PROTECTION BY MONOSPECIFIC GONOCOCCAL ANTISERA OF THE CHICKEN EMBRYO CHALLENGED WITH Neisseria gonorrhoeae

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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 x 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 Kellog supplement (Kellog 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.

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2 weeks after the last injection. The dose of antigen from strain P9(PS) for each injection was for one rabbit 50 μg of pili (Robertson et al., 1977); for the second 100 μg of colony type T4 outer membrane; for the third 50 μg of protein I (m.w. 36 500 daltons) extracted from the outer membrane (Heckels, 1977); for the fourth rabbit 50 μg of protein II (m.w. 24 000 daltons) extracted from the outer membrane; and for the fifth 50 μg 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 x 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 μl 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 × 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-μl 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 μg 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
PROTECTIVE EFFECT OF GONOCOCCAL ANTISERA

TABLE 1

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

<table>
<thead>
<tr>
<th>Antiserum effective against</th>
<th>Experiment 1*</th>
<th>Experiment 2†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number of embryos dead at 48 h/number challenged</td>
<td>Number of embryos dead at 48h/number challenged</td>
</tr>
<tr>
<td>T4 outer membrane</td>
<td>4/11 x2 3.92§</td>
<td>6/11 x2 5.83§</td>
</tr>
<tr>
<td>Outer membrane minus LPS</td>
<td>5/10 x2 1.88</td>
<td>10/11 x2 0.60</td>
</tr>
<tr>
<td>LPS</td>
<td>4/9</td>
<td>...</td>
</tr>
<tr>
<td>Outer membrane protein I</td>
<td>4/6 x2 0.49</td>
<td>9/11 x2 1.62</td>
</tr>
<tr>
<td>Outer membrane protein II</td>
<td>10/10 x2 1.57</td>
<td>11/11 x2 0.002</td>
</tr>
</tbody>
</table>

* In experiment 1 the challenge dose contained gonococci (1.2 x 10⁷) – (5.3 x 10⁷) and monospecific IgG 3 µg. 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 x 10⁶) – (1.2 x 10⁷) and monospecific IgG 1 µg.
‡ 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

<table>
<thead>
<tr>
<th>Amount (µg) of antipilus IgG per embryo</th>
<th>Experiment with piliate gonococci†</th>
<th>Experiment with non piliate gonococci‡</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number of embryos dead at 48 h/number challenged</td>
<td>Number of embryos dead at 48 h/number challenged</td>
</tr>
<tr>
<td></td>
<td>x²</td>
<td>x²</td>
</tr>
<tr>
<td>1.0</td>
<td>5/9 x2 6.25§</td>
<td>4/12 x2 0.04</td>
</tr>
<tr>
<td>0.25</td>
<td>11/13 x2 ...</td>
<td>3/12 x2 ...</td>
</tr>
<tr>
<td>0.05</td>
<td>11/11 x2 ...</td>
<td>6/11 x2 ...</td>
</tr>
</tbody>
</table>

* Challenge dose contained piliate gonococci (2.7 x 10⁶) – (4 x 10⁶)
† Challenge dose contained non-piliate gonococci (3.3 x 10⁵) – (1 x 10⁶)
‡ Normal rabbit serum was used at a concentration corresponding to that of the specific antipilus serum when diluted to contain IgG 1 µg 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-pilate gonococci. The same statistical analysis provided very strong evidence at almost the 1% significance level that 1 µg 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.
The effect of lipopolysaccharide (LPS) antiserum on the multiplication of gonococci in the chick embryo

<table>
<thead>
<tr>
<th>log10 viable counts per embryo* at 20 h when challenge injection contained</th>
<th>absorbed normal rabbit serum</th>
<th>LPS antiserum</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.56</td>
<td>6.24</td>
<td></td>
</tr>
<tr>
<td>6.72</td>
<td>6.34</td>
<td></td>
</tr>
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<td>7.29</td>
<td>6.51</td>
<td></td>
</tr>
<tr>
<td>7.75</td>
<td>6.95</td>
<td></td>
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<tr>
<td>7.83</td>
<td>7.08</td>
<td></td>
</tr>
<tr>
<td>mean 7.23</td>
<td>6.62</td>
<td></td>
</tr>
</tbody>
</table>

* The challenge dose contained gonococci $4.3 \times 10^6$ and either anti-LPS IgG $1 \mu g$ or absorbed normal rabbit serum at equal concentration. At time 0 the mean number of gonococci recovered from control preparations was $7.75 \times 10^2$ 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. Frasch 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 consi-
derations 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 Robert-
son, 1974; Mårghd and Westrom, 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 phagocy-
tosis (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 bromi-
de-activated Sepharose beads. The antisera were standardised for IgG by
solid-phase radioimmunoassay.

Antiserum raised against whole outer membrane was protective and anti-
serum 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.

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REFERENCES


