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Disseminated Gonococcal Infection in Mice

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Gonococci do not readily cause disseminated infection in mice. To simulate some of the conditions leading to disseminated gonococcal infection in women, we suspended gonococci in mucin plus hemoglobin and studied the development of gonococcal bacteremia. The mucin-hemoglobin mixture was used because the menstruum appears to be involved in dissemination of gonococci from the genital tract during menstruation. Mice did not die after massive inocula of 10^9 gonococci given intraperitoneally in broth, but when gonococci were suspended in mucin (15%) alone, the 50% lethal dose was $10^{8.4}$ and in 15% mucin plus 4% hemoglobin (M/H), the 50% lethal dose fell to $10^{6.6}$. Sublethal doses produced local peritonitis and transient bacteremia. With larger inocula the local peritoneal infection progressed to fatal septicemia. Studies of the mechanism by which M/H lowered the 50% lethal dose showed that systemic clearance mechanisms were compromised, but not enough to account for the total decrease in the 50% lethal dose. If gonococci were given intravenously after intraperitoneal inoculation of M/H, sequestration of gonococci in the peritoneal cavity occurred, suggesting an effect on local peritoneal defenses. The effect on neutrophils appeared most significant, since numbers of neutrophils in the peritoneal fluid were decreased in the presence of M/H and neutrophils were destroyed by M/H in vitro. The serum bactericidal system was not affected. We conclude that M/H promotes gonococcal bacteremia by interference with phagocytosis and intracellular killing of gonococci. The model simulates the disseminated gonococcal infection cases in women which follow pelvic inflammatory disease in its progression from local peritonitis to transient or lethal bacteremia and in factors (mucin and hemoglobin) which enhance infection.

The study of the pathogenesis and immunobiology of disseminated gonococcal infection has been greatly hampered by the lack of an animal model which mimics the human disease. Since 1 to 3% of gonorrhea patients develop disseminated gonococcal infection (10), the need for study of this disease is obvious. Much valuable information about disseminated gonococcal infection has been gained from the study of infection of subcutaneous chambers in experimental animals (1) and endocarditis produced by infusing organisms in the presence of a transaortic valve catheter (6). However, these models require surgical manipulation, and the disease produced does not progress from local to disseminated infection as in human cases. Such a disseminated infection in mice was reported by Miller and Hawk in 1939 (21). In their model the virulence of the organisms was enhanced by serial passage through mice and by the suspension of gonococci in mucin (20). However, the gonococcal strains used in this model are no longer available, and the results have not been reproduced. We now present a model of gonococcal bacteremia in female mice which does progress from local peritonitis to transient or lethal bacteremia, depending on the dose of gonococci administered. It is modeled after cases of disseminated gonococcal infection which follow pelvic inflammatory disease in women.

(A preliminary report of part of this work was given at a conference on the Immunobiology of *Neisseria gonorrhoeae*, San Francisco, Calif., January 1978.)

MATERIALS AND METHODS

Animals. Seven- to eight-week-old Caw:CF1 female mice (Carworth Div., Charles River Breeding Laboratories, Inc., Kingston, N.Y.) were used throughout. Animals were fed commercial laboratory animal pellets and water ad libidum and were anesthetized with ether for all procedures.

Bacteria. Gonococci were grown on GC agar base supplemented with IsoVitaleX (BBL Microbiology

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Systems, Cockeysville, Md.). For this model of human infection, cultures with greater than 95% of the colonies being of T_2 morphology were used, since colonial types T_1 and T_2 are associated with human gonococcal infection, and T_3 or T_4 are not (16). The strain used (N24) was recently isolated from a human genital tract infection and was mouse serum resistant and penicillin sensitive.

Infection. For inoculation of mice, gonococci were scraped from plates after approximately 20 h of incubation and were suspended in GC broth supplemented with IsoVitaleX. The concentration of organisms was determined spectrophotometrically and confirmed by plate counts with a drop method (19). To test the effect of the suspending medium (vehicle) on infection, we inoculated a stock suspension of 10^9 gonococci in broth in a series of 10-fold dilutions in the following media: (i) 15% mucin (porcine gastric mucin; Sigma Chemical Co., St. Louis, Mo.); (ii) 4% hemoglobin (Difco Laboratories, Detroit, Mich.); (iii) a combination of 15% mucin and 4% hemoglobin (M/H); (iv) a combination of 15% mucin and iron dextran (500 μg/0.3-ml dose; Imferon; Merrell-National Laboratories, Div. Richardson-Merrell, Inc., Cincinnati, Ohio); and (v) a combination of 15% mucin and 0.02% hemin (type III, crystalline; Sigma Chemical Co.).

Ten mice per group were inoculated intraperitoneally with 0.3 ml of each dilution in each medium via a 23-gauge, 1-in. (ca. 2.54-cm) needle and were monitored for disease for 4 to 7 days thereafter. Criteria of disease included clinical observations, peritoneal culture, blood culture, and death. Blood was obtained by cardiac puncture, and peritonal fluid was obtained with a Pasteur pipette after the peritoneal cavity was aseptically opened. These samples were then streaked on GC agar plus IsoVitaleX.

The kinetics of bacteremia were determined in four groups of 36 mice each inoculated intraperitoneally with high (10^8) or low (10^6) doses of gonococci suspended in M/H or broth. Subgroups of four animals per treatment group were killed at intervals of 0.5, 1, 2, 3, 4, 6, 18, and 24 h after inoculation. Quantitative cultures were done by plate counts (19) with a serial dilution of 0.1 ml of heart blood or peritoneal fluid after flushing with 0.5 ml of broth plus IsoVitaleX. One subgroup from each treatment was kept for determination of lethality.

In vivo studies on the mechanism of action of M/H. The effect of intraperitoneal broth or M/H on the clearance of gonococci from blood was tested by intraperitoneally by inoculating 20 mice with 3 ml of broth and 20 mice with M/H, followed by 10^8 gonococci in Dulbecco solution given intravenously 1 h later. Subgroups of four animals each were killed at 1, 2, 4, and 6 h post-inoculation. Blood and peritoneal fluids were collected for plate counts. A group of four animals from each treatment was kept for 1 week for clinical observation and recording of death.

A second experiment was conducted to determine the in vivo effect of M/H on phagocytes. Ten mice were inoculated intraperitoneally with 0.3 ml of broth, and ten were inoculated with 0.3 ml of M/H. Groups of two mice each inoculated with broth and two inoculated with M/H were sacrificed at 2, 4, 6, 18, and 24 h post-inoculation. Neutrophils in heart blood and peritoneal fluid were counted by hemacytometer and differential leukocyte counts.

A parallel experiment was done to examine the effect of M/H on the serum bactericidal system. Serum and peritoneal fluid were collected from mice at 2, 6, 24, and 48 h after intraperitoneal inoculation with 0.3 ml of M/H or broth. Since mouse complement is extremely labile, all samples were placed on ice immediately and handled in the cold throughout the collection procedure. All samples were stored at −70°C and thawed only once. Complement levels were determined by conventional 50% hemolytic complement (CH_{50}) assays (13). The serum bactericidal system was measured by two techniques. Mouse serum was compared with human serum to standardize the tests. In fresh human serum and initial assays of fresh mouse serum, the bactericidal activity was quantitated by incubating serial dilutions of organisms in whole serum for 1 h before doing plate counts as described previously (17). Controls which had been heated at 56°C for 30 min to inactivate the complement were included in each assay. To conserve serum, in later studies of the mouse bactericidal system we used a plaque assay as described previously (5). In brief, lawns of gonococci were prepared by spreading suspensions of known concentrations of bacteria (determined spectrophotometrically and confirmed by plate count) on GC agar base plus IsoVitaleX containing 0.05 mg of diethylaminoethyl dextran per ml. After the fluid had soaked into the agar, 0.05-ml samples of serum, plasma, or peritoneal fluid were placed on the surface. Plates were incubated and examined for plaques at 24 h.

In vitro studies of the mechanisms of action of M/H. (i) Effect on gonococci. Gonococci were suspended at a concentration of 10^8 or 10^6 bacteria per ml of broth or M/H. The suspensions were incubated at 37°C in 5% CO_2. Samples were removed for plate counts at 1 and 24 h.

(ii) Effect on neutrophils. Murine neutrophils were obtained by flushing vaginas of mice at diestrus with saline. Since mice normally have large numbers of neutrophils in their vaginal secretions at diestrus, we did not need to resort to artificial stimulation. This saline flush was diluted with Dulbecco solution to reach a concentration of approximately 2×10^3 to 3×10^3 neutrophils per mm^3. Then 0.1 ml of the neutrophil suspension was incubated with 0.05 ml of either broth or M/H at 37°C in 5% CO_2 for 1 h. This mixture was diluted with an equal volume of citric acid-crystal violet diluent to stain the nuclei (18), and neutrophils were counted in a hemacytometer.

Statistical treatment of the data. The median effective dose (ED_{50}) and median lethal dose were estimated from the data by a method using the probit transformation (8). This method of estimation allowed us to calculate the standard error of the estimates and thus perform *t*-tests on the comparisons described below.

RESULTS

The ED_{50} was determined for three different criteria of infection: (i) culturally positive peritonitis at 24 h post-inoculation; (ii) bacteremia at 24 h post-inoculation; and (iii) death (median

lethal dose). With each of these criteria, the ED_{50} varied, depending on the suspending medium (Table 1). Positive results were not obtained by any of the criteria when organisms were suspended in broth. When organisms were suspended in 4% hemoglobin, the ED_{50} for culturally positive peritonitis was $10^{9.2}$, whereas with a vehicle of 15% mucin, the ED_{50} was 1.6 logs lower ($10^{7.6}$). The ED_{50} dropped a further 1.6 logs ($10^{6.0}$) when M/H was used in the suspending medium. The differences between ED_{50}s were significant ($P < 0.001$). When bacteremia or death was used as a criterion of infection, similar 1- to 2-log decreases in ED_{50}s were seen between groups (Table 1). Again, the ED_{50} with M/H was significantly lower than the ED_{50} with mucin ($P < 0.001$). Thus, we have lowered the ED_{50} at least 1,000-fold by suspending gonococci in M/H.

To study the role of iron in lowering the dose of gonococci required to produce infection, we combined other iron-containing vehicles with mucin and determined ED_{50}s. Iron dextran was studied because it is known to enhance susceptibility to meningococcal infection (3), and hemin was studied because it would allow separation of the effects of iron in heme from the globin portion of the hemoglobin molecule. Neither iron dextran nor hemin in combination with mucin lowered the ED_{50} over mucin alone.

Clinical observations of mice in the above study of ED_{50}s were recorded throughout the first few hours and at 24-h intervals thereafter. Shortly after inoculation, all mice showed some abdominal discomfort in that they moved hesitantly with hunched backs and pinched abdomens. Those inoculated with M/H but no bacteria were clinically normal by 24 h. Those given low doses of gonococci in M/H (10^5 to 10^6) or high doses in broth appeared a little depressed but usually continued to eat and recovered completely from depression and abdominal discomfort by 48 h. At higher doses in M/H, the mice became progressively worse, developing rough hair coats, inappetence, pinched abdomens, hunched backs, conjunctivitis, and reluctance to move. Most of these animals did not recover. The time of death was related to the dose (Fig. 1).

Since mice inoculated with sublethal doses (Table 1) did develop transient illness, we decided to follow the progression of bacteremia after sublethal and lethal inoculations of gonococci. The results show that high doses of gonococci in broth inoculated intraperitoneally do produce transient bacteremia (Fig. 2), but no deaths. In fact, there was even a very transient bacteremia with the lowest dose of gonococci in broth. When the organisms in M/H were inoculated the level of bacteremia remained high. In the group kept for clinical observation, all animals receiving 10^8 gonococci in M/H died. At the lower inoculum of 10^6 organisms in M/H, the level of bacteremia was initially higher than that in the group with 10^6 organisms in broth, but lower than that in the group with 10^8 orga-

TABLE 1. *Effect of vehicles on the infectivity of gonococci for mice*

Criterion of infection	Log_{10} ED_{50}			
	Broth	Hemoglobin	Mucin	M/H
Peritoneal culture (24 h)	>9	9.2[a]	7.6[a]	6.0[a]
Blood culture (24 h)	>9	>9	9.2[a]	7.0[a]
Death	>9	>9[b]	8.4[a]	6.6[a]

[a] The decreases in ED_{50} between vehicles (hemoglobin and mucin or mucin and mucin plus hemoglobin) were statistically significant ($P < 0.001$).

[b] Three out of ten mice died at the 10^9 dose. All other groups listed as >9 had no deaths or positive cultures.

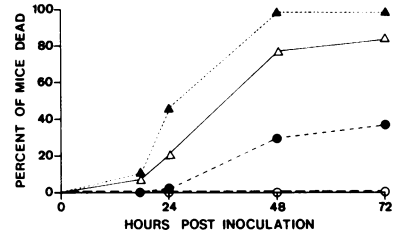

FIG. 1. *Effect of dose on rate of death. Symbols: ▲, 10^8 gonococci; △, 10^7 gonococci; ●, 10^6 gonococci; ○, no gonococci. In all four groups the gonococci were suspended in M/H.*

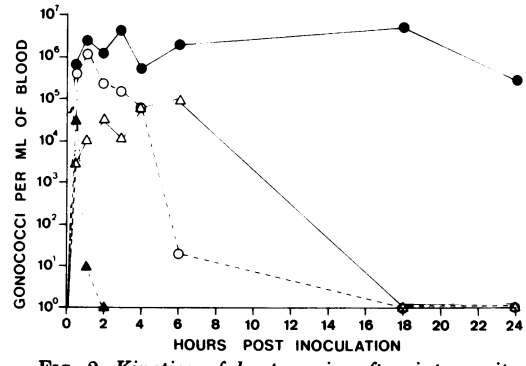

FIG. 2. *Kinetics of bacteremia after intraperitoneal inoculation of gonococci suspended in broth or in M/H. Four mice in each group were killed and bled at each time point. Results given are the average counts from four mice. Dose per mouse: ▲, 10^6 gonococci in broth; △, 10^6 gonococci in M/H; ○, 10^8 gonococci in broth; ●, 10^8 gonococci in M/H.*

nisms in broth. However, the duration of bacteremia was longer in animals inoculated with 10^6 organisms in M/H than in either group inoculated with organisms in broth, and some of the mice died after receiving 10^6 organisms in M/H (see median lethal doses in Table 1).

Studies were then conducted to determine the mechanism by which M/H lowered the ED_{50}. One possibility was that M/H stimulated growth of gonococci, but when the in vitro growth of gonococci in broth was compared with the growth in M/H, the counts were lower in M/H than in broth at 1 h. By 24 h, no viable organisms remained in M/H.

Interference with the clearance mechanisms of the animals was another possibility. To determine whether intraperitoneal inoculation with M/H lowered the ED_{50} by interfering only with local peritoneal defenses or also affected systemic clearance, we performed a second experiment in which the standard amount of M/H or broth was given intraperitoneally, followed by 10^8 gonococci in Dulbecco solution given intravenously 1 h later. Bacteremia did occur in both groups (Fig. 3), but the duration was greater in the group given M/H than in the group given broth. Peritoneal fluid cultures showed that gonococci emigrated from the blood to the peritoneal cavity after M/H had been inoculated intraperitoneally, but not after broth was inoculated intraperitoneally. No deaths occurred in the four animals from each treatment group kept for lethality determinations.

The effect of vehicles on peritoneal exudate cells and peripheral blood neutrophils also was studied in the animals inoculated intraperitoneally with broth or M/H. These animals received no gonococci. In those inoculated with broth, there was a two- to threefold increase in both peripheral blood polymorphonuclear leukocytes and peritoneal exudate cells (primarily neutrophils) at 4 and 6 h, followed by a drop in the peripheral neutrophil counts and the peritoneal exudate cell counts at 18 h. In the animals inoculated intraperitoneally with M/H, the peritoneal exudate cell counts were two to four times lower than those in the broth controls at all time points, and the peripheral neutrophil counts were two to three times higher in M/H-inoculated mice than in broth controls.

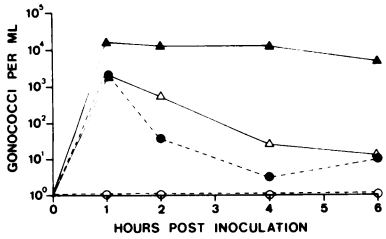

FIG. 3. *Numbers of gonococci in blood and peritoneal fluid after giving broth or M/H intraperitoneally and 10^8 gonococci intravenously 1 h later. Blood counts: △, with broth, intraperitoneally; ▲, with M/H, intraperitoneally. Peritoneal counts: ○, with broth, intraperitoneally; ●, with M/H, intraperitoneally.*

In vitro studies were done to determine whether the low peritoneal exudate cell counts in M/H-inoculated animals were due to a direct effect of M/H. After the standardized suspension of neutrophils had been incubated in broth, the average count was 2,500 neutrophils per mm^3, whereas after 1 h in M/H the average count was 180 neutrophils per mm^3.

The role of the serum bactericidal system was studied with both fresh human serum and mouse plasma or serum. Although strain N24 was killed by human serum, when tested against mouse serum or plasma in either a conventional bactericidal assay (17) or a plaque assay (5), this strain was not killed. Therefore, to test the effect of intraperitoneal inoculation with M/H or broth on the serum bactericidal system, we used gonococcal strain MW, which was sensitive to killing by mouse serum or plasma (Table 2). No differences were found between killing by plasma and killing by serum. Samples of plasma and peritoneal fluid were collected at 2, 6, 24, and 48 h after inoculation with M/H or broth. Two mice per treatment were killed at each time point. In the plaque assay none of the peritoneal fluid samples killed gonococci. However, all plasma samples killed strain MW, regardless of whether the samples were from animals inoculated with M/H or broth. Also, both the fresh uninoculated control sample and the heated control (56°C for 30 min) killed the test strain. Since we did not expect heated plasma to kill gonococci, we then repeated the assay with plasma heated at 60 and 65°C for 30 min. Again, killing occurred.

To check the heat lability of mouse complement, we then did CH_{50} studies with pooled serum from CF1 or C57BL mice. Fresh pooled CF1 serum had 4.4 CH_{50} units per ml, and heated (56°C for 30 min) serum from the same pool had none. Fresh serum from C57BL mice had 12.4 CH_{50} units per ml.

DISCUSSION

The effect of vehicles on the ED_{50} of gonococci for mice was striking. Mucin and hemoglobin both decreased the ED_{50}, but the combination of M/H was much more effective than either mucin or hemoglobin alone (Table 1). Both clinical symptoms and time of death were dose

TABLE 2. *Susceptibility of gonococci to killing by fresh serum*

Serum	Log kill[a] in strain[b]:	
	N24	MW
Human	4.3	<1.0
Mouse	<1.0	4.7

[a] Log of the number of organisms killed (4.3 means $10^{4.3}$ organisms killed by 1 ml of fresh serum).

[b] Strain N24 was isolated from a human genital tract infection, and strain MW was isolated from a human case of disseminated gonococcal infection.

dependent (Fig. 1) as expected. These factors were related to the extent and duration of bacteremia (Fig. 2). Since intraperitoneal inoculation with gonococci in M/H produced prolonged bacteremia, whereas use of a broth vehicle resulted in very transient bacteremia (Fig. 2), it appears that organisms entered the circulation in both cases, but that M/H interfered with peritoneal or systemic clearance or both. This observation is consistent with other studies of these vehicles on host defenses. Both mucin (15, 22) and hemoglobin (14) enhance virulence of bacteria or decrease host resistance. These vehicles both contain iron, which appears to be a critical factor in host-bacterium interactions (24, 28), and can replace mucin in enhancing the establishment of meningococcal infection in mice (3). Yet, when we replaced 4% hemoglobin with an equivalent amount of iron in 0.02% hemin or with 500 μg of iron dextran (which contained much more iron than 4% hemoglobin), there was no decrease in the ED_{50} over that with mucin alone. Thus, the effect of hemoglobin on the ED_{50} was not due to iron alone. Furthermore, since no viable organisms remained after 24 h of in vitro incubation in M/H at 37°C in 5% CO_2, it was concluded that M/H did not function strictly as a growth-promoting nutrient substance.

Others have indicated that mucin acts by interfering with the serum bactericidal system (15) or with intracellular killing by phagocytes (23). Hemoglobin has been shown to inhibit phagocytosis and intracellular killing of *Escherichia coli* by neutrophils (11), as well as killing of staphylococci by leukocytic cationic proteins (27). In fact, it has been suggested that mucin "eliminates complement and reticuloendothelial function in mice" (12). These suggestions led us to study the effect of mucin and hemoglobin on the serum bactericidal system and the phagocytic system of mice in the gonococcal bacteremia model. Studies of the effect of intraperitoneal inoculation with M/H on systemic clearance of gonococci showed that when high doses of gonococci (10^8) were given intravenously after injecting sterile M/H into the peritoneal cavity, bacteremia lasted longer than after injecting broth into the peritoneal cavity (Fig. 3). In both cases the level of bacteremia was much lower than in the previous experiment, in which the same dose of gonococci (10^8) was suspended in the vehicle and given intraperitoneally (Fig. 2 and 3). Also, when the organisms were given intravenously, there was intraperitoneal sequestration of gonococci in mice injected intraperitoneally with M/H but not in mice injected with broth. No deaths occurred even in the mice given 10^8 gonococci intravenously. Thus, it appears that whereas M/H given intraperitoneally may have some effect on systemic defenses, these effects do not account for the whole decrease in ED_{50} with M/H as a vehicle. The fact that some sequestration of gonococci into the peritoneal cavity occurred in the presence of M/H suggests that even when organisms were given intravenously, the interference of M/H with local peritoneal defenses allowed organisms to invade this area and then continually reseed the blood stream. Since clearance was impaired and the reticuloendothelial system is primarily responsible for clearance, it is likely that these results can be explained, at least in part, by a combination of systemic and local reticuloendothelial blockade.

In the studies of other investigators, both neutrophils (26) and complement (2) have been implicated in resistance to gonococci. Since M/H appeared to affect both systemic and local peritoneal defenses, we investigated the effect of M/H on the systemic and peritoneal neutrophil and bactericidal systems. Studies on the serum bactericidal system again suggested that a systemic effect was unimportant since intraperitoneal M/H did not significantly decrease the serum bactericidal effect. No killing of gonococci was observed with peritoneal fluid from M/H- or broth-inoculated mice. Thus, it appears that the effect of M/H on serum killing of gonococci does not account for the decreased ED_{50} with the M/H vehicle.

It was surprising that serum killing was heat stable even though complement activity was eliminated by heating. This stability suggests that unknown factors other than complement were responsible for killing. The CH_{50} levels in fresh CF1 mouse serum were low, as expected in CF1 mice, because of the C5 deficiency in many mice (4) including most CF1 mice (25).

Neutrophils are thought to be important in resistance to gonococci because they dominate the inflammatory response to gonococcal infection. Our data show that peritoneal inoculation with M/H lowers the peritoneal neutrophil

counts while increasing peripheral blood neutrophil counts. Since M/H was found to destroy murine neutrophils in vitro, it is likely that as neutrophils migrated from the peripheral blood to the peritoneal cavity, they were being destroyed by M/H. The rise in peripheral counts in animals receiving intraperitoneal M/H is probably a compensatory response to replace the neutrophils destroyed in the peritoneal cavity. On the other hand, in animals receiving intraperitoneal broth, there was an initial increase in both peritoneal and peripheral blood neutrophils. Since there was no intraperitoneal destruction of neutrophils in animals receiving broth, there was no stimulus for continued high peripheral counts, and by 18 h the numbers had returned to normal. These studies suggest that local destruction of peritoneal exudate cells by M/H is an important factor in allowing bacteremia to persist.

This model resembles human disseminated gonococcal infection in several important respects. The progression of infection from local peritonitis to bacteremia following pelvic inflammatory disease parallels the situation in a significant portion of human female cases. Since one of the important and perhaps frequent (10) complications of pelvic inflammatory disease is perihepatitis, it appears that peritonitis is likely to occur via the ascending route. Furthermore, it is known that menstrual blood refluxes through the fallopian tubes to the peritoneal cavity (7). Since there is a striking association between onset of pelvic pain and menstruation in pelvic inflammatory disease (7), it is likely that gonococci ascend at the time that the menstruum (containing mucin and hemoglobin) enters the peritoneal cavity. The high incidence of pelvic inflammatory disease (10 to 17% of cases [10]), the increased incidence of dissemination at menstruation (9), and the above clinical observations suggest that the peritoneal route is an important one in disseminated gonococcal infection in women. Thus, this small laboratory animal model of disseminated gonococcal infection simulates the infection in women in its progression from local infection to bacteremia, in factors (mucin and hemoglobin) which enhance the infection, and in the transient bacteremia produced with lower doses of gonococci.

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