Evidence that the Serum Resistance Genetic Locus *sac-3* of *Neisseria gonorrhoeae* Is Involved in Lipopolysaccharide Structure

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The gonococcal chromosome contains a sequence of closely linked genes (for example, *sac-1*, *sac-3*, *nmp*) known or presumed to affect cell envelope structure and which appear to influence susceptibility of gonococci to killing by normal human sera (NHS). Previous work has shown that the serum-resistant isolate FA19, and FA899, a serum-sensitive transformant of FA19, differ in outer membrane protein I (PI) and at the *sac-3* genetic locus. However, the *sac-3* locus is separable from changes determined by *nmp-3*, the gene determining PI species. We found that FA19 and FA899 differ in lipopolysaccharide (LPS) molecular size and in reactivity with a monoclonal antibody which recognizes an LPS (L8) epitope. To address the question of whether the changes in LPS were due to the *sac-3* locus, we constructed new transformants of FA19 using donor DNA prepared from FA899. The new transformants could be divided into three groups: (1) those identical to FA19 in serum resistance (>90% survival at 120 min), in LPS molecular size and in expression of the L8 epitope; (2) those identical to FA899 in serum sensitivity (100% killed at 30 min), in LPS molecular size and in lack of expression of the L8 epitope; (3) those significantly killed by 50% NHS at 120 min, whose LPS molecular size was greater than that of FA19 but less than that of FA899 and which did not express the L8 epitope. Except for PI there were no differences in other outer-membrane proteins (e.g. PII, PIII, H.8) among these transformants. Studies of PI using monoclonal antibodies which distinguish between PI serovars confirmed that changes in PI were separate from changes in serum resistance or alteration in LPS chemotype. These data indicate that a genetic locus involved in LPS structure appears to be identical with the *sac-3* locus, which influences serum susceptibility in *Neisseria gonorrhoeae*.

INTRODUCTION

Gonococci isolated from patients with disseminated gonococcal infections are typically resistant to complement-mediated killing by normal human serum (NHS) (Schoolnik *et al.*, 1976). In contrast, strains isolated from patients with uncomplicated gonococcal infections tend to be sensitive to NHS (Brooks & Ingwer, 1978). The molecular basis of serum resistance of gonococci remains unknown. Nevertheless, at least two closely linked genes termed *sac-1* and *sac-3* (*sac*: serum antibody complement) have been described (Cannon *et al.*, 1981; Shafer *et al.*, 1982). Expression of these loci has been suggested to result in the alteration of the gonococcal cell envelope providing for serum resistance (Sac^r).

Gene products of *sac-1* and *sac-3* providing for Sac^r have not been described. Genetic studies (Cannon *et al.*, 1981; Shafer *et al.*, 1982) have separated *sac-1* and *sac-3* from *nmp* (new membrane protein) loci involved in PI synthesis; alterations in PI had earlier been thought to be responsible for Sac^r (Hildebrandt *et al.*, 1978). Changes in lipopolysaccharide (LPS) structure

Abbreviations: Mab, monoclonal antibody; NHS, normal human sera/serum; PI, gonococcal outer-membrane protein I.

have also been associated with Sac^r because spontaneous LPS mutants deficient in core oligosaccharides of Sac^r strain FA19 were found to be Sac^s (Guymon *et al.*, 1982). In this study we show that the gene for an LPS epitope associated with Sac^r appears identical to *sac-3* and distinct from a locus (*nmp-3*) involved in PI synthesis, and describe new genetic and biochemical studies to show that *sac-3* strains differ from *sac-3*⁺ strains in LPS.

METHODS

Gonococcal strains. The strains of *Neisseria gonorrhoeae* used and their genetic construction are listed in Table 1. Strain FA19 (*sac-1 sac-3*) has been well characterized (Cannon *et al.*, 1981; Sparling *et al.*, 1975). We refer to loci dictating serum sensitivity to NHS as either *sac-1*⁺ or *sac-3*⁺ and the resistant alleles are termed *sac-1* or *sac-3*. Strain FA899 is a *sac-3*⁺ transformant of FA19 (Shafer *et al.*, 1982) originally obtained by using DNA from strain FA889 (*sac-3*⁺), a clinical isolate obtained from Inga Lind at the Statens Serum-Institut, Copenhagen. The PI serovar of these isolates and of the new transformants described herein was kindly determined by Dr Joan Knapp (Centers for Disease Control, Atlanta, GA, USA). Colony type T₁ (piliated) vs T₄ (nonpiliated) and colony opacity were determined by techniques previously described (Stephens *et al.*, 1984).

Serum bactericidal assay (SBA). A microdilution SBA was performed as previously described (Stephens & McGee, 1983). Briefly, blood from normal adult subjects without a history of gonococcal or meningococcal disease, and not under antibiotic therapy, was collected under sterile conditions, allowed to clot for 30 min at room temperature (23 °C), and then centrifuged at 500 g at 4 °C for 15 min. The serum was removed, divided into subsamples, and stored at -70 °C until used in the experiments. Total haemolytic activity in the serum [measured by the CH₅₀ value (amount of complement required to lyse 50% of sensitized sheep red blood cells)] was at normal levels. Isolates to be tested were grown on GCISO agar plates [GC agar base (BBL) plus 2% (v/v) IsovitaleX] (Stephens & McGee, 1983) for 14–16 h at 37 °C in 3% (v/v) CO₂. Inocula (Op⁻, Pil⁻) were prepared, diluted to the desired concentration and used immediately in the SBA. In some experiments hypogammaglobulinaemic serum from a patient with acquired common variable immunodeficiency or C2-deficient human serum (Cytotech) were used.

Serum (0.5 ml), bacterial inoculum (0.1 ml) and HEPES-MEM (0.4 ml) were mixed so that the final suspension contained 1 ml with 50% (v/v) serum and 2×10^4 c.f.u. ml⁻¹. The reaction mixture was incubated at 37 °C in 3% (v/v) CO₂ with end-over-end rotation. Triplicate samples (0.01 ml) were withdrawn from the reaction mixture immediately after preparation and after 15, 30, 60 and 120 min incubation. Colony counts were performed as previously described (Stephens & McGee, 1983). After overnight incubation of the plates, the number of c.f.u. per sample was counted and the number of c.f.u. ml⁻¹ in the original mixture at each sampling time was calculated. Data were expressed as percentage survival. The significance of differences between the means of two variables was determined using Student's *t*-test with unpaired values and a two-tailed hypothesis.

Genetic transformation. Transforming DNA used in these experiments was prepared by the method of Marmur (1961). Transforming DNA was prepared from strain FA899 (*sac-3*⁺) and used to transform strain FA19 (*sac-3*) by techniques described previously (Shafer *et al.*, 1981). Limiting concentrations (0.1 µg ml⁻¹) of DNA were used. Spectinomycin resistant (Spc^r) transformants were selected using GC base agar overlays containing 100 µg spectinomycin ml⁻¹ (Shafer *et al.*, 1982).

Biochemical and immunochemical analysis of gonococcal strains. SDS-PAGE of lysates of whole gonococci was done using 15% (w/v) acrylamide gels by techniques previously described (Stephens *et al.*, 1985). Proteinase K digests of the whole cell lysates were prepared (Hitchcock & Brown, 1983) and the gels were silver stained (Tsai & Frasch, 1982).

Western immunoblotting was done by a modification of the method of Towbin *et al.* (1979) as previously described (Stephens *et al.*, 1985). Monoclonal antibody L8, an IgG class immunoglobulin (kindly supplied by Dr Wendell Zollinger, Walter Reed Army Institute of Research) was used in these experiments. L8 recognizes a *Neisseria* LPS epitope (designated L8) associated with Sac^r but not Sac^s gonococcal strains (Schneider *et al.*, 1985). Antibody binding to LPS immobilized on nitrocellulose was detected using staphylococcal protein A conjugated with peroxidase as previously described (Stephens *et al.*, 1985).

RESULTS

Strain FA19 resists killing by 50% (v/v) NHS over a 120 min incubation while FA899 is rapidly killed by NHS (Fig. 1). These strains differ in PI molecular mass (Shafer *et al.*, 1982). The results of PI serovar typing (Table 1) and new SDS-PAGE analysis (data not shown) confirmed that these strains differ in PI. Previous studies (Cannon *et al.*, 1981; Shafer *et al.*, 1982) have also shown that differences in PI are unrelated to Sac^r since changes in PI could be genetically separated from changes in Sac^r .

Fig. 1. Serum bactericidal assay (50% NHS) with gonococcal strains FA19, FA899, SS-2, SS-4 and SS-9. The data are expressed as percentage survival relative to the 0 time values. Strain FA19 (□) resists killing by NHS over 120 min incubation while FA899 (▼) is rapidly killed. Transformant SS-4 (▲) resembles FA19 in susceptibility to NHS, while SS-2 (△) resembles FA899. With transformant SS-9 (●), killing occurred in a delayed fashion over a period of 120 min. Data are the means $\pm$ SEM of three or more experiments with each variable.

Table 1. Characteristics of *N. gonorrhoeae* strains and new transformants

Strain†	Relevant genotype‡	Relevant phenotype§	Serum bactericidal assay (% survival)		PI serovar	L8 LPS epitope	LPS molecular size (kDa)
			30 min	120 min			
FA19	<i>spc-4⁺ sac-1 sac-3</i>	Spc ^s Sac ^r	69	78	IA1	+	5.8
FA889	<i>spc-4 sac-1 sac-3⁺</i>	Spc ^r Sac ^s	0	0	IB9	—	6.6
FA899	<i>spc-4 sac-1 sac-3⁺</i>	Spc ^r Sac ^s	0	0	IB9	—	6.6
SS-1	<i>spc-4 sac-1 sac-3</i>	Spc ^r Sac ^r	71	> 100	IA1	+	5.8
SS-2	<i>spc-4 sac-1 sac-3⁺</i>	Spc ^r Sac ^s	0	0	IB9	—	6.6
SS-3	<i>spc-4 sac-1 sac-3</i>	Spc ^r Sac ^r	83	> 100	IB9	+	5.8
SS-4	<i>spc-4 sac-1 sac-3</i>	Spc ^r Sac ^r	89	> 100	IA1	+	5.8
SS-5	<i>spc-4 sac-1 sac-3</i>	Spc ^r Sac ^r	63	> 100	IA1	+	5.8
SS-6	<i>spc-4 sac-1 sac-3</i>	Spc ^r Sac ^r	68	70	IB9	+	5.8
SS-7	<i>spc-4 sac-1 sac-3</i>	Spc ^r Sac ^r	68	> 100	IA1	+	5.8
SS-8	<i>spc-4 sac-1 sac-3⁺</i>	Spc ^r Sac ^{s*}	35	10	IB9	—	6.2
SS-9	<i>spc-4 sac-1 sac-3⁺</i>	Spc ^r Sac ^{s*}	37	7	IB9	—	6.2
SS-10	<i>spc-4 sac-1 sac-3⁺</i>	Spc ^r Sac ^{s*}	36	14	IB9	—	6.2
SS-11	<i>spc-4 sac-1 sac-3⁺</i>	Spc ^r Sac ^{s*}	30	6	IB9	—	6.2
SS-12	<i>spc-4 sac-1 sac-3⁺</i>	Spc ^r Sac ^{s*}	38	18	IB9	—	6.2
SS-13	<i>spc-4 sac-1 sac-3</i>	Spc ^r Sac ^r	57	92	IA1	+	5.8
SS-14	<i>spc-4 sac-1 sac-3</i>	Spc ^r Sac ^r	52	> 100	IB9	+	5.8

† Strains SS-1 to SS-14 were derived by transformation of strain FA19 with DNA from strain FA899.

‡ *sac*, serum-antibody-complement resistant; *spc-4*, spectinomycin resistant (+ indicates a sensitive allele).

§ Sac^r, resistant to killing by 50% NHS; Sac^s, sensitive to killing by 50% NHS at < 30 min; Sac^{s*}, sensitive to killing by 50% NHS at 120 min.

Fig. 2. (a) SDS-PAGE (15% acrylamide gel) of proteinase-K-digested whole-cell lysates of gonococcal strains SS-14, SS-13, FA899, FA19, SS-12, SS-9, FA899 and FA19. SS-14 and SS-13 are *Sac*^r transformants which have the same LPS molecular size (~5.8 kDa) as the *Sac*^r parent FA19. SS-12 and SS-9 are *Sac*^{s*} transformants whose LPS differs in molecular size (~6.2 kDa) from the LPS of FA19 (~5.8 kDa) or the *Sac*^s transformant FA899 (~6.6 kDa). Similar results were seen with the other *Sac*^r and *Sac*^{s*} transformants. The molecular size of the LPS was calculated in separate experiments using low-molecular-weight protein standards (BRL) and Re, Rd and smooth *Salmonella minnesota* mutants.

(b) Western immunoblot of the electrophoresed lysates in (a), probed with Mab L8. The *Sac*^r transformants SS-13 and SS-14, and FA19, express the L8 LPS epitope while the *Sac*^{s*} transformants SS-9 and SS-12 and the *Sac*^s strain FA899 do not. Similar results were seen with the other *Sac*^r and *Sac*^{s*} transformants.

Differences in LPS have not been previously noted between these strains (Shafer *et al.*, 1982). However, as shown in Fig. 2(a) we found, using techniques developed for examination of LPS by SDS-PAGE (Hitchcock & Brown, 1983), that FA19 and FA899 differed in LPS molecular size. FA19 and FA899 also differ in the capacity of Mab L8 to recognize their respective LPS in Western immunoblots (Fig. 2b and Table 1). This result suggested that the presence of a genetic determinant closely linked or identical to *sac-3* is involved in the synthesis of LPS from *Sac*^r strain FA19.

In order to substantiate the presence of an LPS biosynthetic gene in the *sac-3*⁺ region, DNA was prepared from strain FA899 and used to transform strain FA19. *Spc*^r transformants were selected since *sac-3*⁺ cotransforms with *spc-4* (Shafer *et al.*, 1982). Fourteen *Spc*^r transformants (SS 1-14) of FA19 were studied for changes in serum susceptibility, LPS molecular size, reactivity with Mab L8, outer-membrane proteins, and PI serovar. As shown in Table 1 and Fig. 1, *Sac*^r (>90% survival at 120 min), *Sac*^s (100% killed at 30 min) and *Sac*^{s*} (>90% killed at 120 min) transformants were obtained. Inocula of the parental *Sac*^r strain FA19 and the *Sac*^r transformants exhibited some killing (<50% of inoculum) at 30 min in 50% (v/v) NHS. However, by 120 min multiplication of these strains was occurring, with colony counts occasionally increasing to >100% of the initial inoculum. The *Sac*^s transformant SS-2, like the donor strain FA899, was rapidly killed. Data from earlier time points (not shown) indicated that killing of these strains was rapid, within 15 min of beginning the experiment. Other transformants (e.g. SS-9) displayed intermediate susceptibility to 50% NHS (*Sac*^{s*}). Killing of *Sac*^{s*} transformants occurred in a delayed fashion over a period of 120 min (Fig. 1).

Fig. 3

Fig. 4

Fig. 3. SDS-PAGE (a) and Western immunoblot, probed with Mab L8 (b), of proteinase-K-digested whole-cell lysates of gonococcal strains SS-2, SS-1, FA899 and FA19. The *Sac*^s transformant SS-2 has the same LPS molecular size as FA899 and does not express the L8 epitope.

Fig. 4. Serum bactericidal assay using 50% NHS (●), 50% hypogammaglobulinaemic serum (■) or 50% C2-deficient serum (○), and gonococcal strain SS-9 (*Sac*^{s*}). SS-9 is significantly killed by NHS at 120 min but is resistant to killing by the other two sera. Data are expressed as the means $\pm$ SEM of three or more experiments with each variable.

All *Sac*^r transformants exhibited an LPS molecular size (~ 5.8 kDa) and L8 reactivity profile like that of FA19 (Table 1, Fig. 2). The *Sac*^s transformant (SS-2) had an LPS molecular size (~ 6.6 kDa) identical to the donor strain FA899 and failed to react with the L8 Mab (Table 1, Fig. 3). The molecular size (~ 6.2 kDa) of LPS from the transformants of intermediate serum sensitivity (*Sac*^{s*}) was distinct from that of FA19 and FA899 (Fig. 2a). LPS from *Sac*^{s*} transformants failed to react with L8 Mab (Fig. 2b).

All *Sac*^s and *Sac*^{s*} transformants retained the PI serovar (1B9) of the donor, yet serovar 1B9 transformants (3/8) were recovered from transformants which remained *Sac*^r and reacted with Mab L8 in Western blots (Table 1). The remaining *Sac*^r transformants were PI serovar 1A1. Except for PI no differences in other outer-membrane proteins (e.g. PII, PIII) were noted by SDS-PAGE. The molecular mass of antigen H.8 of FA19, FA899 and transformants SS-1 to SS-14 was also identical as determined in Western immunoblots by monoclonal antibody (kindly supplied by Dr J. Cannon, University of North Carolina).

In order to investigate the mechanism of killing of the intermediate transformants (antibody-dependent or -independent, alternative or classical activation of complement) hypogammaglobulinaemic human serum and C2-deficient human serum were used (Fig. 4). The *Sac*^{s*} transformant SS-9 was resistant to killing by both these sera at 120 min, in contrast to significant killing in NHS at 120 min. This suggests that the killing of strain SS-9 is by an antibody-dependent, classical pathway.

DISCUSSION

In this study we show that a genetic determinant involved in LPS structure appears to be *sac-3*. This area of the gonococcal chromosome in *Sac*^r strain FA19 has been previously associated with the synthesis of cell envelope structures thought to contribute to the *Sac*^r phenotype (Cannon *et al.*, 1981; Shafer *et al.*, 1982). The well-described *sac-1* locus (Cannon *et al.*, 1981), which also gives a *Sac*^r phenotype, is in this region. We were able to separate expression of *sac-1* from *sac-3* by using high concentrations of NHS (Shafer *et al.*, 1982) and by noting in the current study that in isogenic strains differing at *sac-1*⁺, reactivity with Mab L8 was identical (data not shown). Our studies represent the first delineation of a locus (*sac-3*) involved in LPS structure in this region of the gonococcal chromosome.

This work substantiates earlier observations that *sac-3* and *nmp-3* are linked but not identical, thus indicating that changes in PI do not always result in changes in susceptibility to NHS. Less clear, however, is whether acquisition of *sac-3* results in presentation of an LPS epitope defined by Mab L8. Although we were unable to dissociate these phenotypes, the *sac-3* locus and a distinct LPS gene determining L8 reactivity may be closely linked, requiring examination of additional transformants in order to identify recombinants which express only one phenotype. This is particularly important because transformants were obtained which displayed killing by NHS in a delayed fashion. Although such transformants lacked reactivity with Mab L8, they appeared to synthesize an LPS with a molecular size intermediate to that of the parent and donor strains. The molecular basis for this type of recombinant, termed *Sac*^{s*}, is unknown. It may be indicative of two closely linked loci in *sac-3*⁺ strains which provide for distinct levels of susceptibility to NHS. It is important to clarify this issue because in the serum-sensitive transformants (*Sac*^s, *Sac*^{s*}) only one transformant, SS-2, displayed the same phenotypic profile as the donor strain FA899. We are currently examining the possibility that a second gene is responsible for the *Sac*^{s*} phenotype.

It was not surprising that alterations in LPS structure were associated with changes in susceptibility of gonococci to human sera. In *Salmonella* species, deep rough mutants containing truncated LPS are more serum sensitive than the isogenic smooth parental strains (Muschel & Larsen, 1980). Similarly, studies of serum-sensitive gonococcal mutants, selected by pyocins, have associated loss of components of LPS core polysaccharides to increased susceptibility to NHS (Guymon *et al.*, 1982; Morse & Apicella, 1982; Shafer *et al.*, 1984). However, in our study a decrease in LPS molecular size was associated with increasing serum resistance. Recently serum resistance in *N. gonorrhoeae* was shown to be related to the lack of a high molecular mass LPS moiety (Tan *et al.*, 1986). In addition, resistance of certain strains of *N. gonorrhoeae* to the bacteriolytic action of NHS has been shown to result from the absence from their LPS of antigenic loci for the lytic immunoglobulin M that is present in most NHS (Schneider *et al.*, 1982). Taken together these data suggest that a mechanism of serum resistance in *N. gonorrhoeae* may be the loss of LPS epitope(s) recognized by naturally occurring bactericidal antibody.

Using isogenic strains, we confirm and extend this hypothesis. We found that expression of a specific LPS epitope (possibly through loss of components that previously masked this epitope) is related to serum resistance. The L8 LPS epitope has been defined (Schneider *et al.*, 1984, 1985) to be within a ~3.6 kDa component of gonococcal and meningococcal LPS, and to be commonly expressed by *Sac*^r strains of *N. gonorrhoeae*. In a study of 40 randomly selected normal young adults none had evidence of antibody to this epitope (Schneider *et al.*, 1985). We found that the L8 epitope was expressed by the serum-resistant strain FA19 and by all the *Sac*^r transformants, but not by *Sac*^s strains.

The lack of expression of the L8 epitope and the intermediate size of LPS in our *Sac*^{s*} transformants, which exhibit delayed killing to 50% NHS, supports the concept that several alterations in LPS, leading to expression of L8, relate to the ability of gonococci to resist killing by human sera. It is likely that multiple genes are involved in synthesis of gonococcal LPS. The *sac-3* locus may code for an enzyme which modifies LPS structure in such a way as to expose the L8 epitope. In order to identify further the mechanism by which *sac-3* affects LPS, isolation and sequencing of the gene are required. In addition, studies using a panel of LPS monoclonal

antibodies are needed to map the loss or gain of epitopes associated with changes in LPS and serum susceptibility.

Both Sac^s and Sac^{s*} transformants appeared to be killed by an antibody-dependent classical pathway mechanism since neither class of transformant exhibited susceptibility to C2-deficient or hypogammaglobulinaemic serum. Thus, the Sac^{s*} phenotype might be due to fewer sites for bactericidal antibody, sites for bactericidal antibody which are partially masked by LPS or outer-membrane proteins, less efficient activation of serum complement or differences in C5b-9 deposition or insertion.

Elucidation of the relationship between expression of *sac-3*, L8 Mab epitope and the immunological basis for the quantitative differences in Sac^s in the recombinants will require additional study. However, our data indicate that a genetic locus involved in LPS structure appears to be identical with the *sac-3* locus influencing serum susceptibility.

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