INFECTION AND IMMUNITY, June 1975, p. 1352-1361

Vol. 11, No. 6
Printed in U.S.A.

Studies on Gonococcus Infection

X. Pili and Leukocyte Association Factor as Mediators of Interactions Between Gonococci and Eukaryotic Cells In Vitro

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Received for publication 20 January 1975

Two independent gonococcal surface components, pili and leukocyte association factor, appear to mediate in vitro interactions of *Neisseria gonorrhoeae* with tissue culture cells and human peripheral blood leukocytes, respectively.

Virulence of *Neisseria gonorrhoeae* has been correlated with formation of characteristic colony forms in vitro (9, 10), and with pilation of those organisms comprising the "virulent colony types" (7, 18). These demonstrations raised the question of possible causal relationships between pilation and virulence. An obvious possibility is that pili endow the gonococcal surface with characteristics influential in the interactions between these bacteria and cells of the host. Our initial experiments showed that pilated gonococci adhere to tissue culture cells more avidly than nonpilated organisms (15). That observation was extended to show that pilated gonococci also attach to several other types of eukaryotic cells (fallopian tubal epithelium [23], buccal cells [13], and human sperm [6]) more readily than nonpilated gonococci. Those studies suggested that pili enhance the "anchoring" of gonococci to mucosal sites and, hence, promote establishing infection by the organism. Similar mechanisms of virulence have been shown for diverse bacteria including streptococci (4), *Shigella* sp. (3), and *Escherichia coli* (8, 24).

Restriction of phagocytosis was another possible role envisioned for pili with regard to their influence on gonococcal virulence. Two groups have presented evidence to support this hypotheses (12, 13, 21), but our studies suggested that pili play minor roles as determiners of interactions between peripheral blood leukocytes and gonococci (16, 17, 19, 20). Our contention rested largely on demonstration that nonpilated gonococci could exhibit levels of association with leukocytes that were widely divergent; some nonpilated organisms (T4*) showed levels of association with leukocytes higher than those for pilated gonococci whereas other nonpilated forms (T4) exhibited very low level association with leukocytes. These findings led to the suggestion that an additional, nonpilus surface factor was primarily the determiner of interactions between gonococci and peripheral blood leukocytes (19).

The present study provides additional evidence for the existence of the second, nonpilus factor on gonococci. This leukocyte association factor may be present or absent on either pilated or nonpilated gonococci. The factor plays little or no role as a mediator of interactions between gonococci and tissue culture cells. Pili, on the other hand, influence attachment of gonococci to tissue culture cells but do not appear to be major factors as mediators of interactions between these organisms and leukocytes.

MATERIALS AND METHODS

Gonococci. The majority of studies were carried out with gonococci from strains MS11 and F62 whose derivation has been previously described (15, 18, 19). Other gonococci used are noted below. These organisms were propogated as essentially pure cultures of pilated T2 and nonpilated T4 preparations by daily serial passage on GC agar (GC agar base containing 1% IsoVitaleX, Baltimore Biological Laboratories, Baltimore, Md.) and incubation at 36 C in a CO_2 incubator. Daily passage was carried out by inoculation of an agar plate with a single colony chosen for its typical colonial form with a dissecting microscope.

Derivation of nonpilated organisms. Typical type 2 GC colonies were visually selected after 21 to 24 h of growth on GC agar and were marked; these GC agar plates were then reincubated for additional periods. After either 48 or 72 h of total incubation time, organisms from each of the previously selected, marked colonies were transferred with a loop to a fresh GC agar plate which was incubated for 21 to 24 h. After this passage one typical type 4 colony present on each plate was used for passage which, in nearly all instances, resulted in a pure colony type 4 culture as judged by dissecting microscopy inspection.

Incubation of human leukocytes and gonococci. Monolayers of partially purified leukocytes were prepared, as previously described in detail (19), by

sedimenting erythrocytes from heparinized whole blood, washing the leukocytes with both ammonium chloride and Medium 199 (M199) (Grand Island Biological Co., Grand Island, N. Y.), and allowing them to attach to 18-mm round cover slips. After washing off unattached cells, the leukocyte-laden cover slips were overlaid with 1 ml of a suspension of gonococci in M199 containing 0.01% bovine serum albumin. The multiplicity used for these incubations was approximately 50 GC/leukocyte. After 15 min of incubation on a rotating platform at 37 C in a CO_2 incubator, the leukocyte-laden cover slips were washed with 0.5% NaCl, allowed to air dry, fixed in methanol, and stained with Giemsa stain. Association of gonococci with leukocytes was judged microscopically (Zeiss Photomicroscope II equipped with ×63 phase contrast oil immersion objective). Neutrophils that had attached or ingested gonococci were scored and the percentage of such neutrophils in each preparation was assessed.

Incubation of gonococci with HeLa cells. HeLa cells were obtained through the generosity of Donald Summers and were propagated as suspension cultures in Eagle minimal essential medium (Microbiological Associates, Bethesda, Md.) containing 10% heat-inactivated fetal calf serum (Grand Island Biological Laboratories, Grand Island, N.Y.), glutamine, sodium bicarbonate, *N*-2-hydroxyethyl-piperazine-*N'*-*Z'*-ethanesulfonic acid buffer, penicillin, and streptomycin. The cells were washed four times with minimal essential medium containing 2% calf serum, *N*-2-hydroxyethyl- piperazine - *N'* - *Z'* - ethanesulfonic acid buffer, and sodium bicarbonate but devoid of penicillin, streptomycin, and glutamine. The washed cells were resuspended to a concentration of 5.5 × 10^6/ml in the latter medium. Aliquots (0.9 ml) of the HeLa cell suspensions (5 × 10^6 cells) were added to snap-top polypropylene tubes. To these HeLa cells were added 0.1-ml aliquots of suspensions of gonococci (10^3 to 10^4 gonococci) in the same medium. Controls consisted of 0.9 ml of medium plus 0.1 ml of GC suspensions. Immediately after mixing, aliquots were removed from each tube (HeLa cell containing and controls) for plating on GC agar plates. The tubes were then incubated with rotation (18 rpm) at 36 C for 30 min at which time portions were again removed for plating and enumeration of total colony-forming units of gonococci. All tubes were then centrifuged at 45 *g* for 5 min and the supernatants were carefully removed. The same amount of medium removed as supernatant was added to each tube which was then mixed vigorously to resuspend the pellets. Both the supernatants and the resuspended pellets were plated for colony-forming units enumeration.

Light macro- and micrography. Photomicrographs of colonies were taken with a Zeiss Photomicroscope II utilizing a 6.3X objective and bright field illumination obtained after removing the top portion of the substage condenser. Larger portions of agar cultures were photographed with the transmitted illumination obtained from the base unit of a Baush & Lomb dissecting microscope. These photographs were obtained with a Canon FTb camera equipped with a 50-mm Macro FD lens and an FD bellows. All light photographs were recorded on Plus X film (Eastman Kodak Co., Rochester, N.Y.)

Electron microscopy. Electron photomicrographs were obtained with a Siemens Elmiskop 1A after negative staining or freeze-fracture, freeze-etch preparation of specimens as previously described (14, 15).

RESULTS

General comments. The present study was undertaken as an attempt to understand relationships between pilated gonococci and the nonpilated organisms that exhibited two distinctly different levels of association with human leukocytes in vitro. The initial experimental design was to determine leukocyte association characteristics of nonpilated organisms that were derived directly from pilated gonococci. This is easily accomplished as described below. The behavior of these "newly derived" nonpilated organisms was then serially assessed. The study was expanded upon the serendipitious appearance of pilated gonococci within cultures which previously had yielded essentially pure nonpilated progeny for numerous passages. This allowed our study of the leukocyte associating activity of both pilated forms arising from nonpilated parents as well as the converse.

Generation of nonpilated gonococci from type 2 colonies. Selection and passage of 21- to 24-h-old type 2 colonies containing pilated gonococci reproducibly produces essentially pure cultures of type 2 colonies. However, if such typically type 2 parent colonies are allowed to incubate for longer period prior to subculture, the progeny consistently consists of a mixture of pilated and nonpilated forms. This reproducibly allows derivation of nonpilated gonococci from pilated cultures.

During protracted incubation (48 to 72 h), the colonies judged to be type 2 after the initial 21- to 24-h incubation usually show characteristic alterations in morphology, as shown in Fig. 1a–c. The majority of colonies exhibit irregularities in outline. The protruding segments of the colony give rise to a higher yield of type 4, nonpilated organisms than does the remainder of the colony, as shown in Fig. 2a and b. Often these protruding segments are also more lightly pigmented than the bulk of such an "old colony." Subculture of a single type 4 colony arising from old type 2 colonies usually produces a pure type 4 culture after only one passage.

Behavior of nonpilated gonococci arising from original type 2 colonies. The type 4, nonpilated progeny from 26 different old type 2 colonies were serially assessed for their association with human leukocytes. The parent pilated organisms (MS11-2 and F62-2; Table 1) had displayed relatively high-level association with

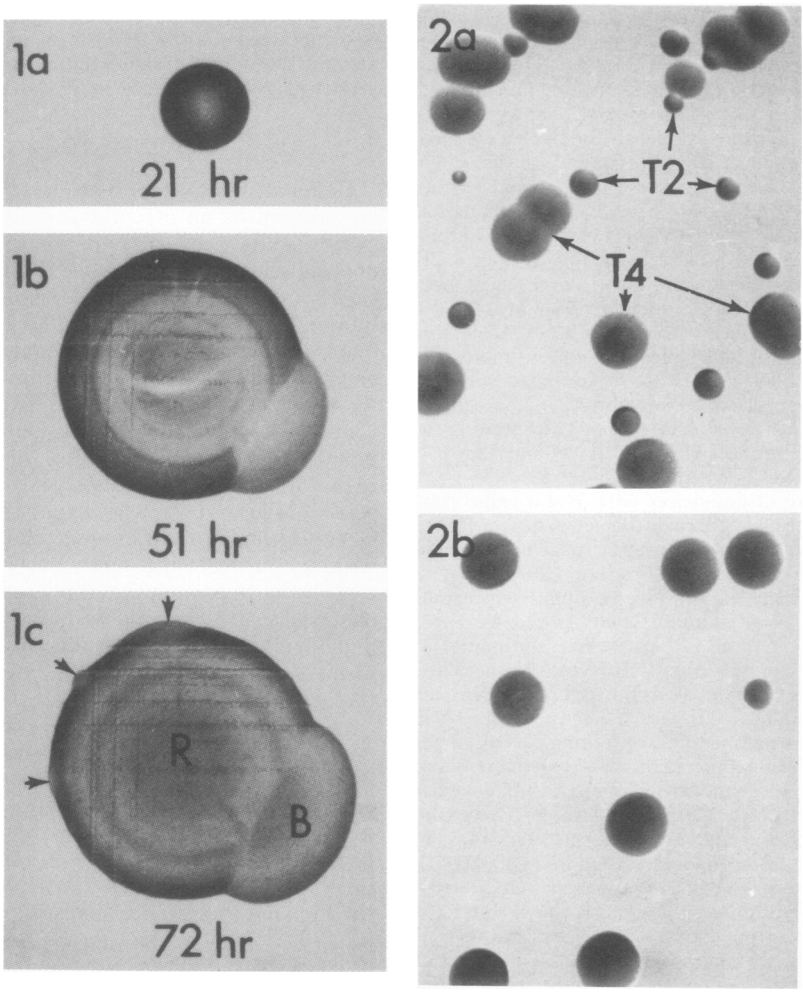

FIG. 1 and 2. *Changes in colony form to a type 2 colony with prolonged incubation. After 21 h of incubation (1a) the colony is round and regular with sharply defined edges. Longer incubation is accompanied by appearance of outline irregularities and one large bulge by 51 h of incubation (1b). This major bulge (B) became larger and the colony showed additional small bulges (arrows) by 72 h (1c). The concentric rings seen in the older colonies appear to be related to cooling the agar plate and the colony during repeated interruption of incubation to obtain photographs. ×30. (2) Progeny on subculture of the 72-h-old type 2 colony shown in 1c. (2a) A representative field of colonies present after subculture of the "R" portion of the colony shown in (1c). Note the mixture of type 2 (T2) and type 4 (T4) colonies present. This contrasts to the nearly "pure" culture of type 4 colonies present after subculture of the "B" portion of the 72-h-old colony. ×10.*

leukocytes in monolayer culture. All of the nonpilated progeny (MS11-4A, -4B, etc., and F62-4A, etc, Table 1) also exhibited high-level, T4*-like activity on initial testing after their isolation.

During serial passage and assay with leukocytes, the nonpilated derivatives showed several patterns of behavior. Some of the colony type 4 preparations retain their high-level (T4*-like) association with leukocytes throughout the study. Others show abrupt changes to low-level (T4-like) interactions with leukocytes either after a few passages or later in the study. These data, shown in Table 1, also include the results of repeated assessment of the leukocyte association of the standard strains used in our laboratory.

Appearance of pilated gonococci with low-level leukocyte association. One of the pilated preparations (MS11-2) that we have studied in the gonococci plus leukocyte system over a prolonged period (18 months) underwent sudden change in its behavior. In contrast to its previous high-level reactivity with leukocytes,

this type 2, pilated preparation changed to display very low association with leukocytes. This strain was being passaged by two individuals in our laboratory, but only one of the serially propagated preparations showed the described change; the other continued its relatively high-level association with leukocytes. Other strains (F62-2, U2-2) of pilated, colony type 2 gonococci which were also undergoing daily, serial passage did not change their behaviors during this period.

Designations for pilation and leukocyte association factor of gonococci. The appearance of pilated, colony type 2 organisms which displayed low-level association with leukocytes necessitated revision of our system for designation of gonococci. This is summarized in Table 2. It should be noted that colony type 2 is a collective term which includes both type 1 and type 2 colonies of Kellogg et al. (9, 10). Similarly, type 4 refers both the Kellogg's type 3 and 4 colonies. We use the simpler differentiation of colony forms based on presence (T2) or absence (T4) of pilation rather than the complete system (T1, T2, T3, T4) proposed by Kellogg et al. On the basis of the new designation system, each preparation is noted as to its state of pilation (P) and the apparent presence or absence of a leukocyte association factor (LA).

Leukocyte associating behavior of gonococci which have undergone P+ → P− or P− → P+ changes. As described above, nonpilated gonococci are easily recovered from pilated colonies subcultured after prolonged incubation. This method was used first to generate nonpilated organisms from the original stocks of pilated organisms; both parents and progeny. on initial isolation and testing, showed relatively high-level association with leukocytes, as shown in Table 1 and described above. This represents a loss of pilation (P+ → P−) with retention of the LA+ characteristic and is summarized as step A in Fig. 3. The same method for generating nonpilated progeny from pilated parents was employed with P + LA− starting preparations which were incubated for

TABLE 1. *Association of newly derived nonpilated gonococci with human neutrophils*

Gonococci (and date isolated)[a]	Gonococci association with human neutrophils[b]							
	6/3[c]	6/7	6/17	6/24	7/1	7/15	7/22	9/3
MS11-4A (5/29/74)	58	29	14	19	30	10	25	
MS11-4B (5/29/74)	93	93	70	87	80	93	97	
MS11-4C (5/29/74)	85	76	76	78	88	88	93	
MS11-4D (5/29/74)	85	65	89	56	64	70	68	
MS11-4E (5/29/74)	88	80	87	92	32	34	56	
MS11-4F (9/1/74)								78
MS11-4G (9/1/74)								87
MS11-4H (9/1/74)								56
F62-4A (6/5/74)		96	10	28	27	14	11	
F62-4B (6/5/74)		72	88	79	16	7	7	
F62-4C (6/5/74)		86	67	67	70	5	5	
F62-4D (6/5/74)		87	63	81	95	92	69	
F62-4E (6/5/74)		86	77	57	17	4	8	
F62-4F (6/5/74)		76	89	18	26	10	9	
F62-4G (6/5/74)		85	4	16	24	17	10	
F62-4H (9/1/74)								81
F62-4I (9/1/74)								83
F62-4J (9/1/74)								80
MS11-2	56	68	48	57	67	58		50
MS11-4/819 (8/19/73)	84	67	76	88		84	90	61
MS11-4/B		13	6	11	28	9	22	12
F62-2				84	67	70	53	75
F62-4/Kel	4	5	8	14	13	7	7	11

[a] Colony type 4 gonococci isolated from MS11-2 or F62-2 colonies after 48 or 72 h of incubation. Behavior of standard strains is also shown.

[b] Association refers to attachment and/or ingestion of one or more gonococci by neutrophils. The data refer to the percentage of neutrophils with gonococci association. Percentage of associations below 40% are underlined.

[c] Date of association.

TABLE 2. *Designation of gonococci*

Colony type[a]	Pilation	Association with leucocytes[b]	Previous designation	New designations[c]
2	+	High	T2	P + LA+ or MS11-2
2	+	Low	-	P + LA- or MS11-2
4	-	High	T4*	P - LA+ or MS11-4
4	-	Low	T4	P - LA- or MS11-4

[a] See text for explainations.
[b] High 40% leucocytes with associated (attach-ingested) gonococci, and low 40% leucocytes with associated (attach-ingested) conococci.
[c] Given, as second designation, for strain MS11.

prolonged periods. Twelve such P + LA- colonies were selected and subcultured. After obtaining pure cultures of nonpilated progeny, their leukocyte association was assessed. In all instances the nonpilated organisms exhibited low-level (LA-) behavior. This change P+LA- → P-LA-) is summarized in step E of Fig. 3.

Two nonpilated gonococcal preparations which had been serially passed for several weeks as pure type 4 cultures exhibited sporadic appearance of type 2 colonies among the predominant type 4 colonies. These type 2 colonies were subcultured and yielded pure cultures of the same type. Fortunately, the nonpilated preparations in which pilated forms appeared represented both P-LA+ and P-LA- gonococci. The P+ type 2 organisms arising in P-LA+ cultures showed LA+ activity whereas those P+ gonococci appearing in P-LA- preparations exhibited LA- behavior. (These steps are summarized in steps C and G of Fig. 3.)

Retention of pilation characteristic with change of behavior toward leukocytes. At present we are unaware of conditions under which changes in leukocyte association characteristics in gonococci can be promoted nor can these transitions be detected except by incubation of the organisms with leukocytes. For this reason, study of the changes in leukocyte association activity without alteration in the state of pilation rests on sporadic, chance alterations that occur during serial passage of the organisms. The first change (LA- → LA+) in nonpilated (P-) organisms occurred during a previous study (19). Transitions from LA+ to LA- are shown in step D of Fig. 3 and in Table 1 for nonpilated organisms. As mentioned previously, the change of P+LA+ to P+LA- occurred in one preparation (step B, Fig. 4). The change from P+LA- to P+LA+ was detected when a lightly pigmented, type 2 colony arose in an otherwise deeply pigmented, type 2, P+LA- culture (step F, Fig. 3). The light colony was selected, passaged to yield a pure preparation, assayed with leukocytes, and showed LA+ behavior. Subsequently, however, other lightly pigmented, type 2 colonies have arisen in P+LA- cultures and have been assessed for leukocyte association, but these have not shown the LA- to LA+ change noted above; thus the change in pigmentation does not necessarily accompany alteration in leukocyte associating behavior.

Association of human leukocytes with gonococci previously assayed for virulence. Several gonococcal preparations that have previously been used for virulence testing were obtained. Two preparations of strain F62 type 4 were obtained from Douglas Kellogg and both had been shown to be avirulent for human volunteers (9, 10). After a single passage in our laboratory one of these F62-4 organisms showed high-level association with leukocytes whereas the other showed negligible attachment/ingestion by leukocytes in vitro. Two pilated, colony type 2 preparations that had been shown to be virulent in chimpanzees (1) were obtained from Steve Kraus. One of these pilated, chimpanzee-virulent organisms exhibited high levels of interactions with leukocytes whereas the other showed low-level association with neutrophils in parallel assays. Two fresh isolates from clinical specimens (strains U3 and 19319) were also incubated with leukocytes after three to four passages in vitro. The former showed high levels and the latter low levels of association with human leukocytes in vitro. Thus, both P+LA+ and P+LA- designations appear appropriate for gonococci with demonstrated virulence and avirulent gonococci appear to be either P-LA+ of P-LA-.

Association of gonococci with HeLa cells. Five gonococcal preparations were assayed for their association with HeLa cells. Parallel experiments on association of the same gonococci with human leukocytes were also carried out and the results are noted in parentheses. Three typical colony type 2 preparations, F62-2 (87%), MS11-2/CT (10%), and MS11-2/ES (48%), were tested as were two colony type 4 preparations, MS11-4/819 (87%) and MS11-4/B188 (20%). On the basis of their colony morphology, absence or presence of pili, and level of association with leukocytes, these organisms bear the following designations: F62-2 (P+LA+); MS11-2/CT (P+LA-); MS11-2/ES (P+LA+); MS11-4/819 (P-LA+); and MS11-4/B188 (P-LA-). The results, shown in Table 3, indicate that pilated (P+) gonococci, regardless of their level of association with leukocytes, displayed much greater association with HeLa cells than did the nonpilated (P-) organisms.

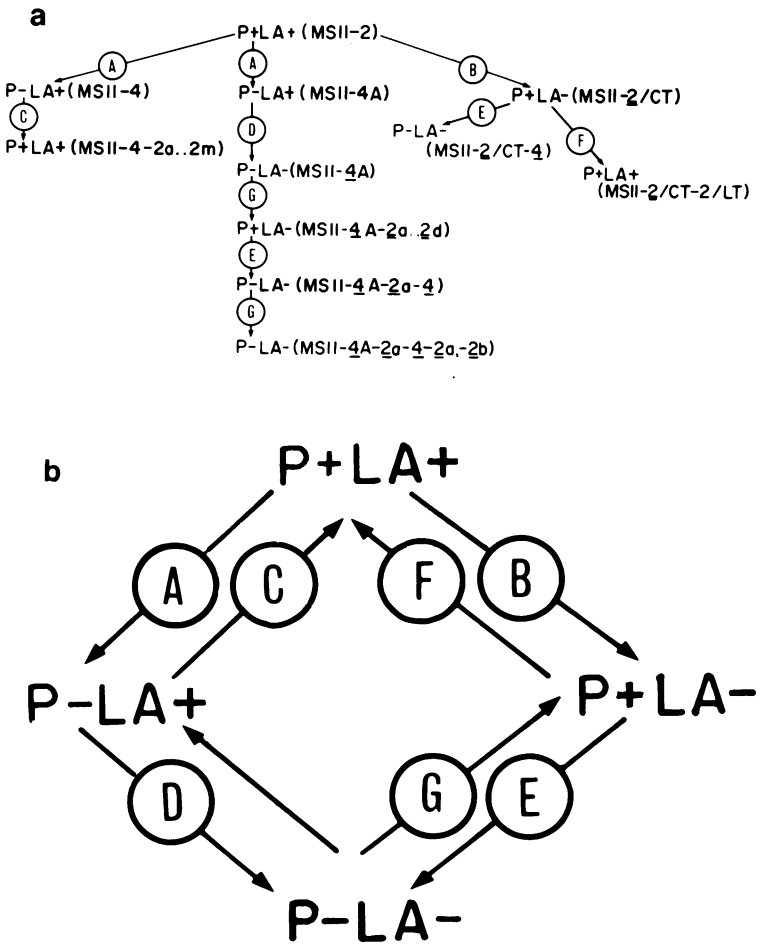

FIG. 3. *Genealogy of gonococci studied. Changes in the states of pilation (P) and association with leukocytes (LA) that occurred during passage of gonococci in vitro are shown in summary form. The designations used are those outlined in Table 2. All organisms noted are from a single strain, MS11, and all derived from a type 2, P+LA+ organism (MS11-2). A change in the state of pilation without loss of leukocyte associating activity is shown (step A) as well as a change in leukocyte associating activity and retention of pilation (step B). In these, as well as other changes found, only one characteristic differed on comparison of progeny and parent organisms. The P−LA− to P−LA+ change, noted during a previous study (19), is not given a letter step designation.*

Electron microscopy observations on LA+ and LA− gonococci. Both pilated and nonpilated gonococci that exhibit LA+ and LA− activity were examined by electron microscopy of freeze-fractured, freeze-etched preparations. The morphologies of these organisms and of the colonies from which they are derived are shown in Fig. 4 and 5. No differences in colony form or ultrastructure are found in comparing P+LA+ and P+LA− gonococci or between P−LA− organisms.

DISCUSSION

Previous studies from this and other groups have indicated that adherence of gonococci to diverse kinds of eukocyotic cells, including tissue culture cell lines, fallopian tubal epithelium, buccal mucosal cells, and sperm, is mediated or enhanced by pili (6, 13, 15, 17, 23). Much less agreement has been forthcoming on the function of pili as mediators or modifiers of interactions between gonococci and human leukocytes. Ofek et al. as well as Thongthai and Sawyer (12, 21) have presented evidence that pilation reduces the attachment and/or ingestion of gonococci by leukocytes. Our previous studies, on the other hand, suggested that pili play a minor role in gonococci-leukocyte associations. Our present studies provide a basis for the synthesis of these apparently contradictory observations.

Pilation and the presence of the leukocyte

TABLE 3. *Attachment of gonococci to HeLa cells in suspension*

Gonococci[a]	HeLa cells[b]	Gonococcal colony-forming units[c]				
		Pellet	Supernatant	Total	Pellet/total	Pellet/total; (HeLa+) − (HeLa−)
F62-2 (P + LA+)	−	2.8×10^2	1.59×10^3	1.87×10^3	0.15	0.28
F62-2 (P + LA+)	+	8.4×10^2	1.13×10^3	1.97×10^3	0.42	
MS11-2/CT (P + LA−)	−	1.2×10^2	7.3×10^3	8.6×10^3	0.15	0.45
MS11-2/CT (P + LA−)	+	5.3×10^2	3.60×10^3	8.90×10^3	0.60	
MS11-2/ES (P + LA+)	−	2.5×10^2	1.58×10^3	1.83×10^3	0.14	0.37
MS11-2/ES (P + LA+)	+	1.0×10^3	9.50×10^2	1.95×10^3	0.51	
MS11-4/819 (P − LA+)	−	3.9×10^2	1.29×10^3	1.68×10^3	0.23	−0.04
MS11-4/819 (P − LA+)	+	3.3×10^2	1.39×10^3	1.72×10^3	0.19	
MS11-4/B188 (P − LA−)	−	3.2×10^2	1.84×10^3	2.16×10^3	0.15	0.09
MS11-4/B188 (P − LA−)	+	5.4×10^2	1.70×10^3	2.24×10^3	0.24	

[a] Strain, colony type, and pilation (P+ or P−) as assessed by electron microscopy and level of interaction with leukocytes (LA+ or LA−) noted.

[b] HeLa cell-free (−) control and HeLa cell-containing (HeLa +) incubation mixtures.

[c] Colony-forming units (CFU) present in each pellet and supernatant specimen were determined and then added to provide the total values shown. The ratios of CFU present in the pellet fractions as compared to total CFU recovered are shown (Pellet/total). These ratios are then used to determine the distribution of gonococci in pellets that is apparently related to their attachment to HeLa cells by substracting the values obtained in the HeLa cell-free controls from the ratios obtained for the HeLa cell-containing specimens.

association-promoting factor occur independently on gonococci. Thus one may observe the following combinations of these traits: P+LA+, P+LA−, P−LA+, and P−LA−. If one were to compare the leukocyte associations of P+LA+ gonococci with those of P−LA− organisms, one might conclude that pili positively influenced attachment/ingestion of the bacteria by leukocytes. Conversely, if one were to compare P+LA− and P−LA+ gonococci, one would conclude that pilation deterred phagocytosis of gonococci by leukocytes. We suspect that the latter combination approximates that used by both Thongthai and Sawyer and by Ofek et al. (12, 21). Having had the opportunity to study the relative interactions of P+LA+, P+LA−, P−LA+, and P−LA− organisms of human neutrophils, we find that pili appear to play a minor, negative role as determiners in these interactions.

The presence or absence of LA factor does not appear to correlate clearly with virulence or avirulence of gonococci. Pilated gonococci of proven virulence in chimpanzees may be either LA+ or LA−. Conversely, gonococci that have been found to be avirulent for human volunteers can show either LA+ or LA− characteristics. Finally, fresh isolates from patients with acute gonorrhea may exhibit either LA+ or LA− behavior. These findings support our previous suggestion that interactions of gonococci with leukocytes play little or no role as determiners of virulence and pathogenicity of *N. gonorrhoeae*. The nature of the LA factor is not clear although the preliminary findings of its tryptic, chymotryptic, heat, and glutaraldehyde sensitivity suggests that it is a cell wall surface protein (19). Current studies in our laboratory are directed toward determining whether this supposition is correct.

The presence or absence of LA factor does not appear to influence the interactions between gonococci and tissue culture cells. Both previous studies on attachment of T4 (P−LA−) and T4* (P−LA+) gonococci to tissue culture cells as well as the studies comparing the HeLa cell attachment of P−LA+, P+LA−, P−LA+, and P−LA− organisms point to this conclusion. Further, the LA factor does not appear to influence interactions between gonococci and mouse peritoneal macrophages in vitro, whereas pili seem to decrease the association of gonococci to these cells (1a). We do not know whether leukocyte association factor might be a determiner of interactions with other types of eukaryotic cells. Though we doubt that leukocyte association factor does play a role in other gonococci-eukaryotic cell associations, the oc-

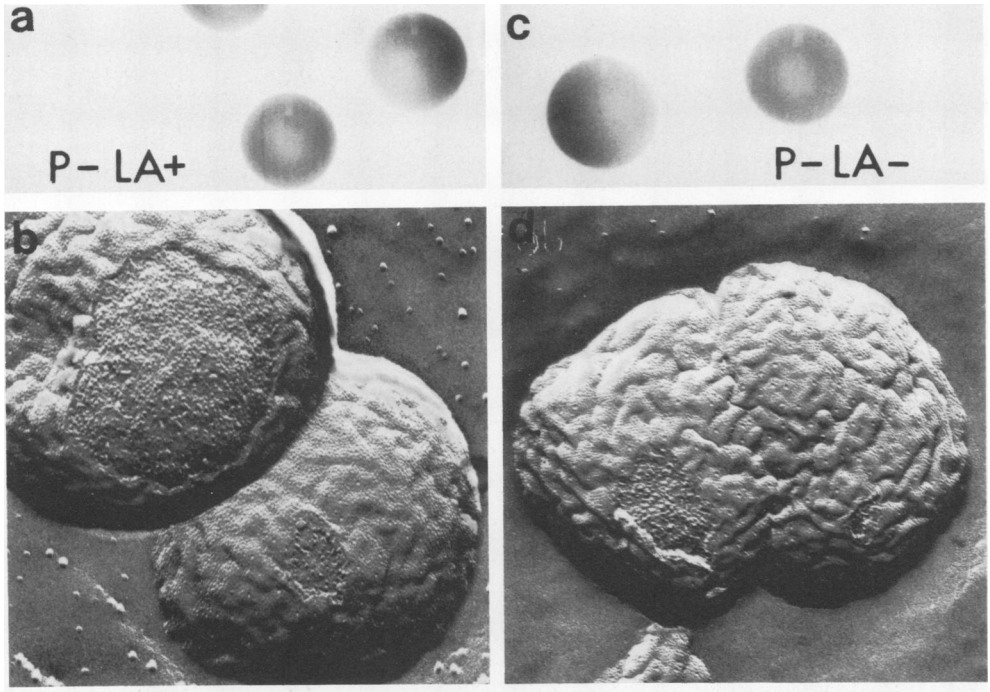

FIG. 4. *Colonial morphology and electron microscopic appearance after freeze-fracture, freeze-etching are shown for P−LA+ (a and b) and for P−LA− organisms (c and d). No differences in size, form, or general appearance of these 21-h-old colonies are noted nor are there observable differences in the morphologies of the cell wall exteriors of these organisms whose behaviors with human leukocytes are distinctly different. Note the absence of pili on the exteriors of these organisms. ×15 (a and c); ×60,000 (b and d).*

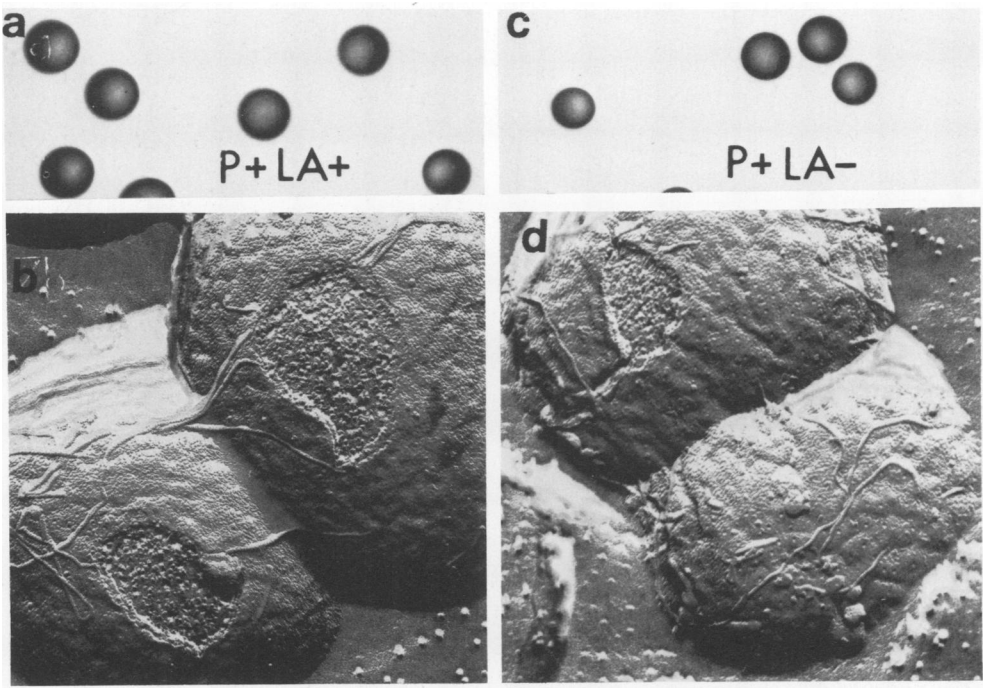

FIG. 5. *Pilated gonococci of designations P+LA+ (a and b) and P+LA− (c and d) exhibit no noticeable differences in colony form nor ultrastructure. Note the characteristic refractile colony edges on the relatively small 21-h-old colonies (a and c) and the pili adherent to the organisms' surfaces (b and d) in contrast to the appearance of colonies and organisms found in Fig. 4. ×15 (a and c); ×60,000 (b and d).*

currence of gonococci with differing combinations of P and LA should certainly be recognized during subsequent studies that attempt to relate gonococcal-eukaryotic cell interactions with virulence of the organisms. On the other hand, preliminary evidence suggests that LA+ and LA− gonococci (both P+ and P−) are equal in virulence when intravenously inoculated into 10-day-old chicken embryos (Blake, M., and J. Swanson, unpublished observations). This model for studying gonococcal virulence has been used by other authors (2) and appears to offer the prospect for studying virulence factors of gonococci by a relatively sensitive method in vivo.

The genetic mechanism(s) responsible for coding and/or expression of pilation and presence or absence of leukocyte association factor are unclear. Recently, changes in state of pilation of gonococci in mammalian cell cultures have been called "phase transitions" (22). It is unclear whether or not those authors intend to imply a mechanism similar to "phase variation" which has been well-documented and studied in *Salmonella* (5). Both from our studies as well as those of others (22) it appears that P− to P+ changes occur with somewhat varying frequencies in different strains of *N. gonorrhoeae*. At present, neither those data nor the other facts available allow for a statement as to the genetic mechanisms responsible for pilation/nonpilation. A recent study (11) indicates that loss of pilation is not accompanied by changes in either the number or size of plasmids found in gonococci. Methods such as transducing phage systems for investigating other genetic mechanisms for pilation are unknown in gonococci. Although differentiation of P+ and P− organisms is easily accomplished by dissecting or electron microscopy examination, large numbers of gonococci cannot be screened due to lack of a selective (P+ versus P−) medium. This problem is more complicated with respect to studies on the leukocyte association characteristic which does not endow gonococci nor their colonies with special morphological characteristics as far as we are aware. Because of the latter problem, we are unable to determine just how stable the leukocyte association characteristic is in gonococcal populations. Changes from LA+ to LA− as well as the reverse occur in our laboratory with variable frequency apparently because we are unable to select, a priori, those colonies with desired characteristics of LA+ or LA−.

ACKNOWLEDGMENTS

We appreciate the help of Becky Felton in preparation of this manuscript.

This work was supported by contract DADA 17 72 2146 of the U.S. Army MDICAL Research and Development Command, and by Public Health Service research grant AI-11073 from the National Institute of Allergy and Infectious Diseases.

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