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Studies on the Virulence of *Neisseria gonorrhoeae*

I. Relation of Colonial Morphology and Resistance to Phagocytosis by Polymorphonuclear Leukocytes

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Colonial varieties of *Neisseria gonorrhoeae* that are associated with virulence, types 1 and 2, were more resistant to phagocytosis by rabbit exudative polymorphonuclear leukocytes than colonial types of lesser virulence, types 3 and 4. Type 1 bacteria were resistant and type 4 gonococci were susceptible to phagocytosis by human polymorphonuclear leukocytes. Recent local type 1 isolates were similar in resistance to type 1 organisms of a standard laboratory strain (F62). Living and Formalin-treated, heat-killed, type 1 gonococci were equally resistant to phagocytosis. The antiphagocytic property of virulent colonial types was independent of leukotoxic action. Phagocytosis of both type 1 and type 4 gonococci by rabbit and human leukocytes was bactericidal. Rabbit leukocytes were superior to human leukocytes in killing gonococci. The results suggest that *N. gonorrhoeae* has virulence properties similar to those of extracellular bacterial pathogens, i.e., virulence is associated with antiphagocytic properties.

Although gonorrhea has been a common human ailment for centuries and *Neisseria gonorrhoeae* has been recognized as the causative agent for nearly a century, little is known of the properties that enable gonococci to invade the human body and to produce disease. The present studies were conducted to evaluate the interaction of gonococci and polymorphonuclear phagocytes (PMN). The work took advantage of the keen observations of Kellogg et al. (7, 8), who associated gonococcal virulence with colonial morphology. They recognized four genetically determined colonial variants of *N. gonorrhoeae* upon growth on suitable media under standard conditions; the varieties were designated as types 1 through 4. Type 1 colonies are small (about 0.05 mm in diameter), round, convex, with an entire edge, translucent, colored dark gold, amorphous, slightly viscid, and easily emulsified. Type 2 colonies are similar to type 1 but have a sharper edge, are slightly crenated, and are friable. Type 3 colonies are larger (about 1 mm in diameter), low convex, flat-edged, granular, viscid, and colored light brown. Type 4 colonies are similar to type 3, but amorphous and colorless. Gonococci of types 1 and 2 were virulent in that standardized intraurethral inocula caused gonorrhea in volunteers. Bacteria of types 3 and 4 were of reduced virulence, for the standardized inocula failed to cause disease in volunteers. Herein, the interaction with leukocytes was compared among the colonial types of the gonococcal strain (F62) used by Kellogg et al. and of strains which were freshly isolated locally from patients with acute gonococcal urethritis. (These studies were part of a thesis submitted by C. Thongthai in partial fulfillment of the requirements for the Ph.D. degree and were reported in part at the 71st Annual Meeting of the American Society for Microbiology, Minneapolis, Minn., 1-7 May, 1971.)

MATERIALS AND METHODS

Media. The solid medium was G C medium base (Difco, Detroit, Mich.) supplemented with 1% IsoVitaleX (BioQuest, Cockeysville, Md.), 1% V-C-N Inhibitor (BioQuest), and 10% of the defined supplement of Kellogg et al. (8; GCBS). Sterile supplements were added to sterile fluid base at 45 C immediately before dispensing to plates. To reduce surface moisture, plates of solidified medium were incubated at 36 C for 2 or 3 days before use.

Liquid media were Tryptose phosphate broth (Difco) as diluent and G C medium base less starch and agar (LGCB) and sometimes supplemented with 1% IsoVitaleX (LGCBI).

Bacteria. *N. gonorrhoeae* strain F62 were provided as lyophilized cultures of colonial types 1 through 4 by D. S. Kellogg, Jr., Center for Disease Control, Atlanta, Ga. The lyophilized cultures

were made from stocks prepared at the time of the volunteer studies (7, 8). Unless otherwise stated, the experiments herein were performed with F62.

Local strains of gonococci were isolated directly from the urethral exudate of males suffering acute gonorrhea. The organisms were grown on GCBS at 36 C in a candle jar for 16 to 20 h and identified by typical colonial and cellular morphology, gram reaction, positive oxidase test with *p*-aminodimethylaniline oxalate (Difco), and fermentation of glucose but not maltose. Colonial type was determined by the technique and criteria of Kellogg et al. (8).

Gonococcal cultures were maintained by serial subculture on GCBS and grown at 36 C in a candle jar for 16 to 20 h; specific colonial types were selected as the inoculum of each subculture.

For experiments, gonococci were grown as above. Only plates on which the desired colony type predominated (>99% of the total colonies) were harvested. Type 4 organisms were directly washed from the surface of the medium with warm (37 C) LCCB. To facilitate preparation of a uniform suspension of bacteria, colonies of types 1–3 were disaggregated with a glass rod before being washed from the surface of the medium with warm LGCBI (types 1 and 2) or LGCB (type 3). The suspension was agitated on a Vortex mixer (Scientific Industries, Inc., Springfield, Mass.) for 3 min to reduce clumping. The bacteria were collected by centrifugation at 1,400 × *g* at 26 C for 20 min and resuspended in medium. Microscope monitoring was performed throughout the harvest procedure, and suspensions were eliminated if (i) any large clumps were observed, or (ii) >5% of the bacteria were in clumps, regardless of the size of the clumps. The viability of gonococci in the suspensions usually exceeded 90%; results were excluded if viability was <85%.

Viable gonococci were enumerated as colony-forming units on GCBS. The total number of bacteria was estimated by direct microscope count, utilizing a Petroff-Hausser chamber (C. A. Hausser and Son, Philadelphia, Pa.).

Killed bacteria were prepared after growth and harvest as above. The bacteria were heated at 65 C for 1 h, collected by centrifugation (1,400 × *g*), and resuspended in modified Hanks solution (9) that contained 0.01% bovine serum albumin (Armour Pharmaceutical Co., Chicago, Ill.) and 0.1% glucose (HBG). Formalin was added to 3% final concentration. Before use, the bacteria were thoroughly washed to remove the Formalin.

Rabbits. Adult rabbits of both sexes were procured from local vendors by and housed in the Animal Care Center, Faculty of Science.

Leukocytes. Rabbit exudative leukocytes were collected in HBG from sterile peritoneal exudates induced in rabbits by injection, 24 h previously, of 250 ml of 0.1% shellfish glycogen (Mann Research Labs, Inc., New York, N.Y.). Rabbit blood leukocytes were aspirated from the buffy coat formed during centrifugation of heparinized (30 U/ml; Normark-werke, Hamburg, Germany) peripheral blood at 1,400 × *g* at 4 C for 30 min.

Human leukocytes were harvested from blood of donors who denied a history of gonococcal infection. The leukocyte-rich fraction was removed after sedimentation of peripheral blood in 25% (vol/vol) of 6% dextran (Pharmacia, Uppsala, Sweden) and 10 U of heparin per ml at 26 C for 90 min.

The leukocytes were concentrated by centrifugation at 230 × *g* at 4 C for 5 min, washed with HBG, resuspended in HBG, and enumerated in a hemocytometer. PMN represented about 95% (range, 90 to 98%), 70% (range, 65 to 85%), and 85% (range, 70 to 90%) of the rabbit exudative and blood, and the human blood leukocytes, respectively. More than 99% of the PMN excluded 0.001% eosin Y.

Phagocytosis. Phagocytosis was measured in vitro in both dilute (10, 11) and concentrated (10, 12) suspensions of leukocytes and gonococci. Briefly, in the dilute system, 6.25×10^8 gonococci in a volume of 0.05 ml and 0.5 ml of HBG were mixed with 1.25×10^8 leukocytes in a screw-capped tube (13 by 100 mm) and tumbled end-for-end at 12 rpm at 37 C for 30 min. The ratio of 5 of bacteria-leukocytes was selected after preliminary experiments. Within limits, the extent of phagocytosis varied with the ratio (Fig. 1).

In the concentrated system, 1.25×10^9 gonococci in a volume of 0.025 ml and 0.1 ml of HGB were mixed with 2.5×10^8 leukocytes, and 0.06 ml was distributed evenly over an area (2 by 2 cm) of a glass slide. The slide was incubated at 37 C for 30 min in a petri dish lined with moistened filter paper. After incubation, the cells were recovered

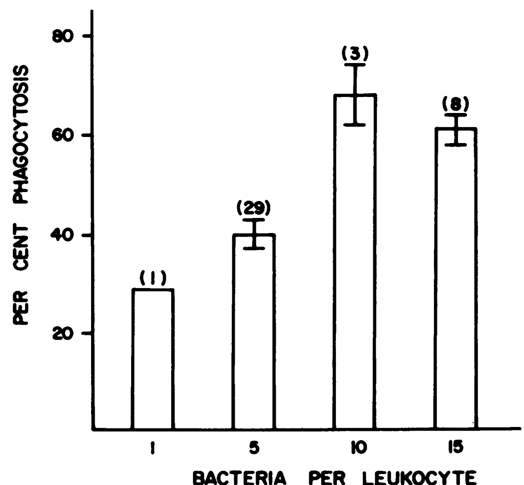

FIG. 1. *Relation of the ratio of gonococci to leukocytes and the extent of phagocytosis. Studies were performed with rabbit exudative leukocytes and type 4 gonococci in the dilute system. Bars and lines represent means, ±2 standard errors, of the number of tests in parentheses.*

from the mixtures, washed with HGB, and concentrated by centrifugation at 180 × *g* at 4 C for 3 min. Smears were prepared, stained with Loeffler's alkaline methylene blue, and examined microscopically for evidence of ingestion of the test organisms by PMN. The results were expressed as the percentage of 400 PMN that contained at least one gonococcus.

Both tests were reproducible. The mean difference between duplicates was less than 10% of the mean percentage of phagocytosis. The 95% confidence level was used for the significance of differences.

Leukocytic bactericidal activity. Mixtures of gonococci and leukocytes were prepared and incubated as in the phagocytic tests. At intervals, the mixtures were recovered, and the viable gonococci were enumerated. All values were expressed as the mean of duplicates. Killing was expressed as the percentage of the number of viable bacteria recovered from samples with 0 incubation time, i.e., $(\text{viable}_0 - \text{viable}_t)/\text{viable}_0 \times 100$ = percent killed. Recovery of viable gonococci from 0 incubation time samples exceeded 70% of theory. As a control in all experiments, gonococci were incubated alone. The number of viable bacteria that were recovered at each time equivalent to the experimental incubation periods was regularly similar to that from 0 incubation time samples, i.e., <25% of gonococci died in the absence of leukocytes.

In early experiments, after the test sample was taken, the mixture was further incubated in 5% saponin at 37 C for 15 min to disrupt leukocytes before dilution and enumeration; gonococci were unaffectd. The yield of viable gonococci was similar to that from untreated mixtures tested in parallel; the saponin treatment was not, therefore, used routinely. Microscope examination indicated that gonococcal clumping was minor during brief periods of incubation. After prolonged incubation, the recovered mixture was agitated briefly on a Vortex mixer to disrupt the few clumps that had formed. Together, these observations indicated that a decline in colony-forming units during incubation with leukocytes was not simply an "apparent" killing of bacteria because of either clumping or sequestration within leukocytes.

The extent of phagocytosis was routinely determined from smears made from mixtures run in parallel with the bactericidal test mixtures.

Urethral exudates. Exudate was collected in calibrated capillary tubes directly from the urethra of males suffering acute, untreated gonorrhea. The leukocyte concentration was determined in a hemocytometer. The extent of phagocytosis of gonococci by PMN that had occurred in vivo was determined by microscope examination of stained smears, as described for phagocytic tests.

RESULTS

Types 1 and 2 gonococci were significantly more resistant to phagocytosis by rabbit exudative PMN in the dilute system than types 3 and 4; type 4 cells were the most susceptible (Fig. 2). The results with human PMN were similar, i.e., type 1 gonococci were more resistant to phagocytosis than type 4. Resistance and susceptibility to phagocytosis were stable characteristics of the colony types over more than 2 years spanned by the present and related experiments. The resistance of type 1 organisms to phagocytosis did not depend on either viability or leukotoxicity. Formalin-treated, heat-killed, types 1 and 4 gonococci were similar to their viable counterparts in resistance and susceptibility to phagocytosis by PMN (Fig. 2). Neither type 1 nor type 4 bacteria were markedly leukotoxic. After incubation with either at 37 C for 30 min, >95% of rabbit and of human PMN excluded eosin Y.

Comparison with freshly isolated local gonococci indicated that the resistance and susceptibility to phagocytosis of types 1 and 4, respectively, were not unique to strain F62 (Fig. 2). The results with rabbit exudative PMN and type 1 organisms of eight local isolates were similar to those with type 1 F62. Type 4 organisms, derived after four to six nonselective subcultures of each of the local isolates, were similar to type 4 F62 in susceptibility to phagocytosis.

In concentrated mixtures, the phagocytosis of gonococci by both rabbit and human PMN (Fig. 3) was greater than in the dilute system (Fig. 2). As expected, type 1 bacteria were more resistant than type 4 gonococci. These results with gonococci resembled closely those obtained with other bacteria with antiphagocytic properties, e.g., pneumococcus (17), in that PMN could partially

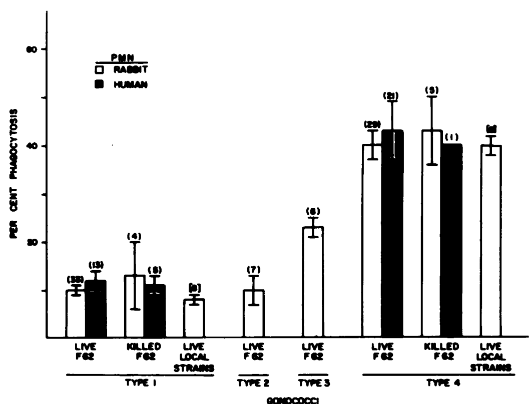

FIG. 2. *Phagocytosis of gonococci by PMN in the dilute test system. Bars and lines represent the means, ±2 standard errors, of the number of tests in parentheses. The number of local isolates tested is in brackets. Viability, strain or source, and colonial type of the test organisms are listed below the bars.*

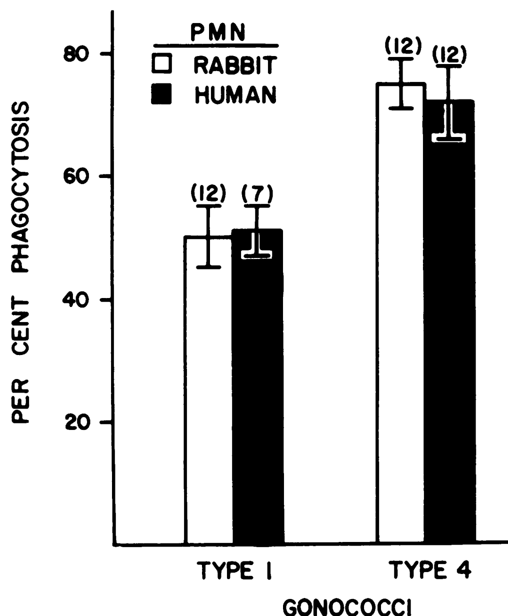

FIG. 3. *Phagocytosis of gonococci by PMN in the concentrated test system. Bars and lines represent the means, ±2 standard errors, of the number of tests in parentheses.*

overcome the antiphagocytic properties of the bacteria if permitted to operate in dense suspensions.

The conditions and results of the concentrated test involving human PMN and type 1 gonococci appeared relevant to gonococcal infection. The mean cell concentration of the urethral exudate from four patients suffering acute gonorrhea was like that in the concentrated test (Table 1). The extent of phagocytosis seen in the exudates and that had occurred in vivo was similar to that observed in vitro in the concentrated test. The comparison is limited because the precise in vivo conditions, e.g., ratio of bacteria-leukocyte, duration of interaction, pH, possible presence of opsonins, etc., were unknown and uncontrolled. Nevertheless, the present observations on the efficiency of phagocytosis of gonococci in concentrated suspensions help to explain the characteristic presence of intracellular gonococci in the concentrated exudate that forms in acute gonorrhea despite the antiphagocytic properties of virulent gonococci.

Phagocytosis by both human and rabbit leukocytes was gonococcidal for both type 1 and type 4 bacteria. Incubation of the gonococci with heat-killed (65 C for 60 min) leukocytes did not result in killing of gonococci. Type 1 bacteria were not appreciably killed by viable leukocytes when reacted in the dilute system, presumably because of the low degree of phagocytosis. When reacted in the concentrated system, however, viable rabbit exudative leukocytes killed most (80%) of the type 1 gonococci in the mixture within 60 min (Fig. 4); the extent of killing did not increase on further incubation. Killing by the rabbit blood leukocytes was somewhat slower than that by exudative leukocytes (Fig. 4). Human blood leu-

TABLE 1. *Concentration of leukocytes and phagocytosis in urethral exudate of acute gonorrhea and in the concentrated in vitro phagocytic test with type 1 gonococci*

Source	Leukocytes/ml × 10^8	Phagocytosis (%)
Exudate of patient		
No. 1	5.1	60
No. 2	4.2	29
No. 3	15	38
No. 4	11	41
Mean ± SE[a]	8.8 ± 0.5	42 ±15
Mean (±2 SE)[b]	6.25	51(±4.5)

[a] SE, standard error.

[b] Mean of in vitro test (concentrated test with human PMN and type 1 gonococci).

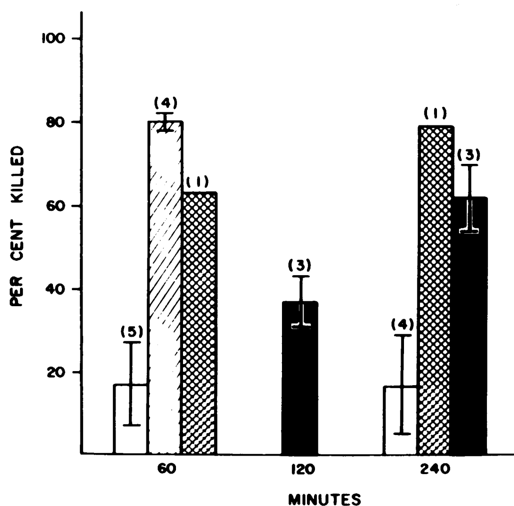

FIG. 4. *Leukocytic bactericidal effect on type 1 gonococci. Tests were performed in the concentrated test system with gonococci alone (□) and together with rabbit exudative (▨) and blood (▩), and human blood (■) leukocytes. Killing is expressed as the percentage of the number of viable gonococci recovered at 0 incubation time. Bars represent the results of single experiments, and bars and lines indicate the mean, ±2 standard errors, of the number of tests in parentheses.*

kocytes were even slower than the rabbit blood leukocytes at killing the gonococci, but managed to kill >60% of the bacteria within 4 h (Fig. 4). Tests with type 4 gonococci using the dilute system also indicated that rabbit exudative leukocytes killed more efficiently than the human cells; the latter killed nearly 80% of the available bacteria within 2 h, however (Fig. 5). Similar observations were made in an earlier comparison of the gonococcidal activity of guinea pig and human leukocytes, i.e., the guinea pig cells killed more efficiently (16). Together, these results indicated that the gonococcidal efficiency of leukocytes varied among species.

To compare the susceptibility of types 1 and 4 gonococci to the leukocytic bactericidal action, it was necessary to modify the method such that a similar proportion of the two types of bacteria was ingested. The concentrated system was used as before, but the ratio of bacteria-leukocytes was reduced to 2 for type 4 gonococci. Under these conditions, the extent of phagocytosis of type 4 bacteria by human cells was similar to that of type 1 gonococci at the usual ratio of 5 (Fig. 6). Examination of smears from the two preparations indicated that the mean number of gonococci within PMN that had ingested gonococci was 3 and 2 gonococci of types 1 and 4, respectively. A slightly, but not significantly, greater proportion of type 4 bacteria were killed. The difference was greater with rabbit exudative cells, cells more efficient in killing than the human blood leukocytes (Fig. 6). The proportion of PMN that ingested type 1 (gonococci-leukocytes = 5) exceeded that which ingested type 4 (gonococci-leukocytes = 2) by 9%; the mean number of bacteria per PMN that ingested bacteria was, however, the same in both preparations: 4 gonococci. Whereas phagocytosis of type 1 gonococci was greater, the proportion of type 4 bacteria that was killed exceeded that of type 1 by 14%, a modest difference. Together, these results suggested that if type 1 gonococci are somewhat more resistant than type 4 bacteria to the leukocytic bactericidal mechanism(s), the difference is modest and much less than the difference between the two types of gonococci in resistance to phagocytosis.

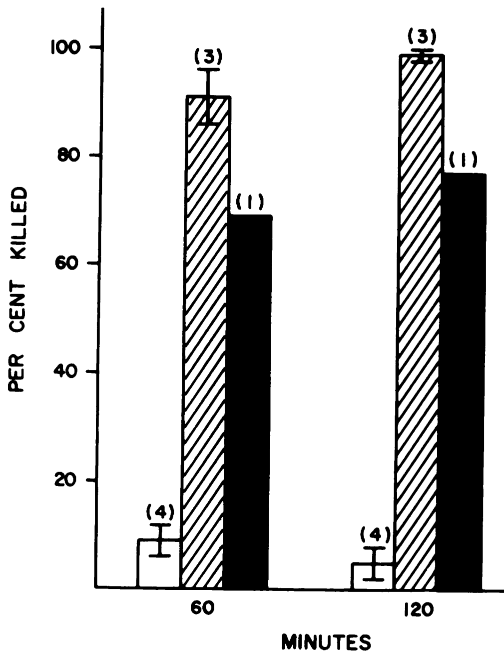

FIG. 5. *Leukocytic bactericidal effect on type 4 gonococci. Tests were performed in the dilute system with gonococci alone (□) and together with rabbit exudative (▨) and human blood (■) leukocytes. Killing is expressed as the percentage of the number of viable gonococci recovered at 0 incubation time. Bars represent the results of single experiments, and bars and lines indicate the means, ±2 standard errors, of the number of tests in parentheses.*

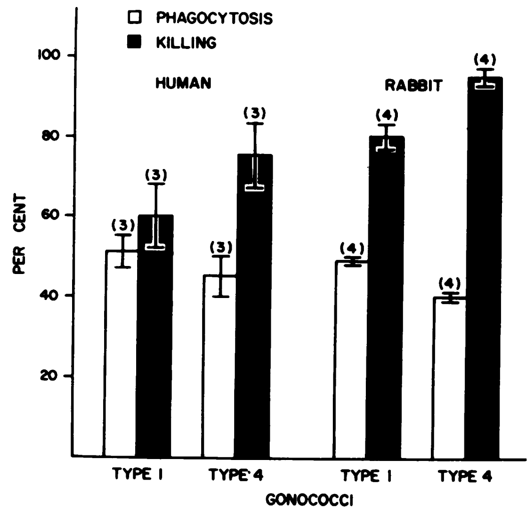

FIG. 6. *The susceptibility of type 1 and type 4 gonococci to intraleukocytic bactericidal activity. Tests were conducted in the concentrated test system and with ratios of bacteria–leukocytes of 5 and 2 for type 1 and type 4 organisms, respectively. Incubation periods were 240 min for human and 60 min for rabbit leukocytes. Phagocytosis was determined from microscope examination of smears and is expressed as the percentage of PMN that contained one or more morphologically typical gonococcus. Killing is expressed as the percentage of the number of viable bacteria recovered at 0 incubation time. Bars and lines represent means, ±2 standard errors, of the number of tests in parentheses.*

DISCUSSION

Genetically determined colonial variation of *N. gonorrhoeae* has been related to virulence, that is, gonococci freshly isolated from patients with acute infection form small colonies, either type 1 or 2, on appropriate media (7, 8, 14). Organisms of these types retain both type-specific colonial morphology and virulence through repeated selective subculture, as demonstrated by the production of gonorrhea upon intraurethral inoculation in volunteers (7, 8). Upon nonselective subculture, organisms emerge that form large colonies, types 3 and 4, and that fail to induce disease when introduced into the urethra of volunteers (7, 8). The present studies extend these observations on colonial morphology and virulence. Organisms of virulent types are more resistant to phagocytosis by PMN than those from the types with reduced virulence. The resistance of virulent bacteria is a property of the gonococcal cell independent of active metabolism and of leukotoxic properties. Thus, virulent gonococci resemble virulent forms of other pyogenic bacteria, e.g., pneumococci (17) and streptococci (1, 2), in that virulent types have genetically determined, antiphagocytic cell structures. The antiphagocytic determinant(s) of gonococci is unknown, but it is probably a surface structure. First, the antiphagocytic properties of bacteria, in general, are related to surface structures, e.g., the pneumococcal capsule (17) and the streptococcal capsule and M protein (1, 2). Second, gonococci of virulent colonial types differ significantly from the less virulent types in a number of properties that probably also involve the cell surface, e.g., autoagglutination (8, 15), competence for genetic transformation (13), resistance to the complement-dependent serum bactericidal reaction (3), and piliation (6, 15). The latter appendages are of particular interest, for they are observed only on gonococci of virulent colonial types, and it is possible that they are antiphagocytic. Studies to this point are in progress.

That the antiphagocytic property of virulent types of *N. gonorrhoeae* is a major determinant of virulence is supported by the present and earlier observations that both the virulent and less virulent types are susceptible to the cellular bactericidal mechanism(s) after they are ingested by leukocytes. Although type 1 gonococci may be somewhat more resistant than type 4 bacteria to the intraleukocytic bactericidal action, the difference is of lesser consequence for gonococcal survival in the presence of leukocytes than is resistance to phagocytosis. That is, killing is more closely related to the extent of phagocytosis than to the type of gonococcus per se. Together, however, the resistance of virulent types of gonococci to phagocytosis and possibly, and to a lesser extent, intraleukocytic killing could account for the ability of virulent gonococci to evade the host cellular defenses, a necessary parameter of virulence.

The present studies indicate one way that the host may overcome the antiphagocytic properties of virulent gonococci, i.e., the formation of a dense exudate via the inflammatory response. In vitro, PMN in a dense suspension ingest virulent gonococci that would escape phagocytosis in a more dilute mixture. The extent of phagocytosis in vitro in mixtures that mimic the leukocyte concentrations of gonorrheal urethral exudate is similar to the proportion of PMN in the exudate that ingest gonococci in vivo. The inflammatory response, formation of a dense exudate, and the resultant phagocytosis and killing of virulent gonococci probably are important in the remission of manifestations and control of infection that typically occur in acute gonorrhea after a few days.

Opsonins, if formed and delivered to the site of infection, could further increase phagocytosis and promote clearance of gonococci from the lesion. Studies to this point are in progress.

The equivalence between rabbit and human PMN in ingesting gonococci is of practical importance, for rabbits are a readier source than men of quantities of PMN. Advantage was taken of the equivalence herein in exploring the general nature of the antiphagocytic properties of the virulent gonococal types. Rabbit and human leukocytes seem dissimilar, however, in the efficiency of killing intraleukocytic gonococci; rabbit leukocytes are superior. Guinea pig leukocytes also seem to be more efficient than human cells in killing ingested gonococci (16). The mechanism(s) of the superior gonococcidal action of the leukocytes of lower animals is unknown and warrants investigation. The difference could conceivably be of significance in the marked resistance of lower animals to infection by *N. gonorrhoeae* (4, 5). Study of species differences in leukocytic bactericidal capacity should be extended to other bacteria, especially if leukocytes of lower animals are to be taken as models in evaluating resistance mechanisms to infections of human importance.

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