SHORT COMMUNICATION

The Complexity of Immunogenicity of *Neisseria gonorrhoeae* in the Guinea Pig Subcutaneous Chamber Model

By N. J. PARSONS, C. W. PENN, D. R. VEALE AND H. SMITH*

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

(Received 6 November 1979; revised 5 February 1980)

The immunogenicity of *Neisseria gonorrhoeae* in the guinea pig subcutaneous chamber model, assessed by serum bactericidal tests and challenge experiments, is complicated by diversity of immunotypes which may or may not show partial cross-reactions, by the need for antibodies to more than one type-specific antigen for full homologous protection, and possibly by the limited accessibility of the relevant antigens on the cell surface.

INTRODUCTION

It is becoming apparent that the basis of the immunogenicity of *Neisseria gonorrhoeae* in the guinea pig subcutaneous chamber model is complex and variable. Different immunotypes have been detected (Arko *et al.*, 1976; Turner & Novotny, 1976; Wong *et al.*, 1976; Penn *et al.*, 1977a, 1978), antibodies to more than one antigen appear to be involved in protection conferred by a single immunotype (Parsons *et al.*, 1979), and strains of one immunotype vary in their capacity to induce immunity (Wong *et al.*, 1979). The results in this paper underline this complexity.

We have attempted to assess the diversity of immunotypes by testing typed strains from two other laboratories, as well as additional clinical isolates, based on the previously described correlation between bactericidal activity in sera of immunized guinea pigs and immunity to infection of subcutaneous chambers.

METHODS

Organisms. Strains AS3, BS3, CS3, DS3, ES3 and FS3, described previously (Penn *et al.*, 1978), are designated Birmingham group 1. Strains α S3, β S3, δ S3, ϵ S3, θ S3 and κ S3 (Birmingham group 2) were obtained from the urethrae of male patients with the help of Dr W. Fowler, Dr J. C. Clay and staff of Birmingham General Hospital. Strains 5766, 5767 and 6611 (Buchanan group) were donated by Drs T. M. Buchanan and J. Knapp, University of Washington, Seattle, U.S.A. Strains A1, B2, C3, D4, F6 and H8 (Johnston group) were donated by Dr K. Johnston, University of Texas, Dallas, U.S.A. Strains within the latter two groups were serologically distinct. Before use, all strains were passaged three times in guinea pig subcutaneous chambers as described by Penn *et al.* (1976, 1978).

Strain BSDH was selected from a parent laboratory strain BS, for 'double highlight' (DH) colonial morphology (Penn *et al.*, 1977b).

Strain BS4PR was a 'pure rough' (autoagglutinating) pilated strain derived from strain BS4R (Parsons *et al.*, 1979) by selection for rough character from single colonies grown from dilute ($< 10^8$ ml⁻¹) suspensions of organisms, vortexed to break up clumps of gonococci. This procedure was essential to eliminate a small proportion of 'smooth' organisms (Parsons *et al.*, 1979). The predominantly 'rough' strain BS4R, lacking the major type-specific antigen *a*, was isolated from a chamber on a guinea pig immunized with rabbit antibody complexes of antigen *a*. Strains CS3PR and ES3PR were rough organisms derived from the pre-

dominantly smooth strains CS3 and ES3 by similar single colony selection for rough character. Surface washes of BS4PR, CS3PR and ES3PR lacked antigen *a* (Penn *et al.*, 1978; Parsons *et al.*, 1979).

Culture media. These were described by Veale *et al.* (1975) and Penn *et al.* (1976).

Bactericidal tests. These were conducted as described by Penn *et al.* (1978), with sera from guinea pigs immunized by chamber infection. In cross-reaction tests, strains were used both for the production of sera and as test organisms. Results are expressed as follows: ++, viable counts decreased by 90 to 100% from initial values after 4 h incubation; +, 70 to 89% decrease; $\pm$, 10 to 69% decrease; -, viable counts decreased by less than 10%, or increased during incubation.

Immunity tests in guinea pigs with live organisms. Limited tests were conducted as described by Penn *et al.* (1978) to investigate the correlation between immunity and bactericidal activity. Between two and nine chambers were challenged in any one cross-immunity test. Results are shown as: (number of chambers immune)/(number of chambers challenged). A 'good correlation' between the bactericidal and immunity tests is reported (i) if for strains showing complete cross-reaction in the bactericidal tests, more than half the chambers were immune in cross-immunity tests or (ii) if for strains not showing cross-reactions in the bactericidal tests, not more than one chamber was immune to challenge in the cross-immunity tests.

Immunity tests in guinea pigs with type-specific antigen a or killed gonococci. Antibody complexes of type-specific antigen *a*, and killed gonococci, were prepared and administered to guinea pigs as described by Parsons *et al.* (1979) and Penn *et al.* (1977a), respectively, except for BS4PR where three injections of 10^9 gonococci were given at 14 d intervals, emulsified with an equal volume of Freund's complete adjuvant on the first two occasions. Challenging of chambers and assessment of immunity was as described previously by Penn *et al.* (1977a) and Parsons *et al.* (1979).

Two-dimensional immunoelectrophoresis. Surface washes for antigenic analysis, rabbit antisera and electrophoresis techniques were as described previously by Penn *et al.* (1978) and Parsons *et al.* (1979). Sonicates of BSDH were prepared by sonicating suspensions [5×10^{10} ml⁻¹ in Trypticase Soy Broth (BBL)] at 20 kHz, 50 W, 30 s, 0 °C (Rapidis 180, Ultrasonics, Shipley, Yorks.) followed by centrifugation at 3000g for 10 min at room temperature.

RESULTS AND DISCUSSION

Cross-reactions between strains in serum bactericidal tests

In the six isolates of Birmingham group 1 two pairs of completely cross-reacting strains, and a partially cross-reacting pair were demonstrated (Penn *et al.*, 1978). Since there was no cross-reaction between the pairs and a good correlation between bactericidal and immunity tests was observed, the conclusion was that there were three immunotypes. Table 1(a) shows the cross-reactions of the strains of Buchanan, Johnston and Birmingham group 2 among themselves and between them and the three immunotypes of Birmingham group 1. Cross-reactions between the groups of Buchanan, Johnston and Birmingham group 2 were not examined. In the tests there were several one-way cross-reactions in which one strain produced a serum bactericidal for another strain, but not vice versa, e.g. between BS3 and κ S3. Within the Buchanan and Johnston groups there was only one cross-reaction (between D4 and H8) and killing was not as great as with most homologous reactions. In Birmingham group 2, four strains cross-reacted completely. The other two (θ S3 and κ S3) showed only one-way cross-reactivity with one (δ S3) of these four strains, and between themselves. All the Buchanan group showed one-way or complete cross-reactivity with one or more of the three immunotypes of Birmingham group 1 but correlation of these bactericidal tests with immunity tests was poor (see below). Of the Johnston group only two cross-reacted with any of the immunotypes of Birmingham group 1. All strains of the Birmingham group 2 cross-reacted with one or more of the three immunotypes of Birmingham group 1 but there was only one complete cross-reaction (ϵ S3 and BS3). However, strains α S3, β S3 and δ S3, which cross-reacted completely with ϵ S3, showed only one-way cross-reaction with BS3.

Cross-reactions between strains in immunity tests

Immunity tests could not be performed on the same scale as bactericidal tests. Sufficient cross-protection tests were done to check whether the good correlation between serum bactericidal activity and immunity to chamber challenge noted previously (Penn *et al.*,

Table 1. *Cross-reactions between strains in serum bactericidal tests and immunity tests*

The extent of serum killing and immunity is expressed as described in Methods. A space indicates that the test was not done.

Antiserum to strain:	(a) <i>Bactericidal tests</i>																						(b) <i>Immunity tests</i>												
	Test strain													Challenge strain																					
	AS3	ES3	CS3	DS3	ES3	FS3	5766	5767	6611	A1	B2	C3	D4	F6	H8	α S3	β S3	δ S3	ϵ S3	θ S3	κ S3														
AS3	++	++	-	-	-	-	-	-	-	++	-	-	-	-	-	-	±	-	±	++	-	++													
BS3	++	++	-	-	-	-	-	-	++	+	-	-	-	-	-	-	±	-	-	++	-	±													
CS3	-	-	++	++	-	-	++	++	++	-	-	-	-	-	-	-	-	-	-	-	-	-													
DS3	-	-	+	+	-	-	++	-	-	-	-	-	-	-	-	-	-	±	-	-	-	±													
ES3	-	-	-	-	++	++	++	-	-	-	++	-	-	-	-	-	-	-	-	-	-	-													
FS3	-	-	-	-	++	++	++	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-													
5766	-	-	++	-	++	++	++	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-													
5767	++	++	-	-	++	++	++	++	-	-	-	-	-	-	-	-	-	-	-	-	-	-													
6611	-	-	-	-	-	-	-	-	++	++	-	-	-	-	-	-	-	-	-	-	-	-													
A1									++	-	-	-	-	-	-	-	-	-	-	-	-	-													
B2									-	+	-	-	-	-	-	-	-	-	-	-	-	-													
C3									-	-	-	++	-	-	-	-	-	-	-	-	-	-													
D4									-	-	-	-	++	-	+	-	-	-	-	-	-	-													
F6									-	-	-	-	-	-	-	-	-	-	-	-	-	-													
H8									-	-	-	-	-	+	+	-	-	-	-	-	-	-													
α S3	++	+	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	-	-													
β S3	++	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	-	-													
δ S3	++	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	-	-													
ϵ S3	++	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	-	-													
θ S3	-	+	++	+	++	+	+	+	+	+	+	+	+	+	+	-	±	±	±	±	±	±													
κ S3	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	±	±	±	±	±	±	±													
Immunizing strain	AS3	BS3	CS3	DS3	ES3	FS3	5766	5767	6611	A1	B2	C3	D4	F6	H8	α S3	β S3	δ S3	ϵ S3	θ S3	κ S3														
AS3	3/3	3/3	0/3						0/2	0/3	0/3	0/3																							
BS3	5/5	10/10	0/4					0/2	0/2	0/3	0/3	0/3																							
CS3	0/8	0/8	6/9	3/5															2/5			3/5													
DS3		0/3	0/3	4/5																															
ES3		0/3	0/3	0/3	9/10	3/3				1/3	0/3	0/3																							
FS3		0/3	0/3	0/4	4/4	10/10																													
5766							2/2																												
5767								1/3																											
6611									1/3																										
α S3		4/4													9/9				4/4		0/5														

1978) held for the new strains (Table 1*b*). Apart from tests on the Buchanan strains (for which only two out of five tests showed good correlation with the bactericidal tests) the correlation was reasonably good (four of six tests with the Johnston strains and five of six tests with Birmingham group 2 showed good correlation).

It is not possible to assess the precise number of immunotypes among the 21 strains because the relationships between the Buchanan strains, the Johnston strains and those of the Birmingham group 2 have not been investigated; additional complications arose from one-way cross-reactions and the incomplete correlation between the serum bactericidal tests and immunity tests. However, there appear to be at least eight immunotypes.

Type specificity of immunity to rough gonococcal strains

Rough strains selected from smooth populations of the three immunotypes of Birmingham group 1 apparently did not share common protective antigens. Immunization with killed strain BS4PR did not protect guinea pig chambers against infection with either strain CS3PR (0/10 chambers protected) or strain ES3PR (0/10) although chambers were completely protected (20/20) against homologous challenge. This implies that there must be type-specific immunizing antigens in addition to antigen *a*.

Evidence for antigens a, b, c and d in strain BSDH

Gonococci selected *in vivo* (strains BS4 and BS4R) were compared with strain BSDH which was selected *in vitro* but had the DH colony morphology characteristic of strains adapted *in vivo* (Penn *et al.*, 1977*b*). Surface washes of strain BSDH contained neither the type-specific antigen *a* associated with smooth strains selected *in vivo*, nor antigens *b*, *c* and *d* associated with both rough and smooth organisms found in guinea pig chambers (Penn *et al.*, 1978; Parsons *et al.*, 1979). However, immunization of rabbits with BSDH produced antibodies against antigens *a*, *b*, *c* and possibly *d*, shown by two-dimensional immunoelectrophoresis. Adsorption of rabbit antiserum to strain BS4 with strain BSDH removed antibodies to antigens *b*, *c* and *d* and diminished those to antigen *a*. A sonicate of BSDH formed *b*, *c* and *d* lines in two-dimensional immunoelectrophoresis with antisera against strain BS4 (agar). Thus, strain BSDH appears to contain antigens *a*, *b*, *c* and *d* in reduced amounts or in a form such that they are not released in surface washes.

Our earlier observation (Parsons *et al.*, 1979) that immunization with killed BSDH organisms did not protect chambers from infection with strain BS4 was repeated and confirmed; but immunization with killed BSDH (only tested at high doses of 10⁹ organisms per injection) and an antigen *a*/antibody complex excised from gels protected 8/10 chambers from challenge with strain BS4. Thus, when used in combination with the antigen *a*/antibody complex, strain BSDH appeared similar in immunizing capacity to strain BS4R (Parsons *et al.*, 1979), and our inference that additional antigen(s) (which may be *b* or *c* or *d* or others not detected) are needed for immunity as well as antigen *a* (Parsons *et al.*, 1979) is unchanged. The poor immunogenicity of strain BSDH alone, against challenge with the smooth strain BS4, may be related to a reduced content of antigen *a* or factors inhibiting its release or activity.

There is no doubt that immunity to the gonococcus is complex. Firstly, a survey of laboratory strains and fresh isolates for cross-reactivity in serum bactericidal tests and in immunity tests has revealed new immunotypes, as well as types that cross-react partially or fully with previously described immunotypes. Secondly, the connection between serum bactericidal activity and immunity noted by us (Penn *et al.*, 1977*a*, 1978) and others (Wong *et al.*, 1976, 1979) holds for many but not all strains of gonococci. Thirdly, the possibility that the immunizing capacity of 'rough' gonococci lacking a major type-specific antigen (Parsons *et al.*, 1979) might be due to the presence of common immunizing antigens was

not supported by tests on three immunotypes. Finally, accessibility or quantity of antigens may be important for immunizing capacity.

REFERENCES

- ARKO, R. J., WONG, K. H., BULLARD, J. L. & LOGAN, L. C. (1976). Immunological and serological diversity of *Neisseria gonorrhoeae*: immunotyping of gonococci by cross-protection in guinea pig subcutaneous chambers. *Infection and Immunity* **14**, 1293-1295.
- PARSONS, N. J., PENN, C. W., VEALE, D. R. & SMITH, H. (1979). More than one antigen contributes to the immunogenicity of *Neisseria gonorrhoeae* in the guinea pig chamber model. *Journal of General Microbiology* **113**, 97-104.
- PENN, C. W., SEN, D., VEALE, D. R., PARSONS, N. J., SMITH, H. & WITT, K. (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., PARSONS, N. J., SEN, D., VEALE, D. R. & SMITH, H. (1977*a*). Immunization of guinea pigs with *Neisseria gonorrhoeae*: strain specificity and mechanisms of immunity. *Journal of General Microbiology* **100**, 159-166.
- PENN, C. W., VEALE, D. R. & SMITH, H. (1977*b*). 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.
- PENN, C. W., PARSONS, N. J., VEALE, D. R. & SMITH, H. (1978). Correlation with different immunotypes of gonococcal antigens associated with growth *in vivo*. *Journal of General Microbiology* **105**, 153-157.
- TURNER, W. H. & NOVOTNY, P. (1976). The inability of *Neisseria gonorrhoeae* pili antibodies to confer immunity in subcutaneous guinea pig chambers. *Journal of General Microbiology* **92**, 224-228.
- VEALE, D. R., SMITH, H., WITT, K. & MARSHALL, R. B. (1975). Differential ability of colonial types of *Neisseria gonorrhoeae* to produce infection and an inflammatory response in subcutaneous perforated plastic chambers in guinea pigs and rabbits. *Journal of Medical Microbiology* **8**, 325-335.
- WONG, K. H., ARKO, R. J., LOGAN, L. C. & BULLARD J. C. (1976). Immunological and serological diversity of *Neisseria gonorrhoeae*: gonococcal serotypes and their relation with immunotypes. *Infection and Immunity* **14**, 1297-1301.
- WONG, K. H., ARKO, R. J., SCHALLA, W. O. & STEURER, F. J. (1979). Immunological and serological diversity of *Neisseria gonorrhoeae*: identification of new immunotypes and highly protective strains. *Infection and Immunity* **23**, 717-722.