REVERSION OF KELLOGG'S COLONIAL TYPES OF *NEISSERIA GONORRHOEAE* IN LIQUID MEDIUM

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PLATE XXIX

DIFFERENCES in the colonial morphology of *Neisseria gonorrhoeae* were first noted by Wassermann (1898) and Lipschütz (1904); since then a great deal has been learned about the variations in colonies of gonococci and their significance.

In 1925 Atkin described two forms of colony and reported that one of these predominated in recently isolated cultures. Kellogg *et al.* (1963) showed a clear relationship between colonial morphology and virulence for human volunteers. It is now accepted that Kellogg's colony types 1 and 2 are infective whereas types 3 and 4 are not. Whilst studying the possible determinants of virulence, Jephcott and Reyn (1971) reported a fifth colonial type. Little was known about the pathogenicity of this type, but it was generally believed to be a degraded type similar to types 3 and 4.

With the development of a liquid medium that supported the growth of gonococci for longer periods than did most solid media, an opportunity arose to study the behaviour of different colonial types in the unusual environment of a liquid culture.

MATERIAL AND METHODS

Media. The liquid medium (Hafiz and McEntegart, 1977) consisted of Proteose Peptone (Difco) 15.0 g, corn or soluble starch 1.0 g, sodium chloride 5.0 g, dipotassium phosphate 4.0 g, potassium dihydrogen phosphate 1.0 g, sodium bicarbonate 0.15 g, glucose 5.0 g and distilled water 1000 ml. It was sterilised by autoclaving at 115°C for 10 min. and was given the media-room code name ANM. The basic medium either alone, with 2% of a defined supplement that consisted of l-glutamine 1.0 g, dextrose 40.0 g, ferric nitrate 0.05 g and cocarboxylase ("Analar", BDH) 0.02 mgm in 100 ml distilled water (White and Kellogg, 1965) or with 0.1% ferric citrate ("Analar", BDH) was distributed in 28-ml, screw-capped, glass Universal containers, in volumes of 15 ml.

The solid medium used throughout the study was Difco GC Base plus 2% defined supplement. Phosphate-buffered saline (PBS) used as diluent was prepared by dissolving Dulbecco "A" Tablets (Oxoid) in distilled water and was sterilised in the same way as the liquid media.

Strains. Twenty strains forming type 4 colonies were used and three strains forming type 5 colonies, one of which was the original type 5-forming strain used by Jephcott and Reyn (1971). The other strains were freshly isolated from patients attending the Special Clinic at Sheffield Royal Infirmary.

All the strains were initially cultured on Difco GC Base with 2% defined supplement and incubated in an atmosphere of air with 10% carbon dioxide for 18–20 h at 35–36°C. Colonies of types 5 and 4 were selectively subcultured on the same medium. The colonial types were checked and pure type 5 and 4 colonies from such plates were used as inocula for liquid medium. Either selected colonies were emulsified in PBS and 0.1-ml volumes of this suspension (containing approximately 10⁵ organisms) were inoculated into 15-ml volumes of

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FIG. 2.—Colonial types of *Neisseria gonorrhoeae* isolated after culture of type 5 in ANM liquid medium. ■ = type 1, □ = type 4, and ▨ = type 5.

ANM and on to standard plates of Difco GC Base plus 2% defined supplement, or 10 colonies of identical type were directly inoculated into 15-ml volumes of the medium and mixed by mechanical agitation.

Although in Universal containers, the liquid cultures were incubated at 35°–36°C in the same carbon dioxide incubator as the solid cultures to ensure the same temperature conditions for both types of culture. Each day during growth, samples were subcultured on to solid medium and viable counts were estimated by the method of Miles, Misra and Irwin (1938). The subcultures were examined after incubation for 18–20 h in the carbon dioxide incubator. The identity of the culture was confirmed by Gram's staining, oxidase reaction, sugar fermentation and fluorescent antibody testing.

RESULTS

Initially, type 1 and type 4 colonies appeared to be stable in ANM and the first indication of change was seen with type 5 strains grown in unsupplemented ANM.

Figs. 1 and 2 show that a type 5 inoculum of *N. gonorrhoeae* changed its colonial type gradually during 26 days in unsupplemented ANM broth culture. Fig. 2 shows the viable counts and percentages of colonial types recovered. Subcultures after 24 h showed about 10% of type 1 colonies and 10% of type 4 colonies. After the 5th day, type 5 colonies were not recovered, and from 9–15 days in broth type 1 colonies only were recovered. Thereafter a rapid increase in the proportion of type 4 colonies was seen; by the 22nd day only type 4 colonies were recovered. This situation persisted until the 26th day, after which time no live organisms could be demonstrated.

When organisms from type 4 colonies were inoculated into ANM broth without defined supplement, they remained in the type 4 phase and survived for 10 days; after that time no viable organisms were recovered. When the procedure was repeated with ANM containing defined supplement, occasional type 1 colonies were recovered.

The iron component of the defined supplement was then tested for its ability to promote reversion of colonial form in ANM. ANM, with and without the addition of 0.1% ferric

COLONIAL TYPES OF *N. GONORRHOEAE*

FIG. 1.—Reversion and progression of *Neisseria gonorrhoeae* type 5 in culture in ANM liquid medium. The colonies illustrated are typical type 5 (top), those in the phase of reversion and progression showing granulation and highlights (centre), typical type 1 (bottom left) and typical type 4 (bottom right). $\times 40$.

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FIG. 3.—Colonial types of *Neisseria gonorrhoeae* isolated after culture of type 4 in ANM liquid medium containing 0.1% ferric citrate. ■ = type 1, □ = type 4, and ▨ = type 5.

citrate, was inoculated with bacteria from type 4 colonies of 23 strains of gonococci, including three known to produce type 5 colonies. The cultures without added iron showed no reversion. In the presence of iron reversion occurred, as shown in fig. 3. In the first 24 h there was an increase in the total count but no reversion of colony type. After 48 hours incubation, 90% of the colonies isolated were of type 1 and the remaining 10% of type 4. On the 4th day, approximately 95% were of type 1 and the remaining 5% were of type 4 except that, as already stated, three strains produced type 5. On the 5th and 6th days, only type 1 colonies were recovered. By the 7th day, the percentage of type 1 recovered had fallen to 80%, and 20% were of type 4; from the 8th to the 14th day only colonies of type 1 were recovered. Occasional type 4 colonies appeared after the 15th day, and after the 22nd day the count began to decline; on the 24th day 100% appeared to be of type 4 and on and after the 26th day no viable organisms were recovered.

DISCUSSION

It has generally been considered that the change in *N. gonorrhoeae* from colonial types 1 or 2 (Kellogg's virulent types) to types 3 or 4 (Kellogg's non-virulent types) is similar to an S to R change and is predominantly unidirectional *in vitro*.

In the present investigation a new liquid medium was used which supported the growth of gonococci for periods of up to several weeks. In this medium changes of colonial type were observed that were not limited to the usual degradation to the non-virulent types, but included significant changes in the reverse direction. Previously such reversions involving more than an occasional colony have only been reported *in vivo*.

Our attention was first drawn to the potential of liquid medium by the finding that colonial type 5 would revert to type 1 in unsupplemented ANM. Type 4 colonies of *N. gonorrhoeae*, however, did not change in unsupplemented ANM. They reverted to a small extent in ANM with defined supplement, but reverted totally to type 1 in ANM with 0.1%

ferric citrate; clearly the addition of iron favoured the change. Recently Payne and Finkelstein (1975) showed that when iron compounds were added to inocula of the relatively avirulent type 3 and type 4 colonies they became more lethal for chick embryos. It remains to be discovered whether this action is on the host and relates directly to the known virulence-promoting effect of ionic iron in many experimental infections (Bullen, Rogers and Griffiths, 1974; Miles and Khimji, 1975), or whether it acts on the organisms and involves a change *in vivo* to virulent colony forms.

No one has yet been able to explain the relationship between the appearance of gonococcal colonies and their predictable virulence. We hope that the discovery of a method that consistently produces reversion of colonial type may assist in solving this problem.

SUMMARY

Colonial type variation of gonococci is well known, but change from type 4 to type 1 is rare except *in vivo*.

By observing quantitatively subcultures from a new liquid medium it was possible to follow the day-to-day changes in the ratio of colonial types present. The results obtained showed that type 1 colonies could be derived from type 5 inocula even in unsupplemented media. In unsupplemented liquid medium, type 4 inocula did not revert to other types and indeed colonial type 4 appeared to be the final form of the organism before it died out. If, however, iron in the form of ferric citrate was added to the medium, reversion occurred and type 1 colonies rapidly came to predominate.

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