PROTECTION BY MONOSPECIFIC GONOCOCCAL ANTISERA OF THE CHICKEN EMBRYO CHALLENGED WITH *NEISSERIA GONORRHOEAE*

JANET N. ROBERTSON

Microbiology—Faculty of Medicine, South Laboratory and Pathology Block, Southampton General Hospital, Tremona Road, Southampton SO9 4XY

RECENT studies with the chick embryo as a model have investigated the infecting ability of virulent and non-virulent colony types of gonococci (Kellogg *et al.*, 1963) and the effect of the inoculation route (Buchanan and Gotschlich, 1973; Bumgarner and Finkelstein, 1973; Foster and Vinson, 1977), but little is known about the pathogenesis of this infection.

To initiate infection after intravenous challenge, gonococci must be resistant to the embryo's phagocytic defence systems and be capable of tissue invasion. Surface components of the gonococcus are presumably responsible and for this reason we have raised antisera to single purified components of the gonococcal surface and investigated the role of these sera in the protection of the chick embryo against gonococcal infection.

MATERIALS AND METHODS

Gonococcus strain. *Neisseria gonorrhoeae* strain P9(PS) was isolated from a patient with gonorrhoea and was freeze-dried after minimum subculture. Colony types T1, T2 and T4 (Kellogg *et al.*, 1963) were selected and stored in liquid nitrogen (Ward and Watt, 1971). The gonococci were grown overnight on solid gonococcal (GC) medium at 36°C in an atmosphere of air with 5% carbon dioxide and at a relative humidity of 100%. A standard inoculum of gonococci was prepared from a suspension containing approximately 1×10^8 organisms per ml in organ culture medium (OCM) followed by centrifugation for 5 min. at 1500 *g* to remove any large clusters of organisms. The number of live gonococci in the inoculum was determined by a viable-count procedure.

Media. Solid GC medium was prepared from GC Base (Difco) with the addition of 1% of Agar No. 1 (Oxoid) and 2% of Kellogg supplement (Kellogg *et al.*, 1963) modified to contain 0.2% ferric citrate. The organ-culture medium (Gibco Biocult, Paisley, Scotland) was Basal Medium Eagle with Hanks salts and Hepes buffer to which was added glutamine to give a final concentration of 2mm, and the concentration of Hepes buffer was raised to 50mm.

Preparation of antigens. Strain P9 (PS) gonococci were grown in bulk on GC Agar Base (Difco) and the pili were purified from them (Robertson, Vincent and Ward, 1977). Outer envelope was prepared from the residual depilated organisms by lithium acetate extraction and the two major outer envelope proteins were purified (Heckels, 1977). Gonococcal lipopolysaccharide (LPS) was extracted by the phenol-water method and further purified by enzyme digestion as described by Stead *et al.*, (1975).

Antisera. Adult New Zealand white rabbits were given a course of five subcutaneous injections each of 1 ml of a pure preparation of a single gonococcal antigen in incomplete Freund's adjuvant; the doses were spaced at fortnightly intervals and then the rabbits were bled

Received 30 Jan. 1978; revised version accepted 27 Nov. 1978.

2 weeks after the last injection. The dose of antigen from strain P9(PS) for each injection was for one rabbit 50 μ g of pili (Robertson *et al.*, 1977); for the second 100 μ g of colony type T4 outer membrane; for the third 50 μ g of protein I (m.w. 36 500 daltons) extracted from the outer membrane (Heckels, 1977); for the fourth rabbit 50 μ g of protein II (m.w. 24 000 daltons) extracted from the outer membrane; and for the fifth 50 μ g of lipopolysaccharide (Stead *et al.*, 1975).

Absorption of antisera. Trace amounts of contaminating antibodies were removed from the rabbit antisera by absorption with the appropriate antigen covalently bound to CNBr-activated Sepharose beads (Pharmacia, Uppsala, Sweden). The antipilus serum was absorbed with outer-membrane components; the antibody to the outer-membrane protein was absorbed with lipopolysaccharide and the normal rabbit serum with a complex of antigens from the gonococcal surface to remove any naturally occurring antibodies. Each serum was absorbed in 5-ml batches with Sepharose beads in a column of 18-cm $\times$ 1.5-cm capacity. The serum was allowed to react for 24 h at room temperature before being pumped out of the column. The IgG antibody titres were determined, after absorption, by solid-phase radioimmunoassay (Lambden and Watt, 1978).

Heat inactivation of antisera. Complement was inactivated by heating the antisera at 56°C for 30 min.

Chicken-embryo protection test. Specific pathogen-free 11-day-old embryonated eggs were obtained from a flock of White Leghorn hens (Wickham Laboratories, Wickham, Hants). Before inoculation the eggs were candled, the shell over the air sac was removed and the eggs were cooled at 4°C for 30 min. A suitable blood vessel was selected for inoculation and 100 μ l of a known number of colony-typed gonococci in a known concentration of rabbit IgG antibody was injected. The eggs were sealed with Sellotape and incubated at 37°C. After 5 h the eggs were examined; embryos that had been damaged by bleeding were discarded and the remaining eggs incubated for 4 days and examined daily.

Challenge dose of gonococci. The LD₅₀ of strain P9 (PS) was determined by the method of Reed and Muench (1938). A challenge dose of 50 $\times$ LD₅₀ was used in the protection test and was prepared immediately before injection.

Homogenisation of embryos. Individual embryos were homogenised for 2 min. in 5 ml of proteose peptone in a screw-capped glass container with a Micro-model Sealed-unit Laboratory Mixer (Silverson Machines Ltd, Waterside, Chesham, Bucks). Viable counts were made by plating 50- μ l portions of tenfold dilutions of the homogenates on to plates of GC medium and incubating at 36°C for 48 h.

RESULTS

The proportion of eggs surviving a standard gonococcal challenge in each series of tests with one of the prepared antisera was compared with the proportion surviving in tests with normal rabbit serum (NRS), and the significance of the result was assessed by a χ -square test incorporating a continuity correction (Yeomans, 1973). Experiment 1 (table I) showed the numbers of chick embryos surviving at 48 h after challenge with an inoculation of gonococci in organ-culture medium (OCM) containing 3 μ g of IgG of each specific antibody. Antiserum raised against whole outer membrane of strain P9 (PS) colony type T4 showed convincing protection when compared with NRS (test statistic corresponding to a significance level of only 5%). There was less evidence of protection for outer-membrane (OM) antiserum that had been absorbed to remove the lipopolysaccharide (LPS) component (test statistic corresponding to a significance level of 15%). Anti-LPS also showed some evidence of protection. Sera raised against protein I and protein II showed no protection at the 10% significance level. The results of a duplicate experiment

TABLE I

Comparison of the protective effect of monospecific antisera and normal serum for the chick embryo challenged with a lethal dose of gonococci

Antiserum effective against	Experiment 1*		Experiment 2†	
	Number of embryos dead at 48 h/number challenged	χ^2	Number of embryos dead at 48h/number challenged	χ^2
T4 outer membrane	4/11	3.92§	6/11	5.83§
Outer membrane minus LPS	5/10	1.88	10/11	0.60
LPS	4/9	2.53	...	...
Outer membrane protein I	4/6	0.49	9/11	1.62
Outer membrane protein II	10/10	1.57	11/11	0.002
Nil (control: absorbed normal rabbit serum)‡	7/10	...	10/11	...

* In experiment 1 the challenge dose contained gonococci (1.2×10^7) – (5.3×10^7) and monospecific IgG 3 μ g. Seven control embryos were challenged with this dose of gonococci in the absence of any serum and all died.

† In experiment 2 the challenge dose contained gonococci (6.3×10^6) – (1.2×10^7) and monospecific IgG 1 μ g.

‡ Normal rabbit serum was used at the same concentration as the lowest titre of monospecific antiserum.

§ Test statistic is significant at the 5% level.

TABLE II

Comparison of the protective effects of antipilus serum and normal rabbit serum on embryos challenged with piliate and non-piliate gonococci

Amount (μ g) of antipilus IgG per embryo	Experiment with piliate gonococci*		Experiment with non piliate gonococci†	
	Number of embryos dead at 48h/number challenged	χ^2	Number of embryos dead at 48 h/number challenged	χ^2
1.0	1/9	6.25§	4/12	0.04
0.25	11/13	...	3/12	...
0.05	11/11	...	6/11	...
Control (normal rabbit serum)‡	5/9	...	2/10	...

* Challenge dose contained piliate gonococci (2.7×10^6) – (4×10^6)

† Challenge dose contained non-piliate gonococci (3.3×10^5) – (1×10^6)

‡ Normal rabbit serum was used at a concentration corresponding to that of the specific antipilus serum when diluted to contain IgG 1 μ g per challenge dose.

§ Test statistic is significant at almost the 1% level.

(experiment 2, table I) confirmed that whole T4 OM antiserum was protective at the 5% significance level.

Table II compares the protective effects of antipilus serum and normal rabbit serum against challenges with piliate and non-piliate gonococci. The same statistical analysis provided very strong evidence at almost the 1% significance level that 1 μ g of specific antipilus IgG protected the embryo from challenge with homologous piliate gonococci. The challenge with non-piliate (type T4) gonococci was unaffected by the presence of the antipilus serum.

TABLE III

The effect of lipopolysaccharide (LPS) antiserum on the multiplication of gonococci in the chick embryo

log ₁₀ viable counts per embryo* at 20 h when challenge injection contained	
absorbed normal rabbit serum	LPS antiserum
6.56	6.24
6.72	6.34
7.29	6.51
7.75	6.95
7.83	7.08
mean 7.23	6.62

* The challenge dose contained gonococci 4.3×10^6 and either anti-LPS IgG $1 \mu\text{g}$ or absorbed normal rabbit serum at equal concentration. At time 0 the mean number of gonococci recovered from control preparations was 7.75×10^2 per embryo. Each log viable count recorded above is the result obtained with one test embryo.

The results of an experiment to investigate the mechanism of protection by anti-LPS antibodies are given in table III. Twenty hours after challenge with gonococci together with LPS antiserum or with normal rabbit serum, the embryos were homogenised individually and viable counts were made to determine the numbers of surviving gonococci. In the presence of anti-LPS antiserum the counts were significantly lower than with normal rabbit serum (*t* test significant at 3%).

DISCUSSION

Neisseria gonorrhoeae strain P9(PS) is a known virulent strain. It is resistant to the bactericidal effect of human serum and has caused an eye infection in a laboratory worker. No attempt was made to lower the LD₅₀ of this strain by passage in eggs lest gonococci selected for their virulence in eggs be atypical organisms. The outer membrane of the cell wall of gonococci, including strain P9(PS), bears surface proteins, lipopolysaccharide and filamentous pili. Protein I comprises approximately half the surface protein of the gonococcus (Heckels, 1978) and is probably important for the structure and integrity of the outer membrane. Antibodies to the major proteins are bactericidal (Buchanan *et al.*, 1977; Ward *et al.*, 1978), and Buchanan has shown that antiserum raised against the outer-membrane components confers protection in the guinea-pig chamber model.

Gonococcal LPS consists of a core of lipid A and a variable sugar side chain (Perry *et al.*, 1975). Antibodies to LPS are bactericidal in serum killing tests, and Glynn and Ward (1970) demonstrated that the bactericidal effect of normal human serum is due to naturally occurring LPS antibodies. However, the chicken embryo has no detectable complement until the 18th day of incubation, so presumably the protective effect of antibodies is a result of opsonic activity. Frasci *et al.* (1976) state that highly bactericidal antibodies are not invariably strongly opsonising and this may mean that, in comparison

with other test systems, the chicken-embryo test underestimates the protective effect of antibodies to outer-membrane components and LPS.

Because antibodies directed against LPS protect against endotoxic shock in other model systems (Brown, Douglas and Braude, 1971; Braude, Douglas and Davis, 1974) it may be that antibodies to gonococcal LPS act as antitoxins in protecting the chick embryo. However, this cannot be the only protective mechanism because the numbers of gonococci recovered in the present study from embryos protected with anti-LPS antibodies were significantly lower than from control embryos (table III). These arguments raise inevitable considerations of the purity of the antigen and antibody preparations concerned in this work in which special efforts were made to ensure specificity.

Another difficulty with the extraction of proteins from the outer envelope of the gonococcus is that immuno-determinants not present at the surface are probably exposed. Antibodies raised against these groups would be detected by the radioimmunoassay used to standardise the sera but could not bind to intact gonococci in the chick-embryo test. A similar problem occurs with the use of whole outer envelope which exposes proteins such as the 60 000 dalton protein on the inner surface of the outer membrane (Heckels and Everson, 1978). This may account for the poor performance in this test of antisera raised against outer-membrane proteins.

Gonococcal pili have been shown by many workers to be involved in the attachment of gonococci to a variety of human cells (Punsalang and Sawyer, 1973; Swanson, 1973; James-Holmquest *et al.*, 1974; Ward, Watt and Robertson, 1974; Mårdh and Weström, 1976). Antibodies to gonococcal pili are only weakly bactericidal (Buchanan *et al.*, 1977), their main mode of action *in vitro* being to block attachment to substrate cells (Buchanan and Pearce, 1976; Tramont, 1976). Pili have also been associated with the inhibition of phagocytosis (Blake and Swanson, 1975). The chicken embryo has an active reticulo-endothelial system and the simple explanation for the protective effect of antipilus serum is opsonisation followed by phagocytosis. However, the related meningococcus attaches to chick endothelial cells and causes vascular injury (Buddingh and Polk, 1939); if the pathogenesis of the gonococcal infection is comparable, antipilus IgG could act by blocking attachment to cell surfaces.

SUMMARY

The protective effects of monospecific gonococcal antisera on 11-day chick embryos challenged with a known lethal dose of gonococci were assessed. The monospecific antisera were prepared by immunisation of rabbits with purified gonococcal antigens, and removal of trace amounts of unwanted antibodies was achieved by absorption with antigen covalently bound to cyanogen bromide-activated Sepharose beads. The antisera were standardised for IgG by solid-phase radioimmunoassay.

Antiserum raised against whole outer membrane was protective and antiserum raised against the lipopolysaccharide (LPS) was moderately protective.

Outer-membrane antiserum from which the LPS component was removed by absorption was less protective than either of these sera. Investigation of the protective mechanism of anti-LPS antibodies indicated that in addition to any antitoxic effect, these antibodies inhibited the multiplication of gonococci. Antisera raised against individual outer-membrane proteins offered no protection in this test. Out of five antisera tested, antipilus serum gave the strongest protection when piliated gonococci were used as the challenge in this model; antipilus serum did not protect against challenge with non-piliated gonococci.

This work was supported by a programme grant from the Medical Research Council.

REFERENCES

- BLAKE, M. AND SWANSON, J. 1975. Studies on gonococcus infection. IX. In vitro decreased association of piliated gonococci with mouse peritoneal macrophages. *Infect. Immun.*, **11**, 1402.
- BRAUDE, A. E., DOUGLAS, H. and DAVIS, C. E. 1974. Treatment and prevention of intravascular coagulation with antiserum to endotoxin. *J. infect. Dis.*, **128**, (supplement), 157.
- BROWN, K. R., DOUGLAS, H. and BRAUDE, A. I. 1971. Prevention of death from endotoxin with antisera. II. Elimination of the risk of anaphylaxis to endotoxin. *J. Immun.*, **106**, 324.
- BUCHANAN, T. M. and GOTSCHLICH, E. C. 1973. Studies on gonococcus infection. III. correlation of gonococcal colony morphology with infectivity for the chick embryo. *J. exp. Med.*, **137**, 196.
- BUCHANAN, T. M. and PEARCE, W. A. 1976. Pili as a mediator of the attachment of gonococci to human erythrocytes. *Infect. Immun.*, **13**, 1483.
- BUCHANAN, T. M., PEARCE, W. A., SCHOOLNIK, G. K. and ARKO, R. J. 1977. Protection against infection with *Neisseria gonorrhoeae* by immunisation with outer membrane protein complex and purified pili. *J. infect. Dis.*, **136**, suppl., 132.
- BUDDINGH, G. H. and POLK, A. D. 1939. Experimental meningococcal infection of the chick embryo. *J. Exp. Med.*, **70**, 485.
- BUMGARDNER, L. R. and FINKELSTEIN, R. A. 1973. Pathogenesis and immunology of experimental gonococcal infection: virulence of colony types of *Neisseria gonorrhoeae* for chicken embryos. *Infect. Immun.*, **8**, 919.
- FOSTER, R. S. and VINSON, J. W. 1977. Chicken embryo as an animal model for Gonorrhea. *Infect. Immun.*, **16**, 568.
- FRASCH, C. E., PARKES, L., MCNELIS, R. M. and GOTSCHLICH, E. C. 1976. Protection against group B meningococcal disease. I. Comparison of group-specific and type-specific protection in the chick embryo model. *J. Exp. Med.*, **144**, 319.
- GLYNN, A. A. and WARD, M. E. 1970. Nature and heterogeneity of the antigens of *Neisseria gonorrhoeae* involved in the serum bactericidal reaction. *Infect. Immun.*, **2**, 162.
- HECKELS, J. E. 1977. The surface properties of *Neisseria gonorrhoeae*: Isolation of the major components of the outer membrane. *J. gen. Microbiol.*, **99**, 333.
- HECKELS, J. E. 1978. The surface properties of *Neisseria gonorrhoeae*: Topographical distribution of the outer membrane protein antigens. *J. gen. Microbiol.*, **108**, 213.
- HECKELS, J. E. and EVERSON, J. S. 1978. The isolation of a new outer membrane protein from the parent strain of *Neisseria gonorrhoeae* P9. *J. gen. Microbiol.*, **106**, 179.
- JAMES-HOLMQUEST, A. N., SWANSON, J., BUCHANAN, T. M., WENDE, R. D. AND WILLIAMS, R. P. 1974. Differential attachment of piliated and non piliated *Neisseria gonorrhoeae* to human sperm. *Infect. Immun.*, **9**, 897.
- KELLOGG, D. S., PEACOCK, W. L., DEACON, W. E., BROWN, L. AND PIRKLE, C. I. 1963. *Neisseria gonorrhoeae*. I. Virulence genetically linked to clonal variation. *J. Bact.*, **85**, 1274.

- LAMBDEN, P. R. AND WATT, P. J. 1978. A solid phase radioimmunoassay on hydrophobic membrane filters: detection of antibodies to gonococcal surface antigens. *J. immun. Methods*, **20**, 277.
- MÅRDH, P. A. AND WESTRÖM, L. 1976. Adherence of bacteria to vaginal epithelial cells. *Infect. Immun.*, **13**, 661.
- PERRY, M. B., DAOUST, V., DIENA, B. B., ASHTON, F. E. AND WALLACE, R. 1975. The lipopolysaccharides of *Neisseria gonorrhoeae* colony types 1 and 4. *Can. J. Biochem.*, **53**, 623.
- PUNSALANG, A. P. AND SAWYER, W. D. 1973. Role of pili in the virulence of *Neisseria gonorrhoeae*. *Infect. Immun.*, **8**, 255.
- REED, L. J. AND MUENCH, H. 1938. A simple method of estimating fifty per cent end points. *Am. J. Hyg.*, **27**, 493.
- ROBERTSON, J. N., VINCENT, P. AND WARD, M. E. 1977. The preparation and properties of gonococcal pili. *J. gen. Microbiol.*, **102**, 169.
- STEAD, A., MAIN, J. S., WARD, M. E. AND WATT, P. J. 1975. Studies on lipopolysaccharides isolated from strains of *Neisseria gonorrhoeae*. *J. gen. Microbiol.*, **88**, 123.
- SWANSON, J. 1973. Studies on gonococcus infection. IV Pili: their role in attachment of gonococci to tissue culture cells. *J. Exp. Med.*, **137**, 571.
- TRAMONT, E. C. 1976. Specificity of inhibition of epithelial cell adhesion of *Neisseria gonorrhoeae*. *Infect. Immun.*, **14**, 593.
- WARD, M. E., LAMBDEN, P. R., HECKELS, J. E., AND WATT, P. J. 1978. The surface properties of *Neisseria gonorrhoeae*: Determinants of susceptibility to antibody complement killing. *J. gen. Microbiol.*, **108**, 205.
- WARD, M. E. AND WATT, P. J. 1971. The preservation of gonococci in liquid nitrogen. *J. clin. Path.*, **24**, 122.
- WARD, M. E., WATT, P. J. AND ROBERTSON, J. N. 1974. The human fallopian tube: a laboratory model for gonococcal infection. *J. infect. Dis.*, **129**, 650.
- YEOMANS, K. A. 1973. Statistics for the Social Scientist. 2: Applied statistics, Bristol, p. 290.