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Studies on Gonococcus Infection

V. Observations on In Vitro Interactions of Gonococci and Human Neutrophils

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The association of in vitro human leukocytes with pilated, type 2 *Neisseria gonorrhoeae* exceeds that for nonpilated, type 4 organisms but is less than that for nonpilated, type 4* gonococci. The two nonpilated forms of gonococci (types 4 and 4*) attach to tissue culture cells to a much lesser extent than do pilated, type 2 organisms of the same strain. Trypsin treatment of either pilated (type 2) or nonpilated (type 4*) gonococci markedly reduces the attachment-ingestion of these organisms with leukocytes, but the same trypsin treatment does not depilate the type 2 organisms nor visibly alter the morphology of their pili. Similar reductions in association with leukocytes are found if the gonococci are pretreated with chymotrypsin, heat, or glutaraldehyde. High levels of association between gonococci and leukocytes are reestablished if the trypsin or chymotrypsin-treated organisms are reincubated in protease-free medium. These data suggest that interactions between gonococci and human neutrophils are mediated through surface characterisitcs of the bacteria, different from those which influence attachment of the organisms to tissue culture cells. In the latter instance, pili appear to positively influence gonococcal attachment, whereas in the former a nonpilus bacterial cell wall nonpilus protein is probably the major determiner in the interaction between leukocytes and gonococci.

Interactions between the surfaces of *Neisseria gonorrhoeae* and cells of the human host are, a priori, likely determiners in the pathogenesis of gonorrhea. Recent studies have begun to elucidate some of the interrelationships between the nature of the gonococcal surface and reactions of the organism with the host and host cells. Studies on these relationships have been based largely on the observations of Kellogg et al. (8, 9) that virulence of gonococci (GC) is correlated with formation of specific colony types by the organisms cultivated in vitro. Extension of that observation has led to the demonstration that "virulent colony forms" contain GC bearing pili, whereas "avirulent colony forms" are constituted of organisms devoid of pili (7, 15). These observations suggested that pili might correlate with virulence of GC through an influence on interactions between the gonococcal surface and host epithelial cells (15). This suggestion has been partially corroborated in recent studies demonstrating enhanced attachment of pilated GC to tissue culture cells (14), buccal mucosal cells (12), uterine epithelial cells (18), and in vitro human spermatozoa (6).

It has also been suggested that pilation of GC might enhance virulence of these organisms by modifying their interactions with leukocytes (15). Inasmuch as other bacterial surface components, such as the pneumococcal capsule and streptococcal M protein, influence virulence by restricting phagocytosis of those bacteria (3, 19) a similar mechanism, i.e., decreasing phagocytosis, was suggested for gonococcal pili. One group of investigators has recently presented evidence to support such a suggestion (12, 17).

Our studies indicate that pili are not major determiners of interactions between GC and in vitro human leukocytes. A significant part of this conclusion has been drawn from demonstration that nonpilated GC may exhibit either greater or lesser levels of association with human leukocytes than do pilated organisms from the same strain. This apparent independence of leukocyte association and gonococcal pilation contrasts with studies on gonococcal attachment to tissue culture cells; in the later instances the presence of pili is correlated with enhanced attachment of GC to the eukaryotic cells. These findings, along with preliminary

studies on modifying the association between GC and human neutrophils, form the basis for this report.

MATERIALS AND METHODS

Gonococci. Two strains, MS11 and F62, were used in this study. The former was isolated from a patient at Mount Sinai Hospital, New York, and the latter was obtained through the generosity of D. Kellogg, Center for Disease Control, Atlanta, Ga. F62 GC were obtained both as a type 2 (T2) culture and as two different type 4 (T4) cultures. The organisms obtained as T4 cultures, as well as T4 colonies selected from T2 cultures in our laboratory, were studied. All GC were propogated as essentially pure cultures of T2 or T4 on G C agar base supplemented with 1% IsoVitaleX (G C agar; Baltimore Biological Laboratory, Cockeysville, Md.). The organisms were utilized for incubation with leukocytes after 18 to 20 h of incubation of 36 C in a 5% CO_2 atmosphere.

Leukocyte preparation. Leukocytes were partially purified from whole human blood by mixing heparinized (2 mg/ml) blood with an equal volume of 2% gelatin (Knox Gelatin, Inc., Johnstown, N.Y.) in 0.9% saline and allowing the mixture to stand at 37 C for 30 min. The plasma was removed and centrifuged at 50 $\times$ *g* for 5 min, after which the pelleted cells were suspended in 0.85% ammonium chloride at room temperature for 5 min. The cells were again pelleted by centrifugation, were washed three times with medium 199 (M-199; Grand Island Biological Co., Grand Island, N.Y.) containing Earle salts, glutamine, and sodium bicarbonate. After counting the cells, the volume of the suspension was adjusted so 5 $\times$ 10^5 cells were contained in 0.1 ml of M-199 containing 0.01% bovine serum albumin (BSA; Schwarz/Mann, Orangeburg, N.Y.). This volume of cell suspension was pipetted onto 18-mm diameter round cover slips in petri dishes (10 by 30 mm), and the leukocytes were allowed to attach at 36 C for 30 min. At the end of this attachment period, the cover slips were washed with 10 ml of M-199, and the M-199-covered cell monolayers were held at room temperature until incubated with GC (maximal duration, 15 min).

Preparation of GC for incubation with leukocytes. For most experiments, GC of the appropriate colony type were removed from G C agar with a Dacron swab and suspended in 0.01% BSA in water, and the optical opacity (blue filter, Klett colorimeter) of the suspension was adjusted to 50 Klett units. This suspension contained approximately 2 $\times$ 10^8 to 4 $\times$ 10^8 gonococci per ml as determined by direct counting in a Petroff-Hausser chamber. These suspensions were then diluted to the desired concentration in M-199 containing 0.01% BSA prior to their incubation with leukocytes. Gonococcal preparations were regularly examined by negative staining and electron microscopy to evaluate the presence or absence of pili on the organisms.

Trypsin treatment of GC. T2, T4, and T4* GC grown for 16 to 18 h on G C agar were suspended in phosphate-buffered saline, pH 7.0 (PBS), and each of these suspensions was divided into two parts. To one part was added trypsin (Worthington Biochemical Corp., Freehold, N.J.) to the desired final concentration, after which both the trypsin-containing suspension and GC without added trypsin were incubated for 15 min at 37 C. At the end of the incubation the organisms were centrifuged at 4 C, washed three times in cold PBS, and resuspended in PBS such that their turbidity was 50 Klett units. These organisms were then diluted in M-100 for exposure to leukocyte monolayers or were utilized in reincubation experiments. In the latter the trypsinized, washed GC, as well as those organisms incubated only in PBS, were reincubated at either 30 or at 37 C in Frantz medium (4), after which they were washed in PBS, diluted in M-199 containing 0.01% BSA, and exposed to leukocytes in trypsin-free medium.

Chymotrypsin treatment of GC. Gonococci were exposed to α-chymotrypsin (Worthington Biochemical Corp., Freehold, N.J.) under conditions essentially identical to those for trypsin as described above.

Heat treatment of GC. Washed suspensions of GC were suspended in PBS and incubated at 56 C for desired intervals, after which they were centrifuged and resuspended in a volume of fresh PBS to an optical opacity of 50 Klett units.

Glutaraldehyde treatment of GC. Washed preparations of GC were suspended in 2% glutaraldehyde (Ladd Scientific Co., Burlington, Vt.) in PBS for 30 min at 4 C, after which the organisms were washed three times in PBS. The fixed, washed bacteria were then resuspended in PBS to an optical opacity of 50 Klett units. In both experiments on heat and on glutaraldehyde treatment of organisms, control GC were prepared for incubation with leukocytes by suspending the bacteria in PBS, allowing them to stand at 4 C for the duration of the heat or glutaraldehyde pretreatment, and washing them under the same conditions as the pretreated GC. The control and pretreated suspensions of GC with optical opacities of 50 Klett units were diluted 1:10 in M-199 with 0.01% BSA just prior to their addition to leukocytes.

Incubation of leukocytes and GC. The M-199 was decanted from the leukocyte-laden cover slips, and GC in M-199 with 0.1% BSA were added to the cover slip-containing petri plates in 1-ml volumes. The petri dishes were incubated at 80 rpm on a platform rotator (Yankee Rotator, Clay Adams, Inc., New York) in a 36 C, 5% CO_2 incubator. After 15 min of incubation, the cover glasses were washed with 10 ml of 0.5% NaCl and were air dried in a vertical position. The dried cover slips were fixed in absolute methanol, washed with water, stained in 1% Giemsa stain for 15 min, water washed, air dried, and mounted on slides.

^{14}C-labeling of GC. A modification of Frantz medium (4) was used for incorporation of ^{14}C-labeled amino acids into GC. The modified medium contained 0.16% dialyzed yeast extract (Baltimore Biological Labs) instead of the usual 0.4% yeast extract, [^{14}C]leucine, and [^{14}C]tryosine (0.1 mCi of each per ml) (New England Nuclear, San Francisco, Calif.). GC of the desired colony type were taken from G C agar (18 h of growth for T2 and 15 h of growth for T4) and inoculated into the modified Frantz medium

which was incubated in a shaking water bath at 36 C for 4 to 5 h, during which time bacterial multiplication was periodically assessed by counting the organisms with a Petroff-Hausser chamber. Portions (25 μm) of the GC suspension in modified Frantz medium were removed at intervals to assess incorporation of ^{14}C into the organisms (1). The portions were pipetted onto two 1-inch (ca. 2.54 cm) diameter Whatman no. 1 filter disks, one of which was quickly immersed in ice-cold 5% trichloroacetic acid. Radioactivity was determined on these acid-washed disks (stored overnight in trichloroacetic acid in refrigerator, 95% ethanol wash, diethyl ether wash, air-dried) as well as on disks which had not been acid washed through use of a Spectrofluor 2,5-diphenyloxazle-1,4-(5-phenyloxazolyl)-benzene toluene solution (Amersham-Searle Radiochemicals, Arlington Heights, Ill.) with a Beckman LS-233 liquid scintillation counter (Beckman Instruments, Inc., Palo Alto, Calif.).

At the end of the desired incorporation-incubation period, GC were collected by sedimentation at 1600 $\times$ *g* at 4 C for 15 min. The pelleted bacteria were resuspended in M-199, centrifuged as above, again suspended in M-199, and subjected to a final centrifugation at 450 $\times$ *g* at 4 C for 5 min to remove any large clumps of bacteria. The GC remaining in the supernatant were utilized for incubation with leukocytes. Incorporation of ^{14}C into GC was determined by monitoring the radioactivity in pelleted bacteria and supernatant fluid in both acid-washed and non-acid-washed portions at each washing and centrifugation step. During the final wash, and in the GC used for incubation with leukocytes, the radioactivity in the acid-washed and non-acid-washed specimens were practically equivalent, indicating that nearly 100% of counts remaining after washing had been incorporated into GC and were in large molecular weight components.

Exposure of leukocytes to ^{14}C-labeled GC. Leukocytes were prepared from whole blood as previously described. For these experiments, portions of a leukocyte suspension were placed in plastic tissue culture dishes, and the cells were allowed to attach for 30 min at 37 C. In the majority of studies 2 $\times$ 10^6 leukocytes were added to the dishes (35 by 10 mm). In one experiment, larger dishes (60 by 15 mm) and more leukocytes (9 $\times$ 10^6) were utilized. After the attachment of leukocytes, they were washed several times with M-199. This wash fluid was replaced with M-199 plus BSA containing the ^{14}C-labeled T2, T4, or T4* GC in multiplicities of 1 or 10. Incubations of leukocytes with these ^{14}C-labeled GC were carried out on a platform rotator at 80 rpm for 30 min at 36 C in a 5% CO_2 atmosphere, and all were done in duplicate. At the end of incubation, portions of the fluid overlying the leukocyte monolayers were removed for counting of radioactivity. The monolayers were then washed with M-199 (seven times, 5 ml each wash) and with calcium and magnesium free Earle basic salt solution (Grand Island Biological Co.) (three times, 5 ml each wash). The monolayer was removed either with trypsin (0.9 ml of a 2.5% solution for 15 min at room temperature) followed by sodium dodecyl sulfate (SOS) (0.1 ml of a 10% solution) or with SDS alone. Samples equivalent in volume to those previously removed were taken for determination of radioactivity associated with the leukocyte monolayer. Either the trypsin-SDS or the SDS alone removed all leukocytes from the plates as judged by phase microscopy. Assessment of radioactivity in the portions was accomplished by dropping the samples on Whatman filter disks which were counted in the scintillation fluor without acid washing.

Exposure of HeLa cells to ^{14}C-labeled GC. HeLa cells (American Type Culture Collection) were propogated on plastic tissue culture dishes in Minimal Essential Medium (Grand Island Biological Co.) containing 10% heat-inactivated fetal calf serum, penicillin, and streptomycin. Before incubation with GC, the HeLa cell monolayers were washed several times with serum and antibiotic free M-199. ^{14}C-labeled T2, T4, and T4* GC, labeled as previously described, were added (3 $\times$ 10^6 GC) in M-199, and the monolayer was incubated for 30 min on a rotating platform at 37 C. ^{14}C-labeled GC in the supernatant and cell-associated fractions were determined as was done with ^{14}C-labeled GC plus leukocytes experiments (see above).

Light microscopy. Light microscopy was carried out on smears or monolayers with a Zeiss Photomicroscope II (Carl Zeiss, Inc., New York) utilizing a 63$\times$ oil immersion objective and phase-contrast optics. Slides were frequently coded to prevent observer bias in scoring. Neutrophils were evaluated as to the percentage which were "associated" with GC. A neutrophil which had GC either within its peripheral outline or touching its periphery was scored as a neutrophil with which GC were associated (attached-ingested). Photo micrographs were recorded on Plus X film (Kodak, Rochester, N.Y.).

Electron microscopy. Pilation of GC was assessed in preparations negatively stained with 1% sodium phosphotungstate or 1% uranyl acetate and examined with a Siemens Elmiskop 1A (Siemens Corp., Iselin, N.J.) as previously described (15).

RESULTS

Leukocytes-GC association: light microscope evaluation. Visualization of GC associated with neutrophils is enhanced through use of phase contrast optics and Giemsa-stained preparations. This technique lends a blue-green hue to GC, which are quite distinct from leukocytic components. However, determination of the exact spatial relationships between bacteria and neutrophils is often ambiguous by light microscopy and it may be difficult or impossible to differentiate attachment from ingestion of the GC (Fig. 1). For this reason, our light microscope data have been tabulated as percentages of neutrophils with attached and/or ingested (attached-ingested) GC. "Association" of GC with neutrophils refers to both attached and ingested GC.

Our initial experiments on leukocytes plus GC were carried out with T2 and T4 cultures of

strains F62 and MS11. These studies consistently showed a higher degree of leukocytic attachment-ingestion of pilated, T2 organisms as compared to nonpilated, T4 GC of the same strains (Fig. 2). Numerous modifications in technique failed to resolve the apparent disparity between our data (T2 > T4) and the converse findings (T4 > T2) of other authors (12, 17). This disparity was partially resolved when a T4 organism we had been studying changed its characteristics during the course of our investigation. Instead of exhibiting the usual low-level association with leukocytes, the T4 GC suddenly showed attachment-ingestion that was equal to or exceeded that of T2 GC. These colony T4 organisms were subsequently designated T4* due to their increased association with neutrophils as compared to that of the first T4 organisms we had studied. The T4* organisms show no differences in colonial morphology as compared to T4 GC. Similarly, the T4* and T4 GC are identical to their cell wall ultrastructure and their lack of pili as judged by repeated electron microscopic study of negatively stained and of freeze-fractured, freeze-etched preparations.

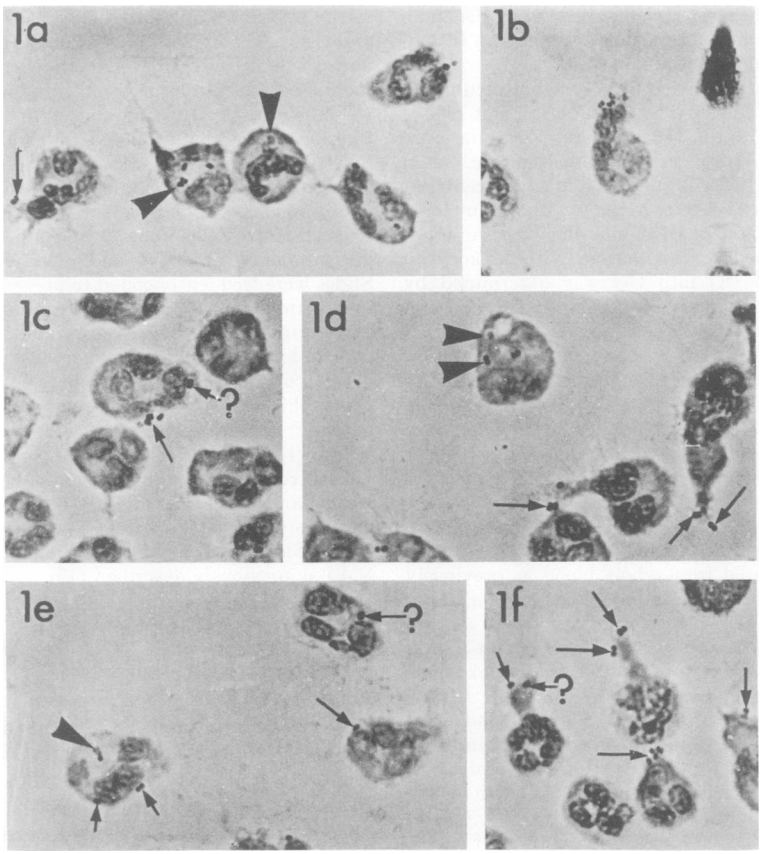

FIG. 1. *Light microscope appearances of neutrophils with attached-ingested T2 GC. Several types of association between GC and neutrophils are shown from an incubation of pilated, colony T2 organisms with leukocytes. Obvious phagocytosis can be easily discerned if a vacuole surrounds the gonococcus whose profile is within the periphery of a leukocyte (large arrows, a, d, and e). In some instances the ingested bacteria have reduced staining (a, neutrophil on right; d, GC near top of cell), and this increases the likelihood that the observed relationship is one of phagocytosis: the intracellular bacterium has apparently lost stainability due to its intraleukocytic destruction. One can be equally certain that the observed relationship between GC and neutrophils is attachment when organisms are found at the periphery of the leukocyte (a, c, d, e, and f, small arrows; and b). In many instances, however, the relationship between bacterium and phagocyte is uncertain (c, e, and f [?]). In these cases the bacteria lie upon or within the peripheries of the neutrophils, but no vacuolation is clearly observed around the bacteria. We are uncertain whether this represents phagocytosis or attachment. Phase contrast; Giemsa stain; ×750.*

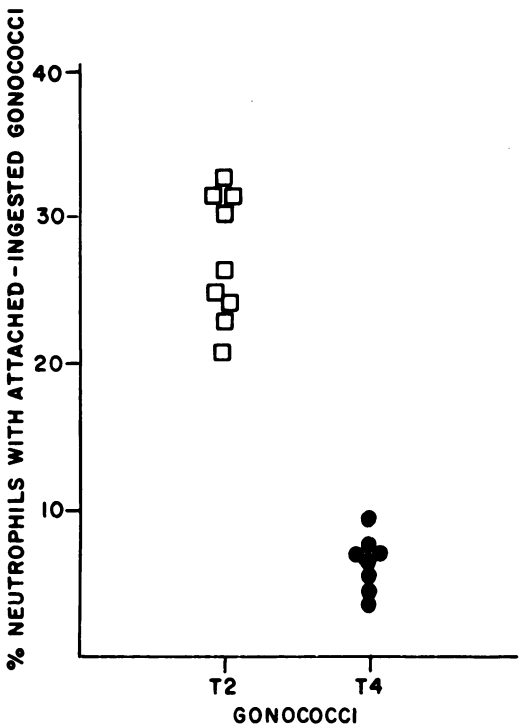

FIG. 2. *Initial results from incubations of GC and human leukocytes. Incubations of partially purified leukocytes and T2 (□) or T4 (●) GC (10 GC per neutrophil) were carried out as described in the text. The increased association (attachment-ingestion) of T2 GC as compared to T4 organisms is clearly demonstrated.*

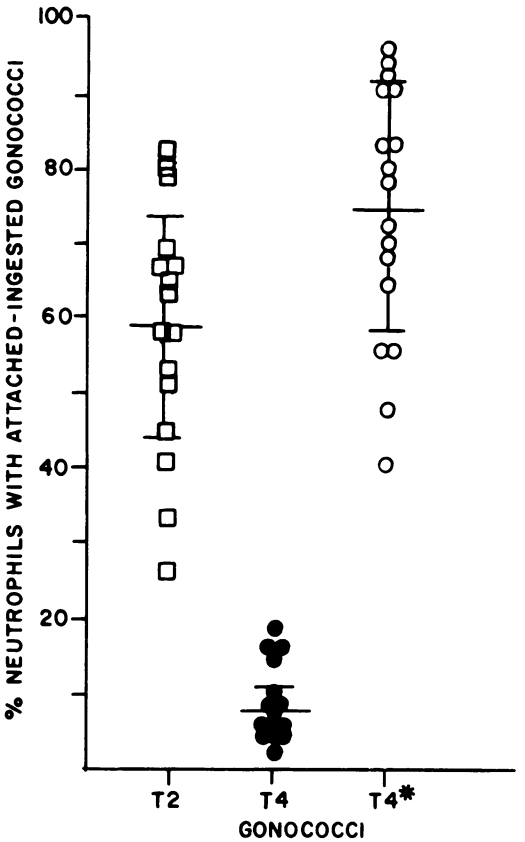

FIG. 3. *Association of T2, T4, and T4* GC with neutrophils in monolayers. The results are from 17 separate experiments which included simultaneous incubations of the three GC types with neutrophils. The percentages obtained from counting at least 300 neutrophils and determining the number with associated (attached-ingested) GC are shown. With T2 (□) GC the mean percentage of such neutrophils is 59% (standard deviation [SD] = 15%), with T4 (●) GC the mean is 8% (SD = 3.5%), and with T4* (○) organisms the mean is 75% (SD = 17%). The differences between the means of T2 and T4 and between T4 and T4* are highly significant ($P < 0.001$), and the means of T2 and T4* are also significantly different ($P < 0.01$). Ratios of GC to leukocytes varied between 10 and 50 in these experiments, but the multiplicity for all three types of organisms was the same in any one experiment.*

Incubation of leukocytes with T2, T4, and T4* GC are summarized in Fig. 3. In each of these experiments, organisms of the three types (T2, T4, and T4*) from a single strain were incubated under identical conditions with duplicate leukocytic monolayers prepared from one whole blood specimen. The results depicted are from 17 such parallel experiments. The mean levels of attachment-ingestion of GC show the following relationships: T4* > T2 ≫ T4. Variations in the levels of association of any one gonococcal type appear to be due, primarily, to small differences in the number of GC used and to minor variations in incubation conditions from day to day. On only two occasions were the levels of association of T2 CG higher than those of T4* organisms in parallel experiments. Representative fields from leukocytes plus GC incubations are shown in Fig. 4.

T4 GC (strain F62) were obtained from D. Kellogg to determine the interaction of these organisms with leukocytes. We obtained two F62-4 cultures which were related to the organisms used by Kellogg et al. (8, 9) in their studies on virulence of GC in human volunteers in which the F62-4 GC were inoculated into humans after 39 and 69 passages; 39-passage T4 showed virulence, whereas the 69-passage inoculum did not establish infection in the human. We assayed both 35-passage and 75-passage F62-4 cultures, and both showed a low level of in

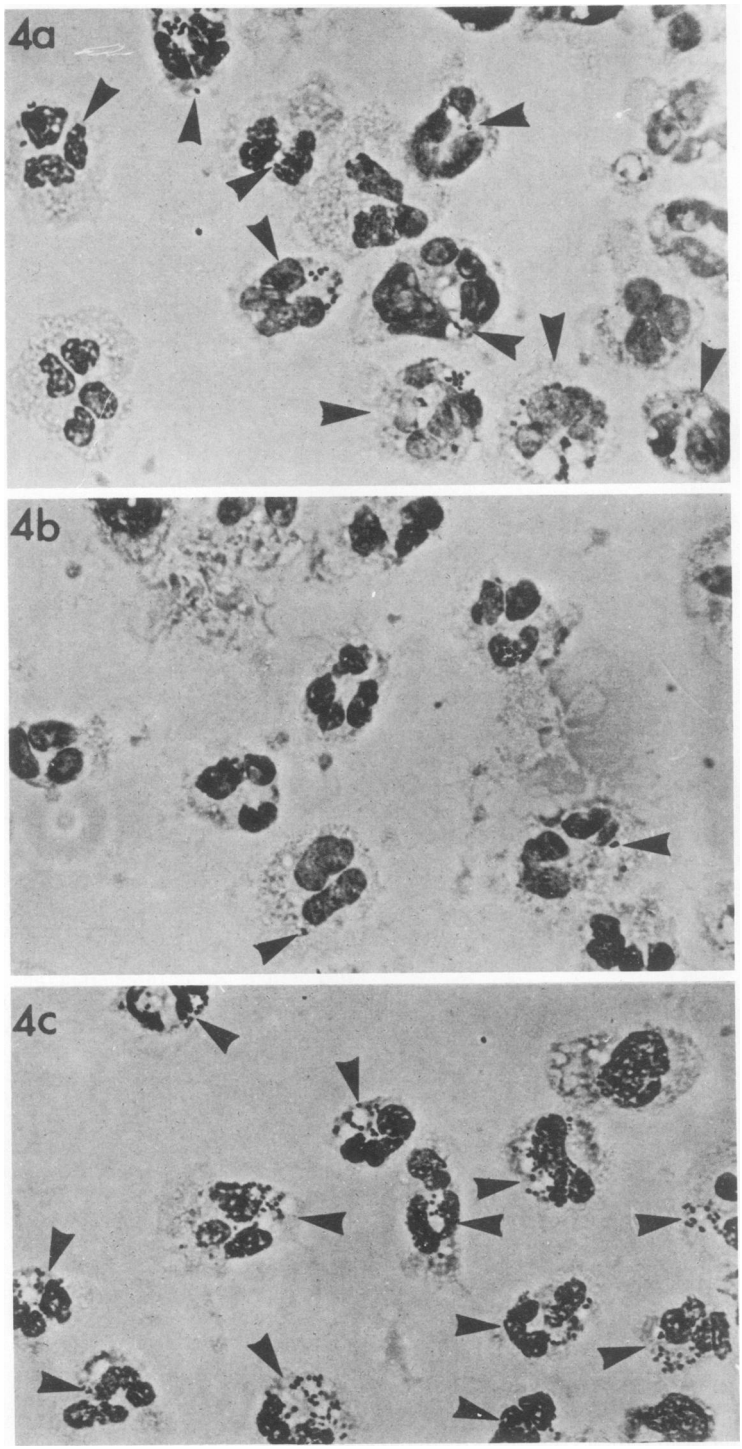

FIG. 4. *Light micrographs of T2, T4, and T4* GC-leukocyte monolayer incubations. Representative fields from incubations of identical numbers (50 GC leukocytes) of T2 (a), T4 (b), and T4* (c) GC with leukocytes are shown. These incubations were part of a single experiment carried out under the same conditions and with the same batch of leukocytes. Few neutrophils (arrows) are associated with T4 GC (b). Nearly every neutrophil in the micrograph of (c) has one or more attached or ingested T4* GC. The number of neutrophils with associated GC after incubation with T2 organisms (a) is somewhat less than in the T4* incubations (c), but it is considerably higher than after exposure of leukocytes to T4 organisms (b).* ×*1,000.*

vitro association (T4-like activity) with neutrophils.

Two areas of potential error in design of the experiments summarized above necessitated obtaining dose-response and time-response data relative to leukocyte-GC associations. First, quantitation of gonococcal number is often imprecise due, primarily, to variable degrees of clumping of the bacteria. For this reason, the number of bacteria added to leukocytic monolayers was varied (Fig. 5). It is clear from these data that minor errors in quantitating GC incubated with leukocytes cannot account for the differences in attachment-ingestion found among the three types of GC.

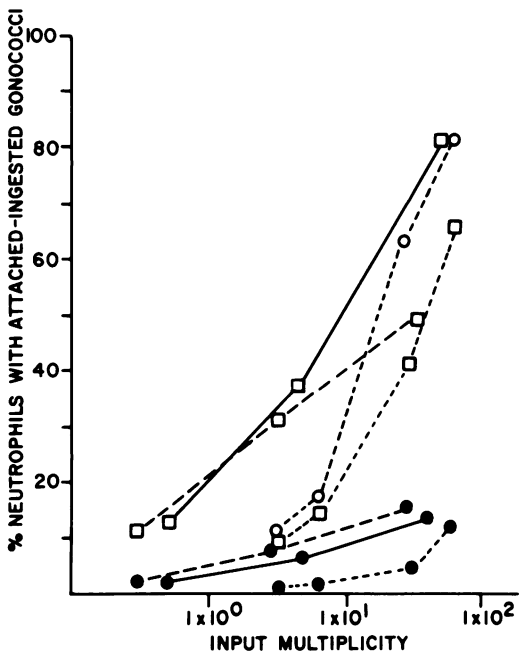

FIG. 5. *Effect of varying input multiplicity of GC on percentage of neutrophils with associated bacteria. Variation in the ratio of GC to leukocytes has the affect shown in this figure which includes data derived from three separate experiments. In two of these, T2 (□) and T4 (●) were compared in suspension (- - - -) and monolayer (——) incubations. In the third experiment (---), T2, T4, and T4* (○) GC were compared in monolayer experiments. Each point shown represents duplicate samples, and a minimum of 200 neutrophils was counted in each sample. Comparing the results in individual experiments shows that the percentage of leukocytes with associated T2 GC exceeds that with T4 organisms at each multiplicity input level in those studies utilizing only these two types of bacteria. In the experiment utilizing T2, T4, and T4* GC, each input multiplicity level showed the following relationship relative to the percentage of neutrophils with attached-ingested organisms: T4* > T2 >> T4.*

A second potential error might arise because the majority of observations on leukocyte-GC association were made after 15 min of incubation. If one colony type was attached, ingested, and destroyed much more rapidly than the other colony types, the former organisms might not be visible at the time points originally chosen for observation. Thus, one would erroneously conclude that the more rapidly ingested and destroyed organism had not become associated with leukocytes. However, the time-response results (Fig. 6) show that this hypothetical difficulty does not pertain in that, at each time point chosen for observation, the association of GC with leukocytes follows the pattern T4* > T2 ≫ T4.

Leukocytes plus ^{14}C-labeled T2, T4, and T4* GC. ^{14}C-labeled GC of the three types (T2, T4, and T4*) were prepared and incubated with leukocytic monolayers, after which the distribution of radiolabel between the supernatant and cell-associated fractions was determined as outlined above (Table 1). Three of the experiments utilized only T2 and T4 GC, as they were carried out prior to the appearance of T4* organisms. The remaining two experiments were performed with T2, T4, and T4* GC. The percentages of GC ^{14}C label found associated with leukocytes (T2, $\bar{x}$ = 62.7%; T4, $\bar{x}$ = 17.2%; T4*, $\bar{x}$ = 77.7%) are relatively similar to the percentages of neutrophils that have attached-ingested GC as judged by light microscopy (T2, $\bar{x}$ = 59%; T4, $\bar{x}$ = 8%; T4*, $\bar{x}$ = 75%). Clearly the same relative degrees of association of GC with leukocytes (T4* > T2 ≫ T4) were obtained in both types of experiments.

^{14}C-labeled T2, T4, and T4* GC plus HeLa cells. ^{14}C-labeled T2, T4, and T4* GC were prepared and exposed to monolayers of leukocytes and of HeLa cells in parallel experiments. Determination of the radiolabel distributed in the supernatant and cell-associated fractions was carried out (Table 2). The percentages of radiolabel in the cell-associated fractions of leukocytes plus GC incubations are similar to those described above. This contrasts sharply with the distribution of ^{14}C label in incubations of T2, T4, and T4* labeled GC with HeLa cells. ^{14}C-labeled T4* GC associate with HeLa cells only to a degree similar to that for ^{14}C-labeled T4 organisms and to a much lesser extent than ^{14}C-labeled T2 GC. This is quite different from results of the ^{14}C-labeled GC plus leukocyte incubations in which T4* exceeds T2, but both T4* and T2 are much higher than T4 in their association with leukocytes.

Pretreatment of GC with trypsin, chymo-

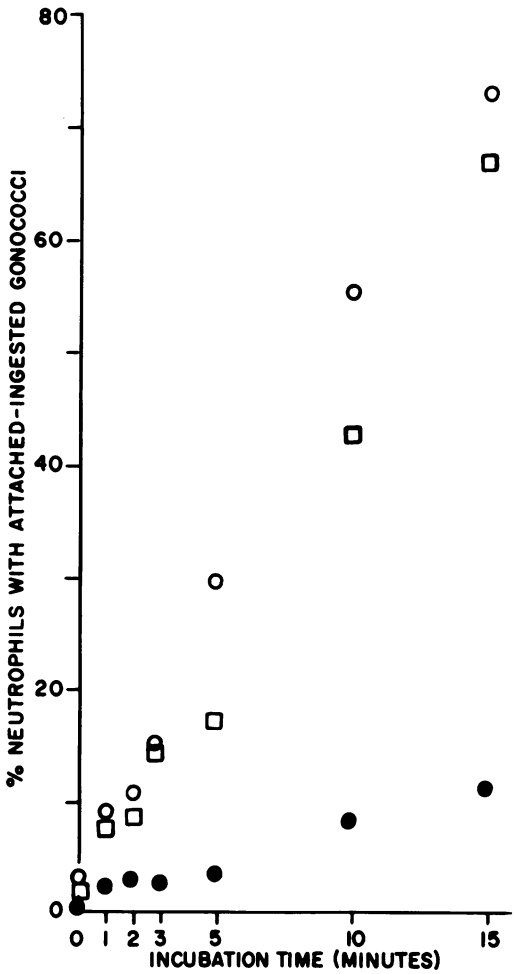

FIG. 6. *Time course of attachment-ingestion of GC with neutrophils. T2 (□), T4 (●), and T4* (○) were added in multiplicities of 50 to leukocyte monolayers at zero time. The cover slips overlaid with the suspension of GC were then incubated at 36 C on a rotating platform. At the desired interval, the cover slips were washed, removed, dried, and stained. At each time point the number of neutrophils with associated GC showed the following relationship relative to the type of GC included in the incubation: T4* > T2 >> T4.*

trypsin, heat, or glutaraldehyde. Incubation of GC with trypsin prior to their exposure to leukocytes drastically reduces the attachment-ingestion of the organisms to the neutrophils. This alteration in reactivity of the organism occurs primarily with T2 and T4* GC (Table 3). The possible affect of such trypsin incubation on T4 organisms is difficult to assess due to the low level of association shown by these organisms under control (no trypsin treatment) conditions.

TABLE 1. *Distribution of radioactivity after incubation of ^{14}C-labeled gonococci (GC) with leukocyte monolayers*

^{14}C-labeled GC	Recovered radioactivity in leukocyte-associated fraction (%)
T2	62.7 (±9.1)[a]
T4	17.2 (±7.4)[a]
T4*	77.7 (±6.6)[b]

[a] Mean and standard deviation of percentages in leukocyte-associated fraction based on five experiments.

[b] Mean and standard deviation based on two experiments.

TABLE 2. *Distribution of radioactivity after incubation of ^{14}C-labeled gonococci (GC) with HeLa cell monolayers*

^{14}C-labeled GC	Recovered radioactivity (%)[a]	
	Expt no. 1	Expt no. 2
T2	58.3	56.4
T4	10.5	17.4
T4*	18.9	19.4

[a] Recovered radioactivity in HeLa cell-associated fractions. Each percentage represents average of duplicate incubation mixtures.

TABLE 3. *Influence of trypsin treatment on association of several gonococcal (GC) strains with human neutrophils*

GC	Trypsin pretreatment[a]	Neutrophils with associated GC (%)
F62-2	0	68
F62-2	+	7
MS11-2	0	69
MS11-2	+	4
F62-4/A50	0	2
F62-4/A50	+	4
MS11-4/B69	0	3
MS11-4/B69	+	2
F62-4*/720	0	65
F62-4*/720	+	5
MS11-4*/819	0	70
MS11-4*/819	+	1
MS11-4*/F4	0	65
MS11-4*/F4	+	2

[a] GC pretreated with trypsin (+) were incubated for 15 min in 0.25% trypsin, washed in PBS, and diluted as described in the text. Control organisms not exposed to trypsin (0) were incubated in PBS for 15 min.

Pili are visible and appear structurally intact on T2 organisms which have been incubated for 15 min in 0.25% trypsin (Fig. 7). Exposure of the

leukocyte monolayers to trypsin, rather than pretreatment of GC with the enzyme, did not change the percentages of neutrophils with associated GC.

Chymotrypsin preincubation appears to exert a similar affect, i.e., decrease in association of GC with neutrophils. With chymotrypsin there appears to be a difference in the amounts of enzyme necessary to decrease the ability of GC to associate with neutrophils relative to the colony type of the GC. T2 organisms show significantly altered reactivity with leukocytes after pretreatment with 1 μm of chymotrypsin per ml, whereas 100 μm of chymotrypsin per ml is needed for a similar effect on T4*.

Reincubation of either trypsin- or chymotrypsin-treated GC results in reestablishment of the propensity of the organisms to associate with leukocytes. This is shown for trypsin treatment in Table 4. Reincubation at either 30 or 37 C after trypsin treatment and washing results in the attainment of levels of association similar to those in nontrypsinized organisms.

Either heating GC to 56 C or pretreatment of the organisms with glutaraldehyde reduced their attachment-ingestion by leukocytes. T2 and T4* GC behave similarly toward leukocytes after either of these pretreatments, and their levels of association with neutrophils were similar to those of untreated T4 GC.

DISCUSSION

The ease with which blood or tissue phagocytes engulf bacteria is, classically, a partial explanation for the relative pathogenicity of several pyogenic microorganisms. The restricted phagocytosis of encapsulated pneumococci or of M protein-bearing streptococci are two instances in which the reduced tendency toward phagocytic uptake correlates with virulence of the organisms. In these examples, specific bacterial surface components are correlated both with the reduced phagocytosis and with the virulence of the microorganisms (3, 19). More recently, streptococcal M protein has also been shown to influence interactions of these bacteria with epithelial cells (2). In this instance, M protein appears to enhance attachment of group A streptococci to buccal mucosal cells.

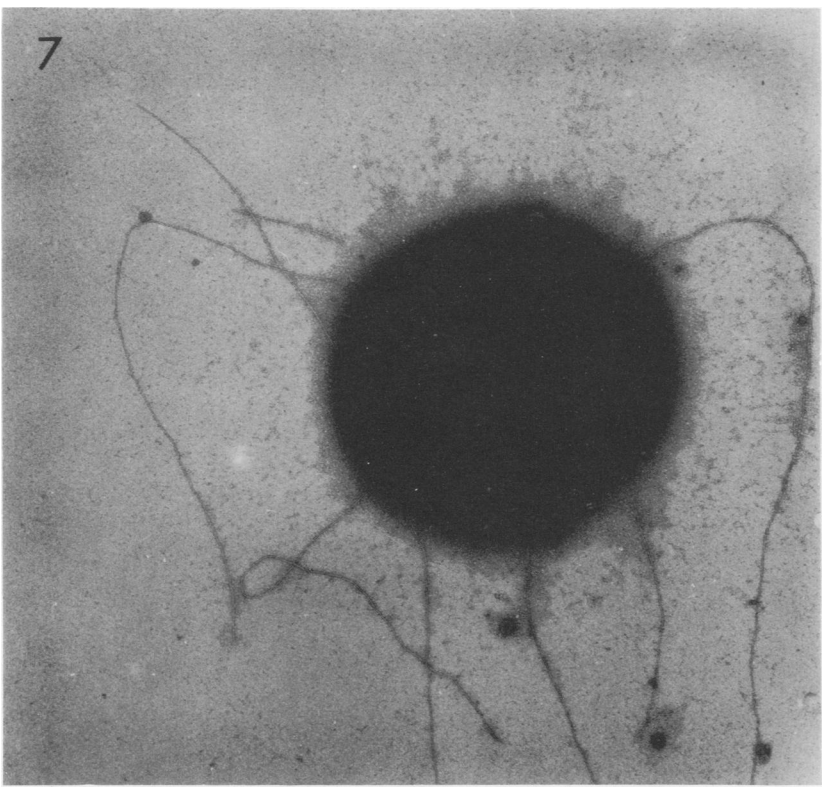

FIG. 7. *By electron microscopy, negatively stained T2 GC exhibit numerous pili after exposure of the organisms to 0.25% trypsin for 30 min. ×30,000.*

TABLE 4. *Influence of trypsin treatment and reincubation on association of T2 and T4* gonococci (GC) with neutrophils*

GC	Trypsin pretreatment[a]	Reincubation in trypsin-free medium	Neutrophils with associated GC (%)
MS11-2	–	–	83
MS11-2	+	–	4
MS11-2	–	2 h, 30 C	67
MS11-2	+	2 h, 30 C	58
MS11-2	–	2 h, 37 C	73
MS11-2	+	2 h, 37 C	72
MS11-4*/819	–	–	77
MS11-4*/819	+	–	12
MS11-4*/819	–	2 h, 30 C	76
MS11-4*/819	+	2 h, 30 C	81
MS11-4*/819	–	2 h, 37 C	72
MS11-4*/819	+	2 h, 37 C	73

[a] GC were pretreated in 0.1% trypsin (+) for 15 min at 37 C, washed, and diluted as described in the text. Control GC not exposed to trypsin (–) were incubated for 15 min at 37 C in PBS.

The virulence of *Neisseria gonorrhoeae* for man has been correlated with formation of in vitro characteristic colony forms, (8, 9), and GC populating those colonies correlated with virulence are pilated (7, 15). These findings led to a hypothesis that pili play a role in the virulence of GC, perhaps through influencing interactions of the organisms with leukocytes, with epithelial cells, or with both (3). Work from this laboratory as well as by others has demonstrated that pili enhance the attachment of GC to some nonleukocyte, eukaryotic cells (4, 6, 12, 18). One group of investigators has reported that pili restrict phagocytosis of GC by human leukocytes (12, 17) in a manner somewhat analogous to the effect of the pneumococcal capsule of streptococcal M protein (3, 19). Our studies on in vitro GC-leukocyte interactions have yielded data which lead to a different conclusion, namely that pilation of GC is of minor importance as a determiner of attachment-ingestion of GC by human polymorphonuclear leukocytes.

Our studies demonstrate that in vitro attachment-ingestion of pilated, colony T2 GC with human neutrophils is intermediate to the levels of leukocyte association for two apparently different forms of nonpilated, colony T4 organisms. Although both of these nonpilated GC have identical colonial morphologies, their reactions with human neutrophils differentiate them quite clearly. One of the nonpilated forms (T4) shows little attachment-ingestion with leukocytes, whereas the other form (T4*) avidly associates with neutrophils. T4 and T4* organisms, as defined by their level of interaction with neutrophils, have been isolated from two strains of GC, MS11 and F62. The propensity of T2, T4, and T4* GC for association with leukocytes has been established through a variety of technical approaches, including use of monolayers and suspensions of leukocytes, different buffered media for incubations, varying time and GC to leukocyte ratio conditions, and microscopic and radioisotope means of assessing GC-leukocyte association.

Attachment-ingestion of T2 and T4* GC by neutrophils is markedly reduced by pretreating the organisms with trypsin, chymotrypsin, heat, or glutaraldehyde. Attachment of trypsin- or chymotrypsin-treated GC to leukocytes is reestablished by incubating the treated organisms in protease-free medium. These findings suggest that a protein on the GC surface promotes in vitro association of these organisms with human neutrophils, and that this material is either present or present in increased amounts of T2 and T4* GC, in contrast to T4 organisms. An alternative suggestion, that the GC-leukocyte association promoting substance is not a protein but is released from the trypsin- or chymotrypsin-treated membranous cell wall of the organisms, cannot be refuted at present. That the GC surface material in question is not pili is certainly clear for T4* organisms which have been extensively examined by electron microscopy. It is possible that T2 and T4* organisms are covered by a similar surface material which is polymerized into pili only in the former; but this seems unlikely. These alternative explanations focus attention on the seemingly enhanced association of T2 and T4* GC with neutrophils. One could argue that T4 GC are not attached-ingested by leukocytes because they possess some surface component, such as a capsule, which reduces their susceptibility to leukocyte association. We have attempted to demonstrate capsules on T4 organisms after growth on G C agar for variable durations (6 to 24 h) by use of India ink and have never found indications of their encapsulation. Although the apparent tryptic removal of surface material which positively influences leukocyte association of T2 and T4* GC prejudices us toward postulating the presence of a hypothetical "leukocyte-association-enhancing surface component," the possibility that T4 organisms possess a unique, phagocytosis-attachment restricting material on their walls cannot be ruled out by the present data.

TABLE 5. *Relative in vitro association of T2, T4, and T4* gonococci (GC) with various types of eukaryotic cells*

Eukaryotic cells	GC			Reference
	T2	T4	T4*	
Human neutrophils	+++	+	++++	
Tissue culture cells				
HeLa cells	+++	+	+	
Amnion cells	+++	+	+	15
Human foreskin cells	+++	+	+	Swanson et al.[a]
Buccal mucosal cells	+++	+	+	12; Swanson et al.[a]
Human sperm	+++	+	+[b]	6

[a] J. Swanson, B. Zeligs, and G. King, manuscript in preparation.
[b] A. N. Holmquest-James and J. Swanson, unpublished observation.

Our previous demonstration of enhanced attachment of pilated GC to tissue culture cells appears to be corroborated by comparing the behavior of pilated, T2 organisms with those of nonpilated T4 as well as the newly recognized T4* organisms toward HeLa cells. The differing behaviors of the T2, T4, and T4* GC in their association with human leukocytes and other eukaryotic cells are summarized in Table 5. The differing patterns in relative association of GC with the various eukaryotic cells suggests that interactions of the organisms' leukocytes are mediated by factors different from those which enhance attachment of the organisms to the other types of animal cells studied. This contrasts somewhat with studies indicating that M protein of group A streptococci both enhances attachment of the organisms to certain epithelial cells (2) and reduces the phagocytosis of these bacteria by human leukocytes (3).

A crucial question regarding GC-leukocyte interactions is the affect of such bacterium-phagocyte associations on the viability of the GC. One recent report indicates that pilated GC resist destruction by human and mouse leukocytes as compared to nonpilated organisms (16). This report is intriguing, but does not contain data relative to the comparative survival of different colony types incubated in the absence of leukocytes. Our preliminary studies on survival of GC when incubated with leukocytes indicate that this is an important consideration, as is use of both T4 and T4* GC for comparison with pilated T2 organisms. We suspect that Thomas et al. (16) have utilized both T4 and T4* organisms in their studies as evidenced by their data (Table 2, reference 16) in which the number of nonpilated GC from two different strains (F62 and RD5) that associate with leukocytes is quite different (90 and 97.5% of organisms recovered associated with leukocytes, respectively). These data suggest that their F62-T4 corresponds to out T4 GC, whereas their RD5-T4 is analogous in its reaction with leukocytes to our T4*. It should also be noted that those authors also find increased numbers of pilated GC associated with leukocytes as compared to T4, nonpilated organisms after an initial attachment period (2.5% of the original inoculum for F62-T1 and 0.21% for F62-T4). It appears that their data, though not to noted by those authors, partially corroborate our findings on relative in vitro association of T2 versus T4 GC with neutrophils.

The present work was undertaken, primarily, as an attempt to understand the role of GC-leukocyte interactions relative to virulence of GC and the pathogenesis of gonorrheal venereal disease. In retrospect, one can turn to a clinical laboratory finding. GC present in smears of urethral or cervical exudates from individuals with acute gonorrhea give rise to colony types associated with pilation (13), are virulent organisms (5, 11), and are primarily found in association with polymorphonuclear leukocytes (17). On the basis of these observations, one could probably conclude that the presence of pili on GC does not restrict phagocytic uptake of the bacteria by leukocytes. Our observations support that conclusion, but they do not yield information relative to the importance of such GC-leukocyte associations for either the organism or the host. Such determinations await further study. The present study also points out the possible hazard of comparing only nonpilated and pilated GC as models for studying factors of GC virulence.

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