Pneumonia in mice produced by *Neisseria gonorrhoeae*

A. P. JOHNSON, D. TAYLOR-ROBINSON, AND G. SLAVIN

From the MRC Clinical Research Centre, and Northwick Park Hospital, Watford Road, Harrow, Middlesex

SUMMARY Gonococci of colony type 1 were introduced by intranasal inoculation into the lungs of mice in an attempt to produce infection. The organisms were eliminated from the three strains of mice used within 24 hours of challenge. Cyclophosphamide treatment of mice failed to render them susceptible although the organisms disappeared more slowly. The lungs of immunologically normal animals that had received either viable or heat-killed gonococci were acutely inflamed with infiltration of bronchioles and alveoli by polymorphonuclear and mononuclear leucocytes. Most of the changes had resolved after about four days. In cyclophosphamide-treated mice pulmonary infiltration by polymorphonuclear leucocytes was not evident, suggesting that these cells were not primarily responsible for the clearance of the gonococci. Despite the failure to produce a sustained infection, this model may be valuable for studying the local inflammatory effect of gonococcal endotoxin.

Introduction

One of the major difficulties confronting investigators in the study of gonococcal infections is the lack of a convenient animal model. Lucas *et al.* (1971) reported that gonococcal urethritis had been produced in male chimpanzees using urethral exudates obtained from human males. Further work with this system (Brown *et al.*, 1972) confirmed the observations of Kellogg *et al.* (1963) that genital infections may be established experimentally if virulent colony types (designated T1 and T2) are used. However, since chimpanzees are not readily available, these studies have been limited. Recently, Arko (1972) reported observations on the fate of gonococci inoculated into artificial chambers implanted subcutaneously in several species of small laboratory animals, and similar studies have been reported subsequently by other workers (Flynn and Waitkins, 1973; Scales and Kraus, 1974; Veale *et al.*, 1975; Turner and Novotny, 1976). Although the immunological and pathological events occurring in this system have been investigated (Arko, 1974; Chandler *et al.*, 1976) their relevance to human infection is not clear, as gonococcal infections in man normally involve infection of a mucosal surface. It is known

that special immunological mechanisms, in particular the production of local secretory antibody (IgA), may be important in infections of mucosal surfaces, and it is unlikely that such host defence mechanisms would be operative in the chamber model. There is, therefore, still a need for a convenient animal model in which infection of a mucosal surface is a central feature. This report describes attempts to produce such a model, by inoculation of gonococci into the lower respiratory tract of mice.

Materials and methods

MICE

Young adult, female, CBA, Balb/C, or A strain mice weighing 15-20 g were used, usually in groups of at least four. All mice were specific pathogen-free.

IMMUNOSUPPRESSION

Mice were injected intraperitoneally with a single dose of cyclophosphamide (300 mg/kg body weight) either three days before, or at the time of inoculation with *Neisseria gonorrhoeae*.

IRON TREATMENT

Ammonium ferric citrate, 625 mg (20.5-22.5% iron; Hopkin and Williams), was dissolved in 100 ml isotonic saline and the solution autoclaved at a temperature of 121°C for 15 minutes. Mice were inoculated intraperitoneally so that they received

Address for reprints: Dr D. Taylor-Robinson, Clinical Research Centre, Watford Road, Harrow, Middlesex HA1 3UJ

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10 mg iron/kg body weight 18 hours and 1 hour before challenge with gonococci.

BACTERIA AND PREPARATION OF INOCULA

The strains of *N. gonorrhoeae* used were F62, 2686, 192A, 203H, and Sal.

The identity of organisms was confirmed by typical colony morphology, Gram-negative stain, positive oxidase reaction and the ability to ferment glucose, but not maltose or sucrose. Colony types were identified according to the criteria of Kellogg *et al.* (1963).

Gonococci were cultured on medium which consisted of GC agar base (Difco) supplemented with 2% volume by volume Isovitalax (BBL). After multiplication for 20–24 hours at a temperature of 37°C in an atmosphere of 2% CO₂ in air, organisms of the desired colony type were scraped from the agar surface with a platinum loop, and suspended in about 4 ml of Eagle's minimal essential medium (MEM) containing 0.05 mol/l HEPES buffer (pH 7.3). The suspension was agitated for 30–60 seconds on a Rotamixer Deluxe (Hook and Tucker Ltd) at maximal amplitude to disperse bacterial clumps.

In one experiment, gonococci were killed by heating a suspension to a temperature of 56°C for 1 hour.

INOCULATION PROCEDURE

Mice were anaesthetised by intraperitoneal injection of pentobarbitone sodium (0.06 mg/g body weight) and then inoculated intranasally with 0.05 ml of the gonococcal suspension. Animals used as controls were inoculated intranasally with an equivalent volume of medium. After all the animals had been inoculated, the titre of the inoculum was determined by making serial 10-fold dilutions, and plating 0.05 ml of each dilution on the solid medium described above.

PROCEDURE FOR RE-ISOLATION OF ORGANISMS

Mice were killed by injecting intraperitoneally 0.1 ml of undiluted pentobarbitone sodium solution followed by bleeding from the axillary vessels. The trachea was exposed and the thoracic cage removed. The lungs were washed *in situ* with 1–2 ml of Eagle's MEM, this being introduced and withdrawn three times through a canula tied securely into the trachea (Taylor-Robinson *et al.*, 1972). Then 0.1 ml of the lung washing was spread on to GC agar medium containing Isovitalax and incubated as described previously to detect bacterial colonies.

HISTOLOGICAL PROCEDURES

After collection of the lung washings for bacterial

isolation, the lungs were redistended with 1–2 ml of buffered formol saline. The lungs were then removed and immersed in the same fixative for at least 24 hours. After fixation, blocks were embedded in paraffin wax, sectioned and stained with haematoxylin and eosin. Histological sections were coded before examination so that they were assessed without knowledge of the treatment which the animals had received. In some experiments, the technique of Sowter and McGee (1976) was used to detect gonococci. The lungs were fixed with formol sublimate and the sections stained with Gram-methyl green-pyronin-light green (Gram-MGPLG).

Results

ATTEMPTS TO RE-ISOLATE GONOCOCCI FROM THE LUNGS

In initial studies, gonococci (strain Sal) of colony type 4 were inoculated into CBA mice. Attempts to re-isolate organisms from their lungs one, two, three, and four days later were uniformly negative. Consequently, in subsequent experiments, gonococci of colony type 1 were used, as this colonial morphology is known to be associated with virulence (Kellogg *et al.*, 1963). Furthermore, in addition to CBA mice, Balb/C and A strain mice were inoculated. The results of attempts to infect the respiratory tract of these mice with four strains of *N. gonorrhoeae* are shown in Table 1. Despite inoculation of at least 10⁷ colony-forming units, gonococci were rarely isolated 24 hours later and appeared to have been completely eliminated from the lungs by 48 hours. Similar results were obtained with mice that had been pre-treated with iron. However, in immunosuppressed animals, the rate of clearance of gonococci from the lungs was slower, organisms being isolated from a larger number of mice after 24 hours (Table 2). Nevertheless, even in these animals, gonococci were rarely recovered 48 hours after inoculation (Table 2).

Table 1 *Recovery of N. gonorrhoeae from lungs of mice inoculated intranasally with type 1 organisms*

Strain of <i>N. gonorrhoeae</i>		Titre of inoculum (colony-forming units/0.05 ml)	Strain of mouse	No. of re-isolations/ No. of mice inoculated	
				Day 1	Day 2
F62	T1	1 × 10 ⁷	CBA	2/8	0/8
2686	T1	7 × 10 ⁷	CBA	0/6	0/6
192A	T1	4 × 10 ⁸	CBA	2/6	0/5
203H	T1	2 × 10 ⁷	CBA	0/9	0/7
2686	T1	1 × 10 ⁷	Balb/C	0/5	0/5
2686	T1	2 × 10 ⁷	A strain	0/4	0/4

Fig. 1 Lung of CBA mouse 48 hours after intranasal inoculation of gonococci. There is a well-marked polymorphonuclear leucocyte and mononuclear cell infiltration of alveoli with pronounced perivascular and peribronchiolar cuffing. H and E, $\times 225$.

HISTOPATHOLOGICAL CHANGES IN THE LUNGS
Introduction of gonococci into the lungs of the three strains of mice produced a transient but severe pneumonia. Earliest lesions consisted of inflammation around respiratory bronchioles and vessels with polymorphonuclear leucocyte and mononuclear cell infiltration of alveoli. The changes were at their severest about 48 hours after inoculation when perivascular cuffing with inflammatory cells was prominent (Fig. 1), and occasionally there was an active vasculitis of small vessels. In severely affected lungs, focal degeneration of bronchiolar epithelium was sometimes seen. In addition, there was pleural inflammatory involvement which was inconstant but focally well marked (Fig. 2). The acute inflammation had largely resolved by day 4, there being only mononuclear cells present in alveoli.

No pneumonia was observed in mice which had been given cyclophosphamide three days before intranasal challenge with gonococci. In such mice

Table 2 Recovery of *N. gonorrhoeae* from lungs of normal and cyclophosphamide-treated CBA mice inoculated intranasally with type 1 organisms

Strain of <i>N. gonorrhoeae</i>		Titre of inoculum (colony-forming units/0.05 ml)	Treatment of mouse	No. of re-isolations/ No. of mice inoculated	
				Day 1	Day 2
192A	T1	4.5×10^7	Untreated CY* 3 days before inocu- lation with <i>N.</i> <i>gonorrhoeae</i>	0/6	0/6
192A	T1	4.5×10^7			
2686	T1	5.2×10^6		4/6	0/6
2686	T1	5.2×10^6		0/4	0/4
2686	T1	2×10^7	Untreated CY 3 days before inocu- lation with <i>N.</i> <i>gonorrhoeae</i>	3/4	1/4
2686	T1	2×10^7		0/6	0/6
			CY at same time as inocu- lation with <i>N.</i> <i>gonorrhoeae</i>	0/6	0/6

*CY = cyclophosphamide (300 mg/Kg body weight).

the only pathological changes observed were slight interstitial thickening of alveolar walls, and occasionally an increase in the number of mononuclear cells in the alveoli. In mice which were given cyclophosphamide and gonococci at the same time, there was a diffuse polymorphonuclear leucocyte infiltration of the lungs after two days, which was less severe than that observed in immunologically normal mice challenged with the same dose of gonococci. Immunologically normal and cyclophosphamide-treated mice inoculated with medium only did not develop lung lesions. In the experiment in which two groups of mice were inoculated with equivalent numbers of viable and heat-killed gonococci, respectively, similar pathological changes were observed in both groups of animals.

Gonococci were not demonstrable histologically in any of the lungs stained with either haematoxylin and eosin or Gram-MGPLG.

Discussion

There have been a number of studies of gonococcal infections in small laboratory animals in which observations have been made on the fate of gonococci introduced into subcutaneous chambers (Arko, 1972, 1974; Flynn and Waitkins, 1973). The pathological changes consist of an intense polymorphonuclear leucocyte infiltration, with tissue necrosis (Veale *et al.*, 1975; Chandler *et al.*, 1976). However, such a model is not entirely analogous to non-disseminated gonococcal infection in man, as there is no interaction of gonococci with a mucosal surface. The pathological changes at a mucosal surface may require initial attachment of the organisms mediated by pili and then toxin liberation or some other, as yet, unidentified event. On the other hand, attachment of organisms to cells may not be an important factor in the development of pathological changes in chambers where the organ-

Fig. 2 Lung of CBA mouse 48 hours after intranasal inoculation of gonococci. There is alveolar wall thickening and a diffuse intra-alveolar infiltrate of mononuclear and polymorphonuclear leucocytes. These are particularly prominent at the pleural surface. H and E, $\times 225$.

isms are already trapped. This may be the reason why Turner and Novotny (1976) found that cross-reactive pilar antibodies did not prevent infection from becoming established in animal chambers. However, it may be that such antibodies might prove successful in preventing infection in a situation where pilar antibodies act to prevent the attachment of gonococci to epithelial cells.

Because the chamber model is not the most appropriate for studying gonococcal infection at a mucosal surface, we attempted to establish infection in the lower respiratory tract of mice. It has been shown by others that strains of mice vary in their susceptibility to pathogenic micro-organisms (Araujo *et al.*, 1976; Plant and Glynn, 1976). However, the three strains of mice used in this study all proved resistant to gonococcal infection. The gonococci were rarely recovered from the lungs and could not be detected histologically. Although the Gram-MGPLG stain is particularly valuable for the detection of gonococci in Fallopian tubes (Sowter and McGee, 1976), it proved less suitable for the study of respiratory epithelium because the cells stained red, the same colour as the organisms. Difficulties in detecting gonococci histologically have also been experienced even by those working with infected chambers (Chandler *et al.*, 1976). Attempts either to enhance the virulence of gonococci or increase the susceptibility of the mice by immunosuppression were also unsuccessful in producing infection. Administration of ammonium ferric citrate to mice was without effect, even though it has been shown that iron promotes the growth of gonococci *in vitro* (Kellogg *et al.*, 1968) and the virulence of gonococci for chick embryos (Payne and Finkelstein, 1975). Cyclophosphamide treatment, known to cause neutropenia (Hunninghake and Fauci, 1976), did indeed prevent the development of a polymorphonuclear leucocyte response in the lungs. However, the organisms disappeared rapidly even though they persisted for a little longer than in immunologically normal mice. This indicates that disappearance is not due solely to phagocytosis and killing by these leucocytes.

Despite the failure of the gonococci to multiply in the lungs of mice, the acute inflammatory response which occurred resembled that seen in infected chambers. Gonococci also failed to multiply in the mammary gland of the mouse (Taylor-Robinson *et al.*, 1975) and goat (Pasioka *et al.*, 1976) but produced an inflammatory response in each. The inflammatory reaction in the mouse lung was also seen in mice inoculated with heat-killed gonococci, suggesting that it occurred in response to the presence of a heat-stable component of the bacterial cell. As the cell walls of gonococci contain endotoxin

(Maeland, 1969), the inflammation may be mediated by activation of the alternative complement pathway. Although inoculation of the mouse lung does not provide a useful model of gonococcal infection it may be of use in studying the local effects of gonococcal endotoxin on the host.

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