SHORT COMMUNICATION

Alteration of Pyocin-sensitivity Pattern of *Neisseria gonorrhoeae* is Associated with Induced Resistance to Killing by Human Serum

By F. P. WINSTANLEY,¹ C. C. BLACKWELL,¹ E. L. TAN,² P. V. PATEL,²
N. J. PARSONS,² P. M. V. MARTIN³ AND H. SMITH^{2*}

¹Department of Bacteriology, University of Edinburgh Medical School, Edinburgh EH8 9AG, UK

²Department of Microbiology, University of Birmingham, Birmingham B15 2TT, UK

³Unité d'Ecologie bactérienne, Institut Pasteur, Paris, France

(Received 1 February 1984)

A laboratory-grown strain of *Neisseria gonorrhoeae*, selected *in vivo*, BS4 (agar), is susceptible to complement-mediated killing by fresh human serum but is relatively resistant to killing by human phagocytes. It can be induced to serum resistance by incubation with a small molecular weight fraction of guinea pig serum. The serum-susceptible and induced-resistant forms show differences in pyocin sensitivity tests. This indicates either differences in the structure of their lipopolysaccharides or masking of some determinant(s). The pyocin sensitivity pattern of BS4 (agar) is only slightly different from that of a closely related strain, BSSH, which is more susceptible to killing by human phagocytes.

INTRODUCTION

A strain of *Neisseria gonorrhoeae* selected from a small colony forming, pilate laboratory strain (BS) by three or four passages through plastic chambers implanted subcutaneously in guinea pigs was resistant to killing by human serum and human phagocytes when examined without subculture (Penn *et al.*, 1976, 1977). When cultured in laboratory media, the *in vivo*-selected strain, BS4 (agar), lost its serum resistance but retained its resistance to killing by phagocytes (Penn *et al.*, 1976, 1977). The serum resistance could be restored phenotypically to BS4 (agar) by incubating it for 3 h at 37 °C in a defined medium (DM) containing 0.1% (w/v) bovine serum albumin (BSA) and small molecular weight fractions of either guinea pig or some human sera (Veale *et al.*, 1981; Martin *et al.*, 1981).

The determinant(s) of the induced resistance of BS4 (agar) to killing by human serum has not yet been investigated. The limited supply of host-derived inducer has prevented the production of sufficient organisms for investigation of outer membrane proteins (OMP) and lipopolysaccharides (LPS) comparable to those studies on the culture-stable type of serum resistance shown by strains from disseminated gonorrhoea (Brooks *et al.*, 1978; Lambden *et al.*, 1979; James *et al.*, 1982). An investigation of the LPS of susceptible and induced-resistant forms of BS4 (agar) seems particularly relevant because LPS appears to be the target antigen for the 'natural' antibody responsible for the bactericidal activity of normal human serum (Brooks *et al.*, 1978; Schneider *et al.*, 1982). A method for indicating changes in LPS of *N. gonorrhoeae* became available when it was shown that partially purified, Kageyama R-type pyocins from *Pseudomonas aeruginosa* (Kageyama, 1975), the receptor sites for which reside on the LPS (Meadow & Wells, 1978), reacted with gonococci and could form the basis of a typing system (Morse *et al.*, 1976; Sidberry & Sadoff, 1977; Blackwell *et al.*, 1979). Only small numbers of gonococci are required and the pyocin method has been used to indicate LPS differences in mutational and epidemiological studies on gonococcal sensitivity to serum killing and attachment to polymorphonuclear leucocytes (Guymon *et al.*, 1982; Blackwell *et al.*, 1983;

Winstanley *et al.*, 1983). This paper reports tests with a range of R-type pyocins on strain BS4 (agar) before and after induction to serum resistance by the low molecular weight fraction from guinea pig serum.

The determinants of the resistance of strain BS4 (agar) to killing by phagocytes have been investigated by comparing it with a closely related phagocyte-susceptible strain, BSSH, selected *in vitro* by colonial appearance from the original parent strain BS (Penn *et al.*, 1977). In this case, sufficient organisms could be obtained for biochemical examination. The putative determinant of resistance was not pili and resided in outer membrane vesicles (OMV) (Parsons *et al.*, 1981). Examination of the proteins of purified OMV from BS4 (agar) and BSSH on sodium dodecyl sulphate polyacrylamide gel electrophoresis associated three OMP with the resistance of BS4 (agar) to killing by phagocytes (Parsons *et al.*, 1982). However, it was thought interesting to include in the pyocin tests a comparison of the phagocyte-resistant [BS4 (agar)] and phagocyte-susceptible (BSSH) strains.

METHODS

Neisseria gonorrhoeae. Strains BS4 (agar) and BSSH were derived, stored, cultured and counted as described previously (Penn *et al.*, 1976, 1977; Parsons *et al.*, 1981, 1982).

Induction of BS4 (agar) to serum resistance. The method used was described by Veale *et al.* (1981): 10^4 – 10^5 gonococci were incubated for 3 h at 37 °C in equal volumes of DM containing 0.1% (w/v) BSA and an ultrafiltrate of guinea pig serum containing molecules of less than 5000 daltons (Veale *et al.*, 1980; Martin *et al.*, 1981). The serum-susceptible BS4 (agar) used for comparison with the resistant organisms in the pyocin tests (Table 1) had been incubated in DM containing 0.1% BSA for 3 h at 37 °C without the inducer.

Pyocins. Representatives of four of the five groups (R1–R5) of R-type pyocins described by Kageyama (1975) were prepared from the following strains of *P. aeruginosa* and partially purified and stored as described by Blackwell *et al.* (1979). These included: R1 pyocins from strains ISD, 2285, IS6 and IS8; R2 pyocin from strain ISB; R3 pyocin from strain ISE; R5 pyocins from strains ISA and ISC; and two unclassified pyocins from strains R205 and 9579.

Tests for pyocin sensitivity. A suspension (15 µl) of partially purified pyocin was added to a suspension of gonococci (100 µl; 10^4 – 10^5 c.f.u. ml⁻¹ in DM (Veale *et al.*, 1981) in a sterile plastic test tube (10 ml capacity). Control tubes received DM (15 µl) but no pyocin. After 30 min incubation at 37 °C, DM (0.5 ml) was added to each tube. The gonococci were deposited by centrifugation (1500 g, 15 min, 37 °C) and the supernatant was removed. The gonococci were resuspended in 0.3 ml DM, which was plated out on haemoglobin agar. After incubation at 37 °C in candle extinction cans the colonies were counted. Results of the experimental samples were recorded as +, ± and – when the viable counts were <5%, <20% (but >5%), and >20%, respectively, of the colony counts for the control tubes. All gonococcal strains were tested against the 10 different pyocins in many tests (see Table 1).

RESULTS

The results are summarized in Table 1. During the 3 h induction period to resistance the serum-susceptible form of BS4 (agar) had lost its sensitivity to the pyocin produced by strain R205 and its partial sensitivity to that produced by strain IS8; also its full sensitivity to the R5 pyocin produced by strain ISC became partial. The serum- and phagocyte-susceptible strain BSSH (Penn *et al.*, 1976, 1977) showed a partial sensitivity to the R1 pyocin from strain IS6 in contrast to the insensitivity of the two forms of BS4 (agar), both of which were relatively resistant to killing by phagocytes (Penn *et al.*, 1976, 1977; N. J. Parsons & H. Smith, unpublished observations). In reactions with the pyocins from strains IS8 and ISC, BSSH behaved similarly to the serum-susceptible form of BS4 (agar). With the pyocin from strain R205, BSSH was less sensitive than the serum-susceptible form of BS4 (agar), whereas the serum-resistant form of BS4 (agar) was fully resistant to this pyocin.

DISCUSSION

The pyocin sensitivity tests suggest that a change in LPS occurs when the serum-susceptible gonococcal strain BS4 (agar) is converted to serum resistance by the low molecular weight inducer from guinea pig serum in the short time of 3 h at 37 °C. Overall, a loss of sensitivity to three pyocins accompanied the increase in serum resistance and this indicates that the LPS

Table 1. Activity of 10 different pyocins on strain BS4 (agar) before and after induction to serum resistance and on strain BSSH

Each result was obtained on at least four different tests with each pyocin and at least eight different tests with pyocins from strains IS6, IS8, R205, 9579 and ISC. The differences between the serum-susceptible and serum-resistant forms of BS4 (agar) are bracketed.

Strain of gonococci	Serum resistance	Resistance to phagocyte killing	Pyocin: Strain:	Inhibition* by pyocins from the Kageyama group									
				R1				Unclassified		R3	R2	R5	
				ISD	2285	IS6	IS8	R205	9579	ISE	ISB	ISA	ISC
BS4 (agar)	—	+		—	—	—	[±]	[+]	+	—	—	+	[+]
BS4 (agar)	+	+		—	—	—	[—]	[—]	+	—	—	+	[±]
BSSH	—	—		—	—	+	+	+	+	—	—	+	+

* Results were recorded as +, $\pm$ and — when the viable counts were <5%, <20% (but >5%), and >20%, respectively, of the counts of the control tubes not containing pyocin.

structure had been altered, since three receptor sites appear to have been removed or modified or masked by a new component. This result complements recent correlations made between the susceptibility of clinical isolates to serum killing and the presence of LPS receptors for the R1 pyocins (Winstanley *et al.*, 1983). Any speculation on the precise changes in LPS structure which accompanied the increase in serum resistance depends on the assumption that observations on pyocin reactions with the LPS of *P. aeruginosa* apply to the LPS of *N. gonorrhoeae*. Mutants of gonococci selected for resistance to R1 pyocins remain sensitive to R5 pyocins, indicating that the receptor for the R5 pyocins is in the portion of the LPS nearest to the cytoplasm and those for R1 pyocins are more distal (Winstanley *et al.*, 1983). These are similar to the results obtained for *P. aeruginosa* (Kageyama, 1975). Little can be drawn from the most marked change in pyocin sensitivity accompanying the increase in serum resistance, namely the complete loss of sensitivity to the unclassified pyocin from strain R205, since its receptor site is unknown even for the LPS of *P. aeruginosa*. It should be noted that in the majority of clinical isolates of gonococci, if the strain is sensitive to R1 pyocins it is also sensitive to the pyocin produced by strain R205 (Blackwell *et al.*, 1979). The smaller losses in sensitivity to the R1 pyocin from strain IS8 and the R5 pyocin from strain ISC suggest that receptor sites both in the polysaccharide side chain and in or near the core of the LPS may have been affected. The receptor sites may not have been produced, or they may have been modified, or even obscured by new structures such as proteins under the influence of the small molecular weight inducing factor whose mechanism of action is unknown. However, one possible masking agency, the formation of a capsule under the influence of the inducer, does not occur; in electron microscopy similar to that which demonstrated capsules on some gonococci (Demarco de Hormaeche *et al.*, 1978), capsules could not be seen on BS4 (agar) organisms either before or after being induced to resistance (P. M. V. Martin, unpublished observations).

It is tempting to suggest that changes in pyocin sensitivity which occur as the result of the action of the small molecular weight inducer indicate changes in LPS which are responsible for the increased resistance to serum killing. However, the evidence at present is only that of association (Smith, 1983) and can only be strengthened by deeper investigations on the connections with serum resistance of the LPS and any other bacterial components (for example, OMP) that may be formed under the influence of the inducer.

REFERENCES

- BLACKWELL, C. C., YOUNG, H. & ANDERSON, I. (1979). Sensitivity of *Neisseria gonorrhoeae* to partially purified R-type pyocines and a possible approach to epidemiological typing. *Journal of Medical Microbiology* **12**, 321–335.
- BLACKWELL, C. C., KOWOLIK, M., WINSTANLEY, F. P., KINANE, D. F., WEIR, D. N., LAW, J. A. & HO WANG CHOK, Y. L. (1983). ABO blood group and susceptibility to gonococcal infection. I. Factors affecting phagocytosis of *Neisseria gonorrhoeae*. *Journal of Clinical and Laboratory Immunology* **4**, 173–178.
- BROOKS, G. F., GOTSCHLICH, E. C., HOLMES, K. K.,

- SAWYER, W. D. & YOUNG, F. E. (1978). *Immunology of Neisseria gonorrhoeae*. Washington, DC: American Society for Microbiology.
- GUYMON, L. F., ESSER, M. & SHAFFER, W. M. (1982). Pyocin-resistant lipopolysaccharide mutants of *Neisseria gonorrhoeae*: alterations in sensitivity to normal human serum and polymyxin B. *Infection and Immunity* **36**, 541-547.
- DEMARCO DE HORMAECHE, R., THORNLEY, M. J. & GLAUERT, A. M. (1978). Demonstration by light and electron microscopy of capsules on gonococci recently grown *in vivo*. *Journal of General Microbiology* **106**, 81-91.
- JAMES, J. F., ZURLINDEN, E., LAMMEL, C. J. & BROOKS, G. F. (1982). Relation of protein I and colonial opacity to serum killing of *Neisseria gonorrhoeae*. *Journal of Infectious Diseases* **145**, 37-44.
- KAGEYAMA, M. (1975). Bacteriocins and bacteriophages in *Pseudomonas aeruginosa*. In *Microbial Drug Resistance: Report of the International Symposium on Bacterial Resistance*, pp. 291-309. Edited by S. Mitsuhashi & H. Hashimoto. Tokyo: University of Tokyo Press.
- LAMBLEN, P. R., HECKELS, J. E., JAMES, L. T. & WATT, P. J. (1979). Variations in surface proteins associated with virulence properties in opacity types of *Neisseria gonorrhoeae*. *Journal of General Microbiology* **114**, 305-312.
- MARTIN, P. M. V., PATEL, P. V., PARSONS, N. J. & SMITH, H. (1981). Induction of phenotypically determined resistance of *Neisseria gonorrhoeae* to human serum by factors in human serum. *Journal of General Microbiology* **127**, 213-217.
- MEADOW, P. M. & WELLS, P. L. (1978). Receptor sites for R-type pyocins and bacteriophage E79 in the core part of the lipopolysaccharide(s) of *Pseudomonas aeruginosa* PAC1. *Journal of General Microbiology* **108**, 339-343.
- MORSE, S. A., VAUGHAN, P., JOHNSON, D. & IGLEWSKI, B. H. (1976). Inhibition of *Neisseria gonorrhoeae* by a bacteriocin from *Pseudomonas aeruginosa*. *Antimicrobial Agents and Chemotherapy* **10**, 354-362.
- PARSONS, N. J., KWAASI, A. A. A., TURNER, J. A., VEALE, D. R., PERERA, V. Y., PENN, C. W. & SMITH, H. (1981). Investigation of the determinants of the survival of *Neisseria gonorrhoeae* within human polymorphonuclear phagocytes. *Journal of General Microbiology* **127**, 103-112.
- PARSONS, N. J., KWAASI, A. A. A., PERERA, V. Y., PATEL, P. V., MARTIN, P. M. V. & SMITH, H. (1982). Outer membrane proteins of *Neisseria gonorrhoeae* associated with survival within human polymorphonuclear phagocytes. *Journal of General Microbiology* **128**, 3077-3081.
- PENN, C. W., SEN, D., VEALE, D. R., WITT, K., PARSONS, N. J. & SMITH, H. (1976). Morphological, biological and antigenic properties of *Neisseria gonorrhoeae* adapted to growth in guinea pig subcutaneous chambers. *Journal of General Microbiology* **97**, 35-43.
- PENN, C. W., VEALE, D. R. & SMITH, H. (1977). Selection from gonococci grown *in vitro* of a colony type with some virulence properties of organisms adapted *in vivo*. *Journal of General Microbiology* **100**, 147-158.
- SCHNEIDER, H., GRIFFISS, J. M., WILLIAMS, G. D. & PIER, G. B. (1982). Immunological basis of serum resistance of *Neisseria gonorrhoeae*. *Journal of General Microbiology* **128**, 13-20.
- SIDBERRY, H. D. & SADOFF, J. C. (1977). Pyocin sensitivity of *Neisseria gonorrhoeae* and its feasibility as an epidemiological tool. *Infection and Immunity* **15**, 628-637.
- SMITH, H. (1983). The elusive determinants of bacterial interference with non-specific host defences. *Philosophical Transactions of the Royal Society* **B303**, 99-113.
- VEALE, D. R., PENN, C. W., PARSONS, N. J. & SMITH, H. (1980). Preliminary studies of a factor in guinea pig serum which induces serum resistance in *Neisseria gonorrhoeae*. *Society for General Microbiology Quarterly* **7**, 188.
- VEALE, D. R., PENN, C. W. & SMITH, H. (1981). Factors affecting the induction of phenotypically determined serum resistance of *Neisseria gonorrhoeae* grown in media containing serum or its diffusible components. *Journal of General Microbiology* **122**, 239-245.
- WINSTANLEY, F. P., BLACKWELL, C. C., WEIR, D. M. & KINANE, D. F. (1983). Blood groups and susceptibility to gonococcal infection. II. The relationship of lipopolysaccharide type to gonococcal sensitivity to the bactericidal activity of normal human serum. *Journal of Clinical and Laboratory Immunology* **11**, 27-32.