Energy Is Required for Maximal Adherence of *Neisseria gonorrhoeae* to Phagocytic and Nonphagocytic Cells

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The possibility that gonococcal energy might play a role in the interaction of *Neisseria gonorrhoeae* with both phagocytic and nonphagocytic cells was examined. Respiratory chain inhibitors including KCN and antimicrobial bile resulted in reduction in gonococcal association with human neutrophils. Similar results were seen with HeLa cells and the human promyelocytic (HL-60) cell line. Identical conditions did not affect the opsonin-dependent association of *Staphylococcus aureus* with the same cell types. New protein synthesis by gonococci did not account for the observed reduction in association. These results suggest that energy is needed for maximal opsonin-independent association of gonococci with mammalian cells.

*Neisseria gonorrhoeae* is a bacterium completely adapted to the human host with no other ecological niche (5). The first step in infection with this organism is its adherence to an appropriate target cell. It seems likely that one or more gonococcal outer membrane proteins (16, 25, 26, 30, 32) and/or pili (11, 28, 30) are involved in this process. Different bacterial surface proteins may be required for adherence to different cell types (16, 25, 28, 30, 32), perhaps related to specific cell receptors (1, 25). It is known that growth conditions may alter the protein composition of the gonococcal outer membrane (3, 7, 14, 22, 35).

We have focused on the role of bacterial metabolic activity in pathogenesis of gonococcal disease. We have demonstrated that gonococcal metabolism is stimulated by human serum (8) and by L-(+)-lactate generated by phagocytic cells during the microbicidal process (6). Enhanced metabolism allows gonococci to compete in vitro for molecular oxygen, thereby limiting the ability of the phagocytes to generate oxygen reduction products (4). In view of the dynamic interaction that exists between neutrophils and gonococci, as well as the phenotypic variability of the gonococcal outer membrane, this study was undertaken to determine whether gonococcal metabolism might also play a role in the process of adherence to mammalian cells.

**MATERIALS AND METHODS**

**Bacterial culture conditions.** (i) Preparation of log-phase gonococci. *N. gonorrhoeae* F62 (a serum-sensitive isolate capable of producing disseminated disease, provided by P. F. Sparling, University of North Carolina [UNC], Chapel Hill) was subcultured daily on GCB agar (GC medium base; Difco Laboratories, Detroit, Mich.) containing 1% (vol/vol) Kellogg defined supplements 1 and 2. Opacity and piliated colony variants of *N. gonorrhoeae* were identified by the criteria of Swanson (29). All studies involved a pilated, opaque strain of F62. Pililated, opaque MS11 gonococci were used for several experiments as well.

For all experiments, 16- to 24-h-old colonies were transferred to GCB containing 2% (vol/vol) supplement 1 and 5 mM NaHCO₃. Flasks were incubated at 37°C in an atmosphere of 95% room air and 5% CO₂ for approximately 5 h. Organisms were pelleted, washed, and suspended in Hanks balanced salt solution with 5.56 mM glucose (pH 7.4; HBSS). Pulse sonication on ice for 2 min at an intensity of 70%, using a Sonic Dismembrator (Fisher, model 300), reduced clumping. Sonication did not affect gonococcal outer membrane structures, and viability was decreased by 5% or less (data not shown).

(ii) Preparation of *S. aureus*. *Staphylococcus aureus* was grown identically to gonococci. Before use in association experiments, the *S. aureus* cells were opsonized in pooled human serum for 30 min at 37°C, pelleted, washed, and suspended in HBSS.

For heat-killed experiments, the organisms were heated to 100°C for 15 min.

**Neutrophil isolation.** Whole venous blood was obtained in heparinized syringes from normal donors without previous gonococcal infection. Neutrophils were separated using Plasmagel (Roger Bellon, Neuilly, France), Ficol-Hypaque (Pharmacia Fine Chemicals, Piscataway, N.J.), and osmotic lysis of contaminating erythrocytes as previously described (8). Neutrophils were suspended in HBSS at the desired concentration as determined by an automated blood cell counter (model D2W; Coulter Electronics, Hialeah, Fla.). Giemsa stain showed that more than 98% of cells were neutrophils, and more than 95% were viable as determined by exclusion of trypan blue dye.

**Cell culture conditions.** (i) HL-60. Human promyelocytic HL-60 leukemia cells (undifferentiated) obtained from the Cancer Research Center, UNC, were grown in suspension culture and passed twice weekly in Dulbecco minimal essential medium-15 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; UNC Tissue Culture Facility) supplemented with 10% heat-inactivated fetal calf serum (UNC Tissue Culture Facility), penicillin (100 U/ml), and streptomycin (100 μg/ml) in a humidified atmosphere of 5% CO₂-95% air. Cells were induced to differentiate along neutrophil lines by the inclusion of 1.3% dimethyl sulfoxide (DMSO; Fisher Scientific, Fair Lawn, N.J.) for 5 days (9).

(ii) HeLa cells. Human cervical carcinoma epithelial cells, obtained from the Cancer Research Center, UNC, were grown in suspension culture and passed twice weekly in Eagle minimal essential medium (UNC Tissue Culture Facility) supplemented with 10% fetal calf serum, gentamicin (50 μg/ml), and amphotericin B (fungizone; 2.5 μg/ml) in a humidified atmosphere of 5% CO₂-95% air.
Association of bacteria with eucaryotic cells. The association (binding plus phagocytosis) of 14C-labeled gonococci was assessed by a modification of a previously described assay (3). Gonococci were grown as described above in the presence of 6 μCi of [14C]adenine (New England Nuclear Corp., Boston, Mass.) per ml. Labeled gonococci (5.0 x 10^6) were added to polypolyrene tubes (12 by 75 mm; Falcon 2063; Becton Dickinson, Lincoln Park, N.J.) containing 0.2 ml of bovine serum albumin, 0.5 ml of polymorphonuclear leukocytes (PMNs; 2.5 x 10^6/ml), and HBSS to make a final volume of 1 ml. The tubes were rotated end over end at 37°C, and binding was stopped by addition of cold HBSS containing 10% fetal calf serum and 1% 0.2 M NaF. Gonococci associated with PMNs were separated by differential centrifugation. The percentage of gonococcal inoculum associated with PMNs was ascertained with a liquid scintillation counter, and results were expressed as the average of duplicate tubes. Background attachment of gonococci to glass was measured in the absence of KCN, but the effects of this compound on this parameter were negligible.

Anaerobic association was performed as described above, but before addition of the gonococci, the suspension of bacteria and the polypolyrene tubes containing the various incubation mixtures were placed in an anaerobic incubator tent (Coy Laboratory Products Inc., Ann Arbor, Mich.). The vessels were first degassed in a vacuum chamber to -20 atm (ca. 2.026 kPa). Nitrogen gas was then bled into the chamber to a pressure of 1 atm (ca. 101 kPa). This process was repeated twice, and after the third evacuation, the chamber was opened to the tent. The remainder of the incubation was performed as described above.

Oxygen consumption. Bacterial oxygen consumption (O_2 consumption) was measured with a Clark oxygen electrode (Yellow Springs Instrument Co., Yellow Springs, Ohio) with a 1-ml volume of HBSS containing 10^8 bacteria. Results were expressed as the maximal rate of O_2 consumption (nanomoles per minute) observed over a 10-min period at 37°C.

LDL. Luminol-dependent chemiluminescence (LDL) was measured in an integrating photometer (SAI Technology Company, San Diego, Calif.) with a 2-ml volume containing 1.0 x 10^6 neutrophils. The final concentration of luminol (Sigma Chemical Co., St. Louis, Mo.) was 1.0 x 10^-3 M in 1% bovine serum albumin. When zymosan was used to stimulate LDL, it was first opsonized using 100% pooled human serum for 30 min at 37°C, pelleted, washed, and suspended in HBSS. A particle-to-neutrophil ratio of approximately 3 was used. When gonococci were used as a stimulus, they were not opsonized, and a ratio of 100 gonococci per neutrophil was used. Controls measuring LDL with neutrophils alone and gonococci alone were carried out. When metabolic inhibitors were employed (see below), control experiments measuring LDL stimulation by phorbol myristate acetate and opsonized zymosan were conducted to assure that these components did not affect PMN metabolism.

Preparation of metabolic inhibitors. 2-Heptyl-4-hydroxy quinoline N-oxide (HQNO, Sigma) and antimycin A (Sigma) were dissolved in DMSO (Sigma). Final concentrations (in the incubation tubes) were 120 μM and 30 μM, respectively. Amobarbital (Sigma; final concentration, 20 μM) and KCN (Fisher Scientific; final concentration, 1.0 mM) were dissolved in distilled water. The addition of KCN increased the pH of the reaction mixture to 9.05. This was corrected by dissolution of the KCN in Tris buffer. Control experiments demonstrated that pH of 9.05 reduced binding in the presence and absence of KCN by 8.0%.

FIG. 1. Association of N. gonorrhoeae with human neutrophils at various time points in the presence (C) and absence (O) of 1 mM KCN. The incubation mixture was 1 ml, including 5 x 10^6 gonococci, 2.5 x 10^6 neutrophils (20:1 ratio), and 0.2 ml of 3% bovine serum albumin. Samples were run at 37°C. The adherence was determined by differential centrifugation, followed by radioactive counting of the supernatant. Adherence is expressed as a percent of standards. Background (A) was determined as described above, except the incubation mixture had no neutrophils; addition of KCN had no effect on background attachment (data not shown). Data are from eight experiments, and bars represent standard error of the mean. KCN significantly (P < 0.05) inhibited attachment of gonococci to neutrophils after 10 min.

Statistics. Data were analyzed using Student's t test. Results were considered significant at P values of <0.05.

RESULTS

First we studied the opsonin-independent binding of N. gonorrhoeae to human neutrophils as measured by the cell association of viable 14C-labeled organisms. It should be emphasized that the assay does not detect whether bacteria have actually been ingested. As shown in Fig. 1, 26% of the bacterial inoculum became cell associated by 40 min of incubation. Adherence increased minimally after this time. KCN (which inhibits gonococcal energy metabolism [21]) significantly inhibited the gonococcal association with human neutrophils (Fig. 1). These results could also be demonstrated with gonococcal strain MS11 (data not shown).

To determine whether these effects were unique to neutrophils, other cell types were examined. The human promyelocytic leukemia cell line (HL-60) can be differentiated toward neutrophil function and morphology with DMSO (9). Association of N. gonorrhoeae decreased with cell differentiation to a level nearly identical to that observed with neutrophils (Fig. 2). KCN inhibited binding of gonococci to both undifferentiated and differentiated cells by 30 and 52%, respectively. Similar results were observed with HeLa cells (data not shown).

To determine whether the effects of KCN were primarily on the bacteria or on the phagocytic cells, we examined binding of heat-killed organisms (Fig. 3). Heat-killed gonococci attached to HL-60 cells in smaller numbers than did viable organisms. However, KCN had no effect on their uptake. To determine whether these results were peculiar to opsonin-independent attachment, we examined the binding of opsonized S. aureus in the presence and absence of KCN. S. aureus cells are bound poorly unless opsonized with...
immunoglobulin and complement (24). KCN had no effect on the association of opsonized S. aureus (Fig. 4).

KCN inhibits several aspects of gonococcal function, including glucose metabolism and electron transport (15, 21). Accordingly, other inhibitors of bacterial electron transport were examined. These inhibitors reduced bacterial O2 consumption by from 70 to 100% (Fig. 5). However, neither antimycin A or HQNO interfered with bacterial attachment. To further determine whether the results observed related to use of O2 as a terminal electron acceptor, we compared attachment of gonococci under aerobic and anaerobic conditions. Binding was reduced by more than 35% under anaerobic conditions (Fig. 6). There was an additional reduction in association when KCN was present, even under anaerobic conditions.

It seemed possible that KCN was interfering with protein synthesis required for formation of ligands important in attachment. However, when the experiments were conducted in the presence of 100 µg of chloramphenicol per ml to block new protein synthesis (13), no effect on binding was noted (data not shown). Similarly, if a continuous supply of ligands was required for attachment, bacteria might be released from phagocytes after addition of KCN. However, KCN did not reverse binding which had already occurred (Fig. 7). Only a small increase in adherence occurred after addition of KCN to gonococcus-PMN complexes. To exclude the possibility that these bacteria had been ingested and the label was degraded, experiments were repeated using undifferentiated HL-60 cells (incapable of phagocytosis); similar results were obtained. Experiments were also

FIG. 2. Association of N. gonorrhoeae with HL-60 cells in the presence and absence of KCN. Experiment was performed as described for Fig. 1 except that HL-60 cells were substituted for neutrophils. The ratio of gonococci to HL-60 cells was 20:1. Symbols: , undifferentiated gonococci; , differentiated gonococci with KCN; , cells differentiated toward neutrophil morphology and function with 1.3% DMSO with KCN; , cells differentiated toward neutrophil morphology and function with 1.3% DMSO without KCN; , background. Bars represent standard error of the mean.

FIG. 3. Association of heat-killed N. gonorrhoeae with undifferentiated HL-60 cells in the presence and absence of KCN. The experiment was performed as described for Fig. 2 except that heat-killed gonococci were subjected to 100°C for 15 min before being added to the incubation mixture. Other symbols: , association of viable N. gonorrhoeae with HL-60 cells; , background.

FIG. 4. Association of opsonized S. aureus cells with human neutrophils in the presence ( ) and absence ( ) of KCN. Bacteria-to-neutrophil ratio was 20 to 1. S. aureus cells were opsonized in pooled human serum for 30 min at 37°C. , background.

FIG. 5. Effects of respiratory chain inhibitors on gonococcal oxygen consumption (open bars) and association of gonococci with human neutrophils at 40 min (hatched bars). Bars are percent inhibition relative to control. The results are the mean of four or five separate experiments in duplicate; standard error was <10% of the mean. Inhibition of binding was significant (P < 0.05) for all agents tested except antimycin A and HQNO.
performed in the presence of cytochalasin B, a compound
known to inhibit phagosome formation by PMNs (27). The
effect of KCN on the gonococcus-neutrophil association was
unchanged by the addition of cytochalasin B (data not
shown).

To confirm the findings obtained using the binding assay
described above, we examined neutrophil LDL. Neutrophil
LDL reflects the magnitude of membrane perturbation,
which is related to the number of particles attached to the
cell (25). Decreased LDL was observed when KCN and

amobarbital were used (Fig. 8). Each inhibitor reduced LDL
in proportion to its effect on association, as judged by the use
of radiolabeled bacteria.

**DISCUSSION**

Binding is a critical step in *N. gonorrhoeae* infection (28,
30; reviewed in references 5 and 34). Many strains of *N.
gonorrhoeae* attach to phagocytic cells in the absence of
host-derived opsonins (16, 25, 30, 32, 33). The identity of
ligands which mediate binding to phagocytic (and nonphagocy-
tic cells) has been the subject of intense interest. Pili
(10-12, 23, 28, 31) and outer membrane proteins (16, 25, 30,
32, 33) have been most vigorously studied. Other data have
suggested that outer membrane protein II (PII) is of critical
importance to the attachment of gonococci to human neu-
rophils (16, 25, 26, 32). This hypothesis has been particu-
larly attractive because of the possibility that the genetic
diversity and phase variation of PII (2, 36) allow gonococci
to escape neutrophil attack. A role for PII, PII, pili, and
lipopolysaccharide in the interaction of gonococci with non-
phagocytic cells is still being explored (reviewed in refer-
ences 5 and 34).

We have previously demonstrated an increase in gonococ-
cal metabolism under conditions of physiologic significance
(3, 4, 6, 8; K.-S. Fu, D. J. Hassett, and M. S. Cohen,
submitted for publication). Specifically, O₂ consumption by
gonococci increases as the cells are forced to interact with

**FIG. 6.** Association of *N. gonorrhoeae* with human neutrophils
under aerobic and anaerobic conditions and in the presence and
absence of KCN. Association was assessed at 40 min. Results are
the mean of four separate experiments. In the absence of oxygen or
with the addition of KCN, binding of gonococci was significantly
inhibited (*P* < 0.01).

**FIG. 7.** Association of *N. gonorrhoeae* with human neutrophils
only (C) and with KCN added at various times during the incubation
period (●: 0, KCN added at the start of incubation; 10, 20, KCN
added 10 and 20 min, respectively, after the start of incubation). ▲,
Background.

**FIG. 8.** Effect of respiratory chain inhibitors on luminol-depen-
dent luminescence of human neutrophils. A 2-ml volume containing
1.0 × 10⁸ neutrophils and 1.0 × 10⁵ M luminol was used. HQNO and
antimycin A could not be evaluated because they affected control
experiments, implying a direct effect on the neutrophil. Data are
representative of three separate experiments. Curves: PMNs plus
gonococci (A) without inhibitor; (B) plus KCN; (C) plus amobar-
bital.

Background.
human neutrophils. In the present study, using piliated, opaque organisms, the role of bacterial metabolism on the attachment of *N. gonorrhoeae* to several different mammalian cells was examined. Inhibition of bacterial oxygen consumption with potassium cyanide, other (but not all) electron transport inhibitors, or anaerobiosis significantly decreased binding to human neutrophils, undifferentiated and differentiated HL-60 cells, and HeLa cells. KCN has minimal effect on neutrophil metabolism (6). KCN had no effect on the association of *S. aureus* (which requires opsonins) or heat-killed *N. gonorrhoeae*.

Experiments were conducted to determine the mechanism of the effect(s) of KCN. Energy is required for protein synthesis (5) and could be involved in the initiation and continuation of gonococcal binding. However, chloramphenicol did not interfere with adherence of gonococci to neutrophils when it was present in concentrations known to inhibit protein synthesis. Furthermore, the addition of KCN after gonococcal binding did not facilitate release of the organism as would be predicted if a continuous supply of ligands was necessary. Gonococcal electron transport is likely involved in the generation of a proton electrochemical gradient through proton motive force (18). Watt and Ward have previously shown that pH and charge can affect binding of gonococci to nonphagocytic cells, with emphasis on individual ligands (34). Although KCN and amobarbital reduced oxygen consumption and binding, antimycin A and HQNO caused a reduction in oxygen consumption while inhibiting cell association little. However, oxygen consumption may not be an accurate measure of the generation of an electrochemical gradient under all circumstances. It seems possible that some (but not all) metabolic inhibitors affect cell surface charge. Gonococci form high-energy "capsular" polyphosphates of unknown biologic importance (22); it seems possible that energy-dependent polyphosphates could also affect attachment.

Our knowledge of the interaction between gonococci and host cells has gained increasing complexity in recent years. Microenvironmental conditions including (but not limited to) growth rate (20), iron (17, 35) and oxygen (4, 7, 14) concentration, and serum (3) all evoke phenotypic changes, some of which may play a role in attachment to phagocytic and nonphagocytic cells. The contribution of bacterial metabolism and metabolism under stress (e.g., during neutrophil attack) is also likely to be relevant. We have demonstrated that beyond simply producing surface ligands, KCN-dependent gonococcal metabolism is required for maximal attachment to several types of mammalian cells.

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**LITERATURE CITED**


