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Ability of *Neisseria gonorrhoeae, Neisseria meningitidis,* and Commensal *Neisseria* Species to Obtain Iron from Transferrin and Iron Compounds

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The ability of *Neisseria* species to use iron compounds and to compete with iron-binding proteins was examined with deferrated defined medium and the iron chelator deferoxamine. All *Neisseria* species were able to assimilate a variety of ferric and ferrous iron salts. They were not able to efficiently solubilize an inorganic iron salt such as ferric nitrate, but were able to use iron chelated by citrate, oxalacetate, pyrophosphate, or nitrilotriacetate. Each of the 95 *Neisseria* isolates examined was able to use hemin as a sole source of iron, and most, but not all, of the isolates were able to obtain iron from hemoglobin. Heated human serum stimulated growth of all gonococci, meningococci, and some commensal *Neisseria* species in iron-deficient medium. All gonococci and meningococci were able to scavenge iron from 25% saturated transferrin, whereas most commensal organisms were inhibited by this iron-binding protein. The ability to compete with transferrin was specific, since partially saturated conalbumin was bacteriostatic for all *Neisseria* species. Although the pathogenic *Neisseria* species were able to compete more efficiently with transferrin for iron than were the nonpathogenic *Neisseria* species, no correlation was observed between the virulence of different strains or colony types of gonococci and the ability to scavenge iron in vitro from transferrin or other chelators.

One mechanism by which an animal host discourages colonization or invasion by microorganisms may be limitation of the availability of an essential nutrient such as iron. In tissues, the concentration of free iron is far below that required for microbial growth. Iron present in the host is sequestered in hemin compounds, as ferritin, or bound to the proteins lactoferrin or transferrin, which function in iron chelation, transport, and exchange (24). The presence of transferrin in serum and of lactoferrin in secretions accounts, in part, for the microbiostatic activity of these host fluids for some bacteria and fungi (4, 5, 24).

The efficiency with which some microorganisms obtain iron in the host may be a determinant of pathogenicity (4, 15, 24, 27). In chicken embryos, Payne and Finkelstein (19, 20) and Payne et al. (21) observed that administration of iron dextran enhanced the infectivity of relatively avirulent colonial type 4 gonococci but did not alter the 50% lethal dose for type 1 organisms. Treatment with conalbumin, the iron-binding protein in chicken egg white, decreased the virulence of most type 1 gonococci isolated from localized genital infections but did not affect the 50% lethal dose of gonococci capable of causing disseminated gonococcal disease. These investigators proposed that the ability of certain strains or colony types of gonococci to scavenge iron in the host correlated with, and might be responsible for, the virulence of the organisms.

The mechanisms by which gonococci obtain iron in the host have not been characterized. The purpose of this investigation was to describe some of the systems by which gonococci obtain iron. For comparative purposes we also examined the ability of *Neisseria meningitidis* and the commensal *Neisseria* species to compete with the iron-binding proteins transferrin and conalbumin and to use iron compounds which might serve as iron sources in vivo.

MATERIALS AND METHODS

Organisms. Strains of *N. gonorrhoeae* were primarily clinical isolates obtained from either Durham County Health Department, Durham, N.C., Duke University Hospital, Durham, N.C., or North Carolina Memorial Hospital, Chapel Hill, N.C. Of the 29 isolates examined for ability to use various iron sources, 7 were isolated from joint or blood cultures of persons having disseminated gonococcal infection (DGI). Laboratory strains of *N. gonorrhoeae* included F62 (obtained from

D. S. Kellogg, Jr., Centers for Disease Control, Atlanta, Ga.) and FA19 (obtained from A. Reyn, Statens Seruminstitut, Copenhagen, Denmark). FA19 has caused disseminated disease in a laboratory-acquired infection. Of the 29 gonococcal isolates, 13 were resistant to the bactericidal activity of serum (8). Twenty strains were amino acid prototrophs, and the remaining isolates represented a variety of auxotypes (6). Four of these gonococcal isolates, FA534, FA852, FA853, and FA854, lacked plasmids detectable by agarose gel electrophoresis (16). Additional gonococcal strains obtained from the Centers for Disease Control; J. S. Knapp, Neisseria Reference Laboratories, Seattle, Wash.; Durham County Health Department; and North Carolina Memorial Hospital were used for determinations of the minimal inhibitory concentrations (MICs) of chelators for isolates from disseminated and urogenital infections. Colonial type 4 organisms were used in all assays, unless otherwise noted, and were selected by the method of Kellogg et al. (13) on Difco GC base agar (GCBA) containing 1% Kellogg defined supplement I and 0.1% supplement II.

Meningococci representing groups A, B, C, X, Y, and Z were obtained from North Carolina Memorial Hospital; North Carolina State Health Department, Raleigh, N.C.; Walter Reed Army Medical Center, Washington, D.C.; and M. H. Mulks, Tufts-New England Medical Center, Boston, Mass. Commensal *Neisseria* species included American Type Culture Collection strains and isolates obtained from J. S. Knapp, Neisseria Reference Laboratories; M. H. Mulks, Tufts-New England Medical Center; North Carolina State Health Department; and North Carolina Memorial Hospital.

Media. The deferrated defined liquid medium (DDL) used in most of these studies was a modification of Catlin's NEDF (6). The original NEDF formula was altered by the omission of ethylenediaminetetraacetic acid, glycerin, polyvinyl alcohol, Tween 80, glucose, hemin, nitrilotriethanol, and ferric nitrate and by the inclusion of 45 mM sodium lactate, 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), and 500 μM dibasic and monobasic potassium phosphate. The medium was prepared at 5× strength, and the pH was adjusted to 7.1. The medium was deferrated by using the ion-exchange resin Chelex-100 (Bio-Rad Laboratories). Chelex-100 (sodium form) was soaked for approximately 2 h in sterile 200 mM NaCl and then rinsed with 10 to 15 volumes of sterile deionized water. The resin was added to sterile medium and mixed very gently with a magnetic stirrer overnight at 4°C. The medium was sterilized by passage through a 0.45-μm filter, which also removed the Chelex-100, and then was treated with fresh Chelex-100 two additional times for 8 to 16 h. Approximately 120 g of Chelex-100 was used to deferrate 1 liter of 5× medium. The deferrated medium was supplemented with 100 μM $CaCl_2$ and 1.0 mM $MgCl_2$, and the pH was adjusted to 7.45. Some lots of Chelex-100 were found to be more satisfactory than others in selectively removing iron without removing other components necessary for the growth of the organisms. Concentrated Chelex-100-treated DDL was stored frozen at −20°C. Before use, DDL was supplemented with 20 mM sodium bicarbonate. The concentration of iron in the 1× deferrated medium, as determined by atomic absorption spectrophotometry, was 0.02 to 0.07 μM.

A defined agar medium (DA) was made from DDL by the addition of 0.8% Sigma type II agarose. DA had a sufficiently high concentration of contaminating iron, approximately 2 μM, that organisms were not iron limited (i.e., addition of iron did not affect the rate or extent of growth). The potent iron chelator, deferoxamine mesylate (DF [Desferal, Ciba Pharmaceutical Co.]), was used in some assays to bind all free iron in the medium. Iron complexed with DF could not be assimilated by any of the *Neisseria* species.

To assess the effects of potential iron-solubilizing compounds on the use of inorganic and organic iron sources, a low-chelate modification of DA (LC-DA) was used. LC-DA was prepared by substituting 28 mM glucose for sodium lactate, using only 300 μM dibasic and monobasic potassium phosphate, and omitting sodium acetate and oxalacetate. LC-DA was not deferrated with Chelex-100.

Except for disposable pipettes (Corning Glass Works), which were found to contribute negligible amounts of contaminating iron, all glass and plastic items used were soaked in 3 N nitric acid and rinsed with deionized water. Plastic items were sterilized by autoclaving, and glassware was sterilized in a hot-air oven.

Iron-binding and iron-containing compounds. Human transferrin (Sigma Chemical Co.) and chicken egg white conalbumin (Sigma) were deferrated and further purified by chromatography, using Sepharose CL-6B (Pharmacia Fine Chemicals) (2). Preparations of transferrin to which no further iron was added were 4% iron saturated as determined by atomic absorption spectrophotometry of samples of known protein concentration. The iron-binding capacity of these proteins dissolved in 10 mM HEPES–150 mM NaCl–10 mM sodium bicarbonate buffer (pH 7.4) was also determined by titration with ferric nitrilotriacetate (9). Deferrated transferrin and conalbumin were made 25% saturated by addition of 1 mM ferric nitrilotriacetate, followed by passage through a Sephadex G-25 (Pharmacia) column equilibrated with the HEPES-NaCl-bicarbonate buffer to remove any unbound ferric nitrilotriacetate (14). Filter-sterilized transferrin and conalbumin were stored at either 4 or −70°C. Purified preparations of transferrin and conalbumin were used in all assays except those used to determine MICs.

Hemin (Sigma) was dissolved in a small quantity of triethanolamine, diluted to the appropriate concentration with deionized water, and then sterilized by filtration. Human hemoglobin (Sigma) was dissolved in deionized water, dialyzed, and sterilized by filtration. Human serum from healthy donors was pooled and stored at −70°C and was heat inactivated by incubating for 30 min in a 56°C water bath.

Plate assays for use of iron compounds. After selective passage on GCBA, organisms were passaged twice on DA. The addition of agarose to DDL resulted in approximately 2 μM Fe in DA, which was sufficient for growth. Organisms from an 18-h culture were suspended in DDL and grown for 2 to 4 h in an incubator shaker at 35°C with an atmosphere of 5 to 10% CO_2. The density of the cultures was adjusted to approxi-

mately 20 Klett units (approximately 10^8 colony-forming units per ml) with a Klett-Summerson spectrophotometer with a 500- to 570-nm filter. The culture was then diluted 1:100 in DDL, and 0.5 ml of the diluted culture was added to 15 ml of molten DA which contained 10 mM sodium bicarbonate and either no additional supplements or 200 μM DF. After the medium solidified, wells of approximately 8 mm in diameter were cut in the agar. The wells were charged with 40-μl samples of either 10 mM $Fe(NO_3)_3$, 75 μM hemin, 200 μM hemoglobin, undiluted heated serum, 500 μM transferrin, or 500 μM conalbumin dissolved in the HEPES-NaCl-bicarbonate buffer. Plates were incubated at 35°C in an atmosphere of 5 to 10% CO_2. Zones of growth stimulation or growth inhibition were measured after 22 to 24 h and after 48 h of incubation.

To assess the effects of potential iron-solubilizing compounds on the use of inorganic or organic iron sources, the same procedure was followed except that LC-DA, rather than DA, was used as the growth medium. Wells were filled with 40-μl samples of 10 mM $Fe(NO_3)_3$ or with 100 mM solutions of oxalacetic acid, nitrilotriacetate, sodium citrate, sodium succinate, sodium maleate, sodium lactate, sodium acetate, sodium pyrophosphate, or dibasic and monobasic potassium phosphate.

Growth kinetics in liquid deferrated medium. After at least two passages on DA, organisms were grown overnight in DDL at 35°C in an atmosphere of 5 to 10% CO_2. Cultures were diluted into fresh DDL and incubated in a CO_2 shaker incubator to obtain logarithmic-phase cells. The cultures were then washed twice in DDL and inoculated into warmed DDL to which various iron sources or iron chelators had been added. The cultures were incubated at 35°C in a shaker incubator with 5 to 10% CO_2, and the turbidity was read with a Klett-Summerson spectrophotometer.

Growth in serum. After selective passage on GCBA, organisms were passaged twice on DA, suspended to an optical density of 30 Klett units, and grown for approximately 2 to 3 h in a shaker incubator in the presence of 5% CO_2. The turbidity of logarithmic-phase cultures was then adjusted to an optical density of 30 Klett units, and serial 10-fold dilutions were made in DDL. A 15-μl amount of each diluted culture was added to sterile microtiter wells containing 150 μl of fresh or heat-inactivated pooled human serum or DDL. All wells were supplemented with 10 mM sodium bicarbonate. After 24 and 48 h, wells were examined for evidence of visible growth. Only wells which showed visible growth yielded viable organisms upon subculture.

MICs of iron-binding compounds. MICs for DF and conalbumin were determined for 44 strains of *N. gonorrhoeae*. After type 1 or 4 colonies had been selected on GCBA, organisms were subcultured at least twice on DA. Subculture back to GCBA revealed that cultures of type 1 gonococci usually still consisted of >90% type 1 organisms. For agar dilution assays, organisms were suspended and adjusted to 30 Klett units in DDL and then diluted 1:50 in DDL. With a calibrated platinum loop, approximately 0.005 ml of the diluted culture was inoculated onto plates of DA either without further additions or with increasing concentrations of DF, unpurified transferrin, or unpurified conalbumin. Unpurified transferrin and conalbumin contained, respectively, 0.001 and 0.015% contaminating iron. Concentrations of these iron-binding compounds tested were as follows: 0.1, 0.2, 0.4, 0.6, 0.8, and 1.0 μM (conalbumin); 0.5, 1.0, 2.0, 4.0, and 8.0 μM (DF); 0.5, 1.0, 2.0, 5.0, and 20.0 μM (transferrin). Medium containing 8 μM DF, 1 or 0.5 μM conalbumin, or 20 μM transferrin to which 40 μM $Fe(NO_3)_3$ had been added was used for growth controls. The lowest concentration of chelator which markedly inhibited growth compared with controls after 48 h was considered the MIC. Each isolate was tested twice, each time with a different batch of medium.

To determine broth dilution MICs, colony type 1 or 4 organisms which had been passaged twice on DA were grown in DDL overnight in a CO_2 incubator without shaking. Cultures inoculated with type 1 gonococci contained greater than 75% type 1 organisms. Cultures were adjusted to a density of 30 Klett units and diluted by 10-fold serial dilutions in DDL, and 20 μl of various dilutions was added to microtiter wells containing 0.2 ml of DDL either without additional supplements or with 0.01, 0.025, 0.05, 0.10, 0.25, 1.0, 2.5, or 5.0 μM conalbumin. MICs were determined by using inoculum sizes of approximately 10^4, 10^5, and 10^6 organisms per ml.

Statistics. The Mann-Whitney two-sample test was used to analyze differences in distribution of growth responses to iron compounds or chelators exhibited by DGI and local isolates or type 1 and 4 gonococci.

RESULTS

Use of iron-containing compounds. Gonococci grew well when inoculated onto DA, which contained about 2 μM iron (Fig. 1, bottom row). However, when they were inoculated onto the same medium containing 200 μM DF, no growth occurred except around wells containing a useable iron source (Fig. 1, top row). Iron salts such as ferric nitrate, ferric chloride, ferric citrate, ferric ammonium citrate, ferrous sulfate, and ferrous ammonium sulfate stimulated the growth of *N. gonorrhoeae* FA19 and F62, a group C *N. meningitidis* isolate, *N. lactamica* ATCC 23970, and *N. perflava* ATCC 14799.

All *Neisseria* isolates were able to use hemin as a sole iron source in the presence of sufficient DF to bind any available free iron. Of 29 gonococcal isolates, 9 of which were serum-sensitive prototrophs, 11 were unable to obtain the iron required for growth from hemoglobin. For many strains of gonococci, growth around wells containing hemoglobin was uneven and was detected only after 48 h of incubation, in contrast to the positive results obtained at 24 h with $Fe(NO_3)_3$ and hemin. When strain FA19 was first grown in the presence of hemoglobin, zones of growth appeared after 24 h of incubation. The delayed growth response suggests that an induc-

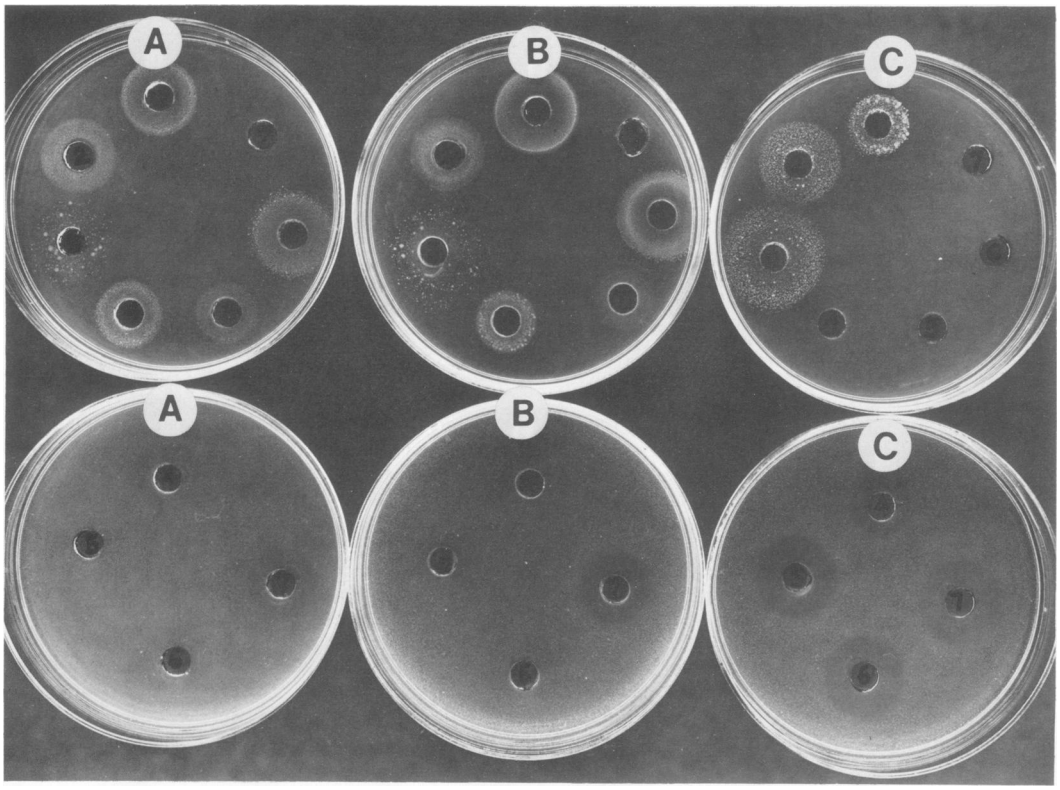

FIG. 1. *Growth response of (A) N. gonorrhoeae FA19, (B) a group C N. meningitidis isolate, and (C) N. perflava ATCC 14799 to iron-containing and iron-binding compounds in DA containing 200 μM DF (top row) and in DA without DF (bottom row). DA contains sufficient iron (ca. 2 μM) to allow matt growth in the absence of DF, although this is not easily seen in the figure (bottom row). Wells in the top row of plates contained the following (counterclockwise from top well): $Fe(NO_3)_3$, hemin, hemoglobin, heated serum, 4% saturated transferrin, 25% saturated transferrin, and 25% saturated conalbumin. Wells in the bottom row of plates contained the following (counterclockwise from top well): heated serum, 4% saturated transferrin, 25% saturated transferrin, and 25% saturated conalbumin. Zones of inhibition are present around well 7 on plates A and B and around all wells on plate C.*

ible system, or possibly a protease, might be involved in obtaining iron or hemin from hemoglobin. Of the 21 meningococcal strains examined, one group A isolate was unable to use hemoglobin as an iron source. Growth of all commensal isolates was stimulated by hemoglobin in medium containing 200 μM DF. The reason for the heterogeneous growth response to hemoglobin exhibited by some isolates is unknown.

Competition with transferrin and conalbumin. The ability of organisms to compete with the iron-binding proteins transferrin and conalbumin for iron was evaluated on iron-sufficient medium (DA, ca. 2 μM iron) and iron-free medium (DA plus 200 μM DF, which chelated all free iron). Results obtained with the two assay systems were complementary. For example, organisms which were stimulated by transferrin in medium totally deferrated by 200 μM DF exhibited growth around wells containing transferrin in iron-sufficient medium. If transferrin or conalbumin was inhibitory in medium lacking DF, no growth stimulation was observed in medium limited in iron by DF (Fig. 1). Heating transferrin (56°C, 30 min) did not affect the results.

When the concentration of DF in plates was decreased, the diameter of the zones of growth stimulation around wells containing a fixed concentration of free inorganic iron increased substantially. Zones of growth around wells containing hemin, hemoglobin, serum, and partially saturated transferrin were unaffected by the concentration of DF (data not shown). Growth stimulation by these iron sources was therefore not due to contamination by free iron.

Transferrin is normally 25 to 35% saturated in

serum (1, 24). Growth of all 29 gonococcal isolates was stimulated by 25% saturated transferrin after 48 h of incubation on DA containing 200 μM DF (Table 1). One strain, FA3003, appeared to be somewhat less efficient than other gonococci in scavenging iron from 25% saturated transferrin, since zones of growth in the presence of DF appeared only after 48 h of incubation rather than at 24 h as observed with most gonococci. All meningococcal isolates were able to obtain the iron required for growth from 25% saturated transferrin. Only one group A meningococcus isolate and two strains of the gonococcus, FA3003 and FA1579, were inhibited by 4% saturated transferrin after 48 h of incubation in iron-sufficient DA. Four percent saturated transferrin was inhibitory at 24 h on DA for four additional strains of the gonococcus, FA852, FA854, FA1513 (local disease isolates), and FA1035 (a DGI isolate), but these organisms overcame the bacteriostatic effects of transferrin and were able to compete successfully with the protein for iron after 48 h of incubation.

In contrast to the pathogenic *Neisseria* strains, most of the commensal isolates were not able to obtain iron required for growth on DA containing 200 μM DF from 25% saturated transferrin and were inhibited by transferrin in iron-sufficient DA (Fig. 1 and Table 1). Of 45 tested nonpathogenic strains, most of which were *N. lactamica* or *N. flavescens* isolates, 10 were able to scavenge iron from 25% saturated transferrin, but growth response to the transferrin for 5 of these isolates was slow. Only 5 of the 45 commensal *Neisseria* species were able to compete with 4% saturated transferrin for iron in the medium.

Conalbumin which was 25% saturated inhibited the growth of all strains of *Neisseria* in iron-sufficient DA and did not stimulate the growth of any of the isolates in iron-limited conditions (DA containing 200 μM DF). The size of zones of inhibition around conalbumin-containing wells did not change with prolonged incubation.

Kinetics of growth in DDL. Gonococci (Fig. 2) and meningococci (not shown) grew normally for a few mass doublings in DDL (<0.07 μM iron), but then were iron limited. More rapid growth inhibition was observed in DDL containing DF or conalbumin. The initial growth in DDL was presumably due to residual iron in intracellular pools, as well as to small amounts of iron remaining in DDL. Kinetics of growth of meningococci were very similar to those of gon-

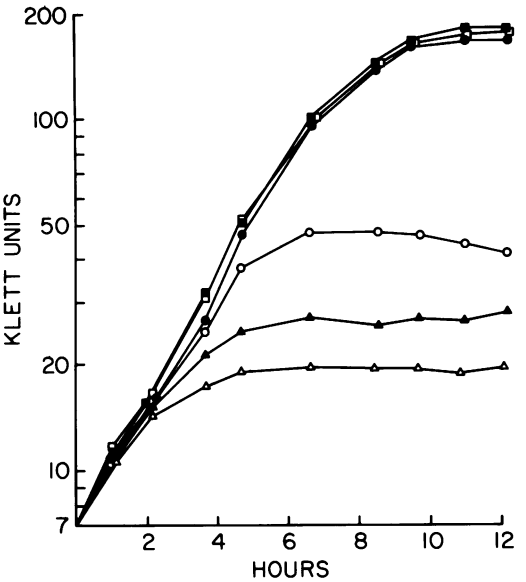

FIG. 2. *Growth of N. gonorrhoeae FA19 in DDL without additional supplements* (○) *or with 10 μM* $Fe(NO_3)_3$ (●), *2.5 μM 25% saturated transferrin* (■), *2.5 μM 25% saturated transferrin and 10 μM DF* (□), *2.5 μM 25% saturated conalbumin* (▲), *or 10 μM DF* (△).

TABLE 1. *Growth stimulation of Neisseria species by iron sources in medium containing excess DF*

Organism	No. of isolates	No. of colonies whose growth was stimulated by the following iron sources:			
		Transferrin (25% saturated)	Transferrin (4% saturated)	Conalbumin (25% saturated)	Heated serum
N. gonorrhoeae	29	29	27	0	29
N. meningitidis	21	21	20	0	21
N. lactamica	6	5	2	0	6
N. flavescens	3	3	2	0	3
N. sicca	9	1	1	0	1
N. flava[a]	11	1	0	0	9
N. mucosa	3	0	0	0	3
N. sicca or *N. flava*[b]	13	0	0	0	3

[a] Includes *N. flava*, *N. subflava*, and *N. perflava*.
[b] Patient isolates from Chapel Hill, N.C., identified as *N. sicca* or *N. subflava*.

ococci under these conditions. The bacteriostatic effects of 25% saturated conalbumin or of DF could be reversed by saturation with excess inorganic iron. The addition of 25% saturated transferrin completely reversed iron limitation in DDL even in the presence of excess DF.

Kinetics of growth exhibited by inoculum sizes of 10^5 or 10^6 organisms per ml of gonococci in either iron-poor DDL or DDL supplemented with 10 μM $Fe(NO_3)_3$ were monitored by determining the number of viable colony-forming units on GCBA. Kinetics of growth were the same regardless of whether organisms previously had been grown in iron-sufficient [10 μM $Fe(NO_3)_3$] DDL or DDL without additional iron. Thus, under these conditions no inoculum-dependent lag was observed for gonococci in iron-poor medium.

In contrast to the results obtained with gonococci and meningococci, the addition of 25% saturated transferrin inhibited growth of *N. subflava* ATCC 19243 in DDL (Fig. 3). Growth inhibition by transferrin was identical to that observed with conalbumin or DF (data not shown). Inhibition by transferrin, conalbumin, or DF could be reversed by adding sufficient iron to the medium to saturate the binding capacity of the chelators.

Growth response to serum. Four serum-sensitive strains of gonococci isolated from local infections and five serum-resistant DGI strains were tested for their ability to grow in human serum. Type 4 organisms of all isolates were able to initiate growth in heated serum even with an inoculum size as low as 10^3 organisms per ml. Plate assays (Fig. 1, bottom row; Table 1) showed that heated serum was not bacteriostatic for gonococci and that all gonococci were able to obtain from serum the iron required for growth. All meningococci and many of the commensal *Neisseria* species also were able to obtain iron from serum in medium containing DDL. Since some of these commensal isolates could not compete with transferrin for iron, other organic iron compounds in serum were presumably serving as iron sources. There was sufficient hemin in serum to support the growth of *Haemophilus influenzae* in plate assays.

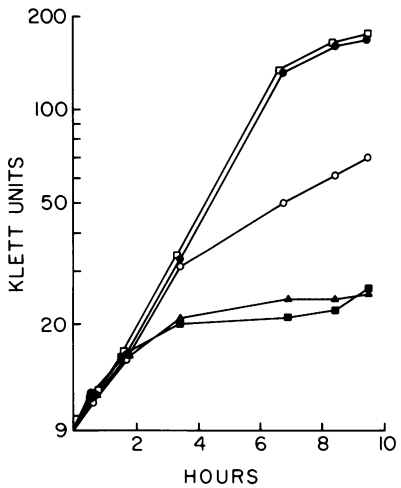

FIG. 3. *Growth of N. subflava ATCC 19243 in DDL without additional supplements* (○) *or with 10 μM $Fe(NO_3)_3$* (●), *2.5 μM 25% saturated transferrin* (■), *2.5 μM 25% transferrin and 10 μM $Fe(NO_3)_3$* (□) *or 2.5 μM 25% saturated conalbumin* (▲).

Iron acquisition by gonococci lacking plasmids. Each of four strains of gonococci which lacked detectable plasmids was able to obtain iron from 25% saturated transferrin and to utilize $Fe(NO_3)_3$, hemin, and iron-containing compounds in serum for growth. Four percent transferrin was somewhat inhibitory for two strains, FA852 and FA854, after 24 h of incubation, but these strains later overcame the bacteriostatic effects of the protein.

Iron scavenging by gonococci having differences in pathogenic potential. Several approaches were used to detect possible differences in the ability of various gonococci to compete with iron chelators in vitro. We reasoned that differences in iron-scavenging ability might be detected by determination of the least amount of conalbumin or DF which would inhibit growth when incorporated into agar medium. Type 1 organisms of each of 12 DGI strains and 16 urogenital isolates were inhibited by 2.0 μM DF and 0.4 μM conalbumin (Table 2). Type 4 gonococci from seven strains had the same MIC for DF or conalbumin as did type 1 organisms from the same isolates (Table 2). All strains grew in the presence of 20 μM transferrin. Each of seven additional DGI isolates and six urogenital isolates which were composed of a mixture of colony types 1 and 4 before being tested also had MICs of 2.0 for DF, 0.4 for conalbumin, and >20 μM for transferrin (data not shown). MICs were determined for conalbumin and DF after 48 h of incubation to allow endpoints to be accurately read for strains of gonococci which grew slowly on DA. However, the concentration of conalbumin or DF which markedly inhibited the growth of each isolate was the same after 24 or 48 h of incubation. Plates containing 0.4 μM conalbumin, which exhibited no growth at 48 h, showed a faint haze of growth for all strains upon prolonged incubation. Growth inhibition by conalbumin was reversed by the addition of $Fe(NO_3)_3$.

Of the total 44 gonococcal isolates tested in these assays, 3 were inhibited by lesser amounts of conalbumin or transferrin than were the other

TABLE 2. *MICs for conalbumin, DF, and transferrin determined by the agar dilution method for colony type 1 and 4 gonococci isolated from local and disseminated infections*

Colony type	Source	No. of isolates	MIC (μM)		
			DF	Conalbumin	Transferrin
1	DGI	12	2.0[a]	0.4	>20
	Urogenital[b]	16	2.0	0.4	>20
4[c]	DGI	2	2.0	0.4	>20
	Urogenital	5	2.0	0.4	>20

[a] MICs for DF, conalbumin, and transferrin were identical for all isolates in each of two experiments.
[b] Strains included two lacking detectable plasmids.
[c] Type 4 organisms were isolated from type 1 colonies of strains included above.

41 isolates, but they failed to grow on control plates containing conalbumin or transferrin and excess iron and were excluded from this study. Presumably, these organisms were sensitive to some component contaminating the unpurified preparations of conalbumin and transferrin used for the MIC assays.

When type 4 organisms of the 10 local disease isolates were compared with 4 DGI isolates on iron-limited medium (DA plus 200 μM DF), sizes of zones of growth stimulation around wells containing $Fe(NO_3)_3$, hemin, and 25% saturated transferrin or 4% transferrin (data not shown) were not significantly different ($P > 0.4$ for each assay). The sizes of zones of inhibition on iron-sufficient DA around wells containing conalbumin were also not significantly different ($P > 0.4$). Similar assays showed no differences between type 1 and 4 organisms of strains FA19 and F62 (data not shown). In microtiter broth dilution assays containing an inoculum of $\leq 10^6$ colony-forming units per ml, type 1 and 4 cells of strains FA19 and F62 had identical MICs for conalbumin (0.1 μM).

Use of iron sources in medium deficient in iron-solubilizing compounds. *N. gonorrhoeae* FA19 and F62, a group A meningococcal isolate, *N. lactamica* NRL 30011, *N. subflava* NRL 30017, and *N. flava* NRL 9993 were tested in assays employing modified DA containing minimal amounts of potential iron-solubilizing components (LC-DA). With DA containing 200 μM DF, zones of growth occurring around wells containing 10 mM $Fe(NO_3)_3$ were approximately 17 to 19 mm in diameter. In contrast, with LC-DA containing 200 μM DF, zones of growth around wells containing 10 mM $Fe(NO_3)_3$ were <10 mm in diameter. In LC-DA, large elliptical zones of growth stimulation were obtained around one side of the $Fe(NO_3)_3$ well when an adjacent well contained sodium citrate, nitrilotriacetate, pyrophosphate, or oxalacetate (Fig. 4). The same pattern of growth stimulation was observed when the concentrations of DF and $Fe(NO_3)_3$ were decreased 20-fold. Sodium lactate, acetate, succinate, maleate, or phosphate did not increase the availability of iron for organisms in these assays. Compounds such as citrate and nitrilotriacetate, which facilitated the use of $Fe(NO_3)_3$, did not stimulate growth of the organisms in LC-DA with or without DF. Presumably, in LC-DA, iron in excess of that required to saturate the DF was not in soluble form and therefore did not diffuse readily in the medium. Citrate, nitrilotriacetate, pyrophosphate, and oxalacetate appeared to effectively solubilize excess iron in LC-DA, making it available for assimilation by organisms seeded into the medium. The growth stimulation observed by $Fe(NO_3)_3$ in DA containing 200 μM DF (Fig. 1) or in DDL (Fig. 2 and 3) was presumably due to the presence of sufficient oxalacetate to solubilize $Fe(NO_3)_3$. In other experiments, the majority of added $^{55}FeCl_3$ (1 μM) was insoluble in low-chelate liquid medium and was trapped on 0.45-μm filters (Gelman GA-6), whereas over 99% was soluble in DDL (data not shown).

On LC-DA, zones of growth stimulation around $Fe(NO_3)_3$-containing wells did not increase with prolonged incubation. Neither filtrates of gonococcal cultures from iron-limited DDL nor a loopful of gonococci grown on DA enhanced the use of $Fe(NO_3)_3$ on LC-DA medium. This suggested that in this assay system gonococci were not secreting sufficient quantities of iron-solubilizing compounds (siderophores) to facilitate use of available iron.

In contrast to the results obtained with $Fe(NO_3)_3$, sizes of zones of growth stimulation around hemin, serum, and transferrin in LC-DA were comparable to those obtained with DA. Therefore, use of these iron compounds was not dependent on the presence of iron-solubilizing agents in the medium.

DISCUSSION

In vitro assays with a deferrated defined medium and the potent iron chelator DF were used to determine some of the mechanisms by which gonococci and other *Neisseria* species acquire iron. Gonococci, meningococci, and the commensal *Neisseria* species assimilated a variety of ferric and ferrous iron salts. These organisms, however, apparently were unable to efficiently solubilize an inorganic iron salt such as $Fe(NO_3)_3$. Compounds such as citrate, oxalacetate, pyrophosphate and nitrilotriacetate facilitated use of $Fe(NO_3)_3$, presumably by solubilizing inorganic iron. Gonococci were able to grow in medium containing as much as 10 mM

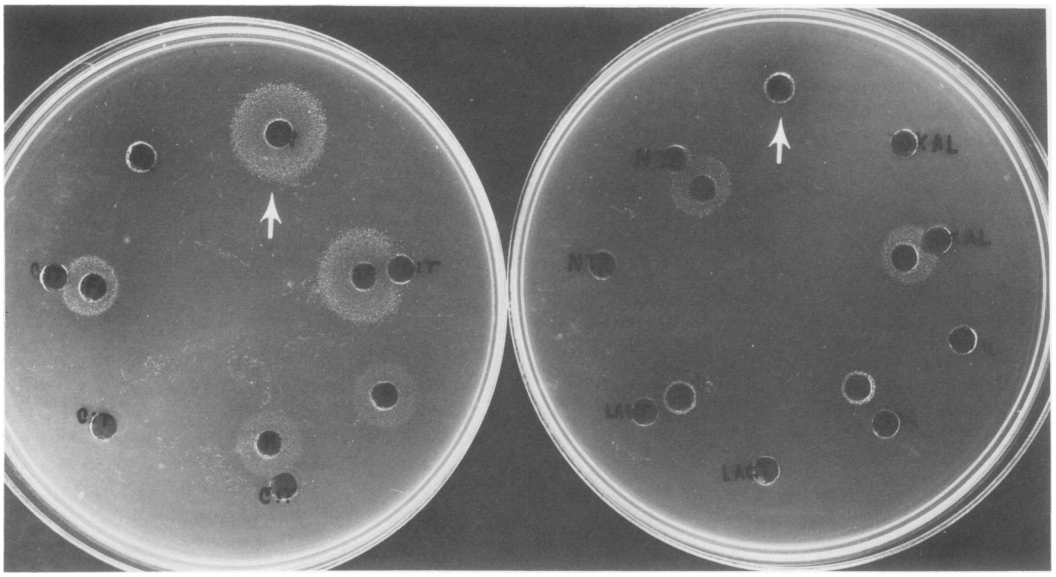

FIG. 4. *Ability of iron-solubilizing compounds to facilitate use of inorganic iron by N. gonorrhoeae F62 in LC-DA containing 200 μM DF. Wells in the plate on the left contained the following (beginning with the well indicated by an arrow and proceeding counterclockwise): 25% saturated transferrin; $Fe(NO_3)_3$; inner well, $Fe(NO_3)_3$, and outer well, sodium citrate; sodium citrate; inner well, hemin, and outer well, sodium citrate; hemin; inner well, 25% saturated transferrin, and outer well, sodium citrate. Wells in the plate on the right contained the following: $Fe(NO_3)_3$; inner well, $Fe(NO_3)_3$, and outer well, nitrilotriacetate; nitrilotriacetate; inner well, $Fe(NO_3)_3$, and outer well, sodium lactate; sodium lactate; inner well, $Fe(NO_3)_3$, and outer well, dibasic and monobasic potassium phosphate; dibasic and monobasic potassium phosphate; inner well, $Fe(NO_3)_3$, and outer well, oxalacetic acid; oxalacetic acid.*

sodium citrate or nitrilotriacetate, suggesting that they either possessed a high-efficiency system for competing with these compounds for iron, were able to mediate release of iron from the chelators, or were able to directly assimilate these ferric chelates.

These observations are similar to those of Archibald and DeVoe (3), who found that chelators such as citrate, pyrophosphate, and lactate facilitate use of relatively insoluble iron compounds by meningococci. These investigators were unable to obtain evidence for a meningococcal siderophore. Yancey and Finkelstein (Abstr. Annu. Meet. Am. Soc. Microbiol. 1980, B83, p. 31), however, have reported the isolation of a dihydroxamate from gonococci and meningococci which has the properties of a siderophore. In view of the low efficiency with which the *Neisseria* species were able to solubilize iron in the LC-DA system, the siderophore described by Yancey and Finkelstein either was not produced in appreciable quantities by the strains that we studied in LC-DA, was cell-bound, or diffused poorly.

All *Neisseria* species were able to use hemin as a sole source of iron. Because most, but not all, gonococcal and meningococcal isolates were able to scavenge iron from hemoglobin, an additional mechanism was probably required for release of hemin from the globin molecule.

Each of 50 tested isolates of *N. gonorrhoeae* and *N. meningitidis* was able to effectively scavenge iron from 25% saturated transferrin even in the presence of excess DF. Archibald and DeVoe (2) also have demonstrated that meningococci are able to obtain iron from transferrin. In contrast, only 10 of 45 isolates of various commensal *Neisseria* species were able to scavenge iron from 25% saturated transferrin; the remainder were growth inhibited by transferrin. Because the pathogenic *Neisseria* species, in contrast to most commensal organisms, possess the capability of scavenging iron from transferrin, it is possible that this is an attribute which contributes to the survival of these organisms in the host. In support of such speculation is a recent study by Holbein (10) in mice which showed a positive correlation between transferrin-bound iron levels in serum and the severity of meningococcal bacteremia. Production of mutants unable to obtain iron from transferrin will be useful in establishing whether acquisition of transferrin iron is an important virulence factor for the pathogenic *Neisseria* species.

Chicken conalbumin, in contrast to human transferrin, was bacteriostatic for all *Neisseria* species. Transferrin and conalbumin are closely related glycoproteins with very similar amino acid compositions, physical properties, and iron-binding affinities (1, 25). This suggests that the mechanism by which gonococci and meningococci obtain iron from host iron-binding proteins is very specific. In view of this specificity, determining whether gonococci and meningococci are able to compete for iron with human lactoferrin or transferrins of other species may increase our understanding of how these pathogens might obtain iron on human mucosal surfaces or in animal model systems.

There was no obvious correlation between the virulence of gonococci and the ability to compete with human transferrin in vitro, since nearly all gonococcal isolates and variants effectively scavenged iron from transferrin. There was also no evidence that virulent strains or colony types of gonococci were particularly efficient scavengers of iron from hemin or serum or that they were able to compete more effectively with the chelator DF or conalbumin for iron under the conditions employed in our experiments. If differences exist in the ability of virulent and avirulent gonococci to scavenge iron, as suggested by Payne and Finkelstein (19, 20) and by Payne et al. (21), they were either too subtle to be detected in the assays used in this study or were not expressed in the in vitro systems that we employed. Although administration of iron has been shown to enhance infections by some meningococci (11), no correlation has been found between the virulence of these organisms in a mouse model and the ability to compete with transferrin, conalbumin, or other chelators in vitro (2, 3). The resolution of apparent differences between our results obtained with in vitro systems and those of Payne and Finkelstein in animal models will depend on a more thorough understanding of the biochemical mechanisms for the acquisition of iron from sources available in the host.

Although it is not possible to directly compare our results with those of Payne and Finkelstein (19, 20) and of Payne et al. (21), the observed effects of iron on the virulence of gonococci in the chicken embryo model may have been influenced by factors other than the ability of the gonococcal isolates to scavenge iron. For instance, levels of available iron may have affected expression of outer membrane proteins (17) to a variable degree in different isolates, or other factors such as colony opacity (22) could have contributed to differences in virulence.

Some gonococci are rapidly killed in fresh human serum (23). Our observations suggest that a heat-labile factor(s), probably an antibody-activated complement, rather than iron deprivation, mediates this bactericidal activity. Gonococci were able to grow in heated serum, and heated serum served as an iron source for gonococci in medium containing DF. The almost immediate killing of gonococci in fresh serum also is inconsistent with the delayed effect of iron limitation on growth of organisms in deferrated medium containing DF or conalbumin. Norrod and Williams (18) and Johnson et al. (12) found that high concentrations of ferric ammonium citrate, but not other iron compounds tested, can inhibit the rapid bactericidal activity of fresh serum for gonococci. The significance of these observations is not clear, but is probably unrelated to the nutritional aspects of iron availability in serum.

Some *Escherichia coli* ColV plasmids (26) and a *Vibrio anguillarium* plasmid (7) carry gene-encoding systems which enhance the ability of organisms to compete with transferrin for iron. Gonococci lacking plasmids were able to utilize transferrin-bound iron and hemin and were able to use these or other iron sources in serum. Genes which code for these iron-scavenging systems in gonococci, therefore, appear to be located on the chromosome.

The pathogenic *Neisseria* species clearly possess several mechanisms for obtaining essential iron from their environment. The ability to utilize human transferrin as a source of iron may contribute to the survival of gonococci and meningococci in the host. However, attributes unrelated to iron acquisition may be primarily responsible for differences in pathogenicity among meningococci (2, 3, 11) and gonococci.

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