SHORT COMMUNICATIONS

Correlation with Different Immunotypes of Gonococcal Antigens Associated with Growth *in vivo*

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

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

(Received 8 November 1977)

INTRODUCTION

Observations on six isolates of gonococci adapted to growth in subcutaneous plastic chambers in guinea pigs (AS3, BS3, CS3, DS3, ES3, FS3) showed that the conditions *in vivo* selected, from a population grown *in vitro*, heavily pilated organisms which formed colonies with a characteristic double highlight (DH) reflection when viewed by a combination of transmitted and reflected light (Penn, Veale & Smith, 1977*b*). Investigation of one of the strains adapted to growth *in vivo* (BS3) showed it to be more resistant to killing by human phagocytes and by human serum than the parent strain grown *in vitro* (BS) (Penn *et al.*, 1976, 1977*b*). Furthermore, surface washes of this strain adapted to growth *in vivo*, after one subculture on agar medium, contained one or possibly two antigens, detectable by gel-diffusion against guinea-pig antiserum, which were not found in similar washes of the parent strain grown *in vitro* or of DH organisms selected from it *in vitro* (Penn *et al.*, 1976, 1977*b*). Thus, the *in vivo* conditions had selected organisms not only showing DH colony character but also having the additional antigens.

Immunological heterogeneity of gonococcal strains has been indicated in active immunity experiments in chimpanzees (Arko *et al.*, 1974) and in guinea pigs (Turner & Novotny, 1976). Preliminary observations on the six gonococcal strains adapted to growth in guinea-pig chambers showed that if animals were infected with strain BS3 in one chamber they became immune to challenge in a second chamber by this strain and only one other (AS3) (Penn *et al.*, 1977*a*). This work and similar observations by others (Wong *et al.*, 1976) indicated that the pattern of the bactericidal action of guinea-pig antiserum might reflect that of the immunotypes. Further investigations reported here showed the presence of three immunotypes in the six strains and strengthened the evidence for a parallel between chamber immunity and serum bactericidal activity. More importantly, surface washes of representative strains of the three immunotypes have shown, in two-dimensional immunoelectrophoresis against rabbit antisera, evidence of antigens associated with adaptation *in vivo*; and these antigens were of similar electrophoretic behaviour but showed no cross-reactivity. The antigens were also found in surface washes of some, but not all, colonies selected from the first or second subculture of human gonorrhoeal pus.

METHODS

Bacteria. Gonococcal strains AS3, BS3, CS3, DS3, ES3 and FS3 were derived (by passage through guinea-pig chambers of laboratory cultures AS, BS and DS and fresh gonococcal isolates CS, ES and FS), stored, cultured and counted (Penn *et al.*, 1976, 1977*a*). After one subculture (24 h) on agar-based medium (Penn *et al.*, 1976, 1977*b*) they were designated AS3 (agar), BS3 (agar) etc. Strain BSDH was also derived (by selecting double highlight colonies from *in vitro* cultures of strain BS), stored, cultured and counted (Penn *et al.*, 1976).

Immunity tests. These were conducted in guinea pigs bearing two subcutaneously implanted plastic chambers; an immunizing infection of chamber adapted organisms was established in one chamber and the challenge dose, again of chamber adapted organisms, was inoculated into the other (Penn *et al.*, 1977*a*).

Results are quoted as:

$$\frac{\text{number of chambers found immune (Penn *et al.*, 1977a)}}{\text{number of chambers challenged}}$$

Guinea-pig antisera. These were prepared and stored as previously (Penn *et al.*, 1977a).

Bactericidal tests. Tests on gonococci with guinea-pig antisera were conducted by the method of Penn *et al.* (1977a) using normal guinea-pig serum as control. On incubation at 37 °C for 4 h in normal serum gonococci multiplied to give viable counts up to 10-fold greater than at the outset. An antiserum was considered to exhibit bactericidal activity (+ in Table 1) if, after this period of incubation, the viable count was less than 1 % of the control, and to be devoid of bactericidal activity (— in Table 1) if the viable count was in excess of 80 % of the control, i.e. multiplication had taken place. In some antisera the count of gonococci was < 10 % but > 1 % of the control; these antisera were considered to have intermediate ($\pm$ in Table 1) bactericidal activity. For each combination of antiserum and test strain two different antisera were examined in a total of two to four tests.

Surface washes for antigenic analysis. These were prepared from gonococcal strains BS3 (agar), CS3 (agar), ES3 (agar) and BSDH by suspending them in Trypticase Soy Broth (BBL) (Penn *et al.*, 1976, 1977b) at a concentration of 10^{11} total organisms ml⁻¹, mixing in a vortex mixer for 30 s with glass beads (3 mm diam.) at room temperature and removing the bacteria by centrifugation (4000 g, 15 min, room temperature).

Rabbit antisera for immunoelectrophoresis. Antisera were raised in rabbits by intravenous injections of live BS3 (agar), CS3 (agar) and ES3 (agar); six injections of about 10^8 organisms were given at 3 to 4 day intervals over 3 weeks and the rabbits were bled 1 to 2 weeks after the final injection.

Two-dimensional immunoelectrophoresis (Axelsen & Bock, 1972). This was performed with surface washes and rabbit antisera in 1 % agarose (Litex HSA, International Enzymes Ltd, Windsor, Berkshire) in barbitone buffer, ionic strength 0.1, pH 8.6. After electrophoresis of the antigen sample towards the anode in one dimension (1 h, 1.6 V mm⁻¹), electrophoresis in the second dimension (3 h, 0.8 V mm⁻¹) was continued towards the anode into a gel containing antiserum (4 μ l cm⁻²).

RESULTS

In immunity tests with the six strains adapted to growth in guinea-pig chambers, three immunotypes emerged (Table 1, columns I). As observed before (Penn *et al.*, 1977a), AS3 and BS3 showed cross-immunity but neither cross-immunized with the other strains. Similarly ES3 and FS3 showed cross-immunity but not with the other strains. The interaction between CS3 and DS3 was more complex although neither showed cross-immunity with the other strains. CS3 immunized against challenge with itself and DS3 but the immunity produced in both cases did not appear as consistent as for the other strains; not all the immunized animals were resistant to infection of the second chamber with either CS3 or DS3. Strain DS3 immunized consistently against challenge with itself but not with CS3. Thus, CS3 was an immunotype distinct from the AS3/BS3 and ES3/FS3 types, with some cross-reactivity with DS3.

The results of the serum bactericidal tests (Table 1, columns B) paralleled those from the immunity tests (Table 1) with one exception; anti-DS3 serum was weakly bactericidal for strain CS3 despite the absence of significant immunity in the appropriate active immunity tests. Anti-DS3 serum also had a weaker bactericidal activity against itself than the other antisera in homologous tests. This cross-activity between DS3 and CS3 in bactericidal tests, although not shown in active immunity tests with DS3-immunized animals, was shown by CS3-immunized animals as regards both chamber challenge and serum bactericidal tests (Table 1). With the more convenient and less expensive serum bactericidal test, the complete pattern of cross-testing between the six strains was accomplished and supported the presence of three immunotypes.

The pattern obtained in two-dimensional immunoelectrophoresis of surface washes of the BSDH colony type, selected *in vitro*, against antisera prepared to the *in vivo* adapted strain BS3 (agar) (Fig. 1a) was similar to that shown by the wash of the *in vivo* adapted strain BS3 (agar) (Fig. 1b), except that the latter showed an additional strong, straight, precipitin line originating from the antigen well and extending towards the anode almost parallel with

Table 1. *Results of cross-immunity tests in guinea-pig chambers with six gonococcal strains adapted to growth in guinea-pig chambers, and of bactericidal tests with corresponding antisera.*

Strain used to immunize by live chamber infection and to prepare antiserum	Strain used for challenge of guinea-pig chambers (I) and test strain for bactericidal tests (B)											
	AS3		BS3		CS3		DS3		ES3		FS3	
	I*	B†	I	B	I	B	I	B	I	B	I	B
AS3	3/3	+	3/3	+	0/3	—	NT	—	NT	—	NT	—
BS3	5/5	+	10/10	+	0/4	—	NT	—	NT	—	NT	—
CS3	0/3	—	0/3	—	6/9	+	3/5	+	NT	—	NT	—
DS3	NT	—	0/3	—	0/3	±	4/5	±	NT	—	NT	—
ES3	NT	—	0/3	—	0/3	—	0/3	—	9/10	+	3/3	+
FS3	NT	—	0/3	—	0/3	—	0/4	—	4/4	+	10/10	+

NT, Not tested.

* No. of chambers found immune to challenge

No. of chambers challenged

† Bactericidal tests designated positive (+), intermediate (±) or negative (—) as described in Methods.

the first dimension of electrophoresis (arrowed in Fig. 1*b*). This line was characteristic of the patterns shown by washes of all the *in vivo* adapted immunotypes against their homologous antisera (arrowed in Fig. 1*b, f* and *j*). This antigen was type-specific since it was not found in the patterns formed by the washes against heterologous antisera (Fig. 1*c, d, e, g, h* and *i*). The three antisera varied in the range and strength of antibodies against antigens common to the three immunotypes (Fig. 1). However, the precipitin line to which our attention has been directed is the only one which, although similar in electrophoretic character between the immunotypes, showed the differences of specificity which paralleled the active immunity tests.

The inability of the BSDH colony type selected *in vitro* to produce the type-specific antigen prompted an investigation of the frequency with which the antigen occurred in clones, derived by single colony subculture on to individual agar plates, of the organisms adapted to growth in guinea-pig chambers. Thirty-five clones out of 41 derived from strain BS3 (agar) formed the antigen, as did 8 out of 13 clones from CS3 (agar) and 9 out of 9 clones from ES3 (agar). Obviously the ability to form the type-specific antigen can be lost.

Strains CS3 (agar) and ES3 (agar) were derived by passage in guinea pigs of organisms (strains CS and ES) obtained from colonies of the first culture of two samples of gonorrhoeal pus. On examining clones from plating the original strains CS and ES, 1 of 30 clones from CS and 10 of 19 clones from ES produced the antigen.

DISCUSSION

Three different immunotypes were found in the six strains of gonococci. The patterns of serum bactericidal activity reflected these immunotypes with the exception of the weak activity of anti-DS3 serum against CS3 which occurred despite the lack of cross-immunity detected in chamber challenge. Both bactericidal and immunity tests with CS3-immunized animals clearly showed some cross-reactivity between CS3 and DS3 but this was weaker than that between the other two pairs of strains. Only three chambers of DS3-immunized animals were challenged with CS3 and a low grade cross-immunity might have been detected if more animals had been immunized and more chambers challenged. This work and that of others (Arko *et al.*, 1974; Turner & Novotny, 1976) clearly indicates that gauging the number

Fig. 1. Two-dimensional immunoelectrophoretic patterns of antigens in surface washes of the *in vitro* colony type BSDH and the *in vivo* adapted strains BS3 (agar), CS3 (agar) and ES3 (agar) against rabbit antisera to these three live strains. Arrows indicate type-specific antigen.

and spread of different immunotypes is a crucial requirement in any assessment of the possibility of a gonococcal vaccine. Serum bactericidal activity may be a useful parameter for this purpose.

An antigen associated with adaptation of gonococci to growth in guinea-pig chambers, and found in organisms of some colonies derived from recent isolates from human gonorrhoeal pus, has shown a type-specificity parallel with three guinea-pig immunotypes. These correlations are more than interesting. The type-specific antigen may be an immunizing antigen and in this connection others (Arko, Bullard & Duncan, 1976) and ourselves (unpublished observations) have noted that gonococci adapted to growth in mouse and guinea-pig chambers, respectively, appear to have a greater immunogenicity than the parent organisms grown *in vitro*.

The frequency of loss of the type-specific antigen on cloning the strains adapted to growth in guinea-pig chambers or those from first culture of urethral pus emphasizes the importance of relating observations on gonococci grown *in vitro* to those grown *in vivo*. It has two implications if the antigen proves to be useful either in vaccination or diagnosis. First, it would be prudent to use for the production of the antigens, cultures not far removed from primary cultures of organisms adapted to growth *in vivo*. Second, frequent monitoring for antigen production would be needed because even at an early stage some colonies might contain organisms which do not form the antigen.

Further research will tell whether the type-specific antigen is an immunizing antigen and whether it is connected with other preparations such as outer membrane materials that appear to be immunogenic (Buchanan & Arko, 1977) or type-specific (Johnston, Holmes & Gotschlich, 1976). It is certainly not a pilus antigen since the BSDH strain providing antigens with the profile shown in Fig. 1(a) produced abundant pili yet it does not show the type-specific antigen.

REFERENCES

- ARKO, R. J., BULLARD, J. C. & DUNCAN, W. P. (1976). Effects of laboratory maintenance on the nature of surface reactive antigens of *Neisseria gonorrhoeae*. *British Journal of Venereal Disease* **52**, 316-325.
- ARKO, R. J., KRAUS, S. J., BROWN, W. J., BUCHANAN, T. M. & KUHN, U. S. G. (1974). *Neisseria gonorrhoeae*: effect of systemic immunisation on resistance of chimpanzees to urethral infection. *Journal of Infectious Diseases* **130**, 160-164.
- AXELSEN, N. H. & BOCK, E. (1972). Identification and quantitation of antigens and antibodies by means of quantitative immunoelectrophoresis. A survey of methods. *Journal of Immunological Methods* **1**, 109-121.
- BUCHANAN, T. M. & ARKO, R. J. (1977). Immunity to gonococcal infection induced by vaccination with isolated outer membranes of *Neisseria gonorrhoeae*. *Journal of Infectious Diseases* **135**, 879-887.
- JOHNSTON, K. H., HOLMES, K. K. & GOTSCHLICH, E. C. (1976). The serological classification of *Neisseria gonorrhoeae* I. Isolation of the outer membrane complex responsible for serotype specificity. *Journal of Experimental Medicine* **143**, 741-758.
- 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. (1977a). 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. (1977b). 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.
- 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.
- 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.