

## Studies on Gonococcus Infection

### VII. In Vitro Killing of Gonococci by Human Leukocytes

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The comparative killing of pilated and nonpilated forms of *Neisseria gonorrhoeae* by human peripheral blood leukocytes was studied in vitro. Some nonpilated gonococci (T2) were killed to a lesser extent than were pilated, T2 organisms, which were killed less readily than another nonpilated (T4\*) form of gonococcus. Thus, the relative order of killing of gonococci by human peripheral blood leukocytes appears to be: T4 < T2 < T4\*. These data suggest that pilation, though correlated with virulence of gonococci, has little influence on the survival or killing of these organisms by human leukocytes.

Virulence of *Neisseria gonorrhoeae* has been correlated with formation of characteristic colony forms on solid medium (6, 7) and with pilation of organisms comprising the "virulent colony types" (4, 12). Pili appear to enhance attachment of the gonococci to epithelial cells as well as to cells of epithelial origin (9, 11, 17). This enhanced attachment to host cells may serve to anchor gonococci at target sites and, hence, may influence virulence by aiding survival of the gonococci.

Interactions between gonococci and human leukocytes represent another relationship between host and microorganism which, potentially, is a determiner of the organisms's pathogenicity. Classically, organisms that resist phagocytosis are more virulent than similar organisms which are more readily engulfed and killed by leukocytes, e.g., pneumococci and streptococci. Our previous studies focused on attachment and/or phagocytosis of gonococci by human leukocytes as defined by light microscopic and radioisotope methods (13, 14). Piliation of gonococci was found to have little or no influence on associations between the bacteria and leukocytes. However, those experiments did not determine the killing of gonococci by leukocytes, which is the point of the present study. Experiments have been designed to assess the relative survival or killing of pilated and nonpilated gonococci incubated with isolated human leukocytes. The results show that the presence or absence of pili does not correlate with killing of *N. gonorrhoeae* by leukocytes.

#### MATERIALS AND METHODS

**Gonococci.** *N. gonorrhoeae* strains F62 and MS11 were identified and propagated as previously de-

scribed (12). In general, the organisms were maintained on a solid medium (GC agar base containing 1% IsoVitaleX; BBL), by daily passage of single colonies of the desired colonial type. For use in killing experiments, gonococci were prepared by removing colonies from the agar with a Dacron swab and suspending the bacteria in phosphate-buffered saline, pH 7.2 (PBS), to an optical opacity of 50 Klett units (blue filter, Klett colorimeter), which yields  $2 \times 10^8$  to  $5 \times 10^8$  gonococci/ml. This suspension was then diluted in PBS to the desired concentration and the suspension was cooled (4 C) until use.

**Leukocytes.** Human leukocytes were prepared, as previously described in detail (13), through sedimentation of erythrocytes from heparinized blood with gelatin, lysing erythrocytes with ammonium chloride, and repeated washing of the remaining leukocytes with PBS. The isolated, pelleted leukocytes were enumerated, and their concentration was adjusted to  $2 \times 10^6$  or  $4 \times 10^6$ /ml with PBS containing 2% fetal calf serum (FCS; Grand Island Biological Co., Grand Island, N.Y.) which had been heat inactivated (56 C, 60 min).

**Assessment of survival/killing of gonococci.** Gonococci of all three types (T2, T4, T4\*) from one of two strains were utilized in parallel incubations for each experiment. Duplicate leukocyte-free control mixtures consisted of 0.9 ml of PBS plus 2% FCS to which was added 0.1 ml of each gonococcal suspension. Duplicate leukocyte-containing mixtures consisted of 0.9 ml of the leukocyte suspension ( $2 \times 10^6$  or  $4 \times 10^6$ /ml) and 0.1 ml of each gonococcal suspension ( $2 \times 10^4$  or  $4 \times 10^7$  gonococci/ml). All solutions and suspensions were cold at time of their addition. Immediately after mixing, 0.1-ml duplicate samples were removed from each leukocyte-free and leukocyte-containing mixture, were pipetted onto warm GC agar, and were spread with a glass rod. The incubation mixtures in snap-top polypropylene tubes (12 by 75 mm, Falcon #2063) were placed on a rotating rack and rotated at 12 rpm in an incubator at 36 C. Duplicate samples (0.1 ml) were removed from each

tube after 1 and 2 h of incubation. In incubations with a high gonococcus-to-leukocyte ratio, these samples were serially diluted and plated. In experiments with low gonococcus-to-leukocyte ratios, the 0.1-ml samples were directly plated onto GC agar plates, which were incubated overnight at 36 C in a 5% CO<sub>2</sub> atmosphere. Colony-forming units (CFU) were enumerated with a Quebec colony counter.

## RESULTS

Gonococcal killing/survival in the presence of human leukocytes was studied through use of two strains of organisms, F62 and MS11. Type 2 and type 4 cultures of the former (F62) were obtained from Douglas Kellogg, Center for Disease Control, Atlanta, Ga., and were derived from the same stocks previously utilized for human volunteer studies (6). Isolation of MS11 has been noted previously (11), and the derivation of type 4\* cultures from these two strains is described in detail elsewhere (J. Swanson and D. Young, manuscript in preparation). The presence of pili on both F62-2 and MS11-2 was regularly assessed by negative staining and electron microscopy, as was the absence of pili on type 4 and 4\* organisms of each strain. The T4 or T4\* designations denote organisms which were classified as type 4 colonies by dissecting microscope examination and whose association with neutrophils in monolayer culture is found to be either low (T4) or high (T4\*) as defined previously (13).

Most experiments on relative killing/survival of gonococci were done with low gonococcus-to-leukocyte ratios (1:1,000) to circumvent serially diluting specimens prior to plating for determination of CFU present. Other experiments were carried out with gonococcus-to-leukocyte ratios of 1:1. There was no difference in the relative survival/killing of T2, T4, and T4\* gonococci with the high versus the low gonococcus-to-leukocyte ratio.

The results of a single experiment conducted with the high (1:1) ratio of bacteria to leukocytes are shown in Fig. 1. The CFU of T4 organisms in both the control, leukocyte-free and the leukocyte-containing tubes were similar, indicating little killing of type 4 gonococci in the presence of leukocytes (leukocyte-containing CFU = 70% of CFU in the leukocyte-free control) after 2 h of incubation. Both T2 and T4\* gonococci exhibited sizable decreases in the CFU present in leukocyte-containing specimens over the 2-h incubation, and the decrease was similar for both types. However, T4\* gonococci multiplied somewhat more in the leukocyte-free control than did T2 organisms. The comparison of CFU in leukocyte-containing and in leuko-

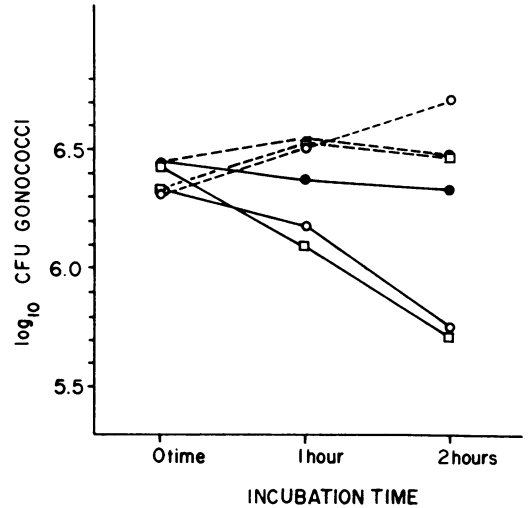


FIG. 1. Survival/killing of strain F62 gonococci in presence of human leukocytes. Type 2 (□), type 4 (●), and type 4\* (○) organisms from strain F62 were added to leukocyte-free, control specimens (dashed lines) and to leukocyte-containing tubes (solid lines) as described in Materials and Methods. The gonococcus-to-leukocyte ratio was approximately 1:1. At each time point, duplicate samples were removed from the incubation specimens and the number of colony-forming units (CFU) was determined by serial dilution and plating. The average CFU in each specimen, expressed as log<sub>10</sub>, is shown for a single experiment.

cyte-free specimens shows that the percentage of T4\* organisms surviving incubation with leukocytes was smaller than that of T2 gonococci (T4\* = 11%, T2 = 17% of controls). The relative killing/survival of gonococci incubated with leukocytes is shown more clearly in Fig. 2, which is a composite from 10 separate experiments each of which utilized T2, T4, and T4\* gonococci incubated in parallel at gonococcus-to-leukocyte ratios of 1:1,000. The relative survival of these organisms was as follows: T4 > T2 > T4\*. These data are based on comparisons between the leukocyte-containing and leukocyte-free specimens, with survival of organisms in the latter being shown in Table 1.

The results described above were obtained by plating gonococcus-leukocyte mixtures. Experiments were carried out to determine whether "disruption" of leukocytes would yield different results. Homogenization of leukocyte-plus-gonococcus specimens (400 strokes in Dounce homogenizers at 4 C) produced a minuscule increase in the CFU from each specimen, but the increments were similar for T2, T4, and T4\* organisms and did not change the comparative values for these gonococci. Saponin treatment

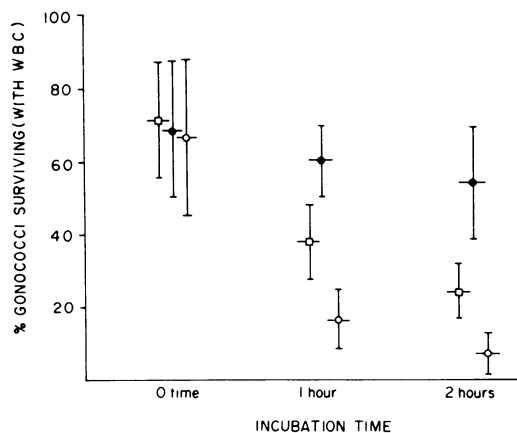


FIG. 2. Percent survival of T2, T4, and T4\* gonococci incubated with human leukocytes. The results of 10 separate experiments utilizing type 2 (□), type 4 (●), and type 4\* (○) gonococci of strains MS11 and F62 are shown. Survival for gonococci of each type was calculated as follows: CFU in gonococcus-plus-leukocyte specimen/CFU in leukocyte-free specimen  $\times$  100. The percent surviving gonococci was determined at each time point through duplicate sampling of duplicate incubation mixtures each of leukocyte-containing and leukocyte-free specimens. Each experiment utilized T2, T4, and T4\* organisms incubated in parallel and added at gonococcus-to-leukocyte ratios of approximately 1:1,000. The means and standard deviations of the percentage of gonococci surviving are shown.

was attempted (5% saponin, final concentration; 5-min incubation; 20 C), but gonococci were extensively killed in both the control and the leukocyte-containing specimens, in contrast to a previous report (16). This bactericidal action of saponin precluded assessing the leukocyte-associated killing of gonococci.

## DISCUSSION

Demonstration that piliation is a characteristic of virulent colony types of *N. gonorrhoeae* (4, 12) has led to speculation and studies on the possible role or roles that pili play as determiners of virulence. Recent reports have suggested that pili enhance attachment of gonococci to several cell types including tissue culture cells (9, 11), buccal mucosal cells (9, 13), fallopian tubal epithelium (17), and sperm (3). This enhanced attachment of gonococci to various eukaryotic cells is similar to the M protein-mediated attachment of streptococci to oral surfaces (2), as well as pilus-mediated attachment of *Escherichia coli* (5) or *Shigella* spp. (1) to intestinal mucosal cells. Sticking of bacteria to host cells is thought to provide advantage to

the bacteria relative to their survival in and colonization of the host (10).

Survival of bacteria, in general, is also enhanced if the hosts' phagocytic leukocytes are unable to ingest and/or destroy the potentially infectious bacteria. On that basis we suggested that pilated, virulent gonococci might exhibit restricted or reduced phagocytosis by leukocytes (12). Two groups of investigators have recently published results interpreted as showing a phagocytosis-reducing influence of gonococcal piliation (8, 9, 16). However, our studies carried out by light microscopic or radiolabeling methods suggested that pili play little or no role as determiners of interactions between gonococci and human neutrophils (13). That conclusion was based on the demonstration that some nonpilated (T4\*) gonococci exhibit greater attachment-ingestion by leukocytes than do pilated (T2) organisms, but other nonpilated forms (T4) are less susceptible to association with leukocytes than are the pilated, T2 gonococci. An additional report suggested that pilated gonococci resist destruction by leukocytes in vitro (15), and that prompted our studies on killing of gonococci by leukocytes. We found that leukocyte-mediated killing of gonococci is consistent with what would be expected on the basis of our studies on gonococcus-leukocyte association, namely, that pilated gonococci are intermediate in their susceptibility to killing by human leukocytes as compared with T4 and T4\* gonococci. Punslang and Sawyer (9) noted that, "although nonpilated gonococci did not detectably adhere to PMN (polymorphonuclear leukocytes), the bacteria were readily ingested. Pilated gonococci on contrast, adhere to PMN but resisted phagocytosis." Our data do not suggest any similar dissociation between the initial interactions of gonococci and leukocytes and the killing of these organisms by the phagocytes. Those organisms that are associated with leukocytes (by light microscopic examination or

TABLE 1. Survival of gonococci in leukocyte-free medium

| Gonococcus type | Percent CFU (0-time specimens as 100%) <sup>a</sup> |                |
|-----------------|---|----------------|
|                 | 1-h incubation                                      | 2-h incubation |
| T2              | 106.6 $\pm$ 9.5                                     | 131.8 $\pm$ 26 |
| T4              | 99.1 $\pm$ 18                                       | 110.6 $\pm$ 25 |
| T4*             | 96.3 $\pm$ 18                                       | 98.4 $\pm$ 16  |

<sup>a</sup> Mean and standard deviation values based on 10 separate experiments utilizing a gonococcus-to-leukocyte ratio of 1:1,000.

through radioisotope studies) are the organisms which are most readily killed by leukocytes. Preliminary experiments utilizing cell-free homogenates of leukocytes show that all three gonococcal types (T2, T4, T4\*) are equally susceptible to the bactericidal action of such preparations (G. King and J. Swanson, unpublished data).

Both our previous and present reports appear to conflict with data obtained by other investigators (8, 9, 15, 16), but these differences are probably apparent rather than real. The conclusion that pili reduce the susceptibility of gonococci to phagocytosis is likely based on comparison of pilated organisms only with nonpilated gonococci that correspond to out T4\* forms, as has been previously discussed (13).

These in vitro studies on killing of gonococci by human leukocytes yield little information relative to the mechanism(s) by which *N. gonorrhoeae* cells attain virulence. Our results indicate that pilation of gonococci does not render the organisms resistant to ingestion and killing by peripheral blood leukocytes in vitro, and suggest that pilation would have a parallel, negligible effect on uptake and killing of the organisms by similar phagocytes in vivo. This does little except give experimental confirmation to the easily observed intraleukocytic presence of gonococci in exudates from patients with acute gonorrhea.

Piliation appears to correlate with virulence of gonococci (4, 12). Although pili do not seem important determiners of the outcome of interactions between gonococci and peripheral blood leukocytes, other studies indicate that pili both enhance attachment of the organisms to epithelial cells (9, 11, 17) and reduce the attachment of these organisms to macrophages (M. Blake and J. Swanson, manuscript in preparation). These may be mechanisms through which pili of gonococci play roles as determiners of virulence of these organisms.

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