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Differential Attachment by Piliated and Nonpiliated *Neisseria gonorrhoeae* to Human Sperm

ANN N. JAMES-HOLMQUEST, JOHN SWANSON, THOMAS M. BUCHANAN, REUBEN D. WENDE, AND ROBERT P. WILLIAMS

Department of Microbiology and Immunology, Baylor College of Medicine, Houston, Texas 77025; Houston City Health Department Laboratories, Houston, Texas 77025; Department of Pathology, University of Utah College of Medicine, Salt Lake City, Utah 84112; The Rockefeller University, New York, New York 10021

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Piliated type 1 *Neisseria gonorrhoeae* attached to 50% of human sperm after incubation of mixtures in vitro for 15 min at 35 C. In contrast, nonpiliated type 4 *N. gonorrhoeae* attached to only 23% of sperm. Similar results were obtained with three different strains of *N. gonorrhoeae*. Treatment with heat or formaldehyde to kill bacteria did not affect the amount of attachment by piliated or nonpiliated types. *Escherichia coli* and *N. subflava*, other species of piliated bacteria, attached to about 40% of sperm, and the nonpiliated species *N. meningitidis* and *N. catarrhalis* attached to a comparable number of sperm, as did type 4 *N. gonorrhoeae*. Prior incubation of type 1 *N. gonorrhoeae* with purified antibody prepared against gonococcal pili reduced the percentage of sperm with attached bacteria to the same level as that for nonpiliated type 4 gonococci. Similar treatment of other piliated organisms or of nonpiliated *Neisseria* did not affect the attachment of the bacteria to sperm.

The basis for virulence of *Neisseria gonorrhoeae* is not well understood. A clue emerged when virulence was associated with colonies of small size, types 1 and 2, of *N. gonorrhoeae* (7, 8, 10). More recently, discovery of pili as a morphological characteristic of cells from these colony types (6, 11) and demonstration of enhanced infectivity of these piliated bacteria for chicken embryos by Buchanan and Gotschlich (1) and by Bumgarner and Finkelstein (Abstr. Annu. Meet. Amer. Soc. Microbiol., p. 126, 1973) related a specific surface characteristic of the gonococcus to virulence. One laboratory reported that pili both promoted adherence of gonococci to eukaryotic cells and reduced phagocytosis of the bacteria (9, 14).

N. gonorrhoeae were attached to epithelial cells scraped from the male urogenital tract (15), a primary site of infection, as well as to other human cells in vitro (13). In the latter experiments, piliated gonococci attached to cells to a significantly greater degree than nonpiliated gonococci. The host cells used for these experiments also phagocytized gonococci.

Attachment per se can be investigated by use of spermatozoa, whose phagocytic ability is nil. Sperm are ubiquitous in the genital tract of normal adult males, and, if gonococci are attached to them, they may be vehicles for transport of the bacteria throughout the male and female reproductive tracts. In this report, we compare attachment to human sperm by piliated and nonpiliated gonococci and explore the role of pili in the attachment.

MATERIALS AND METHODS

Growth and preparation of bacteria. The strains of *N. gonorrhoeae* used were MS11 (12), F-62 (supplied by D. S. Kellogg of the National Center for Disease Control, Atlanta), and WP (5). By use of the method of Kellogg et al. (8), colony types 1 and 4, designated T1 and T4, respectively, were identified from each strain and then were maintained by transfer on GCB medium (GC agar base medium plus 1% IsoVitaleX, Baltimore Biological Laboratory, Cockeysville, Md.). Two other strains of piliated bacteria, *Escherichia coli* 0111:B4 and *N. subflava*, as well as two nonpiliated strains of *Neisseria*, *N. catarrhalis* and *N. meningitidis*, were used for experiments as indicated in the text. These bacteria also were grown on GCB medium.

Neisseria spp. were incubated in candle jars, and *E. coli* was incubated in an air convection incubator at 35 C. After incubating the plates for 18 to 20 h, we scraped the colonies from the agar surface with a cotton swab moistened in medium 199 with Hanks balanced salt solution and sodium bicarbonate (M199; Microbiological Associates, Inc., Bethesda, Md.) and suspended the bacteria in 10 ml of M199. If clumps were seen by microscope examination, the suspensions were centrifuged at 450 × *g* in a centrifuge (model CL clinical centrifuge, International Equipment Co., Needham Heights, Mass.) for 3 min, and the supernatant fluid containing unclumped

bacteria was transferred by pipette to a sterile tube. As a final step, all of the suspensions were agitated on a Vortex Jr. mixer (Scientific Industries, Inc., Greens Village, N.Y.) for 60 s. After the bacteria were counted in a Petroff-Hausser chamber, the suspension was diluted to the desired concentration of organisms per milliliter, as confirmed by serial dilution and plate counts on GCB medium.

In some experiments, gonococci were killed by exposure of diluted suspensions to 65 C in a water bath for 1 h or to 3% (vol/vol) of aqueous formaldehyde for 30 min. Samples of the treated suspensions were streaked on GCB, and the plates were incubated in a candle extinction jar for 48 h at 35 C. Failure of colonies to appear confirmed the absence of viable gonococci in the suspensions.

Electron microscopy of bacteria. All types, species, and genera of bacteria were examined by electron microscopy after negative staining to determine the presence or absence of pili. After incubation of agar cultures for 18 to 20 h, bacteria were taken from an isolated colony by a loop and were suspended in a drop of 0.1 M phosphate-buffered saline (PBS), pH 7.2, on a wax square. A collodion-coated copper grid was laid on top of the drop. Then the grid, with some of the adherent fluid, was quickly inverted and held in air for 1 min. Excess moisture was absorbed with blotting paper, and then the grid was again inverted onto a drop of 1% (vol/vol) aqueous phosphotungstic acid, pH 6.8, for 10 to 20 s. After the moisture was blotted off, the grid was examined in an electron microscope (Elmiskop I, Siemens).

Preparation of sperm. Samples of human semen were furnished by four males with normal sperm counts and by 15 patients seen in the J. Sayles Leach Urological Research Laboratory, Baylor College of Medicine, Houston. All semen spontaneously liquefied before counts were made. Some of the specimens from patients had low sperm counts, but none of the samples used for our experiments contained less than 10^6 sperm per ml. Specimens were kept at room temperature for no longer than 3 h until used.

Seminal fluids were diluted to 5 ml with M199 and then centrifuged at 1,500 $\times$ *g* for 5 min in the clinical centrifuge. The supernatant fluid was decanted, and the pelleted sperm were resuspended in 5 ml of M199 on a Vortex mixer for 1 min. We washed the sperm by centrifuging the suspension again, decanting the supernatant fluid, and resuspending the sperm in M199. This procedure was then repeated to complete washing of the sperm. After the pellet of washed sperm was suspended in M199, the sperm were enumerated by use of a hemocytometer, and the suspension was diluted with M199 to a concentration of 10^6 sperm per ml.

Mixture of sperm with bacteria. Samples (0.5 ml each) of the washed sperm (10^6 per ml of M199) and bacterial suspensions were mixed in polystyrene Snap-Cap tubes (12 by 75 mm) (DiSPo Culture Tubes, Scientific Products, Evanston, Ill.). Suspensions of bacteria were diluted to provide ratios of sperm to bacteria of 1:100, 1:10, 1:1, 1:0.5, and 1:0.1. Tubes were closed and were incubated for 15 min at 35 C on a rack (L/I Lab-Industries, Berkeley, Calif.) rotating end over end at 12 rpm.

Evaluation of attachment of bacteria to sperm. After incubation, 1 drop of the mixture of sperm and bacteria was pipetted onto a glass slide and allowed to dry in air. The slides then were fixed in 95% (vol/vol) aqueous ethanol for 7 min and rinsed with water. For each experiment, we prepared fresh Giemsa stain by diluting 1 part of concentrated stain (Giemsa tissue stain-Wolbach modification, Harleco, Philadelphia, Pa.) with 20 parts of distilled water and then filtering the solution through Whatman no. 1 filter paper. Slides were flooded with the fresh diluted stain for 15 min, washed with water, air dried, and examined by light microscopy.

No fewer than five fields of each slide were examined with a $\times$40 objective. For each slide, a minimum of 100 sperm was counted, and the number with bacteria attached was recorded to determine the percentage of attachment. To check the objectivity of the counts, slides that had been counted were coded by another individual and then recounted by the original examiner. There was no significant discrepancy between the two counts.

Preparation and use of antiserum against gonococcal pili. One part of rabbit anti-pilus antiserum containing specific precipitating immunoglobulin G antibody against gonococcal pili (3.0 mg/ml; reference 2), which had been heat inactivated at 56 C for 30 min, was diluted with 9 parts of 0.1 M PBS, pH 7.2. This diluted antiserum (0.1 ml) was added to 0.5-ml suspensions of bacteria (10^6 or 10^7 per ml of M199). After incubation of these suspensions in the rotating rack at 35 C for 1 or 3 h, 0.5 ml of sperm (10^6 per ml of M199) was added to each tube. Normal rabbit serum was diluted in the same fashion and added to similar mixtures of bacteria and sperm to serve as controls for each experiment. Incubation of the mixtures of serum, bacteria, and sperm, and preparation, staining, and examination of slides were carried out as previously described.

RESULTS

Piliated gonococci from type 1 colonies attach to human sperm, in vitro, more readily than do nonpiliated organisms from type 4 colonies. This phenomenon was demonstrated both in dose-response data obtained with T1 and T4 gonococci derived from a single strain (Fig. 1) and in experiments using piliated and nonpiliated *N. gonorrhoeae* from several strains (Fig. 2).

At the highest ratio of sperm to gonococci (1:100, Fig. 1), nearly 90% of the observed sperm had adherent organisms when incubated with piliated T1 organisms as compared with only 48% of sperm incubated with identical numbers of nonpiliated T4 organisms. At each of the other ratios tested, the percentage of sperm with attached T1 gonococci was approximately twice that found in incubations with T4 organisms. At the highest dose level, many sperm were completely obscured by the numerous, adherent T1 gonococci. At the lower ratios

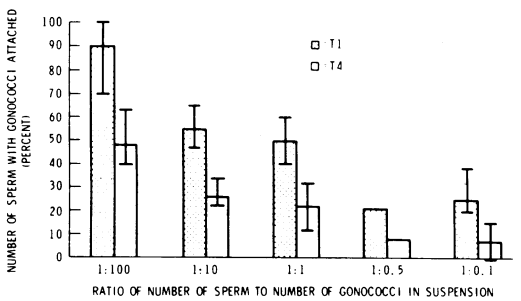

FIG. 1. *Effect of various ratios of sperm to bacteria on attachment by piliated (T1) and nonpiliated (T4) N. gonorrhoeae to human sperm. Suspensions of 10^8, 10^7, 10^6, $10^{5.5}$, or 10^5 gonococci per ml and 10^6 sperm were mixed together in M199 and incubated for 15 min at 35 C on a rotating rack. Mean determinations for ratios of sperm to bacteria with both T1 and T4 gonococci were based on counts of slide preparations from six separate experiments. Data from a single experiment were used as the mean for the reaction mixture of 1:0.5 sperm to bacteria. The mean percent of attachment is expressed by the height of the bars, and the range is indicated by vertical lines.*

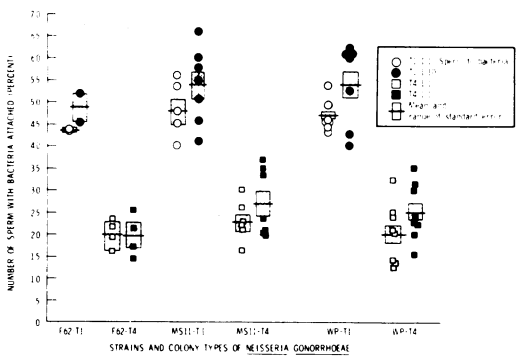

FIG. 2. *Percentage of attachment to human sperm of piliated (T1) and nonpiliated (T4) bacteria from three strains of N. gonorrhoeae. Each circle or square represents an experiment carried out with a different sample of sperm in which a minimum of 100 sperm was counted. Suspensions of 0.5 ml of bacteria (10^6 or 10^7/ml) and 0.5 ml of washed sperm (10^6/ml) were mixed together in M199 to obtain ratios of sperm to bacteria of 1:1 or 1:10. The suspensions were incubated and examined as described in Fig. 1.*

(1:0.5 and 1:0.1), so few T4 gonococci were attached to sperm that accurate measurements could not be obtained. Differences between attachment of T1 and T4 organisms are most readily appreciated at ratios of either 1:10 or 1:1. The percentages of attachment of T1 gonococci or of T4 at these two dose levels are not statistically different. Because of the similarity in values obtained with these two doses, results of later experiments include a combination of both dose levels.

The average number of sperm with attached gonococci, combining ratios of 1:1 and 1:10, was significantly greater for sperm exposed to cells from type 1 colonies of three strains of *N. gonorrhoeae* than for those exposed to gonococci from type 4 colonies of the same strains (Fig. 2). After incubation, the mean percentage of sperm with piliated T1 organisms attached was 49.8%, with a standard deviation of ±4.4%. In contrast, when sperm were incubated with T4 nonpiliated gonococci, the mean was 22.8% and the standard deviation was ±3.7%. The probability of such differential attachment occurring by chance is $P < 0.005$ by the t test.

The percentage of sperm with bacteria attached varied, but not significiantly, among the three strains of *N. gonorrhoeae* (Fig. 2). When individual samples of sperm were compared, variation in the percentage of sperm with gonococci attached could be as much as 25% (MS11, T1, 1:10), but in no case did T4 bacteria attach to as great an extent as T1. We did not investigate the factors involved in this variation, but we suspect that sperm from different individuals may vary in their attraction for bacteria. Because of the variation between individual samples of sperm, untreated controls were always included in experiments to study the effect of various treatments on the attachment of gonococci.

Gonococci of both types attached with apparently equal frequency to heads or tails of sperm (Fig. 3). As many as 20 or as few as two gonococci may adhere to a single sperm.

Attachment to sperm is not affected by killing piliated and nonpiliated gonococci by heat or by formaldehyde (Table 1). These data also demonstrate the importance of including an untreated control to evaluate the results. Live gonococci attach to fewer sperm used for these experiments than for some other experiments (Fig. 2).

Other species of piliated bacteria also attached to sperm (Table 2). Although the percentage of sperm with adherent *N. subflava* or *E. coli* was not as great as that with T1 *N. gonorrhoeae*, the figure was significantly greater than with T4. Selected strains of *N. meningitidis* and *N. catarrhalis* that did not possess pili attached to approximately the same number of sperm as did T4 gonococci (Table 2).

Incubation of T1 piliated gonococci with anti-gonococcal pilus antiserum specifically reduced attachment of these bacteria to sperm by about 50% (Table 3), a percentage of attachment comparable with that for T4 bacteria. Addition of antiserum did not cause clumping of piliated bacteria; therefore, the reduction in

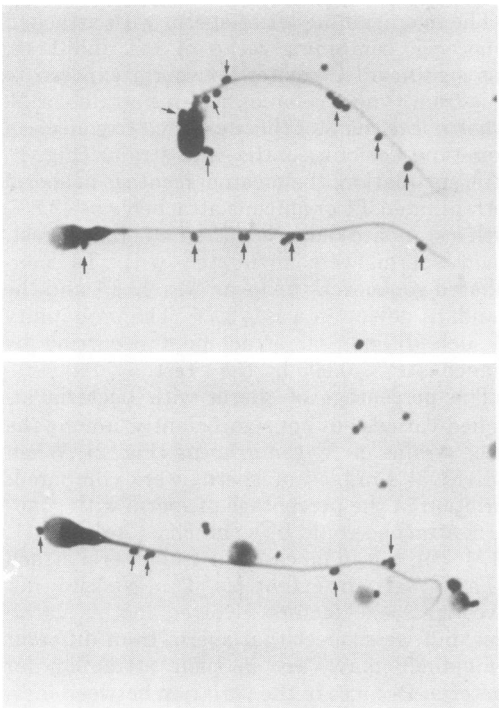

FIG. 3. *Photographs taken by bright-field microscopy demonstrating typical attachment of piliated T1 N. gonorrhoeae strain WP (arrows) to human sperm (×400).*

attachment was not due to a change in effective ratio of sperm to bacteria. In contrast, the antiserum did not affect attachment by another piliated species, *N. subflava*. When analyzed by the chi-square test, the slight reduction in percentage of attachment by T4 is not significant ($P > 0.5$). Attachment by piliated *E. coli* 0111:B4 apparently was not affected by anti-pilus antiserum. These data are not included in Table 3 because *E. coli* multiplied during the period of incubation in antiserum.

DISCUSSION

The interaction between types of *N. gonorrhoeae* and human sperm were similar to those reported for other eukaryotic cells (9, 13, 14). In all of these studies, piliated bacteria of type 1 attached to cells to a greater degree than nonpiliated type 4 bacteria.

Attachment to sperm is not an exclusive property of the gonococci because other species and genera of bacteria also attach. However, some specificity is involved in attachment of type 1 *N. gonorrhoeae*. Other piliated bacteria do not attach to as many sperm as did the piliated gonococci, nor do antisera prepared against pili of gonococci decrease such nonspecific attachment.

The lack of phagocytic ability of sperm provides a singular advantage for investigation of attachment of gonococci to eukaryotic cells. Addition of specific antibody cannot promote phagocytosis by opsonization of the bacteria. Instead, as demonstrated in our experiments, anti-pilus antibody specifically inhibits attachment of gonococci to sperm. By use of our system, the effect of antibody upon various parameters of attachment can be studied exclusive of phagocytosis.

Other factors than pili must be involved in attachment to sperm. Nonpiliated type 4 *N. gonorrhoeae*, *N. meningitidis*, and *N. catarrhalis*, as well as antibody-treated type 1 gonococci, attach to 20 to 25% of sperm. Whether such attachment is a property of the bacteria or of the sperm or is a result of manipulation of either or both types of cells awaits further investigation.

Viability of T1 did not affect attachment of

TABLE 1. *Attachment of live and dead N. gonorrhoeae to human sperm*[a]

Bacteria	Treatment	*N. gonorrhoeae*					
		Type 1			Type 4		
		Total no. of sperm counted	No. of sperm with bacteria attached	Attachment (%)	Total no. of sperm counted	No. of sperm with bacteria attached	Attachment (%)
Live	None	605	242	40.0	512	128	25.0
Dead	Heat	340	140	41.2	400	105	26.3
	Formaldehyde	394	155	39.4	334	92	27.5

[a] Bacteria were killed by heating for 1 h at 65 C or by exposure to 3% formaldehyde for 30 min. After treatment, 0.5 ml of bacteria (10^6 or 10^7/ml) and 0.5 ml of sperm (10^6/ml) were suspended together in medium 199. The suspensions were incubated at 35 C on a rotating rack, and, after 15 min, slides were prepared, stained by the Giemsa method, and counted.

TABLE 2. *Comparison of attachment to human sperm for Neisseria species and for E. coli*[a]

Measurement	Bacteria with pili			Bacteria without pili		
	N. gonorrhoeae (type 1)	*N. subflava*	*E. coli* O111:B4	*N. gonorrhoeae* (type 4)	*N. meningitidis*	*N. catarrhalis*
Total no. of sperm counted	229	243	229	271	208	385
No. of sperm with bacteria attached	119	99	97	56	42	97
Attachment (%)	51.9	40.7	42.4	20.7	20.2	25.2

[a] Suspensions of 0.5 ml of bacteria (10^6 or 10^7/ml) and 0.5 ml of sperm (10^6/ml) were mixed together in medium 199 and incubated for 15 min at 35 C on a rotating rack. Then slides were prepared, stained by the Giemsa method, and counted. Presence or absence of pili was determined by examination in the electron microscope of negatively stained preparations.

TABLE 3. *Effect of antipilus antibody on attachment of piliated and nonpiliated Neisseria to human sperm*[a]

Treatment	Bacteria with pili						Bacteria without pili		
	N. gonorrhoeae (type 1)			*N. subflava*			*N. gonorrhoeae* (type 4)		
	Total no. of sperm counted	No. of sperm with bacteria attached	Attachment (%)	Total no. of sperm counted	No. of sperm with bacteria attached	Attachment (%)	Total no. of sperm counted	No. of sperm with bacteria attached	Attachment (%)
Incubation with antiserum for 1 h	420	106	25.2				334	57	17.1
Incubation with antiserum for 3 h	362	93	25.7	252	108	42.9	227	43	18.9
Incubation with normal serum for 3 h	296	142	48.0	214	84	39.3	229	50	21.8
Incubation with no serum for 3 h[b]	282	136	48.2				367	70	19.1

[a] Rabbit antiserum or normal serum was diluted 1 to 10 in PBS, and 0.1 ml was added to 0.5 ml of bacteria (10^6 or 10^7/ml) suspended in medium 199. The suspensions were incubated at 35 C for the length of time indicated. Then 0.5 ml of sperm (10^6/ml) suspended in medium 199 was added, and the suspensions were incubated and examined as described in Table 2.

[b] PBS replaced serum in these experiments.

bacteria to sperm. Because the methods used to kill the bacteria could alter secondary and tertiary structures of the pilus protein, failure of either treatment to alter ability of pili to mediate attachment suggests either that the precise structure of a protein was not involved in attachment or that the structure of the pilus is highly resistant to heat and chemicals. Inhibition of phagocytosis by piliated gonococci also is not affected by death of the bacteria (14).

Howard (4) speculated that gonococci might attach to human sperm and thus be transported through the female and male reproductive systems to establish infections in the fallopian tubes or the epididymis. The possibility of transmission of *Mycoplasma* from male to female by attachment to sperm also has been suggested (3). Bacteriology laboratories that examine many samples of vaginal or urethral exudate from patients suspected of infection with *N. gonorrhoeae* not infrequently observe gonococci attached to sperm in exudates from both females and males (R. D. Wende, unpublished observation). In the case of the gonococcus, our data provide support for the suggestion that sperm may transport bacterial "hitch-hikers." Further studies will determine the significance of this route in the pathogenesis of gonorrhea.

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