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Ability of *Neisseria gonorrhoeae*, *Neisseria meningitidis*, and Commensal *Neisseria* Species to Obtain Iron From Lactoferrin

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The ability of 107 *Neisseria* isolates to compete for iron with human lactoferrin (LF) was examined. Each of 15 meningococci, 53% of 59 selected gonococci, and 24% of 33 commensal *Neisseria* could use LF-bound iron for growth. Isolates which could not obtain iron from LF were growth inhibited when sufficient LF was added to defined agar medium to bind available free iron. No difference was observed in the ability of colony type 1 and colony type 4 gonococci of the same strain to compete with LF for iron. LF was growth inhibitory for 50% of 22 disseminated disease isolates (DGI strains) and 51% of 35 local urogenital disease isolates (UGI strains). Only 14% of gonococcal isolates requiring arginine, hypoxanthine, and uracil for growth were able to compete with LF for iron, whereas 87% of all other gonococcal isolates could do so ($P < 0.005$). Ability to obtain iron from LF does not appear to be required for survival of *Neisseria* on mucosal surfaces, nor essential for invasion of the bloodstream by gonococci. However, ability to utilize LF as a source of iron may contribute to differences in pathogenicity among certain gonococcal isolates.

Iron is an essential nutrient for almost all organisms. The lack of free iron in the host may serve as a barrier against invasion by some bacteria and fungi (6, 36). Most iron is stored in humans in the reticuloendothelial system as ferritin or hemosiderin, or incorporated into heme or heme-containing molecules such as hemoglobin (28, 36). The iron-binding proteins transferrin and lactoferrin (LF) also function in the absorption, transport, and exchange of iron (28). Transferrin and LF are normally only partially iron saturated, and have high binding affinities for iron (1, 27); they inhibit growth of many bacteria and fungi in vitro (6, 22, 24, 30) and may contribute to the microbiostatic properties of body fluids and secretions by limiting availability of essential free iron (6, 29, 36).

Transferrin and LF are closely related glycoproteins which share considerable amino acid sequence homology but are immunologically distinct (28). Each protein reversibly binds two ferric ions with the simultaneous incorporation of two molecules of bicarbonate (1, 28). Transferrin is found primarily in serum and interstitial fluid (27). LF is produced by acinar cells of the mucosa and is present in very low concentrations in serum but in relatively high concentrations in tears, semen, milk, bile, nasopharyngeal, bronchial, cervical, and intestinal mucosa secretions (23, 24). LF also is found in secondary granules of polymorphonuclear leukocytes and in pus (25). Unlike transferrin, whose iron-binding affinity is diminished below pH 6.0, LF retains its iron-binding properties in the more acidic conditions which often prevail at sites of inflammation (1, 28).

All gonococci (26) and meningococci (3, 26) can obtain iron from transferrin for growth whereas most commensal *Neisseria* are growth inhibited by transferrin (26). This may enable the pathogenic *Neisseria* to circumvent the nutritional defense barrier normally imposed by transferrin. However, since the *Neisseria* colonize mucosal surfaces, LF rather than transferrin is undoubtedly the host iron-binding protein initially encountered. In this report, we examine the ability of various *Neisseria* species to compete with LF for iron.

MATERIALS AND METHODS

Organisms. Clinical isolates of *N. gonorrhoeae* were obtained from several sources, as were strains of *N. meningitidis* serogroups A, B, C, X, Y, and Z and commensal *Neisseria* (26). Strains FA19 and F62 of *N. gonorrhoeae* have been well characterized in this laboratory and elsewhere. Thirty-seven strains of the gonococcus were isolated from urogenital cultures from patients having uncomplicated gonococcal infections (UGI strains), whereas 22 strains were isolated from blood or joint cultures from persons having disseminated disease (DGI strains). Nutritional re-

quirements of gonococci were determined by the auxotyping method of Catlin (7). Methods used for determining the presence of plasmids have been described (33). Twenty-one of 22 DGI isolates and 21 of the 37 UGI isolates were resistant to the bactericidal activity of normal human serum (11). Colony type of gonococci was determined by the method of Kellogg et al. (18) using GC base agar (Difco Laboratories, Detroit, Mich.) containing 1% defined Supplement I and 0.1% of Supplement II (GCBA). Transparent and opaque colonial variants were selected on GCBA using the criteria of Swanson (16, 34).

Media. Deferrated defined liquid medium (DDL) which contained ≤0.07 μM iron and defined agar medium (DA) which contained approximately 2.0 μM iron were prepared as previously described (26). DA contained sufficient iron to support the growth of gonococci, and addition of iron did not further stimulate growth. Organisms grown in DDL were iron starved, but growth could be promoted by adding iron to the medium.

Iron-binding compounds. Human colostrum LF (Sigma Chemical Co., St. Louis, Mo.) was further purified by the method of Querinjean et al. (29). The procedure was modified by not further saturating the commercial LF with iron and by washing and eluting the protein from CM Sephadex (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.) which had been poured in a column rather than by changing supernatants after the beads had settled in a tube. LF was dialyzed against three changes of deionized water and then dialyzed against 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid–150 mM NaCl–10 mM sodium bicarbonate buffer (pH 7.4). The purified LF was approximately 20% iron saturated as determined by atomic absorption spectrophotometry. Further saturation of LF by iron was accomplished using ferric nitrilotriacetate (20). In some studies, LF was made 45% saturated with ^{55}Fe (New England Nuclear Corp., Boston, Mass.) and had a specific activity of approximately 4.6×10^5 dpm/μM. Methods used for preparation of other iron sources or iron-binding proteins have been described previously (26).

MICs of LF. Methods previously used to determine minimal inhibitory concentrations (MICs) of gonococci to conalbumin and desferrioxamine were employed (26). Briefly, a suspension containing approximately 10^4 colony-forming units of organisms which had been passed twice on DA (pH 7.0) was inoculated onto DA supplemented with 10 mM sodium bicarbonate and 20% iron-saturated LF. Studies of *N. gonorrhoeae* employed DA adjusted to either pH 7.4 or pH 6.6 and concentrations of LF of 0.25, 0.5, 0.75, 1.0, 1.25, 1.5, 1.75, 2.0, 3.0, 4.0, 5.0, and 10 μM. MICs were determined only at pH 7.4 for the majority of commensal *Neisseria* and meningococci. Organisms were also inoculated onto DA containing no addition, 50 μM $Fe(NO_3)_3$, 20 μM desferrioxamine (Desferal, Ciba Pharmaceutical Co.), both 50 μM $Fe(NO_3)_3$ and 20 μM desferrioxamine, 5 μM LF and 50 μM $Fe(NO_3)_3$, and both 5 μM LF and 20 μM desferrioxamine. After 48 h of incubation at 37°C in an atmosphere of 5 to 10% CO_2, growth on media containing LF or desferrioxamine was compared to growth on control plates supplemented with iron. Twenty-seven selected strains of *N. gonorrhoeae* including 23 arginine, hypoxanthine, and uracil auxotrophs were also tested for their ability to use partially saturated LF as an iron source on modified GCBA. The modified GCBA lacked iron supplement II, but contained 100 μM desferrioxamine and 5 μM 20% iron-saturated LF.

Uptake of iron from ^{55}Fe-labeled LF. Organisms were grown in DDL to an optical density of 40 Klett units (540-nm filter), and were then added to an equal volume of 37°C DDL containing ^{55}Fe-labeled LF (final concentration 1 μM). During incubation in a 37°C shaker water bath, 0.2-ml samples were collected at intervals on 0.45-μm cellulosetriacetate filters (Gelman GA6; Gelman Sciences Inc., Ann Arbor, Mich.) and washed twice with a total volume of 5.0 ml of minimal medium Davis (Difco). Filters were dried and counted in a liquid scintillation counter.

The amount of ^{55}Fe retained on filters when only uptake medium was collected and washed was considered background. Background counts were subtracted from values obtained in uptake studies with organisms. A sample containing 0.2 μM $^{55}FeCl_3$ was dried on filters and used as a standard for converting counts per minute of ^{55}Fe to moles of ^{55}Fe. Turbidity (Klett units) of cultures used in uptake studies was determined by adding cells to an equal volume of DDL just before commencement of uptake experiments. Optical density (Klett units) of the culture was then converted to micrograms of cell protein using conversion values which had been previously obtained by performing Lowry protein assays (21) on cultures grown in DDL.

Survival of gonococci in the presence of LF. Type 4 organisms of *N. gonorrhoeae* F62 were grown in DDL to an optical density of 40 Klett units, and 0.1 ml of the culture was added to U-bottom microtiter wells (Dynatech Laboratories, Inc., Alexandria, Va.) containing either 0.1 ml of DDL without additions, or 100 μM concentrations of the following: 20% saturated LF, 100% saturated LF, 4% saturated conalbumin (Sigma), or desferrioxamine. Microtiter plates were shaken for 2 min on a rotary shaker to mix the contents of the wells and then placed in a 37°C incubator with an atmosphere of 5 to 10% CO_2. Viability of organisms was determined after 0, 30, and 60 min of incubation by plating dilutions from each well on GCBA.

Statistical analysis. Statistical significance of differences in ability of different groups of gonococci to compete with LF for iron was evaluated using chi-square analysis.

RESULTS

Growth in the presence of LF. A total of 107 *Neisseria* isolates were examined for their ability to compete with human LF for iron using a DA containing increasing concentrations of LF. In DA containing low concentrations of LF, sufficient free iron was present to support growth of all organisms. In DA supplemented with higher concentrations of LF, all iron was bound by the protein and only those organisms which could obtain iron from LF were able to grow.

Each of 15 meningococcal isolates tested was able to compete with LF for iron and grew on DA containing 10 μM LF (Table 1). Although addition of the potent iron chelator desferrioxamine (20 μM) to DA prevented growth of menin-

TABLE 1. Growth of *Neisseria* on DA containing both 20 μM desferrioxamine and 5 μM LF (20% iron saturated)[a]

Species	No. of isolates	No. of strains positive (%)
N. meningitidis	15	15 (100%)
N. gonorrhoeae	59	31 (53%)
Commensal *Neisseria*	33	8 (24%)
N. lactamica	3	3
N. sicca	9	1
N. flavescens	2	0
N. flava	4	3
N. subflava	2	1
N. perflava	3	0
N. mucosa	3	0
N. sicca/subflava	7	0

[a] Identical results were obtained on DA containing 10 μM LF, but no desferrioxamine.

gococci, growth inhibition of all isolates could be reversed by either partially saturated LF (5 μM) or sufficient $Fe(NO_3)_3$ (>20 μM) to saturate the binding capacity of desferrioxamine. Since there was sufficient desferrioxamine in the medium to bind any free iron which may have been present in preparations of LF, meningococci were able to obtain iron for growth from LF.

Thirty-one of 59 (53%) gonococcal isolates were able to obtain iron required for growth from 5 μM 20% iron-saturated LF on DA containing 20 μM desferrioxamine (Table 1). None of the 31 isolates which utilized LF as a source of iron under these conditions was growth inhibited by up to 10 μM LF on DA lacking desferrioxamine. In contrast, 28 of 59 gonococci could not obtain iron from LF on DA containing desferrioxamine, and each of these was inhibited by 1.0 μM to 1.5 μM 20% iron-saturated LF on DA lacking desferrioxamine. Growth inhibition by LF was reversed by addition of sufficient iron to saturate LF. For 39 gonococcal isolates, the effect of pH on utilization of iron from LF was tested; no differences in MICs for isolates were observed at pH 6.6 and 7.4.

Of 33 commensal *Neisseria* tested, only 24% were able to compete with LF for iron. The proportion of isolates from each nonpathogenic species which possessed this capability was highly variable (Table 1). MICs for isolates which were inhibited by LF on DA ranged from 0.75 to 2.0 μM.

Relationship between gonococcal auxotype and utilization of LF. Further studies were undertaken to determine whether ability of gonococci to utilize LF as a source of iron might be correlated with certain variables known or suspected to be related to virulence. A possible relationship between piliation and ability to use LF as a source of iron was examined. No differences were found between colony type 1 (piliated) and colony type 4 (nonpiliated) organisms in 37 of 39 tested isolates. In the two isolates showing differences, colony type 1 organisms were inhibited by 1.5 μM LF, whereas colony type 4 organisms were reproducibly inhibited by 1.0 and 1.25 μM LF, respectively. Isogenic transparent and opaque colonial variants from two isolates, one of which could use LF-bound iron for growth while the other was growth inhibited by LF, showed no differences in ability to compete with LF for iron. There were no differences between isolates from patients with disseminated disease and local urogenital gonorrhea (Table 2), or between serum-sensitive and serum-resistant gonococci (data not shown) in their ability to obtain iron from LF.

Ability to use LF as a source of iron was strongly correlated with auxotype: 19 of 22 (86%) isolates which were nutritional prototrophs could compete with LF for iron, as compared to only four of 28 (14%) isolates requiring arginine, hypoxanthine, and uracil (Arg^- Hyx^- Ura^-) (Table 2). Six of seven (86%) isolates with auxotypes other than Arg^- Hyx^- Ura^- were able to compete with LF for iron ($P < 0.005$). Each of two gonococcal isolates lacking detectable plasmids could obtain iron from LF.

Since most Arg^- Hyx^- Ura^- isolates grew relatively poorly on DA, 27 selected gonococcal isolates including four prototrophs and 23 Arg^- Hyx^- Ura^- auxotrophs were also tested for their ability to use LF as an iron source on a complex medium, GCBA. Only those isolates which were able to compete with LF for iron on DA were able to grow on GCBA containing 100 μM desferrioxamine and 5 μM 20% saturated LF; the remainder were growth inhibited.

TABLE 2. Ability of different auxotypes of *Neisseria gonorrhoeae* isolated from urogenital (UGI strains) and disseminated (DGI strains) infections to obtain iron from LF

Source	No. of isolates	No. of strains positive[a]
DGI strains	22	11 (50%)
Auxotype		
Prototrophs	9	7 (78%)
Arg^- Hyx^- Ura^-	12	3 (25%)
Other	1	1 (100%)
UGI strains	35	18 (51%)
Auxotype		
Prototrophs	13	12 (92%)
Arg^- Hyx^- Ura^-	16	1 (6%)
Other	6	5 (83%)
Plasmidless	2	2 (100%)
Total	59	31 (53%)

[a] Isolates able to grow on DA containing 20 μM desferrioxamine and 5 μM 20% saturated LF.

Uptake of iron from ^{55}Fe-labeled LF. Uptake of iron from ^{55}Fe-labeled LF by several isolates was also examined. *N. gonorrhoeae* F62 and *N. subflava* ATCC 19243 which were growth inhibited by LF failed to take up iron from ^{55}Fe-labeled LF (Fig. 1) even after 4 h of incubation. Strain F62 is known to be able to rapidly incorporate inorganic ^{55}Fe in this medium (unpublished data). The failure of F62 to take up ^{55}Fe from LF indicates that there was little or no free ^{55}Fe in the ^{55}Fe-labeled LF preparation. In contrast, iron was rapidly taken from ^{55}Fe-labeled LF by *N. gonorrhoeae* FA19 and a group C *N. meningitidis* isolate which were able to grow either on DA containing 10 μM LF, or on DA containing desferrioxamine if LF was added as an iron source.

Survival of organisms in the presence of LF. Viability of *N. gonorrhoeae* F62 was monitored for 1 h after the cells were placed in medium containing 20% saturated LF or 100% saturated LF. Although LF was growth inhibitory for this isolate, neither LF preparation decreased viability of gonococci during this relatively brief period. Cells also remained viable in the presence of desferrioxamine and conalbumin which growth inhibit gonococci by binding iron in the medium. We have observed previously (26) that gonococci transferred to DDL containing desferrioxamine and conalbumin can grow for a limited amount of time, presumably using iron stored in intracellular pools.

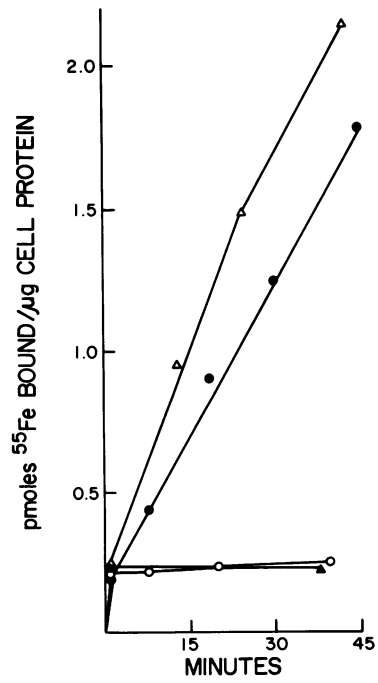

FIG. 1. Uptake of ^{55}Fe from ^{55}Fe-labeled LF by *N. meningitidis* group C-WR (△), *N. gonorrhoeae* FA19 (●), *N. gonorrhoeae* F62 (○), and *N. subflava* ATCC 19243 (▲).

DISCUSSION

Some *Neisseria* were able to remove iron from human LF and use it as a source of iron for growth, since they grew on DA which contained LF in excess of that required to bind free iron in the medium. For these isolates, growth inhibition resulting from addition of the potent iron chelator desferrioxamine to DA could be reversed either by partially saturated LF (5 μM) or by concentrations of inorganic iron sufficient to exceed the binding capacity of DF. Representative isolates of this group of organisms rapidly took up iron from ^{55}Fe-labeled LF.

In contrast, *Neisseria* which could not compete with LF for iron were growth inhibited when sufficient LF was added to DA to bind free iron in the medium (MICs, 0.75 to 2.0 μM). Growth inhibition of these organisms by desferrioxamine could not be reversed by partially saturated LF but could be reversed by excess $Fe(NO_3)_3$. These isolates failed to take up iron from ^{55}Fe-labeled LF. Growth inhibition of LF was due to the iron-binding properties of the protein since saturation of LF with excess iron reversed inhibitory effects. The ability to obtain iron from LF was not noticeably pH dependent since lowering the pH of the medium from 7.4 to 6.6 had no effect on the concentrations of LF required to inhibit gonococci. Whether pH affected the rate with which organisms took up iron from LF was not evaluated. The ability of isolates to obtain iron from LF was not medium dependent, since organisms which were growth inhibited by low concentrations of LF in the defined agar medium, DA, could not obtain iron from LF in the complex medium, GCBA.

Arnold et al. observed that *Streptococcus mutans*, *Streptococcus pneumoniae*, and *Vibrio cholera* were rapidly killed in the presence of LF if organisms were suspended in water (4) but not if suspended in buffer (5). Gonococci which were growth inhibited by LF were not killed during 60 min of exposure to LF (or other chelators) in DDL. Gonococci are highly autolytic (12), and it might be expected that any agent which interferes with growth eventually would be bactericidal. In our assays, LF appeared to inhibit growth of gonococci by limiting availability of iron required for growth rather than by exerting more immediate bactericidal activity.

In previous studies we observed that all meningococci and gonococci could obtain iron from partially saturated transferrin and hemin, but none could compete with conalbumin or desfer-

rioxamine (26). In this study we found that all meningococci obtained iron from LF, but only one-half of tested gonococcal isolates were able to obtain iron from LF. Ability to compete with LF apparently was unrelated to certain variables known to affect virulence, including piliation (17), colony opacity (31, 34), and serum resistance (11, 32).

It is unclear whether ability to scavenge iron from LF contributes to pathogenicity. Certainly, ability to compete with LF is not requisite for invasiveness, since 11 of 22 gonococci isolated from patients with gonococcal bacteremia (DGI strains) were incapable of obtaining iron from LF. Ability to compete with LF for iron also does not appear to be requisite for survival of *Neisseria* on mucosal surfaces, since most commensal *Neisseria* which normally colonize the nasopharynx could not obtain iron from LF. Presumably other iron sources which commensal *Neisseria* can assimilate are present in this environment. These organisms are able to use hemin as an iron source, and it is possible that hemin released by dying cells sloughed from the epithelium may be one source of iron on mucosal surfaces.

There was a significant correlation ($P < 0.005$) between nutritional requirements ("auxotype") of gonococci on defined media and ability to obtain iron from LF. Over 80% of prototrophic isolates could utilize LF as sole source of iron, whereas only 14% of Arg$^-$ Hyx$^-$ Ura$^-$ isolates could do so. It is unlikely that there is a direct biochemical or genetic relationship between the requirement for Arg, Hyx, or Ura and the ability to obtain iron from LF; rather, these are probably independent covariables. Nevertheless, the association between these properties could be biologically important. Arg$^-$ Hyx$^-$ Ura$^-$ isolates are known to be particularly associated with asymptomatic urethritis in males (8). It is possible that such strains are growth limited by LF on urogenital mucosa, and thus do not achieve sufficient mass to stimulate the normal inflammatory response. Arg$^-$ Hyx$^-$ Ura$^-$ strains are rarely isolated from women with salpingitis (10), possibly because of growth limitation by LF in the fallopian tube. Strains with requirement for Arg, Hyx, and Ura are relatively common in patients with DGI (19). These strains might grow better in blood than on the genital mucosa, since all gonococci can obtain iron from serum transferrin (26). Moreover, the association between onset of menses and development of DGI (15) could be due in part to provision of hemin and transferrin, which would presumably abolish any existing limitations imposed by iron availability on mucosal surfaces.

Our ideas about the relationship between ability to utilize LF as a source of iron and the biology of infections caused by Arg$^-$ Hyx$^-$ Ura$^-$ gonococci are highly speculative. We have no information about the symptoms caused by isolates used in this study. Other variables undoubtedly are important in determination of whether gonococci can ascend into the fallopian tube or invade the bloodstream. We do not know whether there is sufficient LF at the urethral, cervical, or fallopian tube sites to limit availability of iron or what other iron sources might be available at these sites. However, ability to scavenge iron from LF is a strain-dependent attribute which conceivably could influence the pathogenic potential of gonococci. Therefore, in future clinical or animal studies, this is a property which should be examined, in addition to other determinants commonly used to characterize gonococcal isolates.

All meningococci could obtain iron from LF for growth. Other studies (2, 3, 13, 14) indicate that attributes other than iron-scavenging capabilities probably account for differences in virulence among different serogroups or strains of the meningococcus. Whether the ability of meningococci to use LF-bound iron for growth contributes to survival of these organisms on mucosal surfaces, at extravascular sites of inflammation, or in the bloodstream during the hypoferremic state which may accompany severe infections (13, 35, 36) is unknown.

Systems which enable *Neisseria* to obtain iron from LF and transferrin are probably independent since all gonococci can compete with transferrin for iron (26), whereas not all can obtain iron from LF. It is unknown whether the recently described gonococcal and meningococcal dihydroxymate siderophores (38) are involved in scavenging of iron from either transferrin or LF. However, *N. gonorrhoeae* F62 has been shown to produce this siderophore (38), but this isolate cannot obtain iron from LF (Fig. 1). This suggests that some additional component is required for removal of iron from LF. We do not know whether differences in ability of gonococci to obtain iron from LF are due to differences in ability to bind LF to the cell surface, to differences in removal of iron from LF, or to other factors. Genes which enable organisms to compete with transferrin for iron have been shown to be carried on some ColV plasmids in *Escherichia coli* (37) and on plasmids of *Vibrio anguillarum* (9). Since gonococci lacking detectable plasmids were able to obtain iron from LF, as well as transferrin, it would appear that these are functions encoded by chromosomal genes.

Delineation of the mechanisms by which the *Neisseria* acquire iron from transferrin, lactoferrin, and other human sources will be greatly facilitated when mutations in specific iron transport systems are obtained. Such mutants may

also allow definition of the role of iron scavenging in pathogenicity.

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