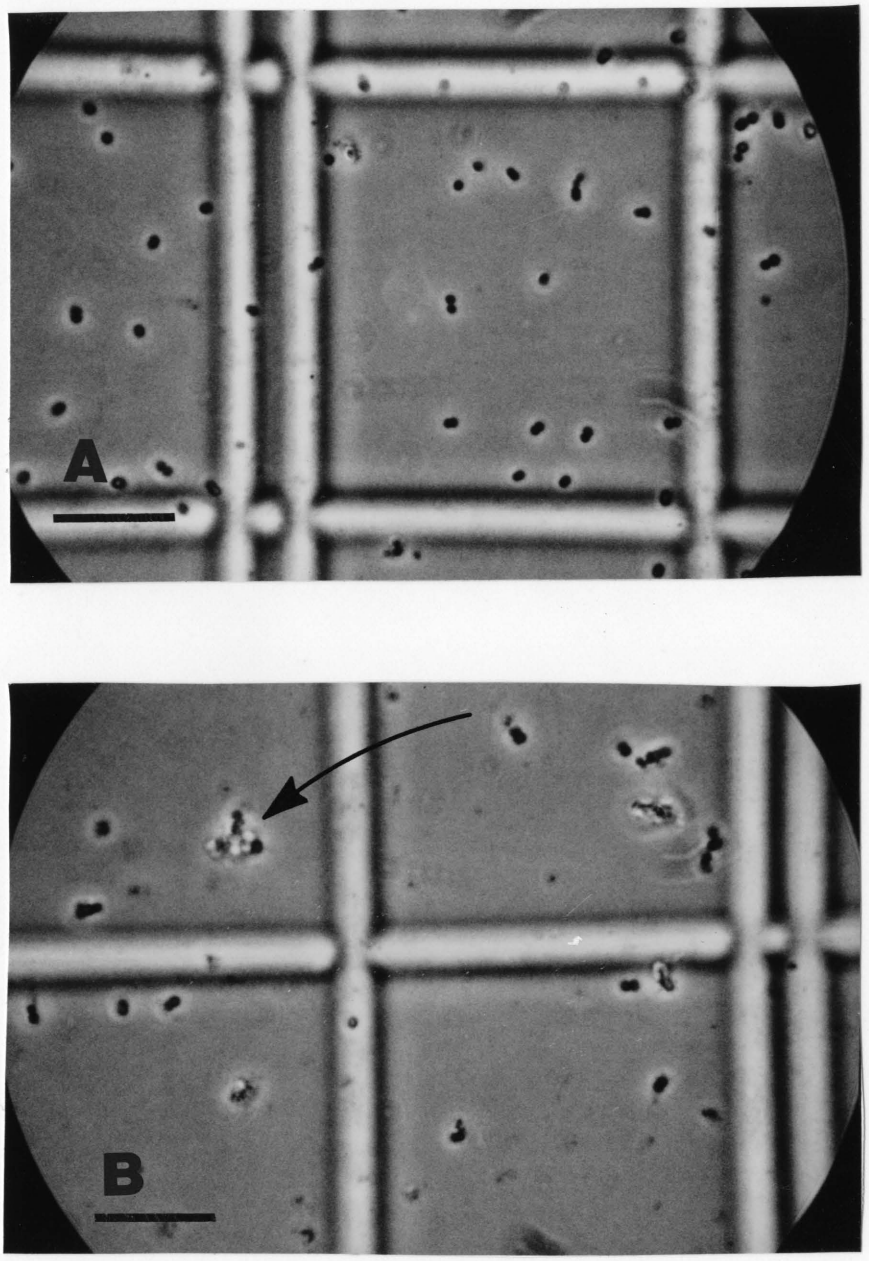

Fig. 6. Light Microscopy of Gonococci

Phase-contrast photomicrographs of Petroff-Hausser counting chambers with (A) PBS-control; and (B) GE-treated gonococci after 60 min of exposure. Note the clumping of the treated cells, and the loss of refractivity (arrow). Bar, 10 μm.

interval (i.e. 60 min) after exposure to GE would not be sufficient to cause the formation of a visible colony.

I was also interested in knowing whether GE-treatment affected the number of cocci per aggregate. An examination of the data in Table 1 confirms what is visually obvious; after 60 min of GE treatment the number of cocci per aggregate was 1.8 times the zero time value. During this same period, the 34% increase in the total number of countable cocci was concurrent with a 26% decrease in observed aggregates. A small amount of membrane-like debris was seen under phase after 60 min of GE treatment. I estimated, from the amount of debris present, that the number of cocci reduced to uncountable debris could account for a maximum error of no more than 15%. Untreated gonococci, on the other hand, maintained a steady number of cocci per aggregate, meaning that the increase in cocci (84%) paralleled the increase in aggregates (78%). When comparing actual numbers of aggregates per ml (visual) to CFU per ml (plate counts) (Table 2) it became apparent that even in untreated gonococci not all aggregates of cocci were capable of forming colonies. Consistently, about 60 to 70% of the observed aggregates formed colonies. Most of the aggregates observed after 60 min of GE treatment were reproductively dead even though they contained cells that microscopically appeared intact.

Table 1. Visual Quantitation of GE-treated Gonococci

Bacteria	Individual cocci*	Aggregates of cocci*	Cocci per aggregate
gonococci + PBS, 0 min,	540 ± 62	335 ± 53	1.61
gonococci + PBS, 60 min	992 ± 97	596 ± 70	1.66
gonococci + GE, 0 min	483 ± 37	294 ± 36	1.64
gonococci + GE, 60 min	648 ± 192	219 ± 119	2.96

* Mean ± standard deviation of three experiments

Table 2. Comparison of CFU by Plate Counts versus Aggregates by Visual Count

Bacteria	Plate count (10^7 CFU/ml)*	Visual count (10^7 aggreg./ml)*	Plate count / Visual count
gonococci + PBS, 0 min,	6.7 ± 0.7	10.0 ± 2.0	0.67
gonococci + PBS, 60 min,	11.0 ± 3.0 (164)%	19.0 ± 2.0	0.58
gonococci + GE, 0 min,	6.7 ± 1.1	9.2 ± 1.1	0.73
gonococci + GE, 60 min,	0.35 ± 0.08 (0.05)%	6.8 ± 3.7	0.05

* Mean ± standard deviation of three experiments

% Per cent viable at 60 min, compared to 0 min

Oxygen Uptake as a Measure of Cytoplasmic Membrane Integrity

After microscopically examining gonococcal division, I assessed cell integrity to determine whether an early breach of the membrane occurred. As a classic indicator of cell integrity, I chose to examine cytoplasmic membrane function for evidence of early effects of GE. Initial attempts to assess membrane integrity by measuring the release of $^{86}Rb^{+}$ (a potassium analog) (Silver and Bhattacharyya 1974) from gonococci failed because gonococci apparently would not transport $^{86}Rb^{+}$ in place of potassium (data not shown). The rate of oxygen uptake was chosen as an indirect measure of cytoplasmic membrane integrity since oxygen utilization is a function of the terminal oxidase of the membrane-associated electron transport chain (Winter and Morse 1975).

The rate of oxygen uptake by control cells increased linearly over time, as would be expected with a concurrent increase in cell number (Fig. 7). GE-treated gonococci, on the other hand, took up oxygen at a slower initial rate than untreated gonococci did, and did not exhibit any statistically significant change in the rate of uptake over the 90 min of the experiment. Comparison of oxygen uptake over time by analysis of variance between control and treated gonococci yielded a significant difference ($P < 0.001$). Multiple regression analysis of the variance data over time gave an even more significant difference in the oxygen

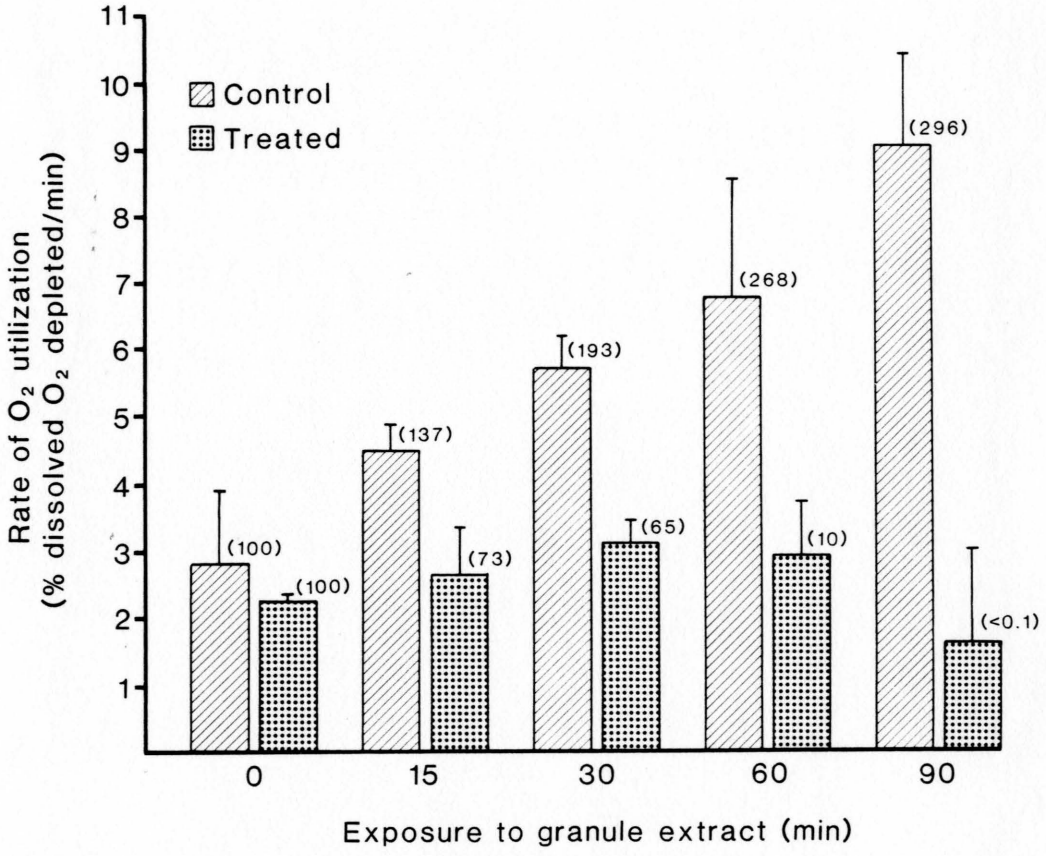

Fig. 7. Oxygen Uptake by Gonococci

Rate of oxygen uptake by control and GE-treated gonococci. Data from each experiment are expressed as the rate of oxygen uptake per 10^8 CFU present at 0 min. Bars represent the means of four experiments; error bars are one standard deviation above the mean. Numbers in parentheses represent gonococcal viability expressed as a percentage of time zero values; numbers are the average of four experiments.

uptake between control and treated gonococci ($P < 0.0001$). Heat-killed gonococci did not take up measurable amounts of oxygen. Cytoplasmic membrane integrity thus appeared to be affected by GE.

Membrane Ultrastructure Visualized by Electron Microscopy

I compared thin sections of control and GE-treated gonococci by transmission electron microscopy. Examination of low power fields (Fig. 8) revealed that 20 min of exposure to GE was sufficient to cause significant aberrations in outer membrane morphology when compared to control cells. Membrane-like fragments were frequently found in fields of GE-treated cells (Fig. 8B, lower left), suggestive of either outer membrane released by blebbing or membrane debris from complete lysis of some gonococci.

The degree of membrane damage observed was related to the time of contact with GE. In treated cells fixed immediately after addition of GE (Fig. 9A), septa appeared normal while outer membranes had a slightly ragged look. As little as 10 min of GE treatment (Fig. 9B) was sufficient to cause significant aberrations in the outer membranes, and septa appeared more electron-lucent although basically intact. An additional 10 min in GE (Fig. 9C) revealed progressively more membrane damage. Thirty minutes of exposure to GE resulted in stripping away of the complete outer

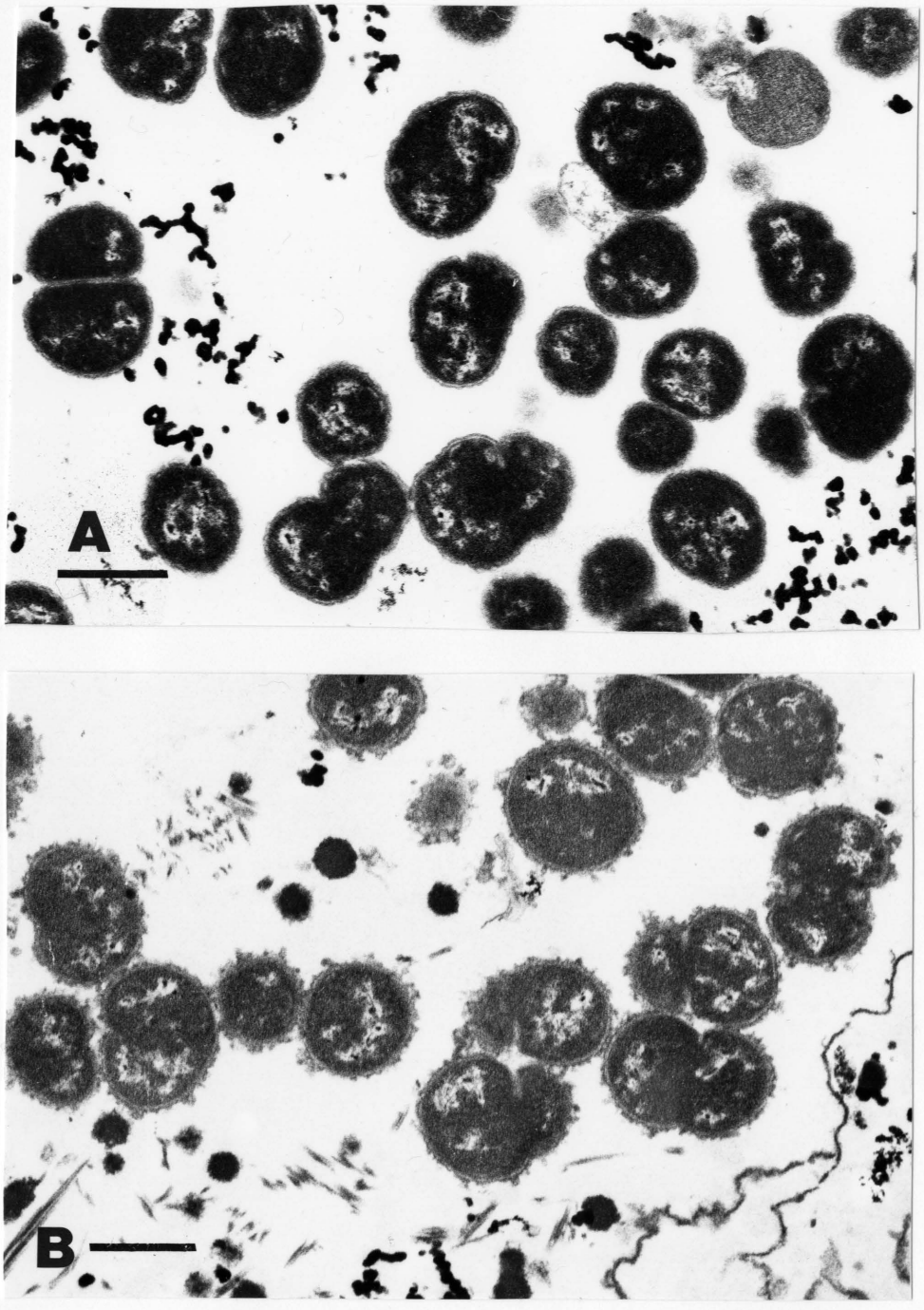

Fig. 8. Electron Microscopy of Gonococcal Populations

Low power fields of (A) PBS-control; and (B) GE-treated gonococci after 20 min of exposure. Note the prevalence and uniformity of membrane damage to treated cells. Bar, 1 μm.

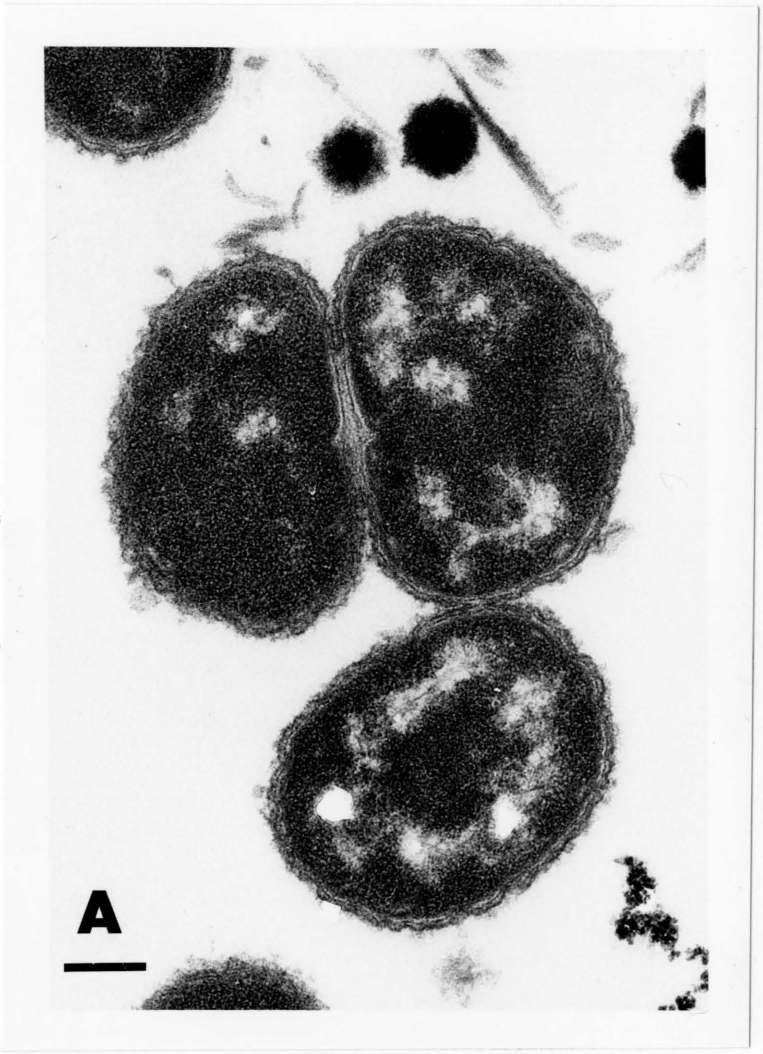

Fig. 9A. Time Sequence of GE Damage to Gonococcal Membranes-Initial

Electron micrograph exhibits immediate effects of GE on gonococci. Bar, 0.25 μm.

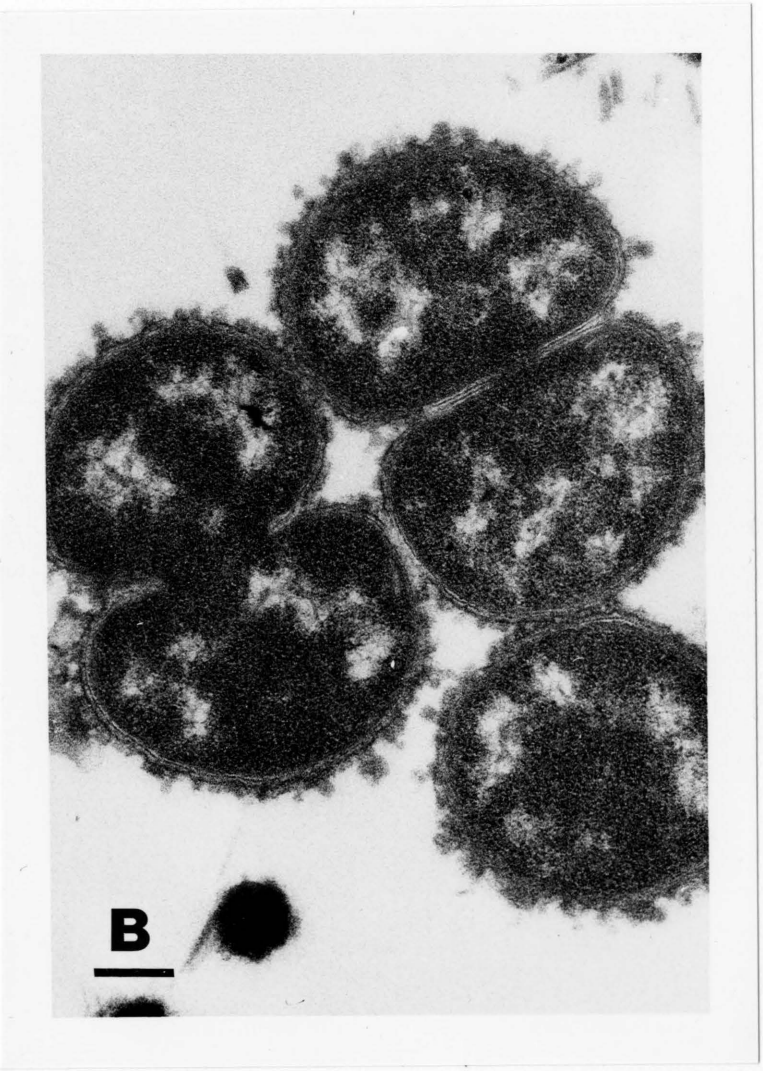

Fig. 9B. Time Sequence of GE Damage to Gonococcal Membranes-10 min

Membrane damage occurring after 10 min of exposure to GE. Bar, 0.25 μm.

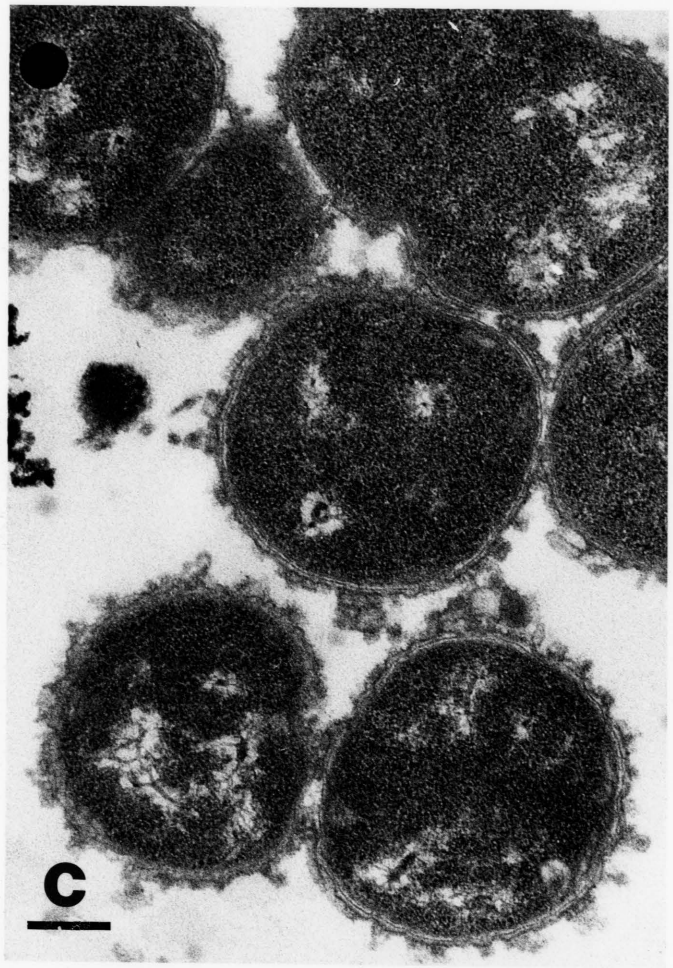

Fig. 9C. Time Sequence of GE Damage to Gonococcal Membranes-20 min

Twenty minutes of exposure to GE causes increasing membrane damage. Bar, 0.25 μm.

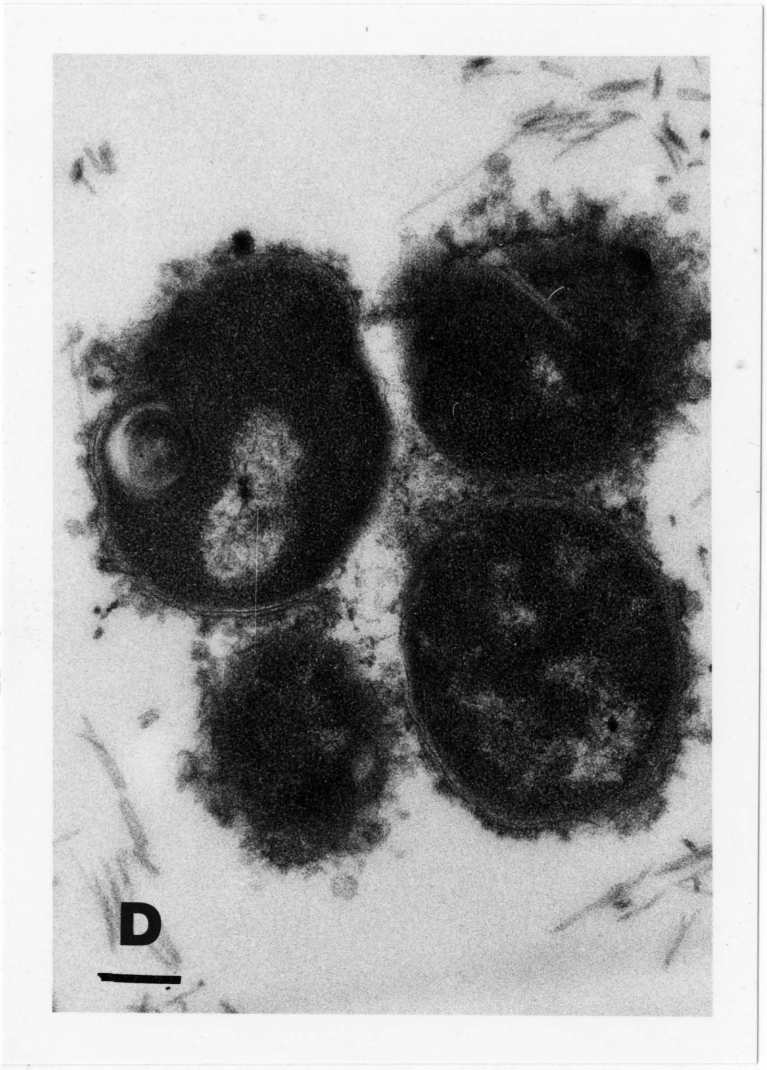

Fig. 9D. Time Sequence of GE Damage to Gonococcal Membranes-30 min

After 30 min of exposure to GE, gonococcal outer membranes are extensively disorganized and partially missing. Bar, 0.25 μm.

membrane structure from large segments of the periphery of cocci (Fig. 9D).

High magnification micrographs of control cells and cells treated with GE for 20 min gave a clearer indication of the effects of GE on membrane structure (Fig. 10). In control gonococci, the peptidoglycan line is clearly visible in the forming septum. Outer membrane material can be seen in the septal invagination in close proximity to the peptidoglycan. The partially formed septa of GE-treated gonococci showed distinct signs of damage. The outer membrane material was seen being peeled away from the invagination, and the peptidoglycan line was less well defined in the area of the forming septum. Differences were also noted between control and GE-treated gonococci in areas of membrane distant from the septum. In GE-treated gonococci, the peptidoglycan line was more linear and much more easily observed than in control cells, where the line was an indistinct part of the whole membrane structure.

It is not apparent at present whether the alteration in peptidoglycan at sites distant from the septum has a cause and effect relationship with the loss of viability.

Discussion

Although much is known about the interactions between gonococci and human neutrophils, there has been a

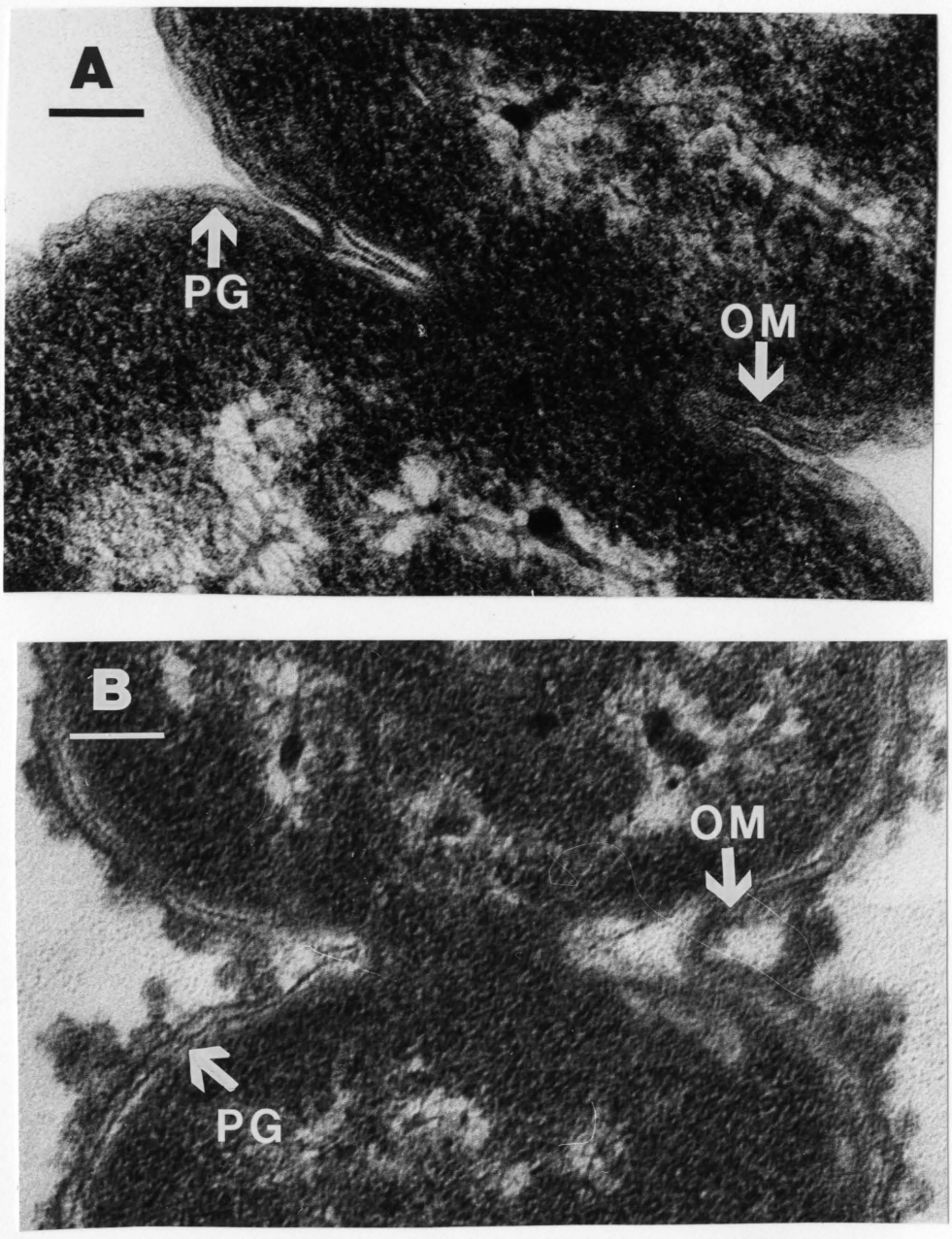

Fig. 10. High Power Electron Micrographs Exhibiting Membrane Damage

Note the structure of forming septa and peptidoglycan in (A) PBS-controls; and (B) GE-treated gonococci after 20 min of exposure. Also, note the changes between untreated and treated gonococci in the definition of the nonseptal peptidoglycan line (PG) and in the proximity of outer membrane (OM) to the forming septum. Bar, 0.1 μm.

dearth of information about the molecular events leading to death once gonococci have been phagocytized.

It is interesting to compare the effects of the penicillins to those of GE on bacterial cells. Neirinck and DeVoe (1981), and Neirinck, DeVoe, and Ingram (1980) examined Neisseria meningitidis treated with subminimal inhibitory concentrations of penicillin G. Penicillin-treated meningococci rapidly lose the ability to form colonies, although cell numbers (assessed from direct counts) do increase slightly. I have found the same results with GE-treated gonococci. Cell volume of meningococci, observed under phase-contrast, and cell mass, measured as dry weight, increase directly with controls for at least the first 90 min of penicillin treatment. I have not seen a notable increase in cell volume with GE-treated gonococci; measurement of cell mass is not feasible at present because the amount of GE required to carry out such experiments would be prohibitive.

Loss by gonococci of the ability to form colonies on agar is more complicated than initially presumed. The GE-induced cell clumping that we observed can certainly, by itself, cause a drop in the number of CFU per ml. The fact that not all aggregates of cells formed colonies complicates the relationship between cell division and increase in CFU. The slight increase in numbers of total cocci counted after 60 min of extract treatment was not significant in terms of

colony formation since the number of cocci increased only 34% whereas viability decreased 95%. It did suggest, however, that cell division is not immediately stopped by exposure to GE.

I feel, because of its simplicity, that using the ability of gonococci to form colonies on agar is still an adequate measure of viability even when the above drawbacks are taken into account. The above noted uncertainties of colony counts are offset by the fact that colony counts are extremely reproducible, which suggests that they represent an accurate measure of the initial effects of GE.

In other studies, I have shown that gonococci exposed to neutrophil GE are rapidly affected (Rock 1985). Peptidoglycan synthesis is quantitatively subnormal in cells immediately after GE treatment, while degradation of peptidoglycan in treated cells is increased to twice control levels. Since peptidoglycan is the major structural component of the bacterial membrane, any alteration in its composition could have direct consequences on outer membrane morphology, septum formation, and cell integrity.

A chymotrypsin-like cationic protein isolated from human neutrophils by Odeberg and Olsson (1976a) inhibits oxygen uptake by Staphylococcus aureus in a dose-dependent manner. On the other hand, penicillin treated-meningococci continue to utilize oxygen from the culture medium at control levels for at least the first 60 min of exposure

(Neirinck, DeVoe, and Ingram 1980). I report here the GE-induced inhibition of oxygen uptake by gonococci. The possible mechanisms of inhibition include (i) direct attack on and degradation of the terminal oxidase by GE, (ii) attack on other components of the electron transport chain upstream from the terminal oxidase, causing a block in electron flow, or (iii) disruption of the cytoplasmic membrane, causing steric hindrance of electron flow.

Weiss et al. (1976) have purified a lysosomal component from rabbit neutrophils that kills E. coli. Upon close examination, thin sections comparing controls with cells exposed to the lysosomal component for 15 min demonstrates (my interpretation) the same manner of outer membrane disruption that we report here. This suggests that the component, later characterized as BPI, may be the compound present in human neutrophil GE causing the membrane damage observed with gonococci. However, van Houte et al. (1977) reported no recognizable ultrastructural changes, observed by freeze fracture electron microscopy, in the outer membranes of another strain of E. coli exposed to rabbit BPI. Differences between the outer membranes of these two E. coli strains could account for this incongruity, as could the different procedures used to prepare specimens for thin section and freeze fracture. Similar information is not available for human BPI.

Rozenberg-Arska et al. (1985) examined the fate of E. coli that have been phagocytized by human neutrophils. The internalized bacteria were killed very rapidly; major structural damage was evident by 15 min after phagocytosis unless the E. coli were encapsulated. Although a capsule has been demonstrated on gonococci grown on special medium in vitro (Hendley et al. 1981), in vivo capsule production would have to be established before any protective effect could be considered. Damage to non-encapsulated, internalized E. coli included agglomeration of cytoplasm, an effect I did not see in GE-treated gonococci. Novotny, Short, and Walker (1975) have shown from thin sections of pus that gonococci inside phagosomes have a notably smooth surface. They also observed, by scanning electron microscopy of negatively stained preparations, that gonococci grown in culture (synonymous with control cells in this study) possess a rough surface. Therefore, the smoothing out of the membrane structure subsequent to exposure to GE has in vivo relevance.

Penicillin has a different effect on meningococcal outer membranes than GE had on gonococcal membranes. Penicillin treatment causes a peeling of the outer membrane, apparently intact, from the underlying layers of the meningococcal cell. Similar results were observed in benzylpenicillin-treated gonococci (Goodell, Fazio, and Tomasz 1978). I show here that GE appears to progressively

disrupt outer membrane structure rather than detach it intact. This results in a distortion of the morphology of the membrane still attached to the cell. Penicillin does cause abnormal orientation during septum formation, similar to what I have seen in GE-treated gonococci (Rock 1985). Another result of penicillin treatment of meningococci and gonococci not seen with GE-treatment is a great thickening of septa, indicating that penicillin and GE do express different mechanisms of damage. Growth of nonseptal cell walls of gonococci is not affected by the presence of penicillin (Lorian and Atkinson 1976). Wilkinson and Nadakavukaren (1983) examined cell wall growth in methicillin-resistant S. aureus. They found that exposure to methicillin causes forming septa to become distorted and thickened. However, they also found inhibition of nonseptal cell wall synthesis to occur. This difference in the effect of similar antibiotics on cell wall synthesis is not unexpected, since gram-negative and gram-positive cells have structurally distinct walls.

In conclusion, I have found several differences in the structural degradation of gonococci caused by unfractionated GE compared to degradation of other gram-negative bacteria by purified GE components. Penicillin and its various analogs mimic some although not all of the effects of GE, which suggests that these antibiotics may be useful probes into further examination of the effects seen here.

CHAPTER 4

QUALITATIVE ASPECTS OF THE EFFECTS OF HUMAN NEUTROPHIL GRANULE EXTRACTS ON GONOCOCCAL PEPTIDOGLYCAN AND PENICILLIN-BINDING PROTEINS

At first I identified GE-induced perturbations of gonococcal peptidoglycan synthesis as candidates for the mechanism of loss of the ability to divide, and showed visual observations of gonococcal membrane damage caused by GE (Chapter 2). Follow-up research examined the effects of GE on the cytoplasmic membrane, and the aggregation of cells and cell division. I also took a more in-depth look at membrane damage by electron microscopy (Chapter 3). In this fourth chapter, I take a qualitative look at how GE affects gonococcal penicillin-binding proteins, as well as both released peptidoglycan and that which remains in the gonococcal membrane. This is the third manuscript to come out of my research, and is being submitted for publication concurrently with the completion of the dissertation.

Abstract

In a continuing effort to determine why gonococci die when exposed to human neutrophil granule extracts (GE), I examined the peptidoglycan structure and penicillin-binding proteins (PBPs) of GE-treated gonococci. PBPs were visualized by treating whole gonococci with saturating

concentrations of tritiated penicillin, subjecting the solubilized, labeled cells to sodium-dodecyl sulfate-polyacrylamide gel electrophoresis, and locating the radio-labeled bands by fluorography of the gel. GE-treatment of gonococci decreased the amount of penicillin that the PBPs bound, but did not change the apparent molecular weight of the PBPs visualized. Gonococcal peptidoglycan, both cell-associated and culture supernatant-associated, was examined by a combination of thin-layer chromatography and molecular sieve, high-pressure liquid chromatography to check for any GE-induced differences. Supernatant-associated peptido-glycan from GE-treated cells was identical to that from control cells when compared by thin-layer chromatography; however, small amounts of high molecular weight fragments were observed by high-pressure liquid chromatography of control supernatants while none were seen in supernatants from treated cells. The converse was seen with cell-asso-ciated peptidoglycan; differences were noticed between controls and GE-treated cells by thin-layer chromatography but no differences were seen by high-pressure liquid chroma-tography.

Introduction

Gonococci are killed when internalized by human neutrophils. They are also killed, very efficiently, when exposed to unfractionated granule extracts of neutrophils.

Several groups are currently involved in attempting to determine which component(s) of the neutrophil arsenal are responsible for the lethal effect. My work has attempted to resolve why GE-treated gonococci die, rather than what is killing them.

I have previously examined the GE-mediated killing of gonococci from the bacterial point of view. Earlier experiments by Buck and Rest (1981) indicate that, although GE treatment causes gonococci to stop dividing almost immediately, they are still metabolically alive. Specifically, cytoplasm-based macromolecular synthesis continues normally for approximately 30 min after GE is added to the cells, even though these cells are reproductively dead.

My initial experiments determined that gonococcal peptidoglycan synthesis is one of the first gonococcal systems to be affected by GE (Rock 1985). Those experiments examined gonococcal peptidoglycan synthesis and turnover quantitatively; in addition, I observed membrane damage. The results of both of these observations suggested that closer inspection of the gonococcal membrane in general and the peptidoglycan sacculus in particular would give additional insight into the "lethal lesion" caused by GE.

Gonococcal peptidoglycan has been extensively studied; its general structure, including degrees of O-acetylation and cross-linking have been determined (Blundell and Perkins 1985; Dougherty 1983a, 1985a, 1985b; Hebeler and

Young 1976a; Lear and Perkins 1983; Rosenthal, Wright, and Sinha 1980), as has peptidoglycan turnover (Greenway and Perkins 1985, Rosenthal 1979, Sinha and Rosenthal 1980). O-acetylation of the carbohydrates comprising the glycan backbone confers lysozyme resistance on gonococcal peptidoglycan (Blundell, Smith, and Perkins 1980; Rosenthal, Blundell, and Perkins 1982; Swim et al. 1983); more specifically, O-acetylated peptidoglycan is highly resistant to human neutrophil lysozyme (Rosenthal et al. 1983). Gonococcal PBPs have been identified (Barbour 1981; Dougherty, Koller, and Tomasz 1980; Dougherty 1985b), and some of the enzyme activities normally associated with PBPs were described (Chapman and Perkins 1983; Gubish, Chen, and Buchanan 1982; Rosenthal 1979; Sinha and Rosenthal 1980).

With all of this background information at hand, I report here on the effects of GE-treatment on the structure of gonococcal peptidoglycan released into the culture supernatant as well as the peptidoglycan still associated with the cell. I also looked at the state of gonococcal PBPs subsequent to exposure to GE.

Materials and Methods

Bacteria

Neisseria gonorrhoeae strain F62 was maintained on GC medium base supplemented with (per liter): dextrose, 4 g; glutamine, 100 mg; cocarboxylase, 0.2 mg; $Fe(NO_3)_3$, 12.5 mg;

and $NaHCO_3$, 400 mg. Cultures were incubated at 36°C in a humidified atmosphere of 5 to 10% CO_2 in air and were subcultured daily to propogate the specific colonial morphology types as identified by Kellogg et al. (1963, 1968) and Swanson (1978). For experiments, type 4 (nonpiliated, protein II$^-$) gonococci were grown to mid-log phase in supplemented (as above) GC broth containing (per liter): Proteose peptone no. 3, 15 g; K_2HPO_4, 4 g; KH_2PO_4, 1 g; NaCl, 5 g; and soluble starch, 1 g. Five milliliters of prewarmed medium were inoculated from a 14- to 18-h plate and vortexed with glass beads. The inoculum was transferred to a 125 ml sidearm flask containing 15 ml of prewarmed, supplemented GC broth. An initial turbidity reading (Klett-Summerson photoelectric colorimeter, green filter) of 10 to 12 U was obtained. The flask was shaken at 225 rpm in a 36°C water bath for 3 to 4 h, until the culture reached a Klett value of 80 to 110, representing mid-log phase. Concentrations of gonococci were adjusted by diluting the log-phase culture with supplemented GC broth to an optical density at 550 nm (Spectronic 20 colorimeter, Bausch and Lomb Inc., Rochester, N.Y.) of 0.30 ± 0.01. This cell density corresponds to 10^8 to 2×10^8 CFU/ml, as verified by plate count.

Preparation of GE

Neutrophils were obtained from healthy adult human volunteers by leukapheresis. Then, as described by Rest

(1979) and Rest, Modrzakowski, and Spitznagel (1977, 1978), the cells were washed in phosphate-buffered saline (PBS) containing (per liter): NaCl, 8 g; KCl, 0.2 g; Na_2HPO_4, 1.15 g; and KH_2PO_4, 0.2 g; pH 7.4. After sufficient washing (150 × g, 10 min, 20°C) to remove serum components, the cells were centrifuged through 6% Ficoll–10% Hypaque (200 × g, 30 min, 20°C); erythrocytes were lysed hypotonically. This yielded approximately 10^{10} neutrophils per donor with no more than 8% contaminating cell types (mostly eosinophils). The neutrophils were disrupted to 90% breakage by nitrogen cavitation (Parr Bomb, Parr Instrument Co., Moline, Ill.), as described by Borregaard et al. (1983). The cellular debris was pelleted (250 × g, 15 min, 4°C) and discarded. The entire granule population was pelleted (20,200 × g, 20 min, 4°C) from the bomb supernatant and extracted by douncing (3 × 10 min, 4°C) in 0.2 M sodium acetate buffer (pH 4.0) containing 10 mM $CaCl_2$. Acetate extracts of granules were clarified by centrifugation (20,200 × g, 20 min, 4°C) and dialyzed against PBS in tubing with an average molecular weight cutoff of 3500. Extracts from the granules of 10^{10} neutrophils yielded approximately 100 mg of protein, as determined by the method of Lowry et al. (1951), with egg white lysozyme as a standard.

The protein concentration required to reduce gonococcal viability by two logs (from 5×10^7 to 5×10^5 CFU/ml) after 60 min of exposure was determined for each batch of

GE. This GE concentration (typically, 500 μg/ml) was used in all experiments.

Assay for Penicillin-binding Proteins

Gonococcal PBPs were examined by the method of Dougherty (1983b). Briefly, gonococci in supplemented GC broth were exposed to GE or an equal volume of PBS for 20 min. A 4 ml sample (5×10^7 CFU/ml) of each was pelleted (1650 × g, 10 min, 20°C). Three milliliters of supernatant were removed, and the gonococci were resuspended in the remaining 1 ml. The samples were immediately exposed to 2 μg of [^{3}H]benzylpenicillin (25 Ci/mmol, generously supplied by T.J. Dougherty, The Rockefeller University, New York, N.Y.) for 15 min at 36°C. The gonococci were again pelleted (12,800 × g, 1 min, 20°C) and rapidly cooled to 4°C. Two additional PBS-control samples were treated with 2 μg of either [^{3}H]benzylpenicillin or unlabeled benzylpenicillin, as above, pelleted (12,800 × g, 1 min, 20°C), and resuspended in fresh, supplemented GC broth which contained 2 μg of unlabeled benzylpenicillin or [^{3}H]benzylpenicillin, respectively. After an additional 15 min incubation at 36°C, these control gonococci were also pelleted as above and then cooled to 4°C. Each pellet was resuspended in 50 μl of 20 mM phosphate buffer, pH 7.2, to which was added an equal volume of 2X treatment buffer (125 mM Tris, 4% sodium-dodecyl sulfate, 20% glycerol, 10% 2-mercaptoethanol, and 0.01%

bromphenol blue, pH 6.8). These mixtures were boiled for 5 min and then loaded on polyacrylamide gels.

Sodium-dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

I essentially used the system of Laemmli (1970) with a 5% stacking gel, 10% separating gel; ratio of acrylamide to bisacrylamide of 30:0.8. The gels were stained in Coomassie blue and destained in several changes of 22.5% isopropanol-10% acetic acid. Gels were impregnated for 90 min with Autofluor (National Diagnostics, Somerville, N.J.) containing 0.5% glycerol, and dried with a model 443 Slab Dryer (Bio-Rad Laboratories, Rockville Centre, N.Y.) at 80°C.

The dried gels were exposed to X-Omat XAR-5 x-ray film (Eastman Kodak Company, Rochester, N.Y.) for 21 days at -70°C. Developed x-ray film was scanned on an LKB 2202 Ultrascan laser densitometer (LKB Instruments, Inc., Gaithersburg, Md.) interfaced to an Apple IIe computer running the LKB Gel Scan Program software (generously offered for our use by B. Rosan of the University of Pennsylvania, Philadelphia, Pa.).

Gonococcal Peptidoglycan

A flask containing 200 ml of supplemented GC broth was inoculated with gonococci from an overnight plate and incubated at 36°C with shaking until a Klett reading of >= 150 was reached. Following growth, the gonococci were pelleted (1500 × g, 20 min, 4°C) and resuspended in 6 ml of

50 mM sodium acetate, pH 5.0. These cells were kept on ice, to be used as "carrier" for cell-associated peptidoglycan purification. Another culture of gonococci, grown in GC broth supplemented with pyruvate instead of glucose, was labeled with 6-[^{3}H]glucosamine (3 μCi/ml; specific activity, 40 Ci/mmol) for the last hour of growth to mid-log phase. When the labeled culture reached mid-log phase, it was centrifuged (4000 × g, 10 min, 20°C) and the pellet resuspended in fresh, supplemented GC broth without radiolabel. After a 30 min incubation at 36°C this culture was centrifuged (as above) and again resuspended in fresh, supplemented GC broth. These gonococci were exposed to PBS or GE for 20 and 45 min. After treatment 6 ml samples of gonococci were centrifuged (12,800 × g, 1 min, 20°C) and the supernatants immediately transferred to separate tubes.

Supernatant-associated Radiolabel. Assay supernatants were concentrated by lyophilization, resuspended in 1 ml of double-deionized water, and desalted by passage over a 13 × 650 mm Sephadex G-10 column, with double-deionized water as the mobile phase (Pharmacia, Inc., Piscataway, N.J.). Following desalting, column fractions containing radiolabel were pooled and again lyophilized.

Pellet-associated Radiolabel. Assay pellets were resuspended in 0.5 ml of 50 mM sodium acetate, pH 5.0. Each suspension was mixed with 1.5 ml of "carrier" cells, and the mixture was added, dropwise, to 2 ml of boiling,

10% sodium-dodecyl sulfate in 50 mM sodium acetate, pH 5.0. After 20 min at 100°C, the tubes were left at room temperature overnight. Insoluble material was pelleted (80,000 × g, 30 min, 20°C), washed 3 times with double-deionized water, and resuspended in 1 ml of 50 mM Tris containing 10 mM NaCl, pH 7.2. One hundred micrograms of Proteinase K (Sigma Chemical Co., St. Louis, Mo.) were added, and the mixture was incubated overnight at 37°C. Insoluble material was pelleted and washed as above and resuspended in 200 µl of 25 mM NaH_2PO_4 containing 0.1 mM $MgCl_2$, pH 6.5. Fifty units of Mutanolysin (Sigma Chemical Co., St. Louis, Mo.) was added to the resuspended pellet, along with NaN_3 (to a final concentration of 0.05%). This mixture was also incubated at 37°C overnight, followed by lyophilization.

High-Pressure Liquid Chromatography

A Bio-Rad isocratic HPLC system was used consisting of a model 1330 pump, Bio-Sil TSK 125 size exclusion column (300 × 7.5 mm) with guard column, and model 1305A UV detector (Bio-Rad, Rockville Centre, N.Y.). The mobile phase was 100 mM KH_2PO_4, with a pump rate of 0.5 ml/min. One-hundred-microliter samples were injected.

Thin-Layer Chromatography

Whatman LK 6D Linear K silica gel plates (Whatman, Clifton, N.J.) were used; plates were activated at 100°C for 20 min immediately before use. Fifty-microliter samples

were spotted on the preadsorbant zones of the plate and dried with an infrared lamp. Plates were developed twice, approximately 7 h each time, with a mobile phase consisting of a 5:3 ratio of isobutyric acid-1 M ammonia, sprayed with EN^{3}HANCE (New England Nuclear Research Products, Boston, Mass.) and subjected to fluorography for 14 d at -70°C.

Results

Previous examination of GE-treated gonococci has shown that peptidoglycan synthesis is decreased compared to controls, while peptidoglycan turnover by treated gonococci is higher than in control gonococci (Rock 1985). A natural continuation of these findings is to determine whether GE has any affect on the PBPs of gonococci.

Effect of GE on gonococcal PBPs

Examination of gonococcal PBPs revealed the same 3 bands, visualized by fluorography of gels (Fig. 11), as were reported by Dougherty, Koller, and Tomasz (1980). Binding of radiolabeled penicillin to these three proteins was specific and irreversible. Labeled penicillin, once bound, could not be competed away by unlabeled penicillin (Control-A lane); the converse was also true (Control-B lane). It is immediately noticeable that the PBPs of GE-treated gonococci do not change in size, although they do bind less penicillin overall than the PBPs of control gonococci.

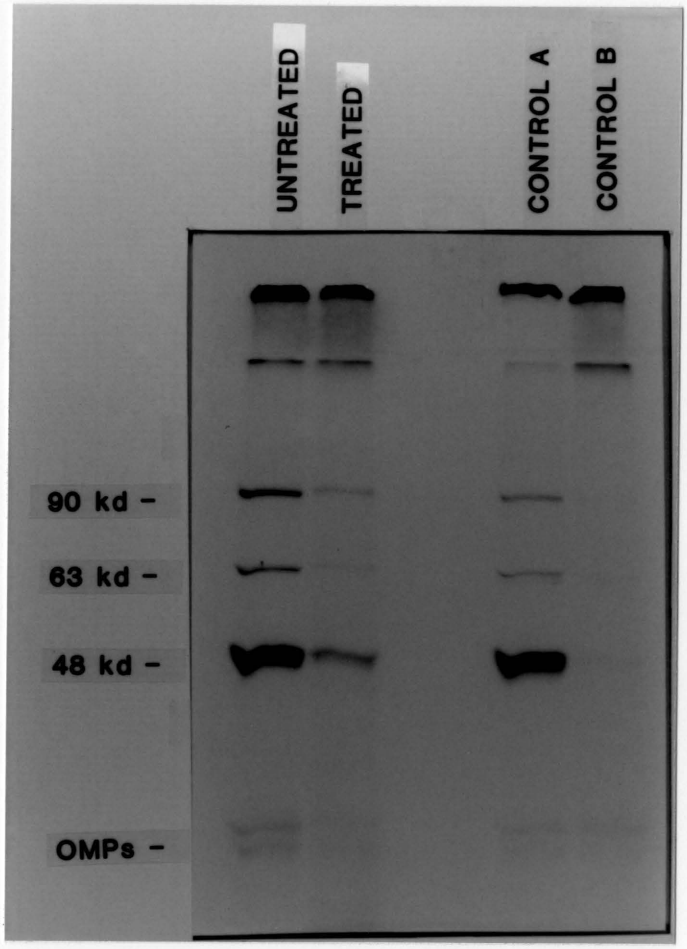

Fig. 11. Gonococcal PBPs

PBPs of gonococci were visualized as indicated in Materials and Methods. Note that GE-treatment does not cause any apparent change in the size of the three PBPs. However, the 3 bands in the treated lane are substantially fainter than the corresponding bands in the control lane. The third lane from the left (Control A) shows PBPs from gonococci that were exposed to tritiated penicillin followed by unlabeled penicillin. In the fourth lane, the gonococci were given the converse treatment. Also note the slight amount of penicillin sticking nonspecifically to the outer membrane proteins (OMP) at the bottom of the fluorograph.

Figure 12, a scan of the fluorograph in Fig. 11, confirms these results. A quantitative measure of the binding of penicillin to the PBPs can be obtained from Table 3, which was generated from the scan in Fig. 12 by the Gel Scan Program. The total intensity of the 3 bands of control gonococci is more than fourfold that of treated cells. There is a small, possibly artifactual, shift between untreated and treated gonococci in the relative amounts of penicillin bound to the individual PBPs. Degradation of the PBPs is not apparent, since there would have to be an associated change in the size of the molecule.

Effect of GE on Supernatant-associated Peptidoglycan

Gonococci normally shed peptidoglycan fragments during logarithmic growth. I have previously demonstrated that GE-treatment of gonococci causes a higher rate of release of prelabeled peptidoglycan than was seen in control cells (Rock 1985). As a follow-up to quantitative measurements of radiolabel release, I examined the culture supernatant for qualitative differences in the products released by treated and control gonococci.

Culture supernatants were examined by high-pressure liquid chromatography to ascertain whether GE caused any alterations in the size of the label released into the supernatant. Line recordings generated by the UV monitor were not useable because other components from the culture

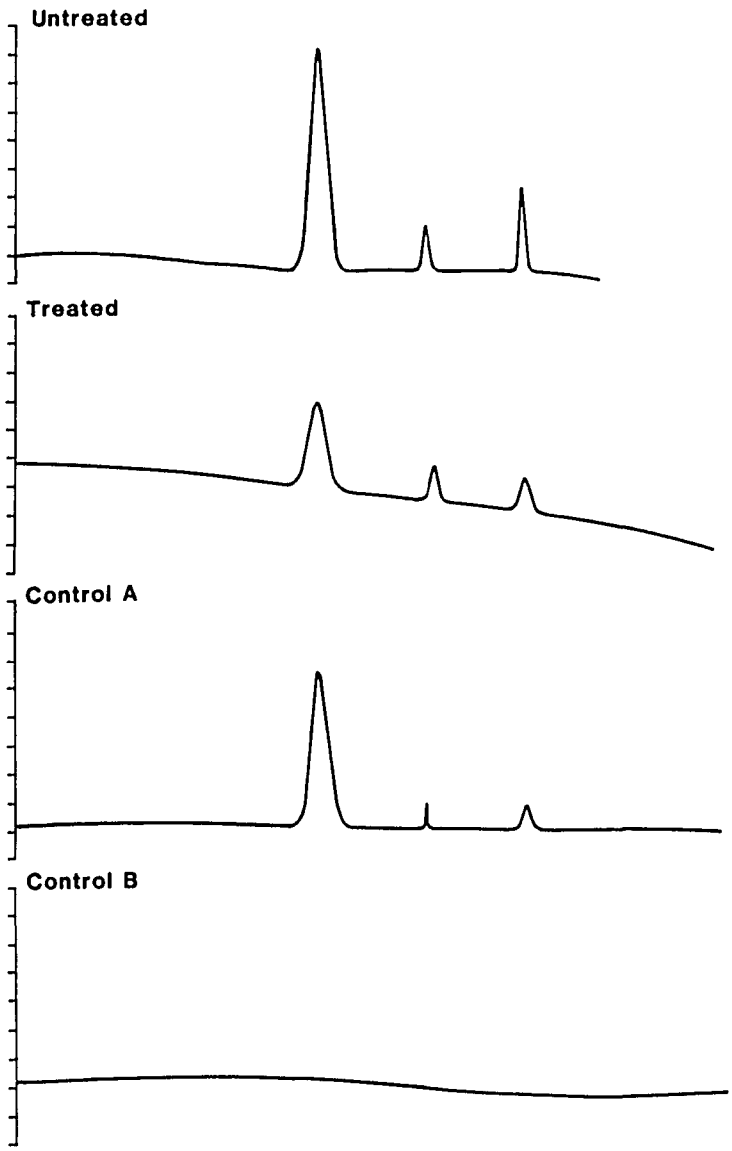

Fig. 12. Densitometer Scan of PBP Fluorograph

The decrease in penicillin binding to each of the PBPs of GE-treated gonococci can again be seen when comparing lanes of treated to untreated cells. The scan also reiterates that there is no shift between control and treated cells in the mobility of any of the 3 PBP bands.

Table 3. Distribution of Radiolabel Among Gonococcal PBPs

	Control		Treated	
MW of band	Intensity of band	% of total intensity	Intensity of band	% of total intensity
90 kd	64.2	12.3	19.4	15.7
63 kd	36.5	7.0	16.7	13.5
48 kd	422.9	80.8	87.2	70.7
Totals	523.6	100.1	123.3	99.9

medium had a much higher absorbence at the 205 nm wavelength than any peptidoglycan fragments present. However, 85% of the radiolabel introduced as [^{3}H]glucosamine is utilized by gonococci for peptidoglycan synthesis, and the other 15%, utilized for lipopolysaccharide synthesis, was removed during peptidoglycan purification. As such, line graphs of the radioactivity associated with each fraction collected from the HPLC column served to indicate the size of the peptidoglycan fragments present (Fig. 13).

Practically all of the radiolabel detected in culture supernatants eluted in a single peak (Fig. 13). This peak matched the elution pattern exhibited by purified peptidoglycan "monomers", which are disaccharide-tetrapeptide molecules (data not shown). There were no observable differences noted between the supernatants of control and treated gonococci collected after 20 min or 45 min of GE exposure. However, supernatants from control gonococci released small amounts of high molecular weight fragments (left side of the graph) after both 20 and 45 min of GE treatment; no high molecular weight radiolabel was observed in supernatants from GE-treated gonococci.

Examination of these same supernatants by thin-layer chromatography did not reveal any striking differences between control and treated gonococci (Fig. 14). A major band was observed in each lane. The identity of this band is uncertain; the mobile phase migrated more slowly in these

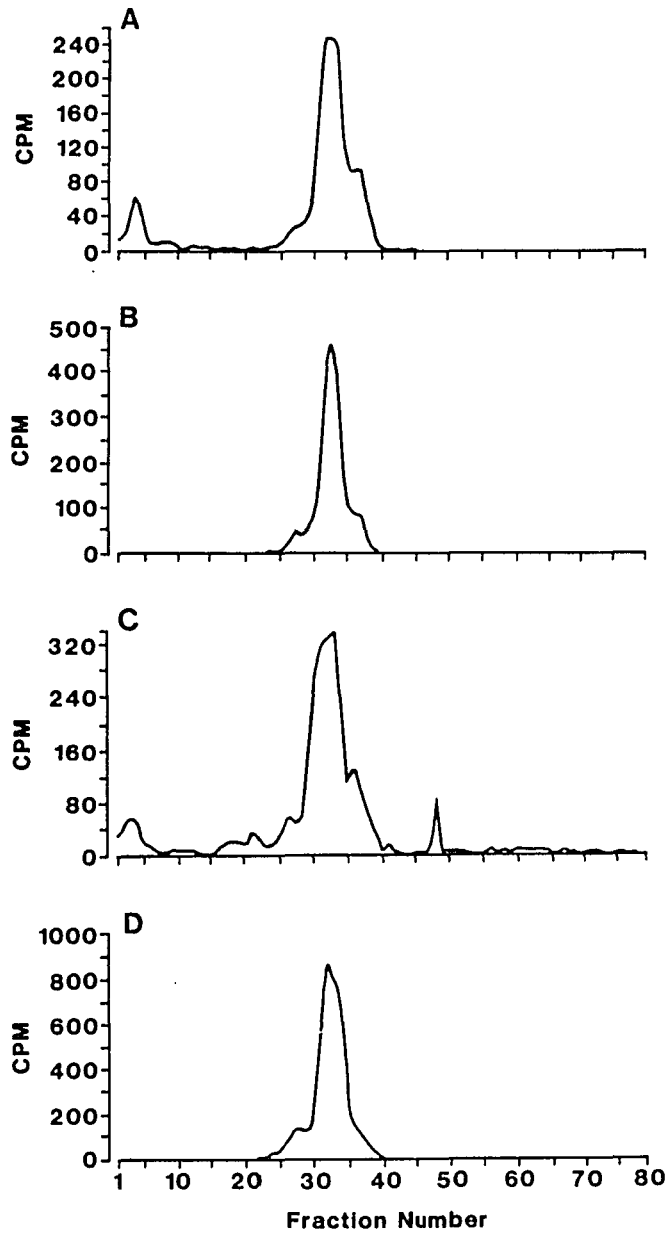

Fig. 13. HPLC-separation of Supernatant-associated Radiolabel

Desalted, lyophilized culture supernatants were analyzed by HPLC. Graphs are plots of the radiolabel associated with each fraction after elution from the HPLC column. Panels correspond to supernatant-associated radiolabel detected after: (A), 20 min exposure to PBS; (B), 20 min exposure to GE; (C), 45 min exposure to PBS; (D), 45 min exposure to GE.

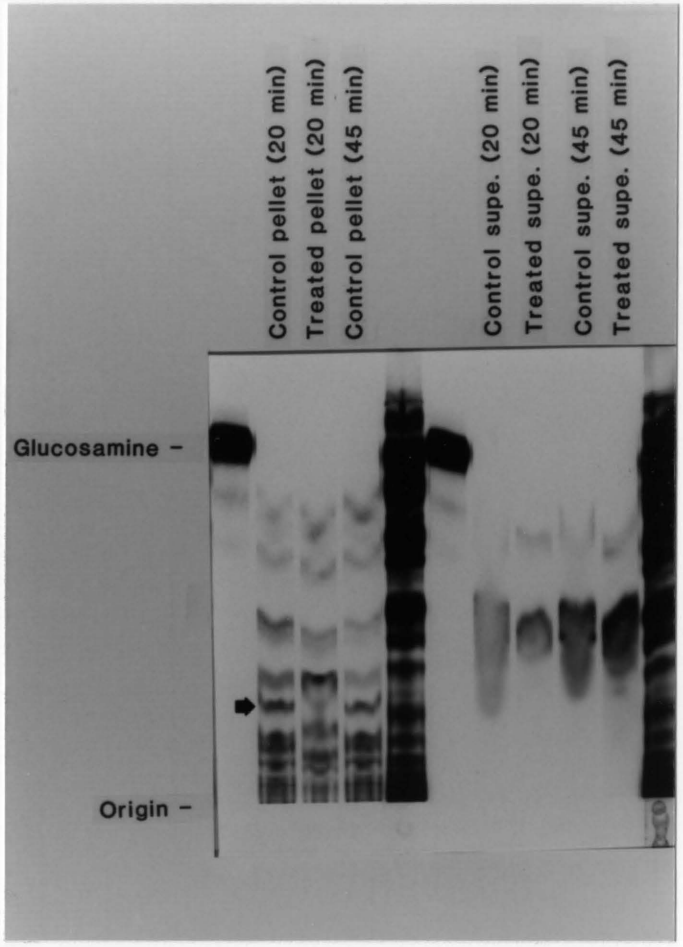

Fig. 14. Thin-layer Chromatography of Supernatant- and Cell-associated Radiolabel

Fluorograph of thin-layer chromatography plate, showing radiolabeled species present after digestion of cell-associated peptidoglycan (left) and present in culture supernatants (right). Note the band, present in the lane labeled "Control pellet", which is absent in the adjacent "Treated pellet" lane (arrow).

lanes than in the lanes of standards, possibly because of the high viscosity of the samples applied to the plate. A faint band was seen above (i.e. of smaller molecular weight) the main band in each lane. The identity of this compound is undetermined at present.

Effect of GE on Pellet-associated Peptidoglycan

Peptidoglycan synthesis in GE-treated gonococci is quantitatively subnormal, beginning immediately after exposure to GE (Rock 1985). The decrease in the amount of penicillin bound to the PBPs of GE-treated gonococci (above) suggests that peptidoglycan synthesis may possibly be qualitatively different as well. I examined cell-associated peptidoglycan after 20 min of exposure to GE to determine if GE affects previously synthesized peptidoglycan.

Pellet-associated peptidoglycan was exhaustively digested with mutanolysin after purification. Fragments of the digestion consist of disaccharide-tetrapeptide units which may be attached to each other through peptide cross-links; various oligomers of the basic disaccharide-tetrapeptide contain 2, 3, or more units.

Examination of the digestion fragments by high-pressure liquid chromatography did not indicate any differences between control and treated gonococci (Fig. 15). Again, the label was found in a single peak which comigrated with the "monomer". However, visualization of fragments by

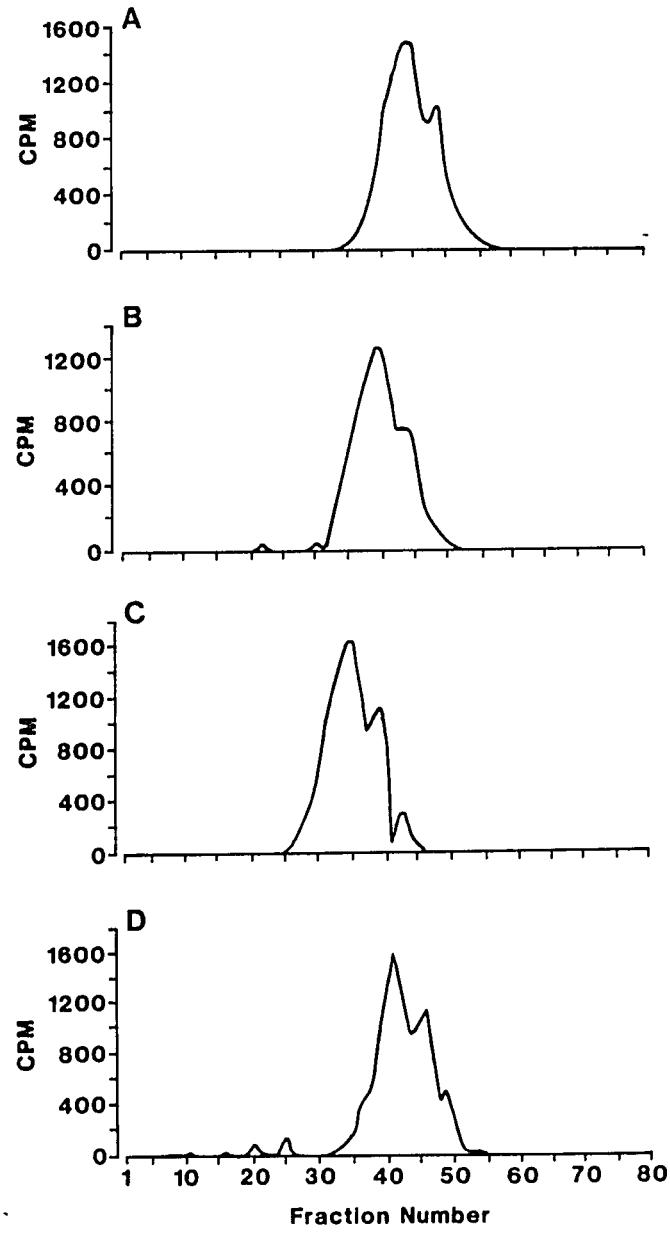

Fig. 15. HPLC-separation of Pellet-associated Radiolabel

Purified, digested, pellet-associated peptidoglycan was analyzed by HPLC. Graphs are plots of the radiolabel associated with each fraction after elution from the HPLC column. Panels correspond to pellet-associated radiolabel after: (A), 20 min exposure to PBS; (B), 20 min exposure to GE; (C), 45 min exposure to PBS; (D), 45 min exposure to GE. The apparent nonalignment of similar peaks between panels is due to fraction collection procedures and is not real.

thin-layer chromatography and subsequent fluorography did reveal a difference between the peptidoglycan of control and treated cells (Fig. 14). One of the bands toward the bottom (i.e. higher molecular weight) of the lane from control gonococci is not present in the corresponding lane of GE-treated cells. In addition, in the lane of GE-treated gonococci the band below the missing one is slightly higher than the corresponding band in the control lane. The identity of these bands has not been determined, although their migration distance indicates that they are larger than "dimers".

Discussion

Gonococcal PBPs from clinical isolates have been examined by Dougherty, Koller, and Tomasz (1980), who reported the molecular weights of the three PBPs to be: PBP 1, 90,000; PBP 2, 63,000; and PBP 3, 48,000. Subsequently, Barbour (1981) looked at gonococcal PBPs and reported slightly lower molecular weights. He also found that intrinsic (non-β-lactamase) penicillin resistance was associated with a decrease in penicillin binding to PBP 2 and to a lesser extent to PBP 1. Penicillin resistance was also found to affect penicillin binding to the PBPs of other bacterial genera (Buchanan and Strominger 1976, Handwerger and Tomasz 1986). Dougherty, Koller, and Tomasz (1981) and later Dougherty (1985b) expanded the knowledge of the

sensitivities of the gonococcal PBPs to different penicillins.

The molecular weights of gonococcal PBPs that I observed were the same molecular weights as seen by Dougherty. In addition, I observed a marked decrease in the binding of penicillin to all 3 PBPs. This could be due to either chemical modification of the active site of the PBPs or blocking of the site by a component of GE. It is probably not a consequence of PBP degradation since no change in molecular weight was observed by gel electrophoresis.

GE causes some subtle differences in the structure and turnover of gonococcal peptidoglycan. According to Sinha and Rosenthal (1980), the peptidoglycan fragments that growing gonococci release into the culture medium are confined to a combination of N-acetylglucosaminyl-β-1->4-1,6-anhydro-N-acetylmuramyl-tripeptide and the corresponding -tetrapeptide. My examination of culture supernatants by TLC also revealed predominantly one product, with no observed differences between the supernatants of control and treated gonococci. Because of decreased mobility of the radiolabel in these lanes, I was not able to correlate the major band with the peptidoglycan "monomers". I also saw a minor band of radiolabel, above (i.e. smaller in size) the major band in all four lanes. An exo-N-acetyl-glucosaminidase has been discovered in gonococci which releases free N-acetyl-glucosamine from peptidoglycan (Chapman and Perkins

1983). It is possible, therefore, that this minor band represents free N-acetyl-glucosamine.

When I looked at supernatant-associated radiolabel by HPLC, most of the label co-eluted with "monomer" standards. However, there was a small amount of high molecular weight label in supernatants from control gonococci. No high molecular weight label was seen by TLC, although the fluorography used to detect the label may not be sensitive enough to pick up such a small number of dpm. More importantly, I did not see any high molecular weight label associated with the supernatants of GE-treated gonococci. It is not likely that the "missing", larger peptidoglycan fragments were released by treated gonococci and then degraded by the lysozyme in GE, since gonococcal peptidoglycan has been shown to be resistant to human neutrophil lysozyme (Rosenthal et al. 1983). The alternative explanation, then, is that the observed difference between control and treated gonococci in peptidoglycan fragments released is real.

Muramidase-digested peptidoglycan breaks down into five fragments which have been resolved by TLC and well-characterized (Dougherty 1985a, 1985b). However, examination of this same digest by reverse-phase HPLC shows a multitude of products, many of unknown composition (Dougherty 1985a, 1985b). I found a subtle difference between control and treated gonococci when cell-associated

peptidoglycan was compared by TLC. Multiple species of radiolabeled digestion products were observed, including several species that are larger than "dimers". Among these large species, a fragment was present in the digest of control gonococci that was not seen with digest from treated cells. The identity of this fragment is unknown at present. To cloud matters, resolution of these same digests by HPLC indicates that all radiolabel resides within species no larger than "monomers". Furthermore, no difference is noted between treated cells and controls when radiolabel is characterized by HPLC.

It appears that GE has some interesting effects on gonococcal peptidoglycan, the structural element of the bacterial membrane. Bacterial cell division must be intimately associated with the structure of the peptidoglycan. The "missing band" from the TLC plate may indicate that a crucial peptidoglycan cross-link may be cleaved by GE, which could have disastrous effects on cell division.

CHAPTER 5

MISCELLANEOUS EXPERIMENTAL RESULTS

"What's true for *E. coli* is true for elephants, but not for gonococci."

Proverb, related by W. Shafer

This chapter encompasses experimental results that are not currently planned for publication, as well as experimental designs and executions that either failed to provide conclusive results or proved unworkable. These items have been included in the dissertation to provide a written record of extraneous information, dead ends, and unusable protocols for future reference by students examining gonococcal-neutrophil interactions. When considering how frustrating gonococci can be to work with, the author is thankful that this chapter is not any longer than presented.

Killing versus Inhibition of Peptidoglycan Synthesis

In an effort to determine whether the GE-induced inhibition of peptidoglycan synthesis was causal for the observed loss of reproductive capability, gonococcal viability was compared to uptake of [^{3}H]glucosamine (a peptidoglycan precursor) over a range of concentrations of GE. It is important to note that the GE used for this series of experiments is unusual in that 1000 µg/ml are

required to reduce gonococcal viability from 5×10^7 to 5×10^6 CFU/ml within 60 min; this is twice the concentration of GE required for killing when compared to GE from any other donor.

Gonococci were grown to mid-log phase, as previously described, and exposed to various concentrations of GE. Peptidoglycan synthesis (uptake of [^{3}H]glucosamine) was measured after 20 min and 60 min of exposure to each concentration of GE tested by pelleting the gonococci and counting the radiolabel associated with each supernatant and pellet. Gonococcal viability, taken from plate counts, was determined from the same samples after 60 min of exposure to GE.

When comparing gonococcal viability, assessed at 60 min, to [^{3}H]glucosamine uptake after 20 min of exposure to GE, it appears that death and inhibition of peptidoglycan synthesis may be separable events at low doses of GE (Fig. 16). This distinction is not as clear when viability at 60 min is compared to uptake after 60 min of exposure to GE (Fig. 17). A repeat of the experiment using GE from another donor and measuring uptake after 60 min of GE-treatment essentially confirms the results obtained with the "abnormal" GE (data not shown). However, these experiments were not repeated enough times to yield to statistical interpretation because other avenues of experimentation seemed to be more fruitful at the time.

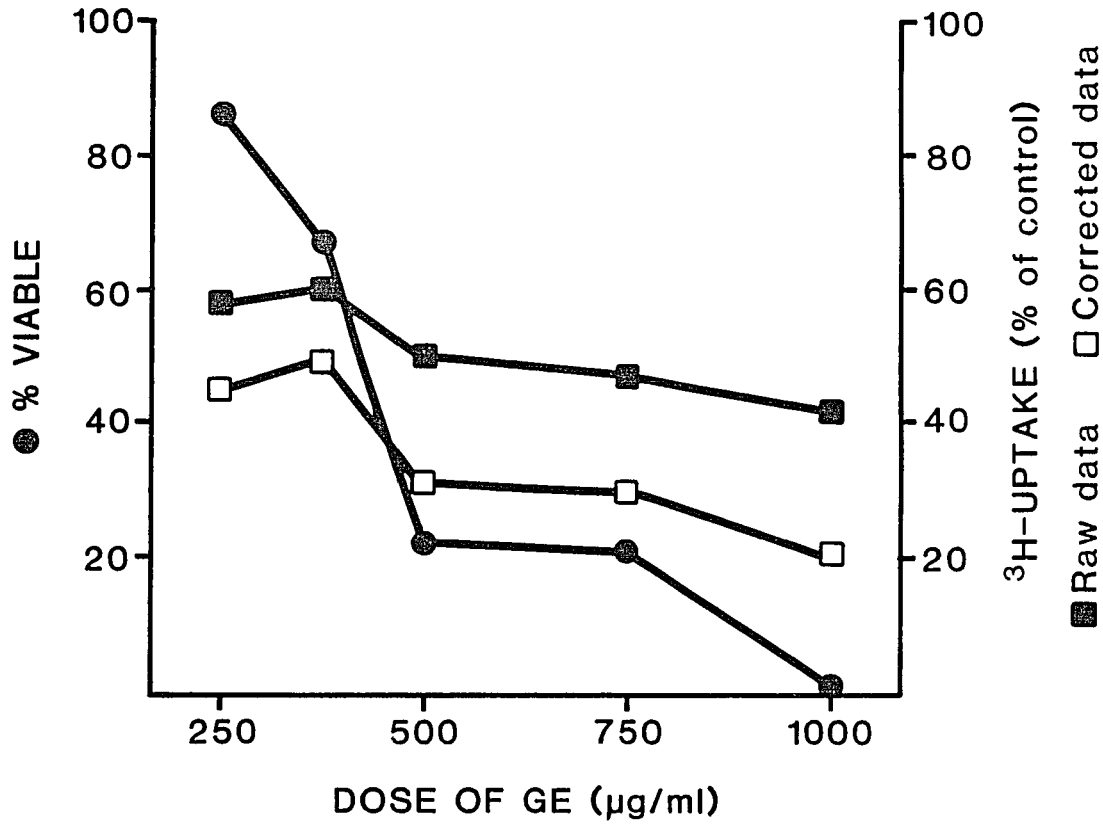

Fig. 16. GE-killing of Gonococci at 60 min versus Glucosamine Uptake at 20 min

Uptake of [^{3}H]glucosamine was measured during a 5-min pulse after 20 min of exposure to the concentration of GE indicated. Uptake data by treated gonococci are expressed as the percent of uptake by analogous controls. Raw data and data corrected for the amount of radiolabel sticking to heat-killed gonococci are both presented. Viability, assessed after 60 min in the indicated concentration of GE, is expressed as the percent of analogous control viability.

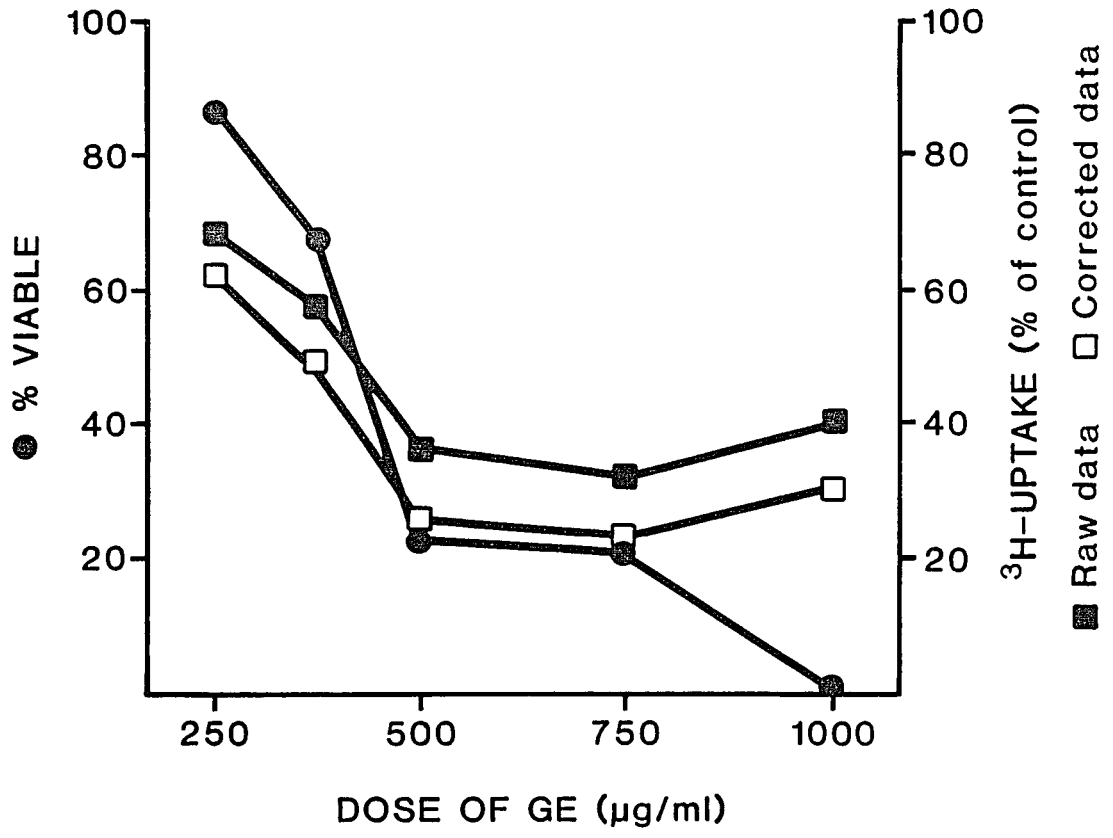

Fig. 17. GE-killing of Gonococci at 60 min versus Glucosamine Uptake at 60 min

Uptake of [^{3}H]glucosamine was measured during a 5-min pulse after 60 min of exposure to the concentration of GE indicated. Uptake data by treated gonococci are expressed as the percent of uptake by analogous controls. Raw data and data corrected for the amount of radiolabel sticking to heat-killed gonococci are both presented. Viability, assessed after 60 min in the indicated concentration of GE, is expressed as the percent of analogous control viability.

Inhibition of [^{3}H]glucosamine Uptake in Salmonella

GE was shown to immediately prevent any time-dependent increase in the uptake of [^{3}H]glucosamine by gonococci (see Chapter 2). Gonococci manifest many differences when compared to other gram-negative bacteria, so it was important to determine whether this affect on peptidoglycan synthesis was peculiar to gonococci. As such, [^{3}H]glucosamine uptake was measured in Salmonella strain TA 1538, a lipopolysaccharide mutant (provided by Dr. Nobuto Yamamoto of Hahnemann University). The lipopolysaccharide mutant was used because the gonococcal outer membrane is structurally closer to that of the mutant than it is to the outer membrane of wild-type Salmonella. Uptake, assessed from a 5-min pulse with radiolabel, was measured as in Chapter 2.

At first, GE concentrations of 500 µg/ml were used, and Salmonella viability at 60 min was reduced to just 0.2% of the initial cell count. Subsequent experiments used lower GE concentrations (300 to 400 µg/ml), although viability reductions of 2 logs within 60 min were maintained. [^{3}H]glucosamine uptake was assayed at 0, 20, and 60 min. All experiments used GE from the same donor.

As can be seen from Table 4, GE completely inhibited the increase in [^{3}H]glucosamine uptake by Salmonella just as it does with gonococci. It is important to note that uptake has not increased after 20 min of extract treatment; this is the period of GE treatment that relates to reproductive

death. There is no reason to believe that uptake at any early time in the experiment would be any greater than that seen here.

Since GE seems to affect peptidoglycan synthesis in Salmonella much the same as it does in gonococci, the question arose whether GE affected other macromolecular synthesis (such as protein synthesis) in Salmonella. Buck and Rest (1981) reported that gonococcal incorporation of leucine into TCA-precipitable material by gonococci was not affected during the first 30 min of extract treatment. An attempt was made to measure uptake of [^{3}H]leucine by GE-treated Salmonella, using the same experimental design employed for [^{3}H]glucosamine uptake. As can be seen from Table 5, this system proved unworkable. Control Salmonella would not take up any increasing amounts of [^{3}H]leucine over time, so the data relating to the GE-treated bacteria is meaningless. It was discovered later that this particular strain of Salmonella is nutritionally deficient and requires a special medium to assess protein synthesis (apparently peptidoglycan synthesis is not affected in the mutant).

Killing of Gonococci by Lysozyme

Some of the early results presented (Chapter 2) indicate that gonococcal peptidoglycan is turned over faster during GE treatment than in controls, and that GE also prevented any growth-related increase in peptidoglycan

Table 4. Uptake of [^{3}H]glucosamine by Salmonella TA 1538

Bacteria	Uptake after 20 min of treatment*	Uptake after 60 min of treatment*
Salmonella + PBS	230	1148
Salmonella + GE	104%	116&

* Uptake is expressed as the percent of uptake at 0 min
% Data generated using 400 µg/ml GE
& Data generated using 500 µg/ml GE

Table 5. Uptake of [^{3}H]leucine by *Salmonella* TA 1538

Bacteria	Uptake after 20 min of treatment*
Salmonella + PBS	75
Salmonella + GE	94%

* Uptake is expressed as the percent of uptake at 0 min

% Data generated using 300 µg/ml GE

synthesis. It was obvious to ask whether the neutrophil lysozyme, an enzyme whose native substrate is bacterial peptidoglycan, was responsible for this increased turnover or lack of increase of synthesis. In a previous report (Rosenthal et al. 1983) purified gonococcal peptidoglycan was found to be fairly resistant to neutrophil lysozyme because the peptidoglycan was structurally modified by the addition of O-acetyl groups. However, it was felt that initial experiments would prove worthwhile towards determining whether human neutrophil lysozyme purified from GE by column chromatography was capable of influencing control on peptidoglycan synthesis.

The first experiments with lysozyme as the antagonist were set up the same as when GE was used. Assays contained 5×10^7 CFU/ml; lysozyme concentrations of 50, 100, and 250 µg/ml were tried in a bactericidal assay. This range of lysozyme concentrations extends well above the amount of lysozyme present in 500 µg/ml of GE (typically 10%, or approximately 50 µg/ml), a GE dose which is very bactericidal.

An examination of the results, summarized in Table 6, would suggest that the lysozyme in GE is not the component responsible for the loss of reproductive capability. However, GE is an extremely complex mixture of dozens of components. The idea that one of these components may be responsible for the bactericidal activity of GE is probably too simplistic a viewpoint. Lysozyme is a case in point; it

would have to penetrate the gonococcal outer membrane just to gain access to the peptidoglycan, which is no guarantee that the enzyme would be active should it reach the substrate. In fact, this mechanism was still plausible for explaining the activity of GE against gonococcal peptidoglycan, and deserved looking into.

As such, EDTA was considered as an agent that may potentiate the activity of lysozyme. EDTA is known to disrupt the structure of the gram-negative outer membrane by chelating away the divalent cations that bridge and stabilize the lipopolysaccharide molecules in the outer membrane. An initial experiment, set up the same as the lysozyme studies, determined the toxic levels of EDTA in relation to gonococci (Table 6).

With this information in hand, gonococci were exposed to a combination of lysozyme and EDTA to test the above hypothesis. The results in Table 7 indicate that perturbation of the outer membrane is not sufficient to reveal any bactericidal activity that lysozyme may express towards gonococci.

Neither of these experiments provided any indication of whether lysozyme is capable of affecting peptidoglycan synthesis or turnover within the context of unfractionated GE. In order to examine this question from a slightly different point of view, the effect of Mg^{+2} on peptidoglycan synthesis was looked into. The reasoning behind this was

Table 6. Bactericidal Activities of EDTA and Purified Human Neutrophil Lysozyme

Effector	Viability after 60 min of exposure*
lysozyme	
none	200
50 µg/ml	208
100 µg/ml	139
250 µg/ml	69
EDTA	
none	235
1 mM	0
500 µM	0
100 µM	194
10 µM	207
1 µM	166

* Expressed as percent viable, compared to initial viability

Table 7. Combined Effects of EDTA and Human Neutrophil Lysozyme

Effector	Viability after 60 min of exposure*
none	200
EDTA (100 μM)	197
lysozyme (60 μg/ml)	226
EDTA + lysozyme	219

* Expressed as percent viable, compared to initial viability

that Mg^{+2} has been shown to inhibit killing of gonococci by GE (Buck and Rest 1981). As such, Mg^{+2} may also be capable of inhibiting entry of the component(s) of GE that are responsible for the perturbations to peptidoglycan synthesis (whether or not lysozyme is involved or not).

Experiments are essentially the same as previous assays (see Chapter 2); 5×10^7 gonococci/ml were exposed to 500 µg/ml GE, with or without 40 mM $MgCl_2$. At times noted, samples of gonococci were pulsed with [^{3}H]glucosamine for 5 min, and after pelleting the amount of radiolabel associated with the supernatant and pellet was determined.

First of all, 40 mM $MgCl_2$ does not kill gonococci; it also does not allow normal growth either, inducing bacteriostasis instead. More importantly, $MgCl_2$ inhibits killing by GE to the same extent, allowing bacteriostasis but not growth (Table 8). $MgCl_2$ exhibits a less clear-cut effect on GE-induced inhibition of [^{3}H]glucosamine uptake (Fig. 18). Magnesium treatment of control gonococci causes a slight reduction in the amount of glucosamine taken up compared to magnesium-free controls. Adding magnesium to GE-treated gonococci does not seem to make any significant difference in glucosamine uptake when comparing to GE-treated gonococci without magnesium.

Table 8. Effect of Mg^{+2} on the Bactericidal Activity of GE

Effector	Viability after 60 min of exposure*
none	206
$MgCl_2$ (40 mM)	71
GE (500 µg/ml)	2.4
GE + $MgCl_2$	91

* Expressed as percent viable, compared to initial viability

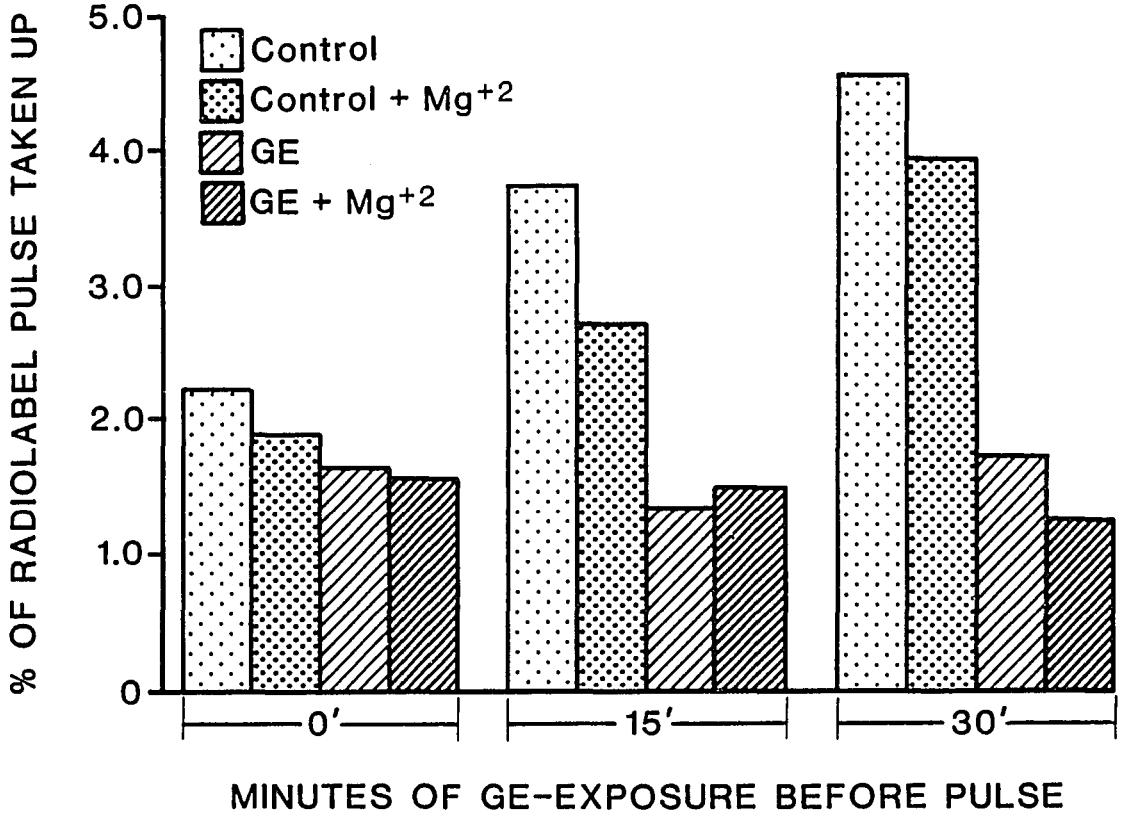

Fig. 18. Effect of Mg^{+2} on GE-induced Inhibition of [^{3}H]glucosamine Uptake

Gonococci were exposed to a 5-min pulse with [^{3}H]glucosamine at the times indicated, and the amount of cell-associated radiolabel was determined. Within each group, bars are (from left to right): control gonococci; control gonococci + 40 mM Mg^{+2}; GE-treated gonococci; and, GE-treated gonococci + 40 mM Mg^{+2}.

GE and Anti-BPI

Purified bactericidal, permeability-increasing protein (BPI), a component of GE, has been shown to kill gram-negative bacteria very rapidly (Weiss et al. 1978). It was not known, however, whether the BPI in unfractionated GE was the major component responsible for the killing of gonococci by GE. An attempt was made to answer this question by trying to block killing by treating GE with anti-BPI (provided by Jerry Weiss and Peter Elsbach of New York University).

Assays contained 5×10^5 CFU/ml; anti-BPI or preimmune IgG were added to the GE and preincubated for 3 h on ice before being added to the gonococci. The GE concentration used in the assay was 300 µg/ml; anti-BPI concentration was 2500 µg/ml; and preimmune IgG concentration was 2000 µg/ml.

As can be seen, anti-BPI does abrogate killing by GE somewhat, although it does not protect gonococci to an extent which would allow growth (Fig. 19). Anti-BPI is somewhat toxic to control gonococci. On the other hand, preimmune IgG has no effect on the growth by controls or killing by GE.

Active Transport in Gonococci

Attempts were made to measure carbohydrate transport in GE-treated gonococci, with the thought that any observed perturbation in transport would have direct consequences in terms of affecting the carbon and/or energy supply necessary

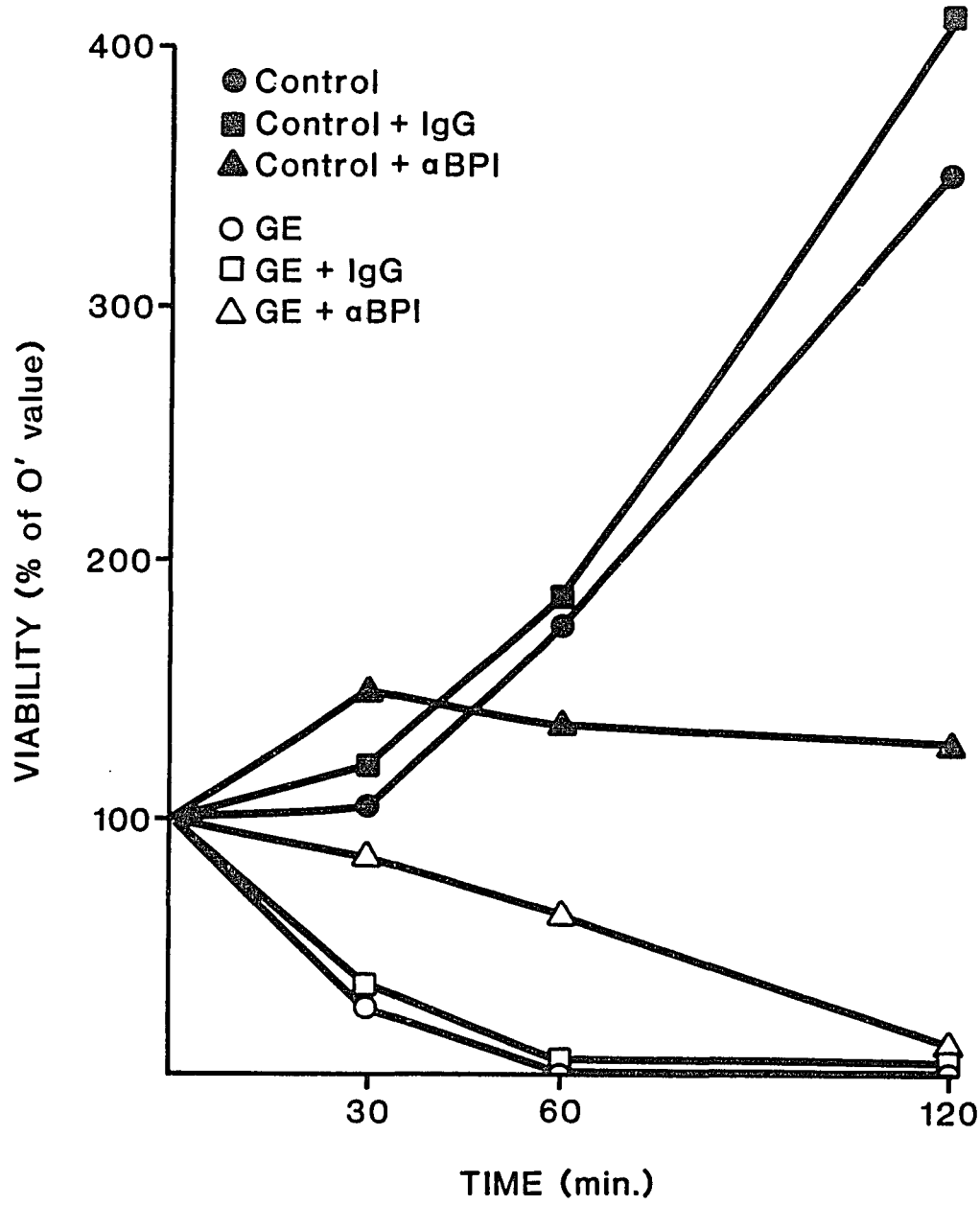

Fig. 19. Effect of Anti-BPI on Killing of Gonococci by GE

Gonococcal viability was assessed at the times indicated. Lines represent (●), PBS-control; (■), PBS-control + preimmune IgG; (▲), anti-BPI; (○), GE-treated; (△), GE-treated + anti-BPI; (□), GE-treated + preimmune IgG.

for cell division. A variety of experimental designs were tried, all with either no success or giving unreproducible results.

"Classic" Transport

The first attempt to measure transport involved the classic method whereby gonococci were pulsed with radiolabeled glucose. Immediately after the radiolabel pulse, samples of bacteria were passed over 0.45 μm Amicon filters. Filters were washed twice with small volumes of ice cold buffer, dried under an infrared lamp, and counted in a liquid scintillation counter. Results obtained from numerous attempts using this technique were very inconsistent; there weren't any obvious changes to the protocol to make it work better, so the method was dropped.

Flow Dialysis

After the classic attempt at measuring transport failed, a second method was tried using a flow dialysis cell (Technilab Instruments, Inc., Pequannock, N.J.) (Colowick and Womack 1969; Kell, Ferguson, and John 1978; Law and John 1981; Ramos, Schuldiner, and Kaback 1976). The cell is an acrylic block divided into two halves, with a cylindrical cavity spanning the junction. A sheet of dialysis membrane is placed between the halves, dividing the cavity into top and bottom chambers with an approximate volume of 3 ml each. The bottom chamber has inlet and outlet connections which

allow a suitable liquid phase to flow through. The top chamber has only one opening, a small diameter acrylic tube which allows the introduction of bacteria, GE, radiolabel, etc. The tube also serves as a snorkel, allowing the whole cell to be submerged in a water bath to control the temperature of the experiment. Both chambers of the cell are mixed by means of stir bars within the chambers.

For a typical experiment, 2 ml of medium was introduced into the upper chamber; enough headspace remained within the chamber for gas exchange to occur. The same medium was pumped through the bottom chamber at a flow rate of 0.5 ml/min, with no headspace. Initial experiments indicated that the amount of label crossing the dialysis membrane was smaller than expected. As such, the amount of radiolabel used, the flow rate through the bottom chamber, and other experimental parameters were varied to obtain maximum label movement across the membrane. This maximum was still not as good as hoped, so it was decided to use Escherichia coli in the initial experiments.

E. coli was used because its carbohydrate transport has already been well characterized, whereas gonococcal transport has proven difficult for other investigators to measure. E. coli were introduced into the system as part of a matrix of bacterial concentrations, radiolabel

concentrations and specific activities, in an attempt to maximize transport.

Although much time was spent to make this technique work, none of these attempts resulted in any reproducible indications of transport. It was felt to be pointless to repeat this technique using gonococci, especially since they are so much more difficult to work with than E. coli. In the future, if it becomes important to determine the effects of GE on carbohydrate transport, this technique may be resurrected by using dialysis membrane from another source and greatly increasing the flow rate in the bottom chamber.

Centrifugation Through Percoll

It was thought that carbohydrate transport could be measured by removing the bacteria from the radiolabel pulse by centrifugation. The medium chosen for this experiment was Percoll (Pharmacia, Inc., Piscataway, N.J.) which can be conveniently adapted to any physiological density range. In addition, Percoll is silica based, which means that it should not have any adverse metabolic effects on gonococci.

Initial experiments, using Percoll diluted to a density of 1.05 g/ml, were set up to determine whether radiolabeled glucose in an overlay would mix into the Percoll during centrifugation. Unfortunately, this is exactly what happened, and no amount of care could overcome the problem.

Centrifugation Through Silicone Oil

Since the Percoll cushion was not immune to mixing during centrifugation, the same basic technique was tried using silicone oil as the underlay cushion for centrifugation. A variety of silicone oils were obtained (Petrarch Systems, Inc., Bristol, Pa.). These oils were mixed in various ways to obtain an oil with the proper density and viscosity. Previous experiments had indicated that log-phase gonococci band at a density of approximately 1.065 g/ml in Percoll gradients, so oil densities between 1.01 and 1.07 g/ml were tried. At this point, a multitude of technical problems arose which eventually proved to be insurmountable. A summary of the problems follows.

Oil density proved to be less of a problem than viscosity and an associated parameter, surface tension. Very low density oil had a tendency to mix with the medium overlay during centrifugation, occasionally even inverting so that the lighter, aqueous medium wound up on the bottom of the tube. As soon as the density was increased sufficiently so that mixing stopped, the combination of viscosity and surface tension of the oil caused the gonococci to pellet at the oil-aqueous interface. This was not usable because the gonococci had to be completely removed from the aqueous layer to stop transport. A longer centrifugation would drive the gonococci through the interface to form a pellet in the oil, but the time of centrifugation was too

long to be useful for measuring a rapid event such as transport. In addition, bacteria pelleted in the oil proved remarkably resistant to resuspension and counting in liquid scintillation cocktail.

CHAPTER 6

CONCLUSIONS

"The dream of a bacterium is to become two bacteria."

F. Jacob

The killing of bacteria by neutrophils is an extremely complex process; real understanding of the process is rudimentary at present. A great deal has been learned about the activities of neutrophil granule components, but understanding individual components is a long way from understanding the intricate interrelationships that must certainly exist when these components act together.

Two examples will serve to illustrate this point. First, neutrophil elastase is not bactericidal by itself, but has been shown to induce sublethal changes in bacteria that render them more susceptible to oxidative killing mechanisms (Odeberg and Olsson 1976b). Second, a phospholipase A_2 has been isolated from neutrophil granules, but it only has enzymatic activity in the presence of the bactericidal, permeability-increasing protein of Elsbach et al. (1979). However, the phospholipase does not mediate the bactericidal activity of Elsbach's protein (Vaara and Viljanen 1983).

As has been mentioned several times, the initial effector of gonococcal death does not appear to be associated with cytoplasm-related events (Buck and Rest 1981). Interestingly, serum killing of Escherichia coli (Taylor and Kroll 1983) exhibits many of the same results as have been seen with GE-killing of gonococci. Purified neutrophil cationic proteins do cause an immediate decrease in macromolecular synthesis in E. coli and Staphylococcus aureus, although these effects are probably related to the associated inhibition of energy-dependent membrane transport (Odeberg and Olsson 1976a). As such, the gonococcal membrane is the structure that is most likely to be involved in the loss of the ability to divide. The outer membrane must, by definition, be the first component of the bacterium to interact with GE. The GE-induced membrane damage, observable in the electron micrographs displayed here, supports this contention.

As an example, the efficiency of killing of Salmonella typhimurium by intact neutrophils is inversely proportional to the complexity of the lipopolysaccharide in the outer membrane (Okamura and Spitznagel 1982). Purified cationic proteins from neutrophil granules are also bactericidal, particularly so against rough mutants exhibiting truncated lipopolysaccharide (Modrzakowski and Spitznagel 1979). The bactericidal activity of these cationic granule fractions could be removed by adsorption with either whole

cells from the smooth parent strain, or with purified lipopolysaccharide.

It appears that stability of the outer membrane structure is important to the well being of the bacterial cell. Disruption of this membrane by the neutrophil could have powerful effects on cell viability and survivability. EDTA treatment of gonococci, which disrupts outer membrane structure, eliminates an antiphagocytic activity that is linked to lipopolysaccharide (Rosenthal et al. 1977). Non-neutrophil proteolytic enzymes can inhibit the growth of gonococci by cleaving outer membrane proteins (Blake, Gotschlich, and Swanson 1981). Since neutrophils have numerous proteases within their granules, some which do degrade outer membrane proteins (Eaton and Rest 1983), it is feasible that the proteins are targets of neutrophil microbicidal activity.

Outer membrane, the surface of gonococci that is exposed to GE, is capable of adaptive changes in response to its particular environment. For example, iron limitation slows bacterial growth (Archibald and DeVoe 1978), and also, more importantly, regulates the expression of outer membrane proteins (Mietzner et al. 1984, West and Sparling 1985). Growth of gonococci on pyruvate and cysteine (Norrod et al. 1983), or in glucose-limited medium (Morse et al. 1983) causes phenotypic changes in lipopolysaccharide, which imparts increased serum sensitivity.

Sites of gonococcal infection typically harbor very low oxygen tension. Gonococci grow much more slowly under low oxygen tension than they do in a laboratory environment (Kellogg, Crawford, and Callaway 1983), although they will grow even in a strictly anaerobic environment if provided with an alternate electron acceptor for the terminal oxidase (Knapp and Clark 1984). Gonococci grown in low oxygen tension express decreased levels of outer membrane proteins (Leith and Morse 1980) which, as noted in Chapter 1, may be specific targets of neutrophil microbicidal activity. Gonococci (Casey Shafer, and Spitznagel 1985, Rest 1979) and Bacteroides fragilis (Wetherall, Pruul, and McDonald 1984) are killed by GE much more efficiently when grown as log phase cells than when maintained in stationary phase, although E. coli and a Salmonella sp. grown anaerobically induce the neutrophil respiratory burst better than aerobically-grown cells do (Maluszynska, Stendahl, and Magnusson 1985).

Although lipopolysaccharide and outer membrane proteins are, as noted above, deeply involved in the interactions of a bacterium and its environment, it is the underlying peptidoglycan layer that has yielded significant indications of being the target of the "lethal lesions" of GE. Since peptidoglycan is the bacterial component directly responsible for maintaining size and shape (Koch 1985), it

is not at all surprising that the neutrophil would target this component when attempting to kill the bacteria.

Sites of peptidoglycan synthesis (Burman, Raichler, and Park 1983) and the mechanism of murein sacculus expansion (Park0) in E. coli have been studied. However, the applicability of these mechanisms to gonococci is uncertain. The structure of E. coli peptidoglycan is directly related to the state of growth of the culture (Pisabarro, de Pedro, and Vazquez 1985).

Very interesting results were obtained by Wegener, Hebeler, and Morse (1977b), and Goodell, Fasio, and Tomasz (1978) when they looked at peptidoglycan synthesis and turnover in penicillin-treated gonococci. They found that penicillin caused an increase in turnover and a decrease in synthesis of peptidoglycan. The increase in turnover, determined to be more than twofold control values (Sinha and Rosenthal 1981), mirrors our findings concerning gonococci and GE. Moreover, they reported that peptidoglycan hydrolysis began immediately but that lysis was delayed, results that correlate well with mine.

Dougherty (1983b), and Blundell and Perkins (1981, 1985) found that penicillin caused a sharp decline in the degree of O-acetylation of gonococcal peptidoglycan, a fact whose importance becomes apparent when the resistance of O-acetylated peptidoglycan to neutrophil lysozyme is considered (Rosenthal et al. 1983). Guymon, Walstad, and

Sparling (1978) found that penicillin caused a significant increase in the degree of peptidoglycan cross-linking.

Peptidoglycan turnover by gonococci occurs normally during growth. This turnover is a consequence of the activity of bacterial autolysins, enzymes that cut incorporated peptidoglycan so newly synthesized peptidoglycan can be inserted and the bacteria grow in size. As a consequence gonococci release peptidoglycan fragments into their surroundings (Rosenthal 1979). Two effects of this release are: (i) damage to epithelial mucosa at the site of infection, contributing to colonization of the site (Melly, McGee, and Rosenthal 1984), and (ii) consumption of complement, which may serve to deplete complement levels to a point where they are not sufficient to harm gonococci. It is interesting to note that E. coli recycle their peptidoglycan (Goodell 1985); they may have no need of the advantages conferred by peptidoglycan release. Autolysin-defective pneumococci continue to synthesize peptidoglycan when treated with penicillin, but the peptidoglycan isn't cross-linked and therefore does not get fully incorporated (Fischer and Tomasz 1984).

With all of this information at hand, plus a little speculation, I propose that initial GE-induced damage to gonococci is manifest as membrane damage, and it is more than likely membrane damage that is responsible for the immediate loss of division capability observed. This

statement is based on several lines of information, which are summarized below.

Cytoplasmic membrane integrity is compromised somewhat (based on oxygen uptake data), but not enough to cause outright cell lysis. If cell lysis did occur immediately, then I would expect macromolecular synthesis and oxygen uptake to also cease immediately. They do not. Light microscopy confirms that GE-treated gonococci do undergo very limited cell division after exposure to GE, which is also inconsistent with immediate lysis.

Peptidoglycan turnover increases when gonococci contact GE. It is tempting, especially in light of the comparison with penicillin, to attribute this increase to the failure of newly synthesized peptidoglycan to cross-link into the matrix. This is consistent with the observed decreased binding of penicillin to the penicillin-binding proteins of treated gonococci. If GE damaged the penicillin-binding proteins enough to curtail their enzymatic activity, then peptidoglycan cross-linking would not occur. The gonococcal autolysins would continue to cleave the peptidoglycan already incorporated, even though no new peptidoglycan was being linked in. Progressive attack by the autolysins could weaken the walls sufficiently for peptidoglycan not destined for immediate removal to be lost anyway. Eventually, the continual activity of the autolysins would weaken the wall to the point where the osmotic

imbalance across the cell wall would rip the cell apart. Qualitative analysis of cell-associated peptidoglycan after GE-treatment agrees with this. The noted absence, from the thin-layer chromatography fluorograph, of one band from the lane of GE-treated cells could be the result of a failed peptide cross-link.

The other side of the peptidoglycan issue, namely the decreased synthesis, has slightly more uncertain implications. GE-treatment does not appear to completely shut down peptidoglycan synthesis. Instead, it causes an immediate decrease when compared to control cells, and does not allow any increase over time as is seen in dividing cells. Unfortunately, the amount of peptidoglycan synthesized by treated cells is insufficient to allow a qualitative analysis by presently available techniques. However, when considering the quantitative data, a plausible explanation comes to mind. This explanation requires a mental, three-dimensional image of an individual, mature coccus dividing into two equal daughter cells, which must then double in size during maturation to repeat the cycle.

For the sake of argument, we can assume that a mature coccus, just before division, approximates the shape of a prolate spheroid, which is a three-dimensional shape formed by the rotation of an ellipse about its major axis. The surface area "s" of this ellipse is described by the formula:

$$s = 2\pi b^2 + 2\pi\frac{(ab)}{e}(\sin^{-1}e)$$

where a and b represent the semimajor and semiminor axes, respectively; e, the eccentricity of the ellipse, is calculated from the formula:

$$e = \frac{(a^2-b^2)^{1/2}}{a}$$

Measurements of gonococcal cross-sections, taken from electron micrographs, give a semimajor axis of 0.4 μm and a semiminor axis of 0.3 μm. Inserting these numbers into the above equations gives an approximate surface area of 1.39 μm^2, which is also the approximate amount of peptidoglycan per cell. One-half of this peptidoglycan area, or 0.70 μm^2 will go to each newly formed daughter cell. Septum formation designed to segregate a mature coccus into two daughter cocci involves peptidoglycan synthesis in the plane swept out by the minor axis of the ellipse as it rotates around the major axis. If we assume that the septum conforms to a roughly circular configuration, then the area of the septum, equivalent to the area of a circle of radius 0.3 μm, is approximately 0.28 μm^2. The newly formed daughter cell must double in size to mature; it must therefore synthesize 0.70 μm^2 of new peptidoglycan during this maturation. Of this new 0.70 μm^2, 0.28 μm^2 or 40% is septal peptidoglycan.

Gonococci, typical of all cocci, do not have diffuse areas of peptidoglycan synthesis such a E. coli does; rather peptidoglycan is synthesized in discrete zones in close proximity to the septum. It seems logical, then, that new zones of peptidoglycan synthesis are added to a population of cells as new septa are formed. I have presented visual evidence that GE has destructive effects on gonococcal septa. If GE indeed inhibits cell division by stopping septum formation, then on the surface it would appear that peptidoglycan synthesis was inhibited. In reality, GE may not affect peptidoglycan synthesis, but only prevent the initiation of new peptidoglycan synthesis sites because they were linked to new septa. Such a GE-treated culture would be expected to be capable of a constant, low level of peptidoglycan synthesis. This level should be approximately 60% of that seen in an analogous control culture capable of adding new peptidoglycan synthesis sites via new septa. A look back to Fig. 3 shows that treated gonococci do indeed maintain a low, steady level of peptidoglycan synthesis. Moreover, the zero minute treated value is very nearly 60% of the zero minute control value. This may be just a coincidence, but there is no conflicting data to support a contrary theory.

In conclusion, I have provided what I feel is a significant contribution to the field of neutrophil killing of gonococci. This comes under the realm of basic research; it

doesn't have any immediate practical applications. However, it does add to the foundation of knowledge necessary to foster the understanding of and, hopefully, eventual eradication of gonorrhea as a disease of man.

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