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Effect of Siderophores on Virulence of *Neisseria gonorrhoeae*

RICHARD A. FINKELSTEIN* AND ROBERT J. YANCEY†

Department of Microbiology, University of Missouri-Columbia, School of Medicine, Columbia, Missouri 65212

The virulence of *Neisseria gonorrhoeae* for chicken embryos can be modified in a predictable manner by the addition of microbial siderophores to the inoculum. "Meningobactin" and "gonobactin," siderophores isolated from iron-limited cultures of meningococci and gonococci, respectively, enhance the virulence of the relatively avirulent colony type 3 (T3) organisms, but have essentially no effect on the virulence of T1 organisms. Both of these compounds were found previously to stimulate in vitro growth of the pathogenic *Neisseria* spp. under conditions made iron limiting by the addition of conalbumin, the transferrin counterpart of chickens. Similarly, ferrated schizokinen and arthrobactin, both dihydroxamate siderophores which stimulated growth in iron-limited conditions in vitro, also enhanced virulence of T3 organisms, whereas desferrioxamine B mesylate (Desferal), a trihydroxamate previously shown to be inhibitory in vitro, decreased the virulence of the T1 colony form. This was due to the iron-binding function of the molecule, as the iron-saturated form, ferrioxamine B mesylate, did not affect virulence. An additional trihydroxamate siderophore, ferrichrome A, which was inactive on *Neisseria* spp. in either the deferri- or ferrated forms in vitro, likewise did not affect virulence in the chicken embryo model. The neisserial siderophores were more effective than the other microbial siderophores in enhancing virulence of T3 gonococci. The results add to the evidence that the ability to acquire iron is an important determinant of virulence.

Neisseria gonorrhoeae, like other parasitic microorganisms, must obtain the iron necessary for its growth from its host. Although the amount of iron present in host fluids is more than adequate for microbial growth (6), the host's iron-binding proteins (transferrin and ferritin in serum, lactoferrin in the secretions) sequester essentially all of the iron in these environments (1, 11), thus limiting its availability to the microbes. Some pathogens, however, have been shown to obtain iron by producing low-molecular-weight, iron-binding compounds, siderophores (6), which compete successfully with the host's iron-sequestering mechanisms. Rogers (10) found that phenolate-type siderophore production influences *Escherichia coli* infection of mice. Yancey et al. (12) determined that the ability of *Salmonella typhimurium* to synthesize the phenolate siderophore enterochelin was essential to the ability of the organism to grow in human serum and to cause lethal infection in mice. However, little is known about the role of siderophores in the virulence of other pathogens.

The siderophores which have been characterized are of two major chemical classes; the hydroxamates (derivatives of hydroxamic acid) and the phenolates (or catechols). We have recently found that the pathogenic *Neisseria* spp. produce siderophores which appeared to be of the hydroxamic acid type. These were designated "meningobactin" and "gonobactin" for the siderophores produced by the meningococcus and gonococcus, respectively (14).

The present paper describes the effect of meningobactin, gonobactin, and other microbial siderophores on the virulence of *N. gonorrhoeae* in the chicken embryo model (2, 8).

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MATERIALS AND METHODS

Bacterial strains. *N. gonorrhoeae* strains F62 and 2686 were as previously described (8). These were maintained by lyophilization and by storage at −70°C in buffered peptone-saline broth (GC medium base formulation as per Difco Laboratories, without agar and starch) containing 20% glycerol. All cultures were incubated at 36°C with 5% CO_2.

Preparation of inocula. Imferon agar (7) was used routinely for growth of the gonococcus. A single colony of the desired colonial type (as described by Kellogg et al. [5]) from an 18- to 20-h culture was streaked for confluent growth onto another plate of Imferon agar. The 20-h growth was harvested from these petri dishes

† Current address: Veterinary Therapeutics Division, The Upjohn Co., Kalamazoo, MI 49001.

in 5 ml of buffered peptone-saline broth, and clumps of bacteria, especially the piliated colony types 1 and 2 (T1 and T2), were dispersed by rapidly passing the cell suspension several times through a 27-gauge needle. This suspension, containing 1×10^9 to 5×10^9 colony-forming units per ml was then decimally diluted in buffered peptone-saline broth to the desired inoculum density. Siderophores, when tested, were added directly to these inoculum tubes. Viable counts on Imferon agar were made by a drop technique immediately after inoculation of the embryos to insure the proper count and purity of the inoculum. Solutions of the various siderophores tested did not significantly affect the viability of the inoculum for a period of up to 3 h.

Chicken embryo virulence determination. Chicken embryos (White Missouri Leghorns) were obtained from the Department of Poultry Science, University of Missouri-Columbia. These were received 48 h before inoculation and were maintained at 37°C in a humidified incubator. Embryos (10 to 11 days old) were inoculated intravenously with 0.1-ml volumes as previously described (2, 4). At least six embryos were used per dose of bacteria or test compound (or both). Viability of the embryos was determined by candling. Embryos that bled beneath the site of inoculation or that died within 2 h of inoculation were discarded. Mean 50% lethal dose (LD_{50}) values were calculated by the method of Reed and Muench (9).

Siderophores. Desferal, the mesylate of iron-free ferrioxamine B (a trihydroxamate), was purchased from Ciba Pharmaceutical Co., Summit, N.J. Schizokinen and arthrobactin (both dihydroxamates) and ferrichrome A (a trihydroxamate) were generously provided by C. E. Lankford, The University of Texas, Austin. Schizokinen was deferrated by the alkaline precipitation of iron (3).

Meningobactin and gonobactin were obtained as phenol-chloroform-ether extracts from *N. meningitidis* B-11 and *N. gonorrhoeae* 6555 as previously described (14). The amount of siderophore in these preparations was quantitated by their activity in the conalbumin agar bioassay (14). One unit of activity was defined as the reciprocal of the dilution required to produce a 10-mm zone of stimulation in the conalbumin agar bioassay for the homologous species of assay organism. One unit was determined to be equivalent to approximately 1 μg of schizokinen (14).

RESULTS

We found previously (13) that of 12 siderophores of exogenous origin tested, the only ones which were stimulatory for *N. gonorrhoeae* in the conalbumin agar bioassay were the dihydroxamates, schizokinen, arthrobactin, and aerobactin. Even these compounds were stimulatory only when in the iron form. In fact some of the compounds, including the trihydroxamate Desferal, were inhibitory, presumably by virtue of their iron-binding ability, as the same compounds were ineffective when saturated with iron (13).

In vivo effects on virulence of *N. gonorrhoeae* F62 closely paralleled those seen previously in vitro (13) (Table 1). Desferrioxamine B mesylate (Desferal), which was inhibitory in the conalbumin agar assay, decreased the virulence of virulent T1 *N. gonorrhoeae* 850-fold. This inhibition was due to the iron-binding function of the molecule, as the iron-bound form of the molecule, ferrioxamine B mesylate, did not affect virulence of the organisms greatly. Desferal was relatively ineffective, but did slightly decrease the apparent virulence of the already iron-limited, avirulent T3 organism (Table 1).

Ferrichrome A, a trihydroxamate siderophore which was previously (13) found to be ineffective even in the ferrated form in vitro, had no effect in vivo. The LD_{50} of the T3 organism was approximately the same, 10^6 colony-forming units,

TABLE 1. *Influence of nonneisserial siderophores on virulence of N. gonorrhoeae F62 for chicken embryos*[a]

Colony type[b]	Siderophore	Amt per embryo (μg)	$LD_{50} \pm SD$[c] (no. of determinations)	Fold increase (decrease) in virulence
T1 (T2)	None		$9.3 \times 10^2 \pm 7.4 \times 10^2$ (3)	
	Desferrioxamine B mesylate (Desferal)	5	$7.9 \times 10^5 \pm 5.5 \times 10^5$ (3)	(849)
	Ferrioxamine B mesylate	5	1.5×10^4 (1)	(16)
T3 (T4)	None		$1.0 \times 10^6 \pm 0.4 \times 10^6$ (5)	
	Desferal	10	3.7×10^6 (1)	(4)
	Ferrichrome A	5	8.9×10^5 (1)	1
	Schizokinen	5	$6.9 \times 10^3 \pm 3.8 \times 10^3$ (3)	145
	Deferri-schizokinen	5	$1.4 \times 10^4 \pm 1.3 \times 10^4$ (2)	7
	Arthrobactin	5	1.0×10^4 (1)	100

[a] LD_{50} assays were performed in 10- to 11-day-old chicken embryos inoculated intravenously as described previously (2, 4). Siderophores were included with the inocula. None were toxic for the embryo at the dose tested.

[b] According to Kellogg et al. (5). These were predominantly (>80%) the type indicated, with the remainder being the colony type in parentheses.

[c] SD, Standard deviation.

with or without the compound. Schizokinen and arthrobactin, both dihydroxamate siderophores, enhanced virulence of T3 organisms in vivo, but as observed in vitro (13) only when in the iron form.

As expected, the siderophores of the *Neisseria* spp., meningobactin and gonobactin had no significant effect on the already high virulence of the T1 organisms, but greatly enhanced virulence of the normally avirulent T3 organisms in a dose-related fashion (Table 2). A dose of 0.2 U of meningobactin enhanced the virulence of *N. gonorrhoeae* F62 (T3) 1,000-fold over the virulence level of the control. A 10-fold-lower dose of either chelator enhanced apparent virulence of the bacteria nearly 100-fold. Since 1 U of activity was found by two separate in vitro assays (14) to be equivalent to approximately 1 μg of schizokinen, the neisserial siderophores were much more effective, on a weight basis, than the previously described dihydroxamate siderophores in enhancing virulence of T3 *N. gonorrhoeae* (cf. Table 2 and Table 1).

The virulence-enhancing or -inhibiting properties of the siderophores were not restricted to a single strain since the compounds exhibited a similar effect on virulence of *N. gonorrhoeae* 2686 (Table 3). A 5-μg dose of Desferal per chicken decreased the apparent virulence of this strain by over 3,000-fold from an LD_{50} of 40 to more than 10^5 colony-forming units, whereas the iron-bound form of this molecule had no significant effect on virulence. A dose of meningobactin equivalent in growth-promoting ability to approximately 40 ng of Schizokinen potentiated virulence of this strain nearly 100-fold.

DISCUSSION

Accumulating evidence (1, 11) supports the concepts that the ability of pathogenic or potentially pathogenic bacteria to acquire iron in vivo is an indispensable attribute of virulence and

TABLE 2. *Influence of neisserial siderophores on the virulence of N. gonorrhoeae F62 for chicken embryos*[a]

Colony type[b]	Siderophore	Amt/Embryo (U)	LD_{50} ± SD[d] (no. of determinations)	Fold increase (decrease) in virulence
T1 (T2)	None		$9.3 \times 10^2 \pm 7.4 \times 10^2$ (3)	
	Meningobactin	0.2	$2.5 \times 10^2 \pm 3.3 \times 10^2$ (2)	3
	Gonobactin	0.2	8.3×10^2 (1)	(1)
T3 (T4)	None		$1.0 \times 10^6 \pm 0.4 \times 10^6$ (5)	
	Meningobactin	0.2	1.0×10^3 (1)	1,000
	Meningobactin	0.02	$1.4 \times 10^4 \pm 0.5 \times 10^4$ (4)	71
	Gonobactin	0.02	1.1×10^4 (1)	91

[a] LD_{50} assays were performed in 10- to 11-day-old chicken embryos inoculated intravenously (2, 4). Siderophores were included with the inocula, and none were toxic at the doses tested.

[b] According to Kellogg et al. (5). These were predominantly (>80%) the type indicated, with the remainder of the colony type in parentheses.

[c] One unit is defined as the reciprocal of the dilution required to produce a 10-mm zone of stimulation in the conalbumin agar assay for the homologous species of assay organism.

[d] SD, Standard deviation.

TABLE 3. *Influence of meningobactin and Desferal on virulence of N. gonorrhoeae 2686 for chicken embryos*[a]

Colony type[b]	Siderophore	Amt per chicken	LD_{50} ± SD[c] (no. of determinations)	Fold increase (decrease) in virulence
T1 (T2)	None		$4.0 \times 10^1 \pm 1.8 \times 10^1$ (2)	
	Desferal	5 μg	1.25×10^5 (1)	(3,125)
	Ferrioxamine B mesylate	5 μg	6.8×10^1 (1)	(1.7)
T3 (T4)	None		$5.4 \times 10^5 \pm 4.5 \times 10^5$ (3)	
	Meningobactin	0.04 U[d]	5.8×10^3 (1)	93.1

[a] LD_{50} assays were performed as described previously (2, 4). Siderophores were included with the inocula and were not toxic at the doses tested.

[b] According to Kellogg et al. (5). These were predominantly (>80%) the type indicated, with the remainder of the colony type in parentheses.

[c] SD, Standard deviation.

[d] One unit is defined as the reciprocal of the dilution required to produce a 10-mm zone of stimulation in the conalbumin agar assay for the homologous species of assay organism.

that this property may also be a major determinant of the nature of the host-parasite relationship, i.e., whether the infection is restricted to surfaces or whether the organisms may disseminate to cause bacteremia. In earlier work (8), a variety of gram-negative organisms examined were found to be distributed into four classes dependent on their ability to acquire iron in the chicken embryo model. Class I organisms, which included meningococci and other bacteria which characteristically are capable of dissemination, were defined as those which were virulent even when the serum iron saturation of the experimental host was markedly reduced by the addition of conalbumin, the transferrin counterpart in embryonated hen eggs or by pretreatment with endotoxin. These manipulations significantly inhibited the virulence of class II organisms, including urogenital isolates of the gonococcus which were virulent for normal chicken embryos. Class II organisms usually caused infections which are restricted to mucosal surfaces. Class III contained those organisms which were relatively avirulent for normal chicken embryos unless the inoculum was supplemented with iron. This class consisted of mutants and clonial variants of class I and II organisms. Class IV was composed of organisms which were avirulent even when iron was added. These organisms, including saprophytic *Neisseria* spp., apparently lacked other essential attributes of virulence. Thus, the ability to acquire iron may be regarded as essential but not sufficient for virulence.

The pathogenic neisserial species, *N. meningitidis* and *N. gonorrhoeae*, appear to be ideal candidates for further evaluation of the role of iron. Even though they are taxonomically related and structurally similar, the two species have vastly different pathogenic potentials. Meningococcal disease characteristically involves severe disseminating infections, meningitis and meningococcemia (in patients lacking the barrier of antibacterial antibody), whereas gonococci are usually associated with more superficial infections of mucosal surfaces and disseminate only rarely. Isolates from disseminated gonococcal infections appear to form a subset which more closely resemble meningococci than do urogenital isolates in their virulence for iron-depleted chicken embryos (8).

In earlier papers of this series (13, 14), the responses of pathogenic *Neisseria* spp. to various iron sources and to exogenous microbial siderophores were found to be similar qualitatively. Further, both meningococci and gonococci were shown to produce hydroxamate siderophores, gonobactin and meningobactin, with similar properties although there was evidence that these were not identical in that each was more effective in stimulating iron-limited growth of the homologous organism. The observations that meningococci produced more siderophore activity than gonococci that strains from disseminated gonococcal infections produced more than urogenital isolates and that virulent T1 colony type strains produced more activity than did avirulent T3 strains are all compatible with the hypotheses proposed that iron acquisition is essential for virulence and that it may also be a determinant of the nature of the host-parasite relationship.

The present paper extends the previous observations with the demonstration that the virulence of gonococci for chicken embryos is affected by microbial siderophores in directions which were predictable from previous in vitro tests. The results add to the evidence that the primary function of the iron sources is to provide iron essential for in vivo growth of the gonococcus rather than to inhibit host defense mechanisms in this model.

Desferal, a trihydroxamate siderophore of microbial origin which is used therapeutically for treatment of iron-overload states, was previously (14) shown to be inhibitory to gonococci in its deferrated, but not its iron-saturated, form. As shown in Table 1, Desferal decreased significantly the virulence of T1 gonococci, but had little effect in its iron-saturated form. It had essentially no effect on the virulence of already iron-limited T3 gonococci. On the other hand, schizokinen and arthrobactin, dihydroxamate siderophores which had previously (14), in their ferrated forms, been shown to support growth of gonococci in iron-limited conditions, enhanced virulence of T3 gonococci in the present study, whereas ferrichrome A, a trihydroxamate previously found to have no effect on gonococcal growth in vitro, likewise did not affect virulence. Both of the previously described (13) neisserial siderophores, meningobactin and gonobactin, enhanced the virulence of T3 gonococci, but had essentially no effect on T1 gonococci which are capable of obtaining the iron essential for their growth (and virulence) in normal chicken embryos. The effective siderophores also elevated the levels of circulating gonococci in infected chicken embryos (Yancey and Finkelstein, unpublished observations).

There are several loci which could result in a deficiency in the ability to acquire iron; in the synthesis of an essential siderophore, in its transport out of the bacterium, or in subsequent recognition and internalization of the siderophore-iron complex. The present and previous results indicate that the defect in the avirulent colony types of *N. gonorrhoeae* lies in the synthesis or transport (or both) of siderophore,

rather than recognition and internalization of the ferrated siderophore as the T3 gonococci (i) produced less siderophore activity, but (ii) were capable of responding to preformed siderophores. Their responsiveness to siderophores which were previously shown to support growth in iron-limited media is also compatible with the conclusion that the primary effect is on the bacteria rather than the host defensive mechanisms. However, it should be recognized that the results obtained thus far are relevant only to the experimental model employed, the chicken embryo. Further studies involving human iron-binding proteins, transferrin and lactoferrin, are indicated. Such studies may reveal novel but useful methods of prophylaxis and treatment of infections caused by pathogenic *Neisseria* spp. and of other bacterial diseases.

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