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Effects of Iron and Culture Filtrates on Killing of *Neisseria gonorrhoeae* by Normal Human Serum

PININA NORROD AND ROBERT P. WILLIAMS*

Department of Microbiology and Immunology, Baylor College of Medicine, Houston, Texas 77030

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Neisseria gonorrhoeae GC9, both colony types T2 and T4, were killed by normal human serum, although populations of colony type T4 were more susceptible. Ferric ammonium citrate prevented the killing of populations of both T2 and T4 colony types. Other iron compounds tested showed no protective effect, nor did ammonium citrate or the divalent cations magnesium or calcium. A filtrate from cultures of an *N. gonorrhoeae* strain grown in a liquid defined medium showed a similar protective effect in the serum assay. The filtrate appeared to chelate iron, as measured by decreased ability of iron-free transferin to bind iron in the presence of the filtrate. However, the two effects did not appear to be related. Neither ferric ammonium citrate nor the culture filtrate sufficiently inactivated complement to account for protection.

Studies in vitro with various bacteria have shown that bacteriostatic and some bactericidal effects of serum (5, 6, 9, 14, 15, 22, 36, 37, 48) can be relieved by addition of iron compounds or siderophores (24), microbial compounds that bind iron. Studies in vivo indicate that the availability of iron to invading bacteria may play a role in virulence. Although the iron requirements of bacteria are small (24, 44), iron is tightly bound to host proteins, such as transferrin in serum (35) and lactoferrin in secretions (28). If bacteria plus iron are injected, or if the bacteria produce iron chelators (siderophores) that can compete with host proteins for iron, their virulence appears to be increased (5, 18, 23, 32, 36).

Killing of *Neisseria gonorrhoeae* by serum, whether by normal or immune, animal or human, has been studied by investigators since the 1930's (1, 39, 40). The killing reportedly is mediated by antibody and complement (39, 42), and in normal human serum, cross-reacting antibody to other *Neisseria* and to *Escherichia coli* appears responsible for killing (13). There are, however, no reports concerning the effects of iron on killing of gonococci by serum. Recent evidence indicates that culture filtrates of gonococci display biological activity attributed to siderophores (32-34), but the isolation and chemical identity of gonococcal siderophores have not been reported.

There is little information about the effects of iron on *N. gonorrhoeae*. One of the first references mentioned increased colony size and coloration when additional iron was added to the growth medium (21). Iron salts increased attachment of gonococci from both T1 and T4 colony types to sperm (19). A recent report showed that the addition of iron to inocula of T3 colony types resulted in increased virulence for chicken embryos (32). Studies in vitro claimed prolonged growth of T1 colony types in media containing ferric citrate (16, 17). There also are reports that high concentrations of iron or of certain iron chelators cause reversion of T4 to T1 colony types (29, 30).

The present report shows that killing of gonococci by normal human serum in vitro is prevented by an iron compound and proposes that gonococci may produce iron chelators. We also report that a culture filtrate of *N. gonorrhoeae* can prevent killing of gonococci by normal human serum. However, we do not believe that the chelating effects of the filtrate are related to its protective effect in the serum assay.

MATERIALS AND METHODS

Maintenance of stock cultures and bacterial strains. Colony types T2 or T4 were maintained on GC agar base (Baltimore Biological Laboratory, Cockeysville, Md. or Difco Laboratories, Detroit, Mich.) plus 1% IsoVitaleX (BBL) or supplement VX (Difco). Colony types were maintained separately and were transferred selectively every 20 to 24 h on this medium (GCB). All cultures were incubated at 35°C in a candle jar. *N. gonorrhoeae* GC9 (31, 47), T2 or T4, was used in all serum assays. Strain CHD-PZ (T4), a genital isolate obtained from the Social Hygiene Clinic of the Houston City Health Department, was grown in a liquid defined medium for production of the protective filtrate. This medium is described below.

Assay for killing of gonococci by serum. After growth on GCB medium for 20 to 22 h, cultures of *N. gonorrhoeae*, T2 or T4, were harvested and then suspended in a modification of Gey balanced salt solution, formulated according to the recipe of Microbiological Associates (Bethesda, Md.), except that the sodium bicarbonate was omitted and 0.01% bovine serum albumin was added. The optical density of the suspension was determined with a Klett-Summerson colorimeter containing a green filter. Then the bacteria were diluted in modified Gey solution to yield about 10^6 colony-forming units (CFU)/ml. Clumping of gonococci from the T2 colony type produced more variation in colony counts than did gonococci from T4 colony type. No special precautions were taken that would select for a particular population of gonococci of T2 types. The presence of bovine serum albumin appeared to reduce clumping. Readings obtained with the Klett colorimeter had been correlated previously to viable count by plating dilutions of the suspension on GCB medium. Modified Gey solution was the first component added to the assay. The amount varied so that the total volume of the assay was always 1.0 ml. In order of addition, the remaining components were as follows: 0.1 ml of any factors being tested for protective activity, 0.1 ml of the standardized suspension of gonococci, and 0.1 ml of normal human serum containing endogenous complement. Assay components were mixed vigorously for 15 s with a Vortex mixer. Then 0.1 ml was removed immediately and serially diluted at 10-fold increments into blanks of 1% proteose peptone (Difco) in 0.85% sodium chloride. Dilutions were plated on GCB medium to determine CFU per milliliter at zero time. After incubation for 60 min at 37°C, the serum assay mixtures again were mixed vigorously for 15 s. The assay mixture was plated directly onto GCB medium and also was diluted as at zero time. Plates were incubated at 35°C in a candle jar for 20 to 24 h. Results are expressed as $\log_{10}$ CFU per milliliter of survivors at 60 min. Each assay was carried out in duplicate and, at a minimum, was repeated on 2 different days. Most experiments were done an even greater number of times.

Preparation of protective factors. Aqueous solutions of the iron compounds were prepared on the same day that they were used. Appropriate dilutions were made so that only 0.1 ml was added to the serum assay.

The filtrate was prepared as follows. *N. gonorrhoeae* CHD-PZ (T4) was grown in the liquid defined medium of LaScolea and Young (25). The conditions for inoculation and growth previously were described in detail (26). Each 250-ml flask contained 30 ml of the defined medium. After incubation for 24 h, cultures were centrifuged at 10,000 × *g* for 20 min. The pellet was discarded, and the supernatant fluid was filtered through a 0.22- or 0.45-μm filter (Nalge, Rochester, N.Y.). The sterile filtrate was stored at 4°C. The crude filtrate was stable for at least 4 months when stored at 4°C.

Tests for possible iron-binding role of filtrate. Colorimetric tests for hydroxamates were performed by a modification (12) of Csaky's method (7) and tests for catechols were performed by Arnow's method (2). The effects of medium and protective filtrate on the binding of iron by iron-free transferrin (Sigma Chemical Co., St. Louis, Mo.) were followed at 470 nm, the wavelength at which the pink transferrin-iron complex absorbs (35). Either 0.2 ml of deionized water, liquid defined medium, or crude filtrate was added to 0.8 ml of iron-free transferrin (2.5 mg/ml) in tris(hydroxymethyl)aminomethane-hydrochloride buffer, pH 7.4. Another buffering system, 10 mM *N*-2-hydroxyethyl piperazine-*N'*-2-ethanesulfonic acid, pH 7.4, also was used and yielded identical results. The absorbance at 470 nm was measured in a Gilford 240 spectrophotometer after each addition of 5 μl of 1.0 mM ferric nitrilotriacetate, an iron compound that reacts stoichiometrically with transferrin (3). The change in absorbance of each mixture was plotted against the amount of ferric nitrilotriacetate added.

Serum. Blood was collected from normal male volunteers with no history of gonococcal infection. It was allowed to clot, was centrifuged to separate clot from serum, and was centrifuged again to remove residual erythrocytes. The serum then was filtered through a 0.45-μm filter, dispensed in small volumes, and frozen at −70°C until used. Sera from two individuals were used, both of which showed killing of *N. gonorrhoeae* GC9. Samples of sera were not pooled. Not all sera samples, even from the same individual, showed the same degree of killing demonstrated in Fig. 1, but all tested showed the differences in killing of T2 and T4 colony types.

Titration of complement. Complement was titrated in the presence of components of the serum assay minus gonococci. The relative volumes of each component were similar to the volumes used in the serum assay. However, the serum dilutions (1:13 to 1:25) used in the titration of complement were greater than the final serum dilution (1:10) used in the serum assay. Therefore, any effects of the protective factors might be greater in the presence of more dilute serum. Sheep erythrocytes (Cordis Laboratories, Miami, Fla.) were washed three times in 10 volumes of modified Gey solution and standardized with a Gilford 240 spectrophotometer to a concentration of 10^9 cells/ml. An equal volume of hemolysin (Cordis) diluted in modified Gey solution was added to sensitize the cells. Each series of titration contained 0.2 ml of sensitized cells, 0.15 ml of protective factor, 0.15 ml of serum diluted 1:13 to 1:25 in modified Gey solution, and modified Gey solution for a total volume of 1.55 ml. After incubation for 60 min at 37°C, the test tubes were centrifuged, and the supernatant fluid was read at 541 nm in a Gilford 240 spectrophotometer. Controls were included to show that lysis did not occur in the absence of serum, with or without protective factors. Results were calculated as outlined by Kabat and Mayer (20) and were expressed as the hemolytic units in 1.0 ml of undiluted serum that would result in 50% hemolysis.

RESULTS

Killing of gonococci by normal human serum. Initial studies showed that bacteria from T2 colony types of *N. gonorrhoeae* GC9 were

more resistant to killing by normal human serum than those from T4 colony types of the same strain (Fig. 1). These data, previously reported by us (P. N. Zey and R. P. Williams, Abstr. Annu. Meet. Am. Soc. Microbiol. 1976, B33, p. 16), agree with those of McCutchan et al. (27), who also reported differences in killing by normal human serum of T2 and T4 types of the same strain. Although not all sera showed the same degree of killing, gonococci from T2 types were more resistant than those from T4 types in all sera tested. Neither serum inactivated for 30 min at 56°C nor modified Gey solution in the absence of serum had an adverse effect on the gonococci.

Prevention of killing by ferric ammonium citrate. As shown in Fig. 2, iron in the form of ferric ammonium citrate (FAC) prevented killing of gonococci from both T2 and T4 colony types. Both types were protected almost completely at a final concentration of 300 μg of FAC per ml, approximately 1.0 mM. FAC was used because of its relative solubility at physiological pH. Because citrate is an iron chelator and in some bacterial systems may induce siderophore production (24), ammonium citrate was tested also. Concentrations of 1.0 mM had no effect on killing when assayed under the same conditions.

Effects of other iron compounds on killing by serum. Three inorganic iron compounds at concentrations equivalent to 30 μg of FAC per ml were used in the assay with bacteria from T4 colony types. Ferric chloride, ferric nitrate, and ferric ammonium sulfate, each at a final concentration of 0.1 mM in the serum assay, had no effect; they gave the same results at 60 min as the control that showed killing in the presence of serum. The effects of serial 10-fold dilutions of ferric chloride (0.001 to 1.0 mM) were tested on bacteria of T4 colony types in the absence of serum and in the complete serum assay. The three lowest concentrations had only a slight effect on the gonococci themselves and showed no protection of the gonococci in the serum assay. The highest concentration was toxic to the gonococci in the absence of serum, possibly due to its low pH.

Effects of other cations on killing by serum. Gey solution, the major component of the assay, contains 0.28 mM of magnesium sulfate, 1.0 mM of magnesium chloride, and 1.5 mM of calcium chloride. Additional amounts of magnesium sulfate and calcium chloride were added to the serum assay so that the added amounts were equivalent in molarity to the highly protective level of FAC (1.0 mM). These additional amounts did not demonstrate a protective effect.

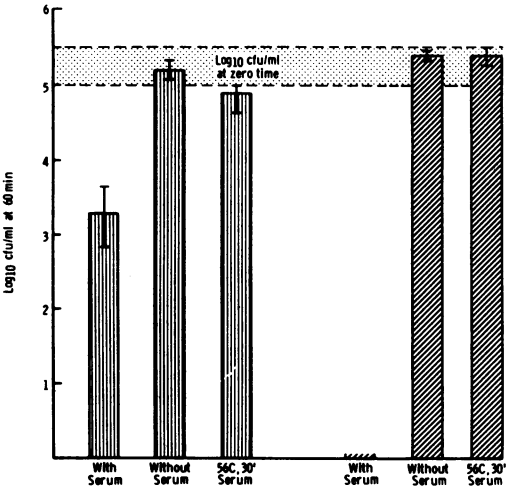

FIG. 1. *Killing of N. gonorrhoeae GC9 by normal human serum containing endogenous complement. Serum assay mixtures were plated for viable counts at zero time and after incubation at 37°C for 60 min. The horizontal, dotted bar represents the range of viable bacteria found at zero time; the vertical bars, survivors at 60 min. Symbols: ▥, GC9-T2; ▨, GC9-T4. Explanations for the vertical bars are as follows: With serum, the complete serum assay that shows killing of the gonococci; without serum, all components of the assay except serum; 56°C, 30-min serum, the complete serum assay containing serum that had been heated for 30 min at 56°C. The vertical lines show the range of results obtained in repeated experiments. The height of the bar indicates the average of the several assays carried out for each experiment. No survivors were detected after exposure of T4 bacteria to this serum.*

Effects of culture filtrate on serum killing and possible relation to iron effect. With other bacteria, either the direct addition of iron or of siderophores found in culture filtrates may have an effect on the bacteriostatic or bactericidal effects of serum. Since FAC showed a protective effect, we tested a gonococcal culture filtrate for a similar effect in the serum assay. The filtrate showed the same protective effect as did FAC, preventing the killing of bacteria from both T2 and T4 colony types. Figure 3 shows the protection of T4 with time by both FAC and the culture filtrate.

The filtrate was tested for catechols and hydroxamates, the known microbial siderophores (24), to determine if siderophores might be involved in the protective mechanism. The results of the colorimetric tests were negative. However, the filtrate did have some ability to chelate iron. When followed spectrophotometrically, the filtrate prevented the binding of iron to iron-free transferrin (Fig. 4). But the iron-binding ability

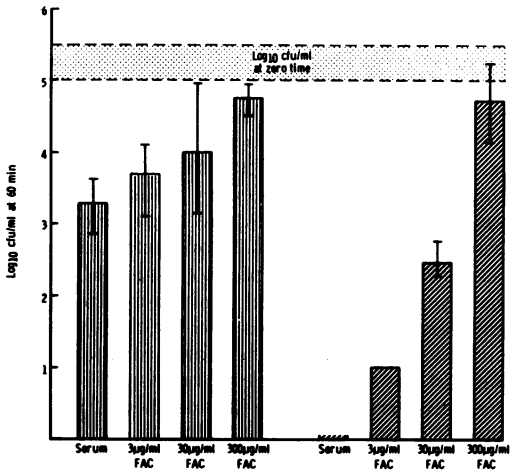

FIG. 2. *Protection from killing of N. gonorrhoeae by FAC. Results of serum assay mixtures are expressed as in Fig. 1. The first vertical bar of each set represents killing due to serum without any additions. Increasing concentrations of FAC were added in the same volume to the serum assay. The other bars represent either 3, 30, or 300 μg of the final concentration of FAC per ml in the serum assay. When 3 μg of FAC per ml was added in the assay with bacteria of T4 colony type, no range of survivors was detected in repeated experiments.*

of the filtrate did not appear to explain the protective effect of the filtrate in the serum assay. The protective effect was lost when the filtrate was diluted 1:8. If the filtrate were solubilizing and chelating iron for the protective effect, then addition of a dilution of filtrate that was not protective plus a level of FAC that showed minimal protection should increase the survival of the gonococci. However, this combination showed only the minimal protection already afforded by the iron alone.

Effect of FAC and filtrate on complement. Complement titers of serum in the presence and absence of FAC, filtrate, or an autoclaved sample of filtrate that was not protective showed that the titers were not sufficiently affected to account for protection (Table 1). Although the titers measured in the presence of FAC or filtrate were lower than the control with no additions, there was still enough complement for killing of the gonococci. This result is evident since the titer in the presence of the autoclaved filtrate showed the same lower levels but the gonococci still were killed in the serum assay upon addition of the autoclaved filtrate.

DISCUSSION

Iron or siderophores can prevent the bacteriostatic and some bactericidal effects of serum, but the mechanisms involved in the bactericidal reactions probably are more complex. The relief of bacteriostasis by addition of iron or siderophores may be due to a greater availability of iron for the bacteria to use for their nutritional requirements (46). Siderophores compete with transferrin for its bound iron and make it avail-

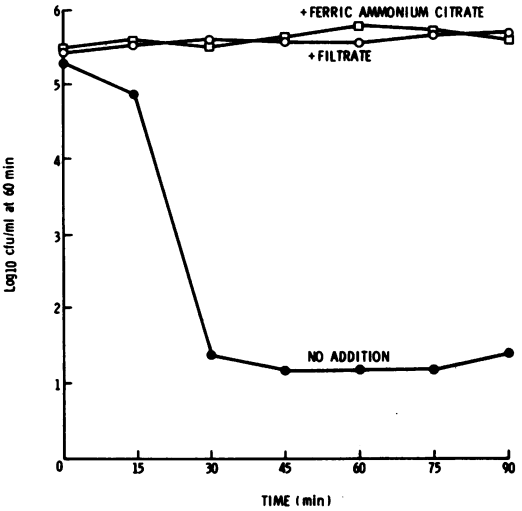

FIG. 3. *Kinetics of killing by serum and of protection by filtrate or FAC. Samples were removed from the serum assay every 15 min for 90 min and plated for viable counts. The vertical axis represents survivors of the population of N. gonorrhoeae GC9-T4. Symbols: (●) serum assay with no additions; (○) serum assay plus filtrate obtained after growth of N. gonorrhoeae CHD-PZ (T4) in liquid defined medium; (□) serum assay plus FAC (300 μg/ml).*

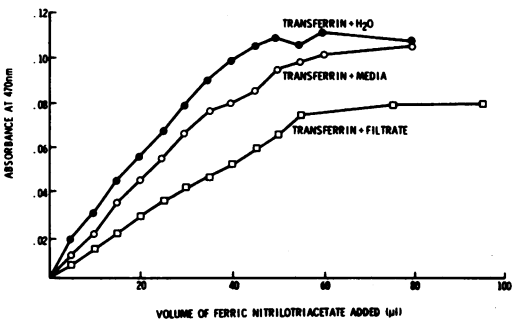

FIG. 4. *Effects of filtrate on binding of iron by transferrin. Iron-free transferrin plus deionized water (●), plus media (○), or plus gonococcal culture filtrate (□) as incubated for 60 min at 37°C. Ferric nitrilotriacetate (5 μl) then was added, and the absorbance after each addition was measured. The vertical axis represents absorbance at 470 nm, the wavelength at which the transferrin-iron complex absorbs; the horizontal axis represents the amount of ferric nitrilotriacetate added.*

TABLE 1. *Effect of protective factors on titer of complement in serum*

Additions	CH_{50}/ml[a]	Protection in serum assay[b]
None	130	–
Filtrate	100	+
Autoclaved filtrate	100	–
FAC (300 μg/ml)	115	+

[a] CH_{50}/ml, hemolytic units in 1.0 ml of undiluted serum giving 50% hemolysis, was determined by titration of complement in the presence of the additions, the factors tested for protective activity in the serum assay.

[b] Protection of *N. gonorrhoeae* from killing by serum was determined by counting the survivors after incubation for 60 min in the serum assay (see Fig. 2 and 3).

able to the bacteria for growth. The role of iron, siderophores, and transferrin in bactericidal reactions in which antibody and complement are involved is not so easily explained. Protection could be due to utilization of iron by the bacteria to prevent lysis. In a system in which antibody and complement were present, one investigator postulated that iron deprivation first leads to bacteriostasis due to either abnormal tRNA or lack of normal tRNA. The result is a shutdown of RNA, DNA, and protein synthesis, with subsequent lysis of the bacteria (14, 15).

Protection does not appear to be due to physical interference, as the most protective level of FAC tested did not inactivate complement sufficiently to account for protection. Since iron does bind nonspecifically to bacteria (11, 44), it could protect by coating the gonococcal surface and blocking sites for attachment of antibody or late-acting components of complement. Or serum proteins attached nonspecifically to the bound iron may coat bacterial surfaces with resultant protection (9). However, iron did not similarly affect the sheep erythrocyte-hemolysin system used to titrate complement. If iron, either alone or through binding of serum proteins, had a major coating effect, then sites for antibody and/or components of complement on the sheep erythrocytes might have been blocked with resultant significant decreases in the titer of complement. Previous reports have shown that iron did not affect complement levels (6, 9), and, by different methods, we also demonstrated that FAC did not affect the titer of complement.

Of the iron compounds tested in this study, the relatively soluble FAC prevented killing of the gonococci by normal human serum. The other iron compounds may have been too insoluble at the pH tested. If iron is added to a serum assay, then it presumably saturates the transferrin by stoichiometric binding to specific sites on the transferrin molecule. Even in the absence of a bacterial siderophore to compete with the transferrin for iron, only very small amounts of iron over that required to saturate transferrin should be needed for utilization by bacteria. However, in our assay, a large excess over that needed for saturation of transferrin was required for protection of the gonococci. There are several possible explanations for the large amounts of iron needed. Because most iron compounds form insoluble complexes at physiological pH (3, 41), the iron does not bind stoichiometrically to transferrin, and amounts greater than the calculated values are required for saturation. Also, some of the added iron binds nonspecifically to the transferrin molecule (3), to other serum proteins (43), and to bacteria (11, 44).

Stabilization of the gonococcal outer membrane does not seem to be the mechanism of protection. Magnesium and calcium, cations known to be important in stabilization of gram-negative outer membranes (10, 38) and in prevention of autolysis of gonococci (8, 45), had no protective effect at concentrations similar to those of FAC that showed protection. Magnesium and calcium did decrease killing of gonococci in serum assays, but the levels used in our assay exceeded those reported (49).

Both ferric iron and siderophores can inhibit killing of some other gram-negative bacteria (5, 9, 14). Siderophores are found in culture filtrates of many bacteria (12, 24, 36). Since we found that FAC prevented killing of the gonogocci by normal human serum, we considered that the gonococcal filtrate might contain siderophores that made iron available to the gonococci in the serum assay. However, the colorimetric tests for the known microbial siderophores were negative. The only indication that siderophores might be present was the ability of the filtrate to inhibit the binding of iron to iron-free transferrin. This inhibition could indicate an ability of the filtrate to chelate ferric iron. The filtrate was not obtained from iron-limited growth, in which large quantities of siderophores are produced by other microbes (24), but low levels of constitutive siderophores might have been present. Low levels could be sufficient to compete with transferrin for iron but not sufficient for detection by the colorimetric tests for the known microbial siderophores. It is also possible that the chelating effect is due to a new class of siderophores.

However, there are explanations other than chelation of iron to explain the inhibition of iron binding to transferrin. Some gonococcal product may bind nonspecifically to the transferrin molecule and thereby block the iron-binding sites of transferrin. Specific binding by filtrate compo-

nents to the iron-binding sites is unlikely due to the high affinity of these sites for ferric iron (35). The gonococcal filtrate may contain a protease capable of cleaving transferrin so that it no longer can bind iron. We are not aware of any bacterial enzymes capable of hydrolysis of transferrin.

Although the gonococcal culture filtrate had the same protective effect as FAC, we do not believe that it is acting as a siderophore in the serum assay. Low levels of iron and filtrate were not additive in their ability to prevent killing of gonococci. The filtrate did not affect the titer of complement, and the mechanism of protection in the serum assay is not known.

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