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Interactions of Gonococci with HeLa Cells: Attachment, Detachment, Replication, Penetration, and the Role of Protein II

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Colony variants of *Neisseria gonorrhoeae* differ in their interactions with eucaryotic cells. When gonococci were cultivated with HeLa cell monolayers, the opacity phenotype (Op) became increasingly dominant in the subpopulation of organisms which adhered to the HeLa cells. Once bound, Op organisms displayed very low levels of detachment. Adherent Op gonococci exhibited generation times up to threefold greater than cultures containing gonococci in the absence of HeLa cells. In addition, the progeny of adherent Op organisms remained bound to the HeLa cell monolayer. Both piliated (P^+) and transparent (Tr) colony types attached to HeLa cells, but their progeny were retained less efficiently. Gonococci bound to HeLa cells were subjected to the bactericidal action of fresh rat serum and approximately 0.5 to 2.5% survived, irrespective of their opacity or piliation phenotype. Incubation with gentamicin resulted in a 10- to 50-fold further reduction in viability. Pretreatment of HeLa cell monolayers with the microfilament-disrupting agent cytochalasin *b* diminished gonococcal survival in either serum or gentamicin by up to eightfold. In contrast, cytochalasin *b* treatment did not decrease survival of the commensal organism *N. sicca*. The data suggest that very few gonococci are completely interiorized and a small proportion of adherent gonococci are partially protected from the soluble-phase environment by HeLa cells.

Attachment of *Neisseria gonorrhoeae* to epithelial cells of the urogenital tract is an early stage in tissue colonization (35). A major pathogenic event following attachment is penetration (invasion) of the mucosal lining, and organisms accumulate in the subepithelial connective tissue (9). Histopathological studies of infected tissue show replicating gonococci within mucosal cells (34, 36, 37) and on the surface of desquamated cervical (4) and urethral cells (34). Organisms attached to the cell surface often appear embedded or partially engulfed. Intracellular gonococci are observed largely within the mucus-secreting, columnar epithelial cells of the urethra, cervix, and fallopian tube. Gonococci appear to enter cells of the mucosal lining by a phagocytic mechanism, and bacteria can be seen surrounded by microvillous projections on the epithelial surface and in membrane-bound vacuoles within the cytoplasm (19, 36).

At least two gonococcal components are purported to facilitate attachment of the organism to epithelial cells: the outer membrane protein II (PII) and pili (27). Each of these adhesins arise from a family of genetically and antigenically related proteins. Both families exhibit phase variation, whereby synthesis of PII or pilin is switched on and off at a high frequency, and switching to different molecular forms occurs as part of this mechanism (22, 28) (for example, see Table 1). Presence of either of these proteins on the gonococcal surface is reflected in the colony forms. PII is often associated with opaque (Op) colonies, which exhibit extensive intergonococcal adhesions (15, 24, 25, 30). The pathogenicity of the gonococcus is influenced by the presence (P^+) or absence (P^-) of pili, opacity-associated PII, or both. Clinical isolates from distinct anatomical sites display a pattern with regard to colony opacity. Fallopian tube isolates and those taken from the cervix at the time of menstruation lack opacity (transparent [Tr]), whereas male urethral and midcycle cervical isolates tend to give rise to Op colonies (3, 11). When tested in human volunteers, it has been shown that piliation confers a significant increase in virulence on gonococci (12).

The fallopian tube organ culture (FTOC) model has been used extensively for in vitro studies of gonococcal invasion (18, 19, 36). Cultivation of gonococci with FTOC reveals that 1% of attached organisms penetrate by 8 h (34). A comparison of piliated and nonpiliated phenotypes show that whereas piliated organisms attach more efficiently, penetration is equally effective for both types once attached. Opacity-associated proteins and pili each provide for increased gonococcal adherence to a variety of host cells in vitro (10, 16, 23, 27). Electron micrographs of gonococci cultivated with monolayers of tissue culture cells show organisms enclosed in membrane-bound vacuoles within the cytoplasm (1, 20, 32, 33). Tissue culture cells provide a model for some of the events which are likely to occur during infection in vivo. In this report, we describe several events which take place during the first 5 h of cultivation of gonococci on HeLa cell monolayers. Gonococci of the Op phenotype predominate on the monolayer, and mechanisms which contribute to their dominance are defined. In addition, we explore the nature and extent of contact between adherent (Ad) gonococci and host cells.

MATERIALS AND METHODS

Bacteria. Strain R10 of *N. gonorrhoeae* and *N. sicca* were grown for 21 to 24 h on GC agar plates prepared as previously specified (24). Colonies were viewed by transmitted illumination from a substage mirror or a substage diffusing surface of a Bausch and Lomb microscope. The colony morphology and PII content of the organisms used in this study are presented in Table 1. The heat-denatured forms of three distinct R10 PIIs bear apparent molecular weights of 29,000, 30,500, and 31,500 and are designated PIIa, PIIb, and PIIc, respectively. The isoelectric points of

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TABLE 1. Colony variants of *N. gonorrhoeae* R10

Organism	PII content[a]
P^-Tr	None
P^+Tr	None
$P^{++}Tr$	None
P^-Op^+c	PIIc (MW 31,500; pI 7.5–8.0)
$P^-Op^{++}a$	PIIa (MW 29,000; pI 9.5)
$P^-Op^{++}ac$	PIIa, PIIc
$P^{++}Op^+b$	PIIb (MW 30,500)

[a] MW, Molecular weight.

PIIa and PIIc are approximately 9.5 and 7.5 to 8.0, respectively; the isoelectric point of PIIb was not determined. The isogenic variants designated $P^-Op^{++}a$ and $P^-Op^{++}ac$ are highly opaque (Op^{++}), dark, and yellow. The variant designated P^-Op^+c is moderately opaque (Op^+), of intermediate darkness, and yellow-gray. Tr colonies are light and clear or slightly bluish and lack PII. Piliated organisms are either P^+ or P^{++} (heavily piliated) as previously described (24).

Tissue culture. HeLa cells (ATCC CCL 2) were derived from a cervical carcinoma of human origin and are epithelial cell-like in morphology. Monolayers were maintained in Dulbecco modified Eagle medium (Flow Laboratories, Inc., McLean, Va.) (pH 7.4) containing 10% heat-inactivated zeta D serum (AMF Biologicals, Seguin, Tex.) with or without antibiotics (zeta-DME).

Gonococcus-HeLa cell cocultures. Monolayers of HeLa cells were grown to confluence in 35-mm (diameter) polystyrene wells (Flow Laboratories). Growth medium was replaced with antibiotic-free zeta-DME 18 h prior to inoculation with gonococci. Gonococcal colonies were swabbed off the agar plates, suspended in zeta-DME, vortexed vigorously to dissociate aggregates, and added to HeLa monolayers in a 1.0-ml volume at bacterium-to-HeLa cell ratios ranging from 10:1 to 1:10. When cytochalasin *b* (CB; Sigma Chemical Co., St. Louis, Mo.) was used, it was added to the HeLa monolayers 30 min prior to inoculation and maintained throughout cocultivation with gonococci at 5 μg/ml. Cocultures were incubated at 37°C with 6% CO_2 for the times indicated. Nonadherent (non-Ad) fluid-phase organisms were removed, and monolayers were washed twice and pooled. Gonococci attached to the HeLa monolayer were collected by addition of 0.25% trypsin (type II crude; Sigma) in phosphate-buffered saline. HeLa cells and Ad gonococci were detached by trypsinization and were mixed with an equal volume of zeta-DME. Pellets were collected after centrifugation for 10 min at 15,000 × *g*, suspended in zeta-DME, and vigorously vortexed for even dispersal. Dilutions of Ad and non-Ad organisms were plated on GC agar and colony forming units (CFU) were enumerated. Colony phenotypes were identified by light microscopy as described above, and CFU measurements were made for the colony phenotype specified.

Bactericidal agents. Serum was collected by cardiac puncture of nonimmune Sprague-Dawley rats, blood was pooled and allowed to clot for 2 h at room temperature, erythrocytes were removed by centrifugation, and serum samples were immediately frozen at −70°C. Following removal of non-Ad gonococci from the HeLa cell monolayer, Ad organisms were subjected to 10% fresh rat serum (FRS) in zeta-DME for 30 min at 37°C. For gentamicin treatment, Ad organisms were incubated for 3 h in 5 to 20 μg of gentamicin sulfate (Sigma) per ml in zeta-DME. After bactericidal treatments, monolayers plus Ad organisms were detached by trypsin and processed as usual.

RESULTS

Net distribution of gonococci. The distribution of P^-Op^{++} gonococci in Ad and non-Ad pools after 4.3 h of cocultivation with HeLa cell monolayers is presented in Fig. 1A. The proportions of gonococci in the Ad fraction were 9, 18, and 88%, at 1, 2, and 4.3 h, respectively. The extent of binding by P^-Tr organisms rose steadily over time but remained below 10% of the total population (Fig. 1B). Using a single inoculum consisting of a mixture of 98.5% P^-Tr and 1.5% P^-Op^{++} gonococci, the distribution of Tr and Op phenotypes in the Ad and non-Ad fractions was measured (Fig. 2). Examination of colony phenotypes in each pool revealed that Op gonococci accounted for a disproportionate share of the attached bacteria. Whereas Op colonies made up only 1.5% of the initial inoculum, they constituted 80% of the CFU in the Ad fraction by 4 h. The results indicated that opacity significantly enhanced the association between gonococci and HeLa cells.

Detachment of gonococci. Several factors can contribute to the overall distribution of a gonococcal phenotype in the Ad and non-Ad subpopulations. We sought to characterize those events which follow initial attachment, namely detachment and bacterial multiplication. Net flux of gonococci from the Ad to the non-Ad fraction was measured. Bacteria were allowed to attach to HeLa cell monolayers for 60 to 150 min, unbound organisms were removed, fresh zeta-DME was added to the monolayer, and incubation was continued for the times indicated (Fig. 3). The proportion of gonococci in the Ad phase decreased within 1 h after removal of the unbound inoculum. The sole exception was the Op^{++} variant $P^-Op^{++}ac$, which remained almost exclusively in the Ad fraction. A steady state was established for each phenotype, with the possible exception of P^+Tr, which showed a slight rise in the Ad pool after the initial drop. The data supported

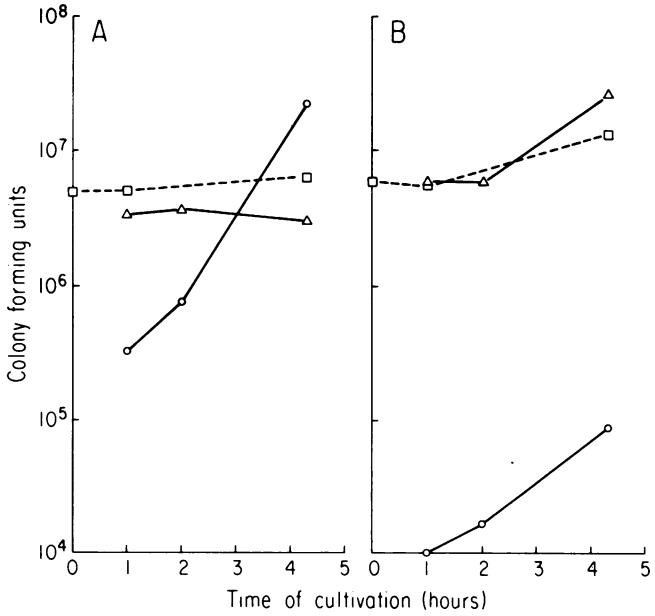

FIG. 1. Distribution of $P^-Op^{++}a$ (A) and P^-Tr (B) gonococci in Ad (○) and non-Ad (△) fractions. Gonococci were incubated on HeLa cell monolayers for the times indicated, and CFU of each fraction were measured. The control (□) represents gonococci in zeta-DME without HeLa cells.

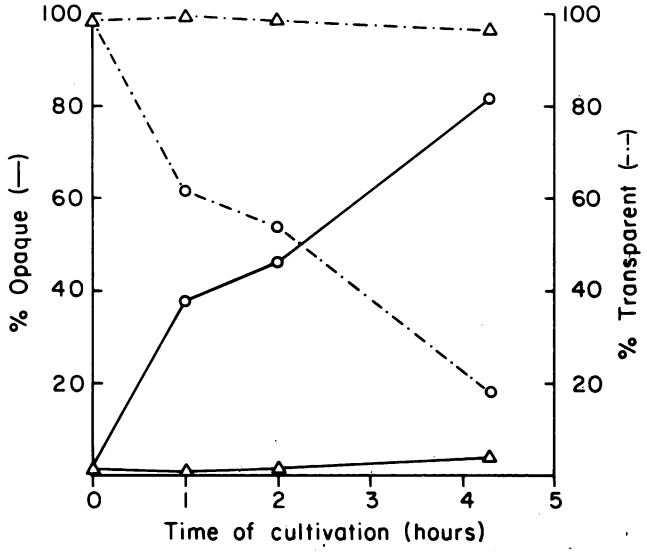

FIG. 2. Distribution of $P^-Op^{++}a$ and P^-Tr gonococci in Ad and non-Ad fractions. A single inoculum containing a mixture of P^-Tr and $P^-Op^{++}a$ gonococci was incubated on HeLa cell monolayers for the times indicated, and the CFU and colony phenotype of each fraction were measured. The proportion of Op and Tr organisms constituting the inoculum is indicated at 0 h. The proportion of Op and Tr organisms constituting the Ad (○) or non-Ad (△) fractions are plotted.

the postulate that redistribution of the Ad subpopulation was a consequence of detachment of gonococci from the HeLa cell monolayer. The Op^+c variant displayed intermediate levels of detachment, suggesting a direct correlation between detachment and opacity-associated PII.

A comparison of P^-Tr and P^+Tr gonococci indicated that piliation did not confer enhanced attachment to HeLa cells, and the percentage of organisms which adhered was less than 10% of the total population (data not shown). Although P^+Tr exhibited significant detachment in our studies, it should not be inferred that detachment is a general property of pilus-mediated adherence. It is possible that a cell abundant in pilus receptors, a gonococcus rich in pili, or both would lead to a more stable interaction.

Multiplication of gonococci. The Ad pool has the potential to increase in CFUs either by attachment of organisms originating in the non-Ad fraction or by replication (and retention) of organisms already bound to the HeLa cell surface. Both Tr and Op gonococci cultured with HeLa cells divide more rapidly than control cultures which lack HeLa cells (Fig. 1). This finding implies that gonococcal replication is stimulated in the presence of HeLa cells. To measure the multiplication of organisms bound to the HeLa cell surface accurately, exchange of organisms between the Ad and non-Ad subpopulations should be minimal. According to Fig. 3, the Op^{++} variant appears to fit this condition best because bound Op^{++} organisms do not readily detach. Bacteria were allowed to adhere to HeLa cell monolayers for 30 min, unbound organisms were subsequently removed and replaced with fresh zeta-DME, and cocultivation was continued (Table 2). Attached gonococci underwent one division between 0.5 and 2.5 h. From 2.5 to 5.0 h, the average generation time dropped to 35 min. Control organisms, which were incubated in zeta-DME without HeLa cell monolayers, displayed an extended lag phase and showed net growth only after 2.5 h. Between 2.5 and 5.0 h the doubling rate of the control culture was significantly slower (by threefold) than the generation time of Ad gonococci during a parallel period. The data indicated that, for the Op^{++} variant, attachment to HeLa cells was coupled to significant stimulation of gonococcal division. Furthermore, daughter cells were retained in the Ad population.

TABLE 2. Multiplication of gonococci on HeLa cell monolayers

Time of infection (h)	Gonococci (CFU/ml)[a]		
	Ad	Non-Ad	Control[b]
0.5	7,682	0	179,000
2.5	15,531	1,400	184,000
5.0	295,000	7,400	471,000

[a] The colony variant was $P^-Op^{++}a$. HeLa cells were inoculated at 0 min, and unbound gonococci were removed at 30 min (non-Ad is 0). This was a representative experiment.

[b] Controls were gonococci in zeta-DME lacking HeLa cells.

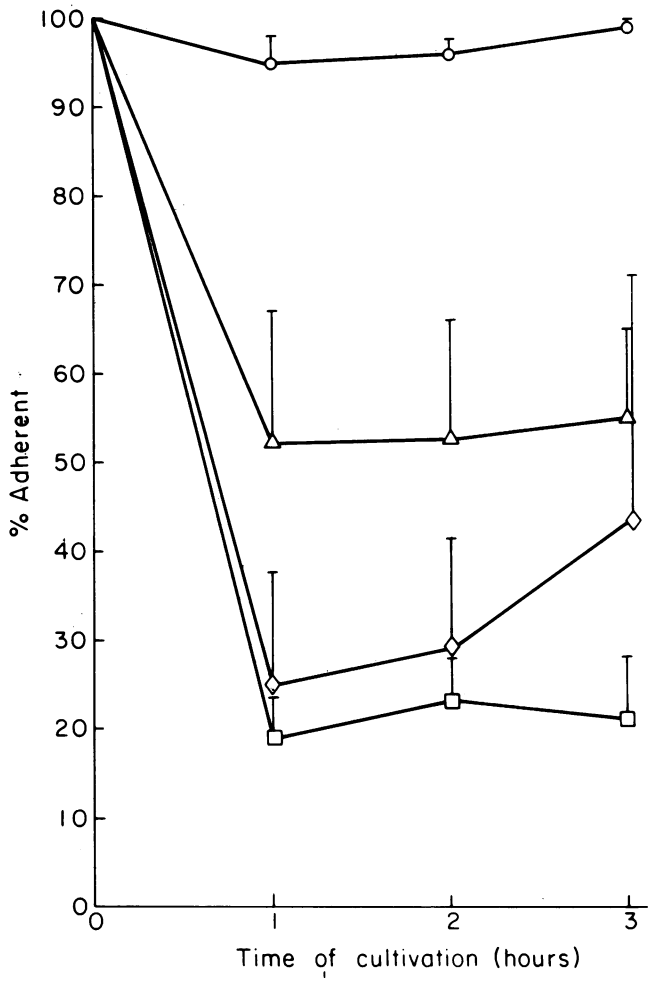

FIG. 3. Proportion of organisms in the Ad fraction after removal of unbound organisms at 0 h. At 0 h, 100% of gonococci were Ad; cultivation was continued, and the percentage of the total population which was Ad at various times is indicated. The colony variants represented are: $P^-Op^{++}ac$ (○), P^-Op^+c (△), P^-Tr (□), and P^+Tr (◇). Each point represents the mean of four experiments, and the SDs are indicated by bars.

The gonococcal colony forms each attained a different level of distribution between the Ad and non-Ad subpopulations (Fig. 3). There was a correlation between the propor-

TABLE 3. Correlation of adherence and replication[a]

Organism	Mean % (± SD) Ad	Mean % (± SD) growth increase per h	Generation time (min)
P⁻Op⁺⁺ac	96.2 ± 2.4[b]	196.3 ± 83.1[c]	61.5
P⁻Op⁺c	52.5 ± 12.3	170.9 ± 93.4	77.6
P⁻Tr	19.6 ± 5.1	163.1 ± 85.3	85.0

[a] Gonococci were allowed to attach to HeLa cells for 150 or 60 min, unbound organisms were removed, and cultivation was continued. Measurements were made beginnng 1 h after removal of unbound organisms and continued for 1 or 2 h, respectively (150 or 60 min initial attachment period). The percent growth increase per hour and generation times were calculated for the total population (Ad plus non-Ad fractions). The means and SDs of four experiments are presented.

[b] Differences between P⁻Op⁺⁺ac and P⁻Tr, and between P⁻Op⁺c and P⁻Tr were significant ($P < 0.001$) by the t test.

[c] The difference between P⁻Op⁺⁺ac and P⁻Tr was significant ($\alpha = 0.10$) by the Mann-Whitney U test.

tion of the population in the Ad phase and the growth rate of the total (Ad plus non-Ad) population (Table 3). Opaque organisms multiplied faster than Tr organisms in the presence of HeLa cells.

Nature of the association with HeLa cells. Gonococci invade host epithelium in vivo (9), and tissue culture monolayers have served as valuable in vitro models for the study of invasion by other microorganisms, such as *Shigella*, *Salmonella*, and *Chlamydia* spp. (5, 6, 14). Bacteria which completely penetrate epithelial cells are no longer accessible to microbicidal agents which do not permeate across the eucaryotic plasma membrane (7, 17). FRS is extremely cytolytic for *Neisseriae* spp., and evidence indicates that its action on the cell surface is mediated by complement (29). We sought to determine whether Ad gonococci are protected from the bactericidal action of FRS (Table 4). A small percentage of Ad gonococci retained their viability after treatment with FRS. Neither piliation nor opacity conferred a survival advantage (or disadvantage) on the organism.

If HeLa cells ingest gonococci and thereby render them inaccessible to FRS action, one would expect that disruption of the endocytic process would lead to fewer survivors of serum action. CB disrupts microfilament function and impairs endocytosis by professional phagocytes (2, 21, 38) and by epithelial cells presented with invasive bacteria (8, 13). HeLa cells subjected to CB rounded up and cell extensions retracted (data not shown). Immunofluorescent staining of Ad gonococci showed accumulation above the perinuclear region of untreated HeLa cells. In contrast, the majority of gonococci adhered close to the cell perimeters of CB-treated cells. Resistance to serum-mediated killing of gonococci bound to CB-treated and untreated HeLa monolayers is given in Table 5. The percentage of survivors was consistently lower if monolayers were pretreated with CB. CB did not hamper overall levels of adherence and had no effect on gonococcal viability. The ratio of percent survivors without CB (−CB) to percent survivors with CB (+CB) exceeded one and typically fell in a range between two- and eightfold. In contrast, a ratio of less than one (about 0.4) was observed with the nonpathogenic commensal *N. sicca* (Table 5). Ad gonococci were treated for 3 h with microbicidal concentrations of gentamicin, and less than 0.1% of the organisms survived (Table 6). This represents 10- to 50-fold less survival than that following serum exposure. Treatment of monolayers with CB caused an additional fivefold decrease in gentamicin resistance. The results indicated that resistance to both serum and gentamicin was depressed when HeLa cell cytoskeleton function was impaired. However,

TABLE 4. Survival of gonococci in FRS[a]

Organism	% Survivors in FRS among:	
	Ad gonococci[b]	Suspension controls[c]
P⁻Op⁺⁺a	1.22	<0.0016
P⁻Tr	1.37	<0.0016
P⁺⁺Tr	0.48	<0.0016
P⁺⁺Op⁺b	1.89	<0.0016

[a] Gonococci were cultivated with HeLa cell monolayers for 4 h before unbound organisms were removed and monolayers were treated with FRS. the percentage of organisms which survived FRS treatment was calculated from the number of adherent organisms before and after FRS treatment.

[b] There was a significant ($P < 0.001$) difference between the mean of adherent survivors in FRS and controls by the t test.

[c] In controls, gonococci were suspended in growth medium at a concentration of 10^6 CFU/ml, FRS was added to a concentration of 10%, incubation proceeded for 30 min at 37°C, and the number of surviving organisms was determined. Addition of HeLa cells at the time of FRS addition to control mixtures had no effect on the viability of gonococci (data not shown).

TABLE 5. Effect of CB on survival of adherent *Neisseria* spp. after FRS treatment

Organism (MOI)[a]	CB	CFU, 10^3		% Survival	−CB/+CB ratio[c]
		Ad[b]	Survivors in FRS		
P⁻Op⁺⁺a (0.32)	−	150	5.5	3.67	5.02
	+	350	2.6	0.73	
P⁻Op⁺⁺a (0.13)	−	950	6.2	0.65	1.10
	+	1,600	9.5	0.59	
P⁻Op⁺⁺a (1.0)	−	1,470	17.9	1.22	7.63
	+	3,290	5.3	0.16	
P⁻Op⁺⁺a (4.8)	−	21,000	456.0	2.17	4.43
	+	28,600	139.0	0.49	
P⁻Op⁺⁺a (5.0)	−	22,700	382.0	1.68	1.89
	+	37,300	332.0	0.89	
P⁻Tr (2.1)	−	195	2.6	1.37	1.62
	+	379	3.2	0.85	
P⁺⁺Tr (0.8)	−	35,100	169.0	0.48	3.43
	+	17,500	24.5	0.14	
P⁺⁺Op⁺b (0.6)	−	5,560	105.0	1.89	2.28
	+	10,200	85.0	0.83	
N. sicca (0.15)	−	1,870	6.2	0.33	0.38
	+	2,250	19.8	0.88	
N. sicca (0.02)	−	117	0.07	0.06	0.38
	+	117	0.18	0.16	

[a] MOI, Multiplicity of infection of bacterium to HeLa cells in inoculum.

[b] *N. gonorrhoeae* or *N. sicca* was allowed to adhere to CB-treated or untreated monolayers for 4 to 5 h, unbound bacteria were removed, and Ad organisms were subjected to FRS as described previously. The colony variants of *N. gonorrhoeae* are indicated.

[c] −CB/+CB represents the ratio of the percentage of Ad organisms surviving FRS treatment on HeLa cells without (−) or with (+) CB treatment. The percentage of Ad gonococci surviving FRS treatment was significantly greater in the absence than in the presence of CB by the Wilcoxon matched-pair test ($\alpha = 0.01$).

the actions of serum and gentamicin were distinct since the level of survival was significantly lower following gentamicin treatment.

We sought to determine whether protection of Ad gonococci could be increased by extending the cocultivation period. In these studies, gonococci were allowed to bind to monolayers for 2 to 3 h, and unbound organisms were removed and replaced with fresh zeta-DME. Cultivation continued for 1 or 2 days, after which time monolayers were subjected to FRS or gentamicin. Survival following gentamicin treatment remained unchanged from that of short-term cultures, at about 0.1 to 0.01% Ad gonococci (Table 7). However, an increase in the proportion of organisms surviving bactericidal FRS was apparent. Mean averages for percent serum survival of P^-Op^{++} gonococci in short-term (Table 5) and long-term (Table 7) cultures were 1.88% (standard deviation [SD], 1.15%) and 4.67% (SD, 1.45%), respectively.

TABLE 7. Effect of FRS and gentamicin on survival of Ad gonococci in long-term cultures

Expt no.	% Survival[a]	
	FRS[b]	Gentamicin
1	5.73	0.078
2	2.94	0.034
3	4.02	ND[c]
4	6.00	ND

[a] Gonococci ($P^-Op^{++}a$) were allowed to attach to HeLa cells for 2 to 3 h, unbound organisms were removed, and cocultivation was continued for an additional 19 h (experiments 3 and 4), or unbound organisms were again removed after 25 h, and cocultivation was continued for an additional 19 h (experiments 1 and 2). Treatment with bactericidal agents was as previously described. Gentamicin was used at 5 μg/ml.

[b] Percentage of gonococci surviving FRS in short-term (Table 5) and long-term (Table 7) cultures of Ad $P^-Op^{++}a$ were significantly different ($\alpha = 0.05$) by the Mann-Whitney U test.

[c] ND, Not done.

DISCUSSION

Monolayers of the epithelial cell-like HeLa cell line were used as a model for the study of interactions between gonococci and epithelial cells. Several factors contributed to the enrichment of Op gonococci in the Ad phase: high avidity for HeLa cells, low levels of detachment, HeLa cell-stimulated replication, and retention of daughter cells. Once organisms were attached, HeLa cell-mediated protection from microbicidal agents occurred independent of opacity and piliation.

Enumeration of gonococci by measuring CFU was chosen because it is a highly sensitive analysis (as few as 50 CFU can be detected), and it allows one to discern colony phenotypes readily. Since Op gonococci clump (25), and aggregation could influence CFU measurements, several controls were performed to ensure that single organisms were being quantified. The pattern of interaction of Op and Tr gonococci with HeLa cells was unaltered by filtration of the inocula through 1.0 μm pores. Neither trypsinization of organisms nor passage through a 30-gauge needle had an effect on the CFU yield. In addition, uptake of radiolabel by Ad gonococci after serum treatment displayed the same pattern of survival on CB-treated and untreated monolayers as that revealed by the CFU measurements reported above.

The colony variants under study exhibited strong association with HeLa cells when the basic PIIa was present. The role of PIIc and pili in mediating HeLa cell association was less well defined, but neither provided the adherence level attributable to PIIa. Inspection of $Op^{++}ac$ and Op^+c colonies by light microscopy revealed differences in morphology, with the Op^+c type being lighter and less intense in color. Although the analysis was subjective, it appeared that Op^+c was less opaque than $Op^{++}ac$. Detachment occurred most readily when PII was absent, and PIIc allowed for greater detachment than PIIac. Therefore, as the opacity of gonococci decreased, detachment from HeLa cells increased.

TABLE 6. Effect of CB on survival of Ad gonococci after gentamicin treatment

Gentamicin (μg/ml)	CB	% Gentamicin survivors among[a]:		+CB/−CB ratio[b]
		Ad gonococci	Controls	
5	−	0.0447	<0.0005	5.45
	+	0.0082		
20	−	0.0571	<0.0005	6.96
	+	0.0082		

[a] Gonococci (colony variant $P^-Op^{++}a$) were allowed to adhere to CB-treated or untreated monolayers for 4 to 5 h, unbound bacteria were removed, and Ad organisms were subjected to gentamicin for 3 h. The percentage of organisms which survived gentamicin was calculated from the number of Ad organisms before and after treatment. Controls for survival of gonococcal suspensions in gentamicin are indicated.

[b] −CB/+CB represents the ratio of the percentage of Ad organisms surviving gentamicin treatment on HeLa cells without (−) or with (+) CB treatment.

The Op phenotype exhibited an altered growth rate in our tissue culture model. Growth of both Op and Tr gonococci was enhanced in the presence of HeLa cells; however, replication of Op organisms exceeded that of the Tr type. There was an inverse correlation between the proportion of the population in the Ad phase and the net generation time of the total (Ad plus non-Ad) population. Opacity and adherence were inseparable in this study, and therefore it is difficult to distinguish between two reasonable interpretations for the enhanced growth of Op gonococci: a microenvironment favorable for reproduction of all gonococci adherent to the HeLa cell surface or opacity-specific stimulation arising from HeLa cells. The progeny of Ad and replicating Op gonococci remained bound to the monolayer. This could be the consequence of intergonococcal adhesions, which give rise to the Op colony form (30). The daughter cell has the option of attaching directly to the HeLa cell surface or adhering indirectly by binding to another gonococcus, which in turn makes direct contact with the monolayer. Although this concept is not new (26), our experiments provide a working model. In conclusion, low levels of detachment, combined with enhanced multiplication and retention of daughter cells, contribute to the ability of Op gonococci to colonize the HeLa cell surface extensively.

Tissue culture cells have served as valuable tools for studying the invasive capacity of several other microorganisms (5, 6, 8, 13), and for this reason we sought to determine whether HeLa cells provide a useful model for gonococcal invasion. The data indicated that a small proportion (0.5 to 2.5% or more) of Ad gonococci survived the bactericidal action of FRS. Although the various colony phenotypes attached to HeLa cells with different levels of efficiency, once they attached, survival in serum was approximately the same for all types. In FTOC, both P^+Tr and P^-Tr colony types penetrate at comparable levels once the organism is firmly attached to the surface (19, 34), and in this

way, our findings parallel the FTOC invasive model. However, less than 0.1% of organisms adhering to HeLa cells survived gentamicin treatment, which is substantially lower than the 1.0% survival rate reported for Ad gonococci cultivated in FTOC and subjected to spectinomycin (34). In addition, long-term cultivation of gonococci with HeLa cells failed to increase the proportion of Ad organisms resistant to gentamicin. If survivors of gentamicin represent organisms which have completely penetrated HeLa cells, then the level of invasion is significantly less than that observed with columnar epithelium of the FTOC. Our data support some degree of penetration of HeLa cells by gonococci. However, the level of invasion is low and therefore limits the usefulness of HeLa cells as an experimental model for gonococcal invasion. One distinction between the two microbicidal agents used in this study is that long-term cocultivation of gonococci on HeLa cells led to a small but significant increase in the extent of survival in serum but not in gentamicin. Another difference between gentamicin and the complement components of FRS is molecular size, and this may have bearing on their accessibility to partially engulfed or deeply embedded gonococci. The results obtained with CB support a key role for the HeLa cell cytoskeleton in protection. Gentamicin-resistant organisms may represent those few which have completely invaded the HeLa cell, whereas those surviving serum are more likely to be present on the cell surface in a partially engulfed state.

The action of CB on professional phagocytes is well documented. By disrupting microfilament function, pseudopod extension is blocked and phagocytosis is diminished (2, 21, 38). Uptake of invasive bacteria by epithelial cell monolayers is retarded in the presence of CB (8, 13), and treatment of FTOC with CB reduces gonococcal penetration by fivefold (34). This drug had profound effects on HeLa cell morphology, causing cells to round up and edges to retract. The survival of Ad gonococci after either FRS or gentamicin treatment was reduced on CB-treated monolayers by up to eightfold. CB treatment did not increase the susceptibility to FRS of the commensal *N. sicca*. Our data indicated that the function of the HeLa cell cytoskeleton enhanced the survival of the attached gonococcus. The distinctive behavior of nonpathogenic *Neisseria* spp. provides further support that the association between gonococci and HeLa cells is highly specific.

A speculative scenario for uncomplicated gonorrhea has been proposed whole or in part by several investigators. The sites of infection under consideration are the male urethra (31, 34, 35) and the ectocervix or squamocolumnar junction (4, 26, 31). Gonococci are embedded and partially engulfed on the epithelial cell surface (4, 26, 31, 34, 35). The attached organisms multiply (4, 26, 34), forming microcolonies on urethral cells (34). Daughter cells are retained by those organisms possessing opacity-associated PII (26). An effective mode for transmission to a new host may be desquamated epithelial cells studded with gonococci (4) which are replicating (31). There is controversy as to whether squamous cells of the cervix are truly invaded by gonococci (4, 9, 34). Our data appear to resemble those events best which histopathological studies have suggested to be the hallmarks of urethral and cervical cell infection (4, 26, 31, 34, 35). We observed increased multiplication of adherent gonococci, and the daughter cells of the Op variant were retained in the Ad fraction. The data suggest that some degree of partial engulfment occurs on the HeLa cell surface. The preponderance of Op colony types in clinical isolates taken from the urethra or cervix at midcycle (3, 11) further supports the parallels between the gonococcus-HeLa cell model and events in vivo. The value of an in vitro model for the interaction of gonococci with epithelium is that it can potentially lead to a better understanding of molecular mechanisms which underlie gonococcal infection.

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