Role of endotoxin in the pathogenicity of *Neisseria gonorrhoeae* colonial types 1, 4 and 5 determined by chicken embryo model

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Summary. The pathogenicity of *Neisseria gonorrhoeae* is a subject of considerable interest. It is believed that *N. gonorrhoeae* of colonial type 1 are pathogenic while those of type 4 are not. This is based on experimentation in human volunteers. The object of this study was to determine the reasons for the differences of susceptibility of chicken embryos to *N. gonorrhoeae* strains of colonial types 1, 4, 5 and 1R (a type-1 revertant from a non-pathogenic type 4 strain originally tested in human volunteers). Colonial types 1, 5, 1R and 4 caused mortality rates of 80, 70, 85 and 20% respectively. This variation in lethality appeared to depend upon the availability of free extra-cellular endotoxin and this was confirmed by chicken-embryo inoculation results and electronmicroscopy of normal and heated colonial types 1, 4 and 5. Similar results were obtained by inoculating purified endotoxins from these types into chicken embryos. The results of this study suggest that endotoxins play a major role in the pathogenicity of *N. gonorrhoeae* and that the variations in virulence of the colonial types depends on the stability of their cell walls.

Introduction

Colonial variation in *Neisseria gonorrhoeae* was reported by Kellogg *et al.* (1963), who described four colonial types and established a clear relationship between colonial morphology and virulence for human volunteers. It is now generally accepted that colony types 1 and 2 are virulent whereas types 3 and 4 are not. A fifth colonial type was reported by Jephcott and Reyn (1971), and Hafiz *et al.* (1978) suggested that it was a pathogenic type on the basis of its effect after intravenous inoculation into 10-day-old chicken embryos (Finkelstein, 1964). The intravenous injection of pathogenic and non-pathogenic colonial types of gonococci into 10-day-old chicken embryos has produced results consistent with, and parallel to, those obtained by intra-urethral inoculation in human volunteers (Bumgarner and Finkelstein, 1973; Diena *et al.* 1975) *N. gonorrhoeae* isolates from clinical specimens are of colonial types 1 and 2, and they degrade to type 3 and 4 on subsequent subcultures. Hafiz *et al.* (1977)

showed that colonial type 4 could revert to type 1 in the presence of iron. Berry (1975) stated that the endotoxin is the only clearly established toxic component in gonococci as well as in other gram-negative bacteria such as meningococci and brucellae. Although the factors responsible for virulence of *N. gonorrhoeae* are still ill-understood, Berry went further and suggested that, by a process of elimination, it could be assumed that endotoxin is responsible for host damage. It is also interesting to note that Scholz in 1900 introduced killed gonococci into the human urethra which then became transiently inflamed (cited by McEntegart, 1975). This observation further implied the importance of gonococcal endotoxin as one of the possible agents responsible for host damage. Ovčinnikov and Delektorskij (1971) reported that *N. gonorrhoeae* secreted endotoxin blebs.

In an attempt to show that changes observed in in-vitro colonial morphology of gonococci in reverting from type 4 to type 1 are associated with changes in virulence, the chicken-embryo model has been used for pathogenicity studies. The role of endotoxin in the pathogenicity of several colonial types has also been investigated. Experiments were performed with whole live cells, cells inactivated by heat or formalin, and with extracted endotoxin.

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Materials and methods

Organisms

N. gonorrhoeae strain F62, colonial types 1, 4 and 5 were all derived from a single clinical isolate obtained from Dr D. S. Kellogg, C.D.C., Atlanta, GA, USA. Type 1R (a revertant type 1 derived from the type-4 culture of F62) was also studied. The four colonial types were grown for 18–20 h at 36°C on GC medium (Difco) with defined supplement (Kellogg *et al.*, 1963) 2% and the identity of the colonial types confirmed by plate microscopy with a combination of incident and transmitted light. For injection into the chicken embryos the growth on this medium of each type was suspended, separately, in phosphate-buffered saline, pH 7.2 (PBS), and the suspensions were diluted to give an inoculum of *c.* 10⁶ cfu/ml as assessed by nephelometry (EEL Nephelometer). The nephelometer had been standardised by comparison of a series of readings with total viable counts (Miles *et al.*, 1938) of gonococcal suspensions.

Heat treatment of *N. gonorrhoeae*

A suspension of 10⁶ cfu/ml in PBS of each type was heated at 60°C for 30 min; subsequent subcultures were sterile.

Formol-saline treatment

Cells of each type were harvested in PBS and centrifuged at 600 *g* for 30 min, and the deposit was treated with 10% formol-saline (formaldehyde 10% in saline) overnight. The killed cells were washed three times in PBS and resuspended in PBS to give a concentration equivalent to *c.* 10⁶ cfu/ml as assessed by nephelometry.

Preparation of endotoxin

Endotoxin was extracted from all four colonial types of gonococci. Each colonial type was grown on GC medium and harvested into a universal container (*c.* 1 g wet weight) and 20 ml of 45% aqueous phenol was added (Maeland, 1968). The gonococcal suspensions in aqueous phenol were incubated at 37°C for 30 min and then centrifuged at 3000 *g* for 45 min. After centrifugation three layers were distinct. The contents of the centrifuged containers were allowed to stand at 4°C for 20–24 h. The clear top aqueous layer, containing free endotoxin only, was removed gently with a Pasteur pipette into a clean, sterile universal container. To ensure complete removal of volatile phenol, the endotoxin preparation was heated in an open container in a hot-water bath at 55°C for 1 h and then the product was treated with 4 mg of ribonuclease A (Koch Light Laboratories Ltd) and incubated at 37°C for 18 h (Perry *et al.* 1975). The endotoxin extract, in approximately 20-ml volumes, was dialysed against cold running tap water for about 48 h in Visking® cellophane tubing with an inflated diameter of 6.3 mm (Scientific Instruments Centre Ltd) and finally lyophilised in a high-

vacuum freeze drier (Model EF03, Edwards, Manor Royal, Crawley, Sussex). The resultant white powder of purified endotoxin was weighed and then dissolved in PBS to give a final concentration of 0.1 mg/ml for each preparation.

Lethality test

Series 1. Embryonated eggs, 10 days old, were obtained from a local farm. While candling, rectangular markings approximately 4 mm × 6 mm were made over a prominent allantoic vein. The egg shell was cut with a dental drill along the markings and the shell flap was lifted off with an 18 gauge needle without damaging the chorio-allantoic membrane. The eggs were placed in a humidified incubator at 37°C for 2 h to allow fixation of the veins before inoculation and to allow the embryos to settle, thus helping to prevent any haemorrhage. Whilst monitoring by transillumination, the chicken embryos were given intravenous injections of 0.1 ml of the appropriate suspension (*i.e.*, 10⁵ cfu or equivalent of live or killed *N. gonorrhoeae* or 10 µg of purified endotoxin) with a 27 gauge needle; control eggs received 0.1 ml of PBS. After inoculation the eggs were returned to the incubator at 36°C and candled daily for 3 days. Suspected deaths were confirmed by opening the eggs. Dead embryos were fixed in formol saline and thin sections of the brain and the visceral organs were cut and stained by Haematoxylin and Eosin and by Gram's staining methods.

Series 2. In further experiments, *N. gonorrhoeae* of the four colonial types were injected into a total of 48 embryos. Two eggs were opened at hourly intervals and the dead embryos were cut into two. One half was fixed and embedded; thin sections were cut and examined histologically for pathological changes. The other halves of the chicken embryos were chopped, emulsified in PBS and 0.1 ml spread on plates of GC medium for recovery of viable gonococci.

Electronmicroscopy

The various cell suspensions were examined by electronmicroscopy. Cells were harvested by centrifugation at 1000 rpm (150 *g*) for 5 min to form a pellet, washed three times with PBS, fixed with glutaraldehyde 3% in cacodylate buffer, and then post fixed with 1% aqueous osmium tetroxide. The material was dehydrated with increasing concentrations of ethyl alcohol and then embedded in low viscosity epoxy resin (Spurr, 1969). Ultrathin sections were cut on a Reichert Ultra-microtome and stained with uranyl acetate and lead citrate. The sections were examined with a AEI EM6B electronmicroscope at magnifications up to 50 000.

Results

Effects of whole live cells on chicken embryos

The deaths of the chicken embryos after inocula-

Table. The effect of intravenous inoculation of *N. gonorrhoeae* preparations into chicken embryos

Colonial type	Cell preparations	Number of chicken embryos used	Number of deaths of chicken embryos after			Percentage mortality after 72 h
			24 h	48 h	72 h	
1	Live cells	30	24	24	24	80
	Heated	20	16	16	16	80
	Formalised	20	2	6	6	30
	Freeze-dried Endotoxin	30	21	21	21	70
4	Live cells	30	7	7	7	23
	Heated	20	16	16	16	80
	Formalised	20	1	4	4	20
	Freeze-dried Endotoxin	20	14	14	14	70
5	Live cells	30	6	9	21	70
	Heated	20	16	16	16	80
	Formalised	20	2	5	5	25
	Freeze-dried Endotoxin	20	14	14	14	70
Reverted type 1 (1R)	Live cells	20	17	17	17	85
	PBS (negative control)	30	0	0	0	0

tion of various preparations of *N. gonorrhoeae* (Series 1) are shown in the table. Most deaths occurred between 18 and 24 h. The percentage mortality up to 72 h with whole live cells of colonial types 1, 4, 5 and 1R of gonococci varied from 85% (reverted type 1) to 23.33% (type 4). In series 2, no organisms were recovered after 6 h or on the deaths of the embryos, although in the first 5 h viable organisms were recovered from the embryos killed at hourly intervals.

Effect of heat- and formalin-treated cells or endotoxin of gonococcal types 1, 4, and 5

When cells of three colonial types (1, 4 and 5) were heated at 60°C and injected into chicken embryos (20 were given each preparation) the mortality rate was 80% with all three types. The same suspensions, treated with formalin but without heating, produced reduced mortality rates of 20–30% in sets of 20 embryos. There were no deaths in chicken embryos that had received PBS as negative controls. The mortality after injection of 10 µg of freeze-dried purified endotoxin of colonial types 1, 4 or 5 was 70% with each.

Histopathological findings in dead embryos

The typical gross pathological picture in chicken

embryos was that of haemorrhagic lesions. The first minimal haemorrhagic signs appeared in the brains of chicken embryos after 10 h and by 21 h there were gross haemorrhagic lesions and engorgement of sinusoids. Deaths of embryos given purified endotoxin occurred within 24 h. The embryos showed the same typical pathological changes seen in the embryos given live or killed *N. gonorrhoeae*; signs of these changes were first noticeable 6 h after inoculation.

Electronmicroscopy

The electronmicroscopic appearance of heated and unheated cells of *N. gonorrhoeae* colonial types 1, 4 and 5 are shown in figs. 1–6. Endotoxin blebs were seen on cells of unheated virulent colonial type 1 (fig. 1); by contrast the unheated avirulent colonial type 4 had a more stable outer cell wall with no distinct endotoxin blebs evident (fig. 3). There were no differences between the appearances of the heat-killed gonococci of colonial types 1, 4 and 5 (figs. 2, 4 and 6); with each type the outer cell walls were partially disrupted and there were endotoxin blebs lying free from the cells.

Discussion

This study confirmed that the gonococci of

colonial types 1 and 5 were virulent for the chicken embryos whereas gonococci of colonial type 4 were relatively avirulent (Bumgarner and Finkelstein 1973; Diena *et al.*, 1975; Hafiz *et al.*, 1978). The morphological change from colonial type 4 to colonial type 1 *in vitro* (Hafiz *et al.*, 1977) was shown to be a true reversion because the virulence of the reverted colonial type 1 for chicken embryos was increased to at least the same as that of the original type-1 culture.

The failure to recover viable gonococci from the cultured specimens of the dead chicken embryos 6 h after inoculation, and the histological findings of

severe haemorrhage and engorgement of sinusoids in dead embryos suggest that death of the chicken embryos was due to endotoxin. This was further supported by the findings that the pathogenicity of heat-killed colonial types 1 and 5 was preserved while formalin-fixation considerably reduced pathogenicity. This suggests that heating liberates endotoxin whereas formalin treatment preserves the cell wall, thus reducing the liberation of endotoxin. The increased virulence of heat-killed colonial type 4 cells was surprising. This interesting finding could mean that heating altered the cell-wall stability of colonial type 4 gonococci in such a way

Figs. 1–6. Electronmicrographs of *N. gonorrhoeae* colonial types 1, 4 and 5 before and after heating: 1, unheated type 1; 2, heated type 1; 3, unheated type 4; 4, heated type 4; 5, unheated type 5; and 6, heated type 5. Bar = 1 µm.

as to release endotoxin more easily and make it available to cause the same effect on chicken embryos as was found with the unheated and heated cells of the virulent types. Electronmicroscopic examination of heated and unheated cells of the three colonial types of gonococci revealed that unheated cells of virulent colonial types 1 and 5 carry small blebs of endotoxin which confirmed the findings of Grimble and Armitage (1974). The heated cells of all three colonial types released

endotoxin. Inoculation of purified endotoxin extracted from the four colonial types had the same effect; all were equally potent. Chicken embryo lethality tests suggest that the pathogenicity of *N. gonorrhoeae* colonial types is related to the presence of free endotoxin, which may thus play an important role in the pathogenicity of *N. gonorrhoeae*.

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REFERENCES

- Berry L J 1975 Metabolic effects of endotoxin. In: Schlessinger D (ed) *Microbiology* 1975. American Society for Microbiology, Washington D.C., pp. 315.
- Bumgarner L R, Finkelstein R A 1973 Pathogenesis and immunology of experimental gonococcal infection: virulence of colony types of *Neisseria gonorrhoeae* for chicken embryos. *Infection and Immunity*, **8**:919–924.
- Diena B B, Lavergne G, Reyn A, Ashton F E, Wallace R, Perry M, Daoust V 1975 The chick embryo in studies of virulence and immunity with *Neisseria gonorrhoeae*. *Revue Canadienne de Biologie*, **34**:213–220.
- Finkelstein R A 1964 Observations on mode of action of endotoxin in chick embryo. *Proceedings of the Society of Experimental Biology and Medicine* **115**:702–707.
- Grimble A, Armitage L R G 1974 Surface structures of the gonococcus. *British Journal of Venereal Diseases* **50**:354–359.
- Hafiz S, McEntegart M G, Jephcott A E 1977 Reversion of Kellogg's colonial types of *Neisseria gonorrhoeae* in liquid medium. *Journal of Medical Microbiology* **10**:377–380.
- Hafiz S, Jennings R, McEntegart M G, Jephcott A E 1978 *N. gonorrhoeae*: pathogenicity of colonial type 5. *Journal of Clinical Pathology* **31**:437–438.
- Jephcott A E, Reyn A 1971 *Neisseria gonorrhoeae*. Colony variation I. *Acta Pathologica et Microbiologica Scandinavica, Section B* **79**:609–614.
- Kellogg D S, Peacock W L, Deacon W E, Brown L, Pirkle C I 1963 *Neisseria gonorrhoeae*. I. Virulence genetically linked to clonal variation. *Journal of Bacteriology* **85**:1274–1279.
- Maeland J A 1968 Antigenic properties of various preparations of *Neisseria gonorrhoeae* endotoxin. *Acta Pathologica et Microbiologica Scandinavica* **73**:413–422.
- McEntegart M G 1975 Fundamental research developments. In: Morton R S, Harris J R W (eds) *Recent advances in sexually transmitted diseases*. I Churchill Livingstone, Edinburgh, p. 59.
- Miles A A, Misra S S, Irwin J O 1938 The estimation of the bacterial power of blood. *Journal of Hygiene* **38**:732–749.
- Ovčinnikov N M, Delektorskij V V 1971 Electron microscope studies of gonococci in the urethral sections of patients with gonorrhoea. *British Journal of Venereal Diseases* **47**:419–439.
- Perry M B, Daoust V, Diena B B, Ashton F E, Wallace R 1975 The lipopolysaccharides of *Neisseria gonorrhoeae* colony types 1 and 4. *Canadian Journal of Biochemistry* **53**:623–629.
- Spurr A R 1969 A low-viscosity epoxy resin embedding medium for electron microscopy. *Journal of Ultrastructure Research*, **26**:31–43.