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Attachment and Ingestion of Gonococci by Human Neutrophils

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Previous studies have indirectly shown that type 1 gonococci are more resistant to phagocytosis by human neutrophils (PMN) than type 3 gonococci. Using phase contrast, fluorescent, and light microscopy, we directly quantitated PMN-gonococcal interaction, with emphasis on separating ingestion from attachment. PMN monolayers were incubated on slides with type 1 or type 3 gonococci. The slides were iced to stop phagocytosis and stained with anti-gonococcal fluorescent antibody (FA). After methanol fixation, the FA-stained gonococci associated with PMN were counted. Since the live PMN excludes FA, the FA-stained gonococci represent only extracellular gonococci. Methylene blue was then added to the same slide to stain both ingested and surface attached gonococci. Using these methods, intracellular and extracellular cell-associated gonococci were quantitated under varying conditions. The numbers of methylene blue-stained cell-associated gonococci were similar for types 1 and 3. The proportions of cell-associated gonococci that were ingested were: with normal serum, 3.7 ± 4.1% for type 1 and 56.2 ± 3.5% for type 3 ($P < 0.001$); with heat-inactivated serum, 1.0 ± 3.0% for type 1 and 52.6 ± 3.7% for type 3 ($P < 0.001$); with high-titer anti-gonococcal antibody serum, 4.8 ± 4.3% for type 1 and 64.0 ± 1.6% for type 3 ($P < 0.001$). Thus, most type 3 organisms were ingested, but most type 1 gonococci were bound on the PMN surface.

The virulence of *Neisseria gonorrhoeae* in human volunteers has been correlated with colony morphology by Kellogg et al. (5, 6); types 1 and 2 small-sized colonies produced infection, whereas types 3 and 4 large-sized colonies did not. Several studies have since suggested that types 1 and 2 gonococci may be more resistant to phagocytosis by human neutrophils than types 3 and 4. However, the techniques used to measure phagocytosis have not separated ingestion from cell surface attachment.

Punsalang and Sawyer (10) and Thongthai and Sawyer (18), after incubating polymorphonuclear neutrophils (PMN) with gonococci for 30 min, prepared stained smears of the mixture and expressed the results as the percentage of PMN that contained at least one gonococcus. Ofek et al. (9) incubated gonococci for 15 min with PMN adherent to cover glasses and expressed the results as the percentage of PMN ingesting bacteria. Both groups noted a significantly lower percentage of types 1 and 2 gonococci associated with PMN compared to types 3 and 4. They concluded that types 1 and 2 gonococci were more resistant to phagocytosis by the PMN than types 3 and 4. However, since their procedures stain both intracellular and extracellular bacteria, one cannot separate ingestion from surface attachment of gonococci to PMN.

Thomas et al. (17) measured the number of viable extracellular and cell-associated types 1 and 4 gonococci after 2 h of incubation with PMN adherent to cover glasses. They noted that although types 1 and 4 bacteria appeared equally cell associated by light microscopy, the viability of type 4 gonococci declined in the PMN cultures whereas that of type 1 remained constant. The addition of bactericidal serum had little effect on type 4 viability, but significantly reduced type 1 viability. The authors concluded that the majority of type 1 gonococci either adhered to the PMN surface or were located such that they could interact extracellularly with the bactericidal serum.

Watt (22), without referring to colony types, measured the viability of gonococci associated with PMN. After 1 h, 80 to 85% of the bacteria were killed but there was little subsequent killing over the next 3 h. To exclude intracellular survival, Watt added penicillin to the mixture and noted prompt killing of the remaining gonococci. He concluded that "the persisting organisms were extracellular and that failure [of the PMN] to kill all the gonococci was due to a

relative inability to phagocytize the bacteria."

The stickiness of types 1 and 2 gonococci has been studied microscopically. They have been shown to autoagglutinate (7, 14) and to agglutinate erythrocytes of different mammalian species (10). In addition, type 1 gonococci have been shown to attach to buccal epithelial cells (10), human amnion cells (13), urethral mucosal cells (20), human sperm cells (2), and fallopian tube epithelium (21). Punsalang and Sawyer (10) also reported adherence of type 1 gonococci to PMN treated with sodium fluoride to inhibit phagocytosis. Sodium fluoride significantly alters PMN metabolism and function (4), and one must question the relevance of observations on sodium fluoride-treated cells.

We studied gonococcal-PMN interaction with special effort to distinguish ingestion from attachment quantitatively. Techniques used included: (i) phase contrast microscopy of live preparations; (ii) fluorescent antibody (FA) stains of live PMN, which will stain only extracellular bacteria; and (iii) methylene blue stains of killed leukocytes, which stain both intracellular and extracellular organisms.

MATERIALS AND METHODS

Gonococci. Strains of *N. gonorrhoeae* were isolated from urethral exudates. Colony types 1 and 3 were identified by the method of Kellogg et al. (6) and stored before use in 1% proteose peptone no. 3 (Difco) with 8% glycerol at −70 C in a Revco freezer (19). When thawed, 90% of the original inoculum was viable. Prior to an experiment, the gonococci were either thawed for use that day or cultured overnight on GC agar base medium with 1% IsoVitaleX (Baltimore Biological Laboratory) for 16 to 18 h in 6% CO_2 at 37 C. Gonococci were then harvested with Hanks balanced salt solution without bicarbonate (HBSS, Microbiological Associates, Inc., Bethesda, Md.), centrifuged at 3,000 × *g* for 15 min, and washed in HBSS one time. The thawed gonococci were similarly treated. The final concentration of bacteria was about 2 × 10^8/ml as determined with a Petroff-Hauser counting chamber. Experimental results were similar with thawed or fresh 18-h cultures of gonococci. If clumps of bacteria were seen, the suspension was agitated on a Vortex mixer for 3 min. If the clumps persisted, the suspension was discarded.

Polymorphonuclear neutrophils. Two to three drops of peripheral venous blood from a single normal donor were placed on clean glass slides or cover glasses and incubated in a moistened petri dish for 30 to 45 min at 37 C to allow PMN attachment to the glass surface. The clot was then washed off with 37 C HBSS. This technique yields about 10^6 PMN per cover glass or slide. Homologous serum (10%), either fresh or heat inactivated at 56 C for 30 min, was used for most experiments. Serum from a patient recently recovered from gonococcemia with an anti-gonococcal antibody titer of 1:2,056 by indirect immunofluorescence (performed by N. Jacobs, Center for Disease Control, Atlanta, Ga.) was also used; this serum was stored at −70 C and thawed immediately before use.

Studies of gonococcal-PMN interaction. (i) Phase contrast microscopy. Five-tenths milliliter of bacterial suspension (abour 10^8 bacteria) was dropped onto a PMN monolayer prepared on a cover glass. The mixture was immediately inverted onto a clean glass slide and the sides of the cover glass were rimmed with melted paraffin. The slide was kept at a constant 37 C with a thermostatically controlled air blower (air curtain incubator, model 279, Sage Instruments) while being viewed under a phase contrast microscope. Electronic flash photomicrographs were made of the interaction between living PMN and types 1 and 3 gonococci.

(ii) Fluorescent microscopy. Bacterial suspension (0.5 ml) was added to a PMN monolayer on a glass slide. The mixture was incubated at 37 C for 30 min and then gently rinsed with iced HBSS to stop phagocytosis. Two drops of an iced 1:4 dilution in FA buffer (Difco) of commercially prepared fluorescein-tagged rabbit anti-gonococcal antiserum (FA *N. gonorrhoeae*; Difco) were then placed on the slide. After 5 min in the cold, the FA stain was rinsed off with iced HBSS and the slide was fixed in 100% methanol and rapidly air dried. The number of FA-staining gonococci associated with 50 PMN were counted using the oil immersion lens of a fluorescent microscope (only PMN with some cell-associated gonococci were counted). Photomicrographs of representative cells were taken with a 35-mm camera using Ektachrome high-speed daylight film.

(iii) Methylene blue stain. After the FA count was completed, the overlying cover slip was carefully removed and the same glass slide monolayer was counter-stained with a drop of 0.15% methylene blue in saline. The number of methylene blue-staining gonococci associated with 50 PMN was recorded and photomicrographs of representative cells were taken (only PMN with some cell-associated gonococci were counted).

Calculation of ingestion and cell attachment. The percentage of cell-associated gonococci that were extracellularly attached to the PMN surface was calculated from:

$$\left[\frac{\text{FA count (extracellular gonococci)}}{\text{methylene blue count (intra- and extracellular gonococci)}}\right] \times 100$$

The percentage of cell-associated gonococci that were ingested by PMN was calculated from:

$$\left[1 - \left(\frac{\text{FA count}}{\text{methylene blue count}}\right)\right] \times 100$$

The total numbers of cell-associated gonococci were similar for types 1 and 3 in all experiments. Statistical comparisons were made with Student's *t* test.

RESULTS

Less than 5% of type 1 gonococci were ingested by PMN as compared to more than 50% of type 3 gonococci ($P < 0.001$, Table 1).

Experiments performed with 10% fresh normal serum, high-titer anti-gonococcal antiserum, heat-inactivated serum, and no serum all gave similar results. The high-titer antiserum did not enhance phagocytosis of type 1 gonococci, although it did slightly increase ingestion of type 3 gonococci ($P < 0.05$). Conversely, more than 94% of cell-associated type 1 gonococci were attached to the PMN surface compared to less than 48% of type 3 gonococci (Table 2).

Figure 1 shows type 1 gonococcal-PMN interaction using phase contrast microscopy. Although this technique readily demonstrated extracellular cell-attached gonococci, it did not easily demonstrate intracellular bacteria and therefore did not lend itself to quantitation of ingestion and cell attachment.

Figure 2 shows a methylene blue-stained PMN with type 1 gonococci. It is apparent that ingested bacteria cannot be distinguished from cell-attached organisms.

Figure 3 shows a PMN with type 1 gonococci stained with FA. The gonococci that stain are attached to the PMN surface and are extracellular.

DISCUSSION

The living PMN does not pinocytize macromolecules (8) and is impermeable to antibacterial antibodies (12) and most other proteins (1). After PMN interacted with bacteria at 37 C, phagocytosis was stopped with iced HBSS. These iced cells were then stained with FA in the cold to prevent any further phagocytosis (or internalization of FA) from occurring. Therefore, the FA stain, as used in our experiments on live PMN-gonococcal preparations, stained only extracellular bacteria, allowing us to quantitate extracellular cell-associated bacteria. Once PMN are fixed, however, they become permeable to stains which can now color both intracellular and extracellular organisms. The methylene blue count thus reflects both intracellular and extracellular cell-associated gonococci. Counts of cell-associated gonococci utilizing these two stains allow unequivocal quantitative separation of ingestion from PMN attachment. It should be reemphasized that the FA stain used here was performed on live preparations, whereas normally the FA stain for identifying gonococci in clinical specimens is employed on fixed material, thereby demonstrating both intracellular and extracellular gonococci (23).

The presence of pili on types 1 and 2 gonococci distinguishes them from types 3 and 4 gonococci, which lack pili (3, 14). It has been stated that pili play an important role in the resistance to phagocytosis of types 1 and 2 gonococci (10). Also, pili appear to be important in the attachment of gonococci to nonleukocytic, eukaryotic cells (2, 10). Pili may have only a minor role, if any, in attachment to PMN, since 50% of type 3 nonpiliated gonococci attached to PMN in our studies.

Swanson et al. (15, 16) recently studied the phenomenon of gonococcal attachment to leukocytes. They demonstrated attachment of nonpiliated type 4 gonococci to leukocytes, which appeared to be mediated by a surface protein. These investigators also commented on the inability of standard light microscopy to separate attachment from ingestion among PMN-associated bacteria; indeed they used the term

TABLE 1. *Percentage of ingestion of gonococci by human PMN*

Determination	Type 1	Type 3	*P*
Normal serum	3.7 (±4.1)[a]	56.2 (±3.5)	<0.001[b]
Heat-inactivated serum	1.0 (±3.0)	52.6 (±3.7)	<0.001
No serum	4.2 (±2.8)	52.2 (±2.9)	<0.001
High-antibody titer serum	4.8 (±4.3)	64.0 (±1.6)	<0.001

[a] Mean ± standard error of the mean.
[b] Student's *t* test.

TABLE 2. *Percentage of attachment of gonococci to human PMN*

Determination	Type 1	Type 3	*P*
Normal serum	96.3 (±4.1)[a]	43.8 (±3.5)	<0.001[b]
Heat-inactivated serum	99.0 (± 3.0)	47.4 (±3.7)	<0.001
No serum	95.8 (±2.8)	47.8 (±2.9)	<0.001
High-antibody titer serum	95.2 (±4.3)	36.0 (±1.6)	<0.001

[a] Mean ± standard error of the mean.
[b] Student's *t* test comparison.

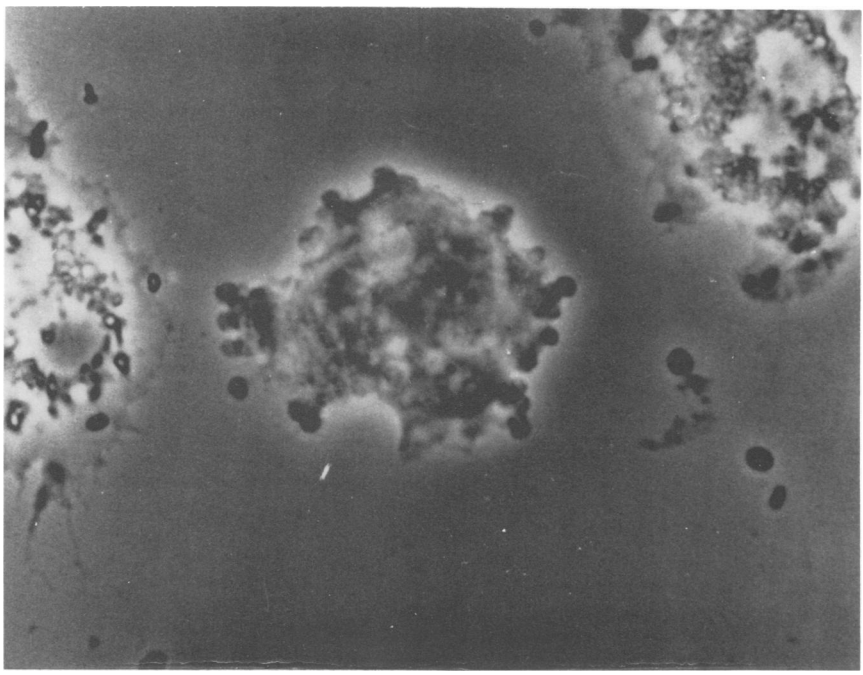

FIG. 1. *Phase contrast photomicrograph of type 1 gonococci and PMN. Several cell-attached extracellular gonococci can be seen on the periphery of the cell (original magnification ×1,000).*

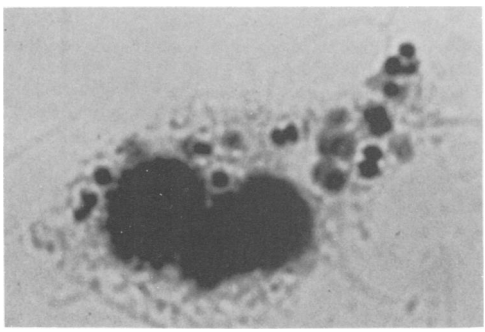

FIG. 2. *Methylene blue stain of type 1 gonococcal-PMN interaction. All gonococci are stained and intracellular bacteria cannot be distinguished from extracellular bacteria (original magnification ×1,000).*

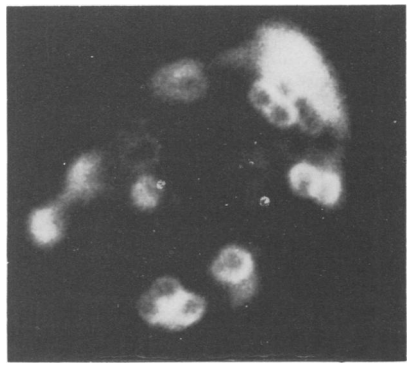

FIG. 3. *FA stain of type 1 gonococcal-PMN interaction. The FA stain demonstrates nearly all cell-associated gonococci, indicating that the majority of gonococci that are cell associated are cell attached extracellularly (original magnification ×1,000).*

"attached-ingested" to refer to cell-associated gonococci. Their elegant electron microscope techniques demonstrated both ingestion and attachment.

Swanson and Zeligs (16) stated that the ratio of ingested and attached bacteria, determined by thin-section electron microscopy, was approximately the same whether piliated type 2 or nonpiliated type 4 gonococci were used. This is similar to our findings with nonpiliated type 3 gonococci but is at variance with our data using type 1 piliated gonococci. A possible explanation for this difference may be found in the intriguing electron micrographs of Swanson and Zeligs, showing protrusion of pili from PMN that appear to have ingested piliated type 2 organisms. Pili protruding from inside the PMN would allow FA to stain the gonococcus and thus it would appear extracellular by our fluorescent antibody technique. Thin sectioning for electron microscopy, on the other hand, might show many of these bacteria to be intracellular.

Some cell-attached extracellular gonococci may be killed by PMN. Salin and McCord (11) presented data that suggest that the potential antibacterial substance, superoxide, is produced on the outer surface of the PMN cell membrane. This may explain why some investigators have found greater killing of type 1 gonococci by PMN than would be expected from the low percentage of type 1 ingestion found in our study.

It has been suggested that stickiness of the gonococcus may be important in the pathogenesis of clinical disease by causing attachment to urethral mucosa (20) or to sperm (2). Attachment to PMN may not only spread gonococci to contiguous noninfected sites but also may interfere with ingestion by altering the surface characteristics of the PMN. Further studies are needed to delineate the mechanisms of cell attachment and the response of the PMN to such attachment.

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