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Cell Envelope of *Neisseria gonorrhoeae*: Phospholipase Activity and Its Relationship to Autolysis

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The relationship between conditions which permit or inhibit cell lysis and those which promote phospholipid hydrolysis in *Neisseria gonorrhoeae* was investigated. Suspension of exponential-phase gonococci in buffer in the absence of divalent cations resulted in autolysis but not in phospholipid hydrolysis. The addition of Ca^{2+} or Mg^{2+} to the buffer inhibited autolysis and markedly stimulated the hydrolysis of phosphatidylethanolamine. Incubation of cells in buffer at pH 6 inhibited both autolysis and phospholipid hydrolysis.

The autolysis of *Neisseria gonorrhoeae* has been intensively investigated (3, 4, 6, 12), but the biochemical mechanisms of the autolytic process have not been resolved. When exponential-phase cultures of *N. gonorrhoeae* JW-31 are suspended in buffer at an alkaline pH, cell lysis occurs. Cell lysis can be prevented either by adding divalent cations to the buffer or by decreasing the pH to 5.5 to 6.0 (12). Although divalent cations and acid pH prevent cell lysis, they do not prevent loss of viability (manuscript in preparation). When cultured in media containing limiting glucose, gonococci lose viability and as a result of glucose depletion, often exhibit lysis after growth ceases (6). Gonococci release free fatty acids (FFA) and lysophosphatidylethanolamine (LPE) into the medium during growth (11), which suggests activity of phosphatidylethanolamine (PE) phospholipase. The concentration of LPE in lipid extracts of cells increases with increased culture age (2). The relationship between loss of viability, cell lysis, and hydrolysis of membrane phospholipids after the cessation of growth has not been assessed. The present study was undertaken to investigate the relationship between phospholipid hydrolysis and autolysis of *N. gonorrhoeae* in buffer and to determine the effect on phospholipid hydrolysis of conditions such as pH and the presence of divalent cations which influence the autolytic process.

N. gonorrhoeae JW-31 (colonial type 4) was employed in these studies. The properties and maintenance of this strain have been previously described (6, 8). The procedures for determining the rates of autolysis and peptidoglycan hydrolysis were those described by Wegener et al. (12).

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For studies on phospholipid hydrolysis, cells were grown at 37°C on a gyratory shaker to the log phase (125 Klett units; no. 54 filter) in liquid basal medium containing a growth factor supplement (1%, vol/vol), $NaHCO_3$ (420 mg/liter), and glucose (0.5%, wt/vol), as previously described (7). The final pH of the medium was 7.2. Cells were harvested by centrifugation, suspended in fresh, sterile glucose-containing medium containing 5 μCi of [^{3}H]acetate (sodium salt) per ml (686 mCi/mmol; New England Nuclear Corp., Boston, Mass.), and incubated for 40 min. The cells were again harvested by centrifugation, resuspended in fresh, sterile glucose-containing medium, and incubated for an additional 10 min to facilitate incorporation of any residual label in the intracellular pools. In the presence of glucose, gonococci use exogenous acetate primarily for lipid biosynthesis (8). In the present study, 97% of the ^{3}H label in phospholipids was found in the fatty acid acyl groups after deacylation by mild alkaline methanolysis as described by White (13).

To assess the extent of phospholipid hydrolysis, 10-ml portions of the labeled culture were centrifuged, and the cell pellets were suspended in 50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer (pH 8.5 or 6.0) with appropriate additions. After incubation for 2 h at 37°C, the suspensions were centrifuged and the cell pellets were extracted by shaking with 10 ml of a 1:1 (vol/vol) mixture of chloroform-methanol in 125-ml capped flasks at 25°C for 60 min. Residual cell material was removed by centrifugation, and the chloroform-methanol extract was evaporated to dryness at room temperature. The residue was dissolved in 0.12 ml of chloroform-methanol-water (2:1:0.1, vol/vol) and applied to precoated, glass-backed silica gel plates (5 by 20 cm, 0.25 mm thick; E. M. Labo-

ratories, Inc., Elmsford, N.Y.). The vials were washed twice with 0.05-ml portions of the solvent to insure maximal recovery of ^3H, and the washes were applied to the chromatogram.

Chromatograms were developed for 2 h in chloroform-methanol-water (65:25:4, vol/vol) and then air dried. A spot which migrated near the solvent front (R_f = 0.95) was identified as FFA by the thin-layer chromatography procedures of Kates (5) and by gas-liquid chromatography. FFA comprised >96% of the radioactivity in this spot. Fatty acids were visualized by short-wave ultraviolet light or by spraying with bromocresol green indicator. Phospholipids were detected by exposure to iodine vapor. PE, phosphatidylglycerol (PG), LPE, and linoleic acid (Sigma Chemical Co., St. Louis, Mo.) were employed as standards. Spots, visualized by iodine, that corresponded in R_f to the authentic standards were scraped off and placed in scintillation vials. These spots have been previously identified as PE, PG, and LPE by Senff et al. (9). One milliliter of methanol-water (2:1, vol/vol) and 10 ml of Triton X-100 scintillation fluid (1) were added to the vials, and radioactivity was determined in a liquid scintillation spectrophotometer. Samples were counted with an efficiency of 27 to 28%; no additional quenching was observed in the presence of unlabeled phospholipids and FFA.

The percentage of total ^3H present on the chromatograms that was associated with each of the lipid classes was determined before (zero time) and after incubation of cells. Essentially all of the ^3H on the chromatogram (ca. 98%) was localized in four major spots having R_f values corresponding to those of PE, PG, LPE, and FFA. The relative amounts of PE, PG, LPE, and FFA in strain JW-31 were similar to those reported for other strains of *N. gonorrhoeae* (9, 10). At zero time, cells contained 73% PE, 22% PG, 2% LPE, and less than 1% FFA.

Table 1 shows the relationship between autolysis, peptidoglycan hydrolysis, and phospholipid hydrolysis in *N. gonorrhoeae* JW-31 suspended in HEPES buffer. Autolysis occurred at pH 8.5 and at pH 6.0 in the presence of 10 mM ethylenediaminetetraacetic acid, disodium salt. The addition of Mg^{2+} or Ca^{2+} (20 mM) prevented cell lysis at pH 8.5. No cell lysis was observed at pH 6.0 in the presence or absence of divalent cations. However, peptidoglycan hydrolysis was observed under both autolytic and nonautolytic conditions (12).

The data indicate that transfer of exponential-phase cells to buffer results in hydrolysis of membrane phospholipids as evidenced by a decrease in the percentage of PE and PG and by an increase in the percentage of LPE and FFA. However, phospholipid hydrolysis and autolysis are separable events. The phospholipase activity of cells suspended in HEPES buffer at pH 8.5 in the absence of divalent cations was minimal, whereas these conditions permitted cellular autolysis to occur. Addition of Ca^{2+} or Mg^{2+} to this buffer markedly stimulated phospholipid hydrolysis but inhibited autolysis. Phospholipid hydrolysis and the accumulation of FFA under nonautolytic conditions may have been responsible for the marked decrease in viability previously observed (3, 12). The stimulation of phospholipase activity in intact cells by Ca^{2+} was in agreement with studies by Senff et al. (9) on phospholipase A activity in cell envelope preparations of *N. gonorrhoeae* 2686. In such preparations, phospholipase A was more active with PE than with PG as a substrate, exhibited optimum activity at pH 8 to 9, and required Ca^{2+}. Our results also showed a preferential hydrolysis of PE. The absence of phospholipid hydrolysis at pH 6.0 in the presence or absence of divalent cations suggests that phospholipase A was in-

TABLE 1. *Relationship between autolysis, peptidoglycan hydrolysis, and phospholipid hydrolysis in N. gonorrhoeae JW-31*[a]

Conditions[b]	Autolysis	Rate of autolysis $k \times 10^{-3}$	Peptidoglycan hydrolysis (%)	[^3H]acetate recovered (%) as:[c]			
				PE	PG	LPE	FFA
Time zero	−			72.6	22.4	2.5	1.1
HEPES (pH 8.5)	+	17.0[d]	20[d]	71.0	21.7	1.3	4.5
HEPES (pH 8.5) + $MgCl_2$	−	<0.2[d]	19[d]	62.1	21.7	2.8	12.0
HEPES (pH 8.5) + $CaCl_2$	−	<0.2	19	36.2	17.4	7.3	36.9
HEPES (pH 6.0)	−	<0.2[d]	17[d]	72.6	23.5	1.3	2.0
HEPES (pH 6.0) + $MgCl_2$	−	<0.2[d]	17[d]	ND	ND	ND	ND
HEPES (pH 6.0) + EDTA	+	11.4	ND	70.5	25.1	1.2	2.2

[a] [^3H]acetate-labeled cells were incubated at 37°C for 2 h under conditions described in the text. After extraction of the cell pellets, ^3H distribution in lipids was determined as described in the text. ND, Not determined.

[b] $MgCl_2$ and $CaCl_2$ each at 20 mM, and ethylenediaminetetraacetic acid (EDTA) at 10 mM when present.

[c] Values represent averages of four experiments.

[d] Data from Wegener et al. (12).

active at this pH. This is of interest because gonococci can grow in media buffered with HEPES at pH 6, and it suggests that phospholipase A may not be essential for growth. In summary, both hydrolysis of peptidoglycan and hydrolysis of membrane phospholipids can occur when gonococci are suspended in buffer, and hence may cause loss of viability and loss of integrity of the cell envelope. Neither event, however, can account for autolysis, and the mechanism by which divalent cations and acid pH inhibit cell lysis remains unknown.

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