Identification and comparative analysis of the lactoferrin and transferrin receptors among clinical isolates of gonococci

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Summary. *Neisseria gonorrhoeae* expresses receptors for both lactoferrin (LF) and transferrin (TF). To determine whether qualitative or quantitative changes in these receptors, or both, correlate with the pattern of disease due to *N. gonorrhoeae*, a dot binding assay with whole cells was used to measure the absolute receptor levels expressed during iron-limited growth of strains isolated from asymptomatic patients and patients with urethritis, cervicitis, pelvic inflammatory disease and disseminated infection. Lactoferrin and transferrin receptor complexes were purified by affinity chromatography and their protein profiles were compared. The molecular weights of the LF and TF receptor complexes from a representative sample of gonococci were homogeneous. We conclude that there is no relationship between the LF and TF receptor levels and disease manifestation, auxotype or serotype.

Introduction

Iron acquisition is a microbial virulence factor (Bullen, 1981; Finklestein *et al.*, 1983; Weinberg, 1984) but the availability of iron in the human host is limited by binding to lactoferrin (LF) and transferrin (TF), respectively present in exocrine secretions and the intravascular compartment (Bullen, 1981; Finklestein *et al.*, 1983; Weinberg, 1984). These high-affinity iron-chelating proteins may be sources of iron for *Neisseria gonorrhoeae* (Micklesen and Sparling, 1981; Micklesen *et al.*, 1982).

Certain phenotypic traits characterise gonococcal strains that cause disseminated infection. These include serum resistance, the transparent Protein II variant, the AHU⁻ auxotype and the Protein 1A serogroup (Britigan *et al.*, 1985). In view of the importance of the ability of *N. gonorrhoeae* to capture iron from TF and LF, the question arises whether the ability of the gonococcus to utilise iron derived from these carriers is important in governing the type of disease produced. The recently described gonococcal LF and TF receptors (Lee and Schryvers, 1988) provided an opportunity to examine the hypothesis that the differential expression of these receptors might reflect differential

abilities to acquire iron from LF and TF and determine the type of clinical disease produced.

Materials and methods

Bacterial strains

A total of 40 clinical isolates of *N. gonorrhoeae* was maintained as stock cultures by freezing at -70°C . They were kindly provided by Dr R. C. Brunham (University of Manitoba) and were derived from a previous prospective study to correlate Protein 1 serotype and auxotype with disease expression (Brunham *et al.*, 1985). The strains were isolated from patients with the following clinical diagnoses: Seven with asymptomatic disease, seven with urethritis, five with cervicitis, 12 with disseminated gonococcal disease, and 9 with pelvic inflammatory disease. Twenty-four of the isolates expressed the Protein 1A serotype and 16 expressed the Protein 1B serotype. Twenty-one of the strains were nutritional auxotrophs comprising five distinct auxotrophic groups.

Organisms were identified as *N. gonorrhoeae* if they had typical morphology on Gram's stain, were oxidase positive, produced acid from glucose and gave negative reactions with maltose, sucrose and *o*-nitro-phenyl- β -D-galactoside by the Minitek method (Morse and Bartenstein, 1976). β -Lactamase production was assessed by the chromogenic cephalosporin assay (O'Callaghan *et al.*, 1972). All isolates were β -lactamase negative.

Strains were categorised as Protein 1A or 1B in a coagglutination assay with a panel of monoclonal

antibodies according to the classification system of Knapp *et al.* (1984). Auxotyping was performed by the method of Hendry and Stewart (1979).

Lactoferrin and transferrin receptor assay

The whole-cell assay used to identify the LF and TF receptors has been described previously (Schryvers and Morris, 1988a). Briefly, *N. gonorrhoeae* strains were grown overnight on heated blood agar at 37°C in CO₂ 5% in air. Non-fimbriate cells were then inoculated into prewarmed SJ-GC broth (Shockley *et al.*, 1980) containing 40 µM desferrioxamine, a potent iron chelator, to an initial optical density of 10 Klett units, as determined with a Klett-Summerson colorimeter with a green filter (no. 540) and grown in a shaking incubator at 37°C in the presence of CO₂ 5% until mid-exponential growth was achieved (60–70 Klett units). Samples of 1 ml from these cultures were centrifuged in a Beckman microfuge and the cell pellets were resuspended in 50 mM Tris-HCl buffer (pH 7.5) to give a final protein concentration of 1 mg/ml as determined by the method of Lowry *et al.* (1951). Dots of 2-µl volumes were applied to nitrocellulose paper (0.45 µm, Millipore Corp., Bedford, MA, USA), air dried, and blocked with skimmed milk (Johnson *et al.*, 1984). Peroxidase-conjugated LF or TF (Jackson ImmunoResearch Lab., Inc., Avondale, PA, USA) was added to the blots in skim milk and the blots were incubated for 1 h at 37°C, washed and developed with chloronaphthol reagent (HRP Color Developing Reagent, Bio-Rad Lab., Richmond, CA, USA).

Quantitation of lactoferrin and transferrin receptor activity

Concentrations of TF and LF were measured with a Bio-Rad Model 620 Densitometer interfaced with an IBM PC computer incorporating the Bio-Rad I-D Analyst software package (Bio-Rad Lab.). Values obtained from individual experiments were compared with standardised controls of known concentrations of LF and TF. Equimolar concentrations of LF and TF produced equal intensities when measured.

Statistical analysis

Mean LF and TF values among the different groups were compared by a one-way analysis of variance. The difference between mean LF and TF receptor values was evaluated by the paired Student's *t*-test.

Affinity-chromatography of membranes for lactoferrin and transferrin receptor

Total membranes were prepared by the method of Schryvers and Morris (1988a) from cells grown in iron-rich (SJ-GC broth alone) and iron-limited (SJ-GC broth with the addition of 40 µM desferrioxamine) conditions. These preparations were enriched for LF and TF receptor

by affinity chromatography with streptavidin-sepharose bound with its respective ligands (Schryvers and Morris, 1988b). The proteins were separated by sodium dodecyl sulphate (SDS-PAGE) (Laemmli and Favre, 1973) and the resultant bands stained with silver (Oakley *et al.*, 1980).

Growth experiments

For the growth experiments, organisms were streaked onto heated blood agar from stock cultures that were frozen at -70°C and the plates were incubated for 12–14 h at 37°C in CO₂ 5% in air. Cells were then suspended in SJ-GC broth containing 50 µM desferrioxamine to an initial optical density of 10 Klett units. Cultures were shaken at 37°C in an atmosphere containing CO₂ 5% until mid-exponential growth was achieved (60–70 Klett units). Samples were removed and used to inoculate fresh prewarmed SJ-GC broth containing 50 µM desferrioxamine to which were added the various iron sources. Human LF and TF purified by high performance liquid chromatography were added to a final concentration of 1.25 µM available iron. Ferric chloride was used at a final concentration of 50 µM. Growth was monitored hourly for 8 h with a Klett-Summerson colorimeter.

Results

LF and TF receptor values of gonococci isolated from each of the five disease categories and for each of the six different auxotypes are shown in the table. All gonococci expressed detectable levels of both LF and TF receptor, irrespective of auxotype, disease manifestation, or Protein 1 serotype (data not shown). Mean values for the TF receptor exceeded those for the LF receptor in each of the five disease categories ($p < 0.01$) and in each of the seven auxotypes ($p < 0.01$). There were no significant associations between the LF and TF receptor values and disease manifestation, auxotype, or Protein 1 serotype (data not shown).

To determine whether a putative relationship may have been obscured by confounding distributional effects, such as arbitrary clinical assignment, the disease data were segregated into two classifications—localised (urethritis and cervicitis combined) and disseminated. Similarly, the OHU⁻ auxotype was used as a surrogate marker for dissemination (Knapp and Holmes, 1975) and these strains were compared with non-OHU⁻ bearing strains. These manipulations did not accentuate or reveal any differences as the comparative analyses yielded results identical to the original unadjusted analysis.

Two other observations, which did not achieve statistical significance because of their small sample size, are intriguing. Unexpectedly, cervicitis strains

Table. Lactoferrin and transferrin receptor values of *N. gonorrhoeae* strains isolated from patients with asymptomatic disease, urethritis, cervicitis, disseminated gonococcal infection and pelvic inflammatory disease

Auxotype	Number of isolates (n)	Mean receptor value* (and SD) in strains from																	
		asymptomatic carriers			urethritis			cervicitis			disseminated infection			pelvic inflammatory disease			all		
		n	LF	TF	n	LF	TF	n	LF	TF	n	LF	TF	n	LF	TF	n	LF	TF
Proto	19	1	1.205	2.127	3	0.872	0.910	2	0.967	2.646	9	0.816	1.343	4	0.960	1.651	19	0.929 (0.395)	1.455 (0.491)
PCU ⁻	8	1	1.396	1.270	2	0.801	1.089	2	1.276	1.729	0	—	...	3	0.947	1.243	8	1.050 (0.420)	1.330 (0.597)
Pro ⁻	3	2	0.914	2.036	1	0.935	2.067	0	...	...	0	...	...	0	...	...	3	0.921 (0.262)	1.673 (0.630)
OHU ⁻	5	2	0.614	1.275	1	0.177	0.423	1	0.234	0.685	0	...	...	1	0.298	1.139	5	0.398 (0.265)	0.960 (0.388)
Orn ⁻	4	1	0.900	0.988	0	...	...	0	...	...	3	1.269	2.100	0	...	...	4	1.196 (0.248)	1.823 (0.640)
CU ⁻	1	0	...	...	0	...	...	0	...	...	0	...	...	1	0.985	1.714	1	0.985	1.714
All	40	7	0.944 (0.335)	1.573 (0.476)	7	0.763 (0.413)	1.057 (0.630)	5	0.944 (0.575)	1.647 (0.719)	12	0.929 (0.405)	1.532 (0.486)	9	0.964 (0.419)	1.465 0.477	40		

Proto prototrophic, PCU⁻ proline, citrulline and uracil-requiring, Pro⁻ proline-requiring, OHU⁻ ornithine, hypoxanthine and uracil-requiring, Orn⁻ ornithine-requiring, CU⁻ citrulline and uracil-requiring.

* Mean ng of conjugate/ μ g wet weight total cell protein.

possessed the highest TF receptor values. Paradoxically, the OHU⁻ auxotype displayed the lowest LF and TF receptor levels among the auxotypes scrutinised.

An examination of the silver-stained SDS-PAGE preparation (figs. 1 and 2) disclosed no major differences between the protein profiles of the LF and the TF receptor complexes among a representative selection of isolates from localised and disseminated gonococcal infection. A prominent 101-Kda protein, the putative LF receptor, was present in all the LF receptor affinity-enriched iron-limited preparations (arrow, fig. 1, lanes d–g). This band was absent from both the control sample grown in iron-rich conditions (fig. 1, lane b), indicating the iron-regulated nature of the protein, and from the samples processed for the TF receptor (fig. 2, lanes d–g), thus demonstrating the ligand specificity of the affinity chromatography method.

Fig. 1. SDS-PAGE of crude total membranes showing lactoferrin-receptor complexes of five clinical isolates of gonococci. The protein profiles are shown in lanes d–g, where lane d is W003 and lane e W006 (both strains from disseminated infections), lane f W202 (a urethritis strain), and lane g W159 (a cervicitis strain). For comparison, lanes a and c represent the starting crude total membranes of strain W003 cultured in iron-rich and iron-limited SJ-GC broth respectively. Lane b is the corresponding affinity-enriched sample prepared from the starting material in lane a. Strains W003, W006 and W202 expressed the Protein 1A serotype whereas strain W159 expressed the Protein 1B serotype. All the strains were prototrophic auxotrophs. Molecular weight markers (Kda) are displayed on the left side of the gel. Arrows (◄) designate the protein bands representing the putative lactoferrin receptor. Dots (•) indicate contaminating bands present in the polyacrylamide gel matrix.

Fig. 2. SDS-PAGE of crude total membranes showing transferrin receptor complexes of five clinical isolates of gonococci. The protein profiles are shown in lanes d–g, where lane d is W003 and lane e W006 (both strains from disseminated infections), lane f W202 (a urethritis strain), and lane g W159 (a cervicitis strain). For comparison, lanes a and c represent the starting crude total membranes of strain W003 cultures in iron-rich and iron-limited SJ-GC broth respectively. Lane b is the corresponding affinity-enriched sample prepared from the starting material in lane a. Strains W003, W006, and W202 expressed the Protein 1A serotype whereas strain W159 expressed the Protein 1B serotype. All the strains were prototrophic auxotrophs. Molecular weight markers (Kda) are displayed on the left side of the gel. Arrows (◄) designate the protein bands representing the putative transferrin receptor. Dots (•) indicate contaminating bands present in the polyacrylamide gel matrix.

Similarly, five bands of 98, 74.5, 68.5, 66.5, and 37 Kda were either co-purified or enhanced in the iron-limited samples subjected to the TF-receptor affinity chromatography protocol (arrows, fig. 2, lanes d–g) when compared with the iron-rich control membrane sample (fig. 2, lane b) and the samples prepared for the LF receptor (fig. 1, lanes d–g). Other polypeptide bands that were present were common to both the affinity purified iron-rich and iron-limited preparations and represent contaminating fractions present in the acrylamide gel matrix (dots, figs. 1 and 2) or proteins binding nonspecifically to the affinity gel. The latter proteins can be preferentially eluted with stringent washing conditions, without disturbing the appearance of the enhanced bands (data not shown).

Functional differences were not identified in the ability of iron-limited cultures of isolates from urethritis and disseminated gonococcal infection to

utilise either LF or TF as the sole exogenous source (data not shown).

Attempts to identify either the LF or the TF receptor by a binding assay after SDS-PAGE and electroblotting (Schryvers and Morris, 1988*b*) failed, indicating that the LF and TF receptor binding sites are conformationally dependent (data not shown).

Discussion

This survey of LF and TF receptor expression in clinical isolates of gonococci did not support the concept that qualitative or quantitative differences in the TF receptor alone can predict intravascular invasion. Analysis of the TF-receptor values, functional status, or molecular weight failed to discriminate between strains from localised and disseminated gonococcal infection. This can be explained because it is understood that the process of bacterial virulence is complex and involves, sequentially or in concert, an array of bacterial virulence determinants (Sparling, 1983). In this context, the interaction of Protein II and serum resistance, two other phenotypic traits epidemiologically linked to strains from disseminated gonococcal infection (Britigan *et al.*, 1985), with TF-receptor levels may prove instructive.

However, despite the lack of an association, two tantalising observations emerge from this investigation. Firstly, the low expression of LF receptor in the OHU⁻ strains may be the molecular correlate of the recognised inability of such auxotypes to acquire iron from LF (Micklesen *et al.*, 1982) and may account, in part, for the frequent association of this auxotype with asymptomatic gonorrhoea (Knapp and Holmes, 1975). Additional explanations, such as qualitative differences in the LF receptor or differences in processing of the ligand-receptor complex cannot be excluded.

Finally, the LF and TF receptor complexes enriched by affinity-chromatography correspond

with iron-regulated gonococcal proteins of similar molecular weight previously described (West and Sparling, 1985; Meitzner *et al.*, 1986). The enhancement of a 101-Kda protein band by LF affinity-chromatography identifies this protein as the putative LF receptor. In contrast, several bands were co-purified by the TF process. Two of these proteins of 98 and 37 Kda were represented in all the gonococcal strains examined, whereas the third co-purified protein exhibited interstrain molecular weight heterogeneity, varying from 66.5 to 74.5 Kda. This suggests that the TF receptor consists of a functional complex of three proteins. One of these, a 37-Kda protein, may correspond with a previously shown iron-regulated protein that is antigenically conserved among pathogenic *Neisseria* spp. (Meitzner *et al.*, 1986). The co-elution of this protein as a component of the TF-receptor complex tentatively supports the contention that it is involved in iron uptake (Meitzner *et al.*, 1986). Alternatively, the use of more stringent methods of affinity purification might have permitted the definitive assignment of the TF receptor to a single protein band. The possibility that these bands represent degradation products of the TF receptor cannot be excluded. The relative molecular weight homogeneity of all these binding proteins suggests a functional conservation, consistent with their important role in iron acquisition. Implicit also from these results is the existence of separate receptors for LF and TF. Their uniform presence in iron-limited conditions suggests coordinate expression.

Thus, the exact contributions of these receptors in governing diseases expression remain to be elucidated. Since ability to acquire iron is a trait critical to virulence, it is to be expected that their roles will be significant.

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