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A new colonial type of *N. gonorrhoeae*

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Kellogg, Peacock, Deacon, Brown and Pirkle (1963) described four colonial types of *Neisseria gonorrhoeae*. Types 1 and 2 were virulent when tested in human volunteers, and when viewed under diffuse transmitted light after 20 hrs growth, were 0·5 mm. in diameter, dark gold and convex with an entire edge. Avirulent Types 3 and 4 were light brown or colourless, about 1 mm. in diameter, somewhat more flat than Types 1 and 2, and also entire. These observations have been confirmed by a number of investigators. Recently, Jephcott and Reyn (1971) have observed a fifth colonial type, the colonies of which resemble Types 3 and 4 in size and colour. Type 5 in contrast with Types 3 and 4 is shiny, granular, and opaque, with an uneven surface and crenated edge. Other types undoubtedly exist, particularly after cultivation in the laboratory, and the situation is further complicated if antibiotics are present in the media on which type differentiation is effected (James, Wende, and Williams, 1973).

We have observed an additional new colonial type of *N. gonorrhoeae* which has been designated Type 1[1] on the basis of its visual appearance and virulence in the chick embryo, and in the present report we compare its characteristics to those of known types.

Material and methods

Maintenance and preservation of strains

Two strains of *N. gonorrhoeae*, F62 and 3956, were investigated in this study. The F62 strain was obtained from Dr. D. S. Kellogg of the Communicable Diseases Center in Atlanta, Ga., and 3956 was isolated from a patient by the staff of the Manitoba Provincial Laboratories in Winnipeg.

The strains were grown on GCBD medium (Kellogg and others, 1963), which is composed of Difco GC medium base supplemented with dextrose (2 per cent.), L-glutamine, and cocarboxylase. Inoculated plates were incubated for 20 hrs at 37°C. in a Hotpak CO_2 incubator under 5 per cent. CO_2 tension and 95 per cent. relative humidity. Colonial types were identified with the aid of a Bausch and Lomb Model 7 stereomicroscope and selectively subcultured daily onto fresh GCBD medium. The microscope was equipped with a substage source of diffuse light. This arrangement, coupled with oblique overhead illumination by means of a Bausch and Lomb illuminator (cat no. 31–35–47) was similar to that described by Kellogg and others (1963). Differentiation of colonies under these lighting conditions was made according to criteria set out by the Kellogg group and by Jephcott and Reyn (1971).

Isolated strains, in addition to daily subculturing, were also preserved in ampoules submerged in liquid nitrogen according to the method of Ward and Watt (1971). Strains to be frozen were suspended in a solution of 8 per cent. (v/v) glycerol and 1 per cent. (w/v) proteose peptone in water.

Saline auto-agglutinability

A loopful of gonococci grown on GCBD medium for 20 hrs was suspended in 1 ml. saline which contained 1 per cent. formalin, in a tube measuring 1 × 7 cm. The suspension was gently shaken for a few seconds and examined for agglutination of the cells.

Haemadsorption tests

These procedures were performed according to the methods of Punsalang and Sawyer (1973). Growth of gonococci on GCBD medium was flooded with 1 ml. of a suspension of rabbit erythrocytes (3 per cent. v/v) in saline. The plate was held at 37°C. for 15 min. and the surface gently washed with saline after which colonies were examined under the microscope by reflected light at a magnification of 40 × for adherence of red cells.

Haemagglutination

A suspension of gonococci (0·1 ml. containing 10^9 cells) in saline was mixed with 0·1 ml. of a 3 per cent. (v/v) suspension of sheep or rabbit erythrocytes in a small 1 × 7 cm. test tube, as described by Punsalang and Sawyer (1973). The tubes were sealed with 'Parafilm' (Canlab, Toronto, Ont.) and incubated at 37°C. in a water bath for 20 min. followed by a period of 16 hrs at 4°C. The settling patterns of the red cell-bacterial suspensions were compared with controls.

Electron microscopy

The procedure of Swanson (1971) was used in the examination of gonococci for pili. Gonococcal cells harvested after

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20 hrs growth on GCBD plates were suspended in saline. A drop of this suspension (10^{10} organisms/ml.) was placed on a 200-mesh formvar-coated copper grid. After 10 sec. the excess was extracted with filter paper. The grid was then washed for 10 min. with 0·15 M sodium-potassium phosphate buffer (pH 7·0) by the method of Webb (1973). The grid was stained for 10 sec. with 2 per cent. uranyl acetate (pH 3·5). Observations were made with the Phillips EM 201 electron microscope.

Chick embryo inoculation

Fertilized hens' eggs were obtained from a single Leghorn flock reared in the Poultry Laboratory of the University of Manitoba. The birds were fed on an antibiotic-free diet. On receipt, eggs were placed in a Jamesway single-stage egg incubator for 11 days.

Intravenous inoculation of 0·1 ml. volumes of a suspension of gonococci in 1 per cent. proteose peptone saline was performed as described for eggs by Beveridge and Burnet (1946). After inoculation, embryos were examined daily by candling. Blood samples when required were withdrawn from embryos, dead or alive, according to the method of Beveridge and Burnet, and plated onto GCBD medium. These plates were incubated at 37°C. under 5 per cent. CO_2 for 20 hrs, and colonial morphology examined as described above.

For LD_{50} determinations, eleven eggs per dilution of organisms were used.

Mean lethal (LD_{50}) dose determination

Probit analysis of the data was performed on the computer according to a program set up by the Health Sciences Computer Department of the University.

Preparation of antisera

Types 1 and 4 cells of *N. gonorrhoeae* strain 3956 were treated with 1 per cent. (v/v) formalin-saline for 2 hrs, washed three times with sterile phosphate buffered saline at pH 7, and re-suspended to a concentration of 10^{10} cells/ml. Two American Dutch female rabbits were inoculated intravenously with the Type 1 cells, and two with Type 4 cells, according to the immunization procedure of Rudbach (1971). This method was modified to the extent that the inoculum size was increased ten-fold. The animals received 10^8 cells on Days 0, 3, and 5. On Days 7, 9, and 11, the dose was increased to 10^9 cells, and on Days 14, 17, and 19, the inocula were further increased to 10^{10} cells. 2 days after administration of the final dose, the rabbits were bled from the heart. Sera were separated from erythrocytes and stored at —20°C. in 1–2 ml. volumes.

Titration of antisera in the presence of whole cells

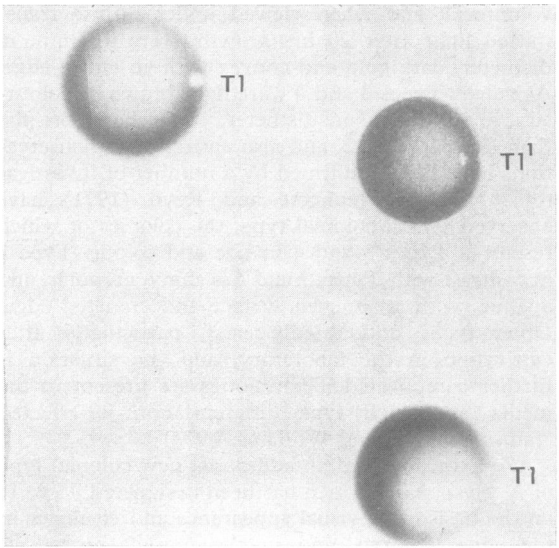
Antigen preparation and agglutination titrations conformed to the methods described by Hurvell, Ahvonen, and Thal (1971), except that antigen was autoclaved only for 90 min. Growth, harvested from GCBD plates in saline and autoclaved at 120°C. for 90 min., was washed three times with saline and diluted to an optical density of 0·3 at 540 nm.

Serial doubling dilution of 0·2 ml. antiserum in phosphate buffered saline at pH 7 were made in tubes (1 × 7 cm.) and 0·2 ml. antigen suspension was added. The tubes were sealed with 'Parafilm' and incubated for 14 hrs in a water bath at 52°C. The end-point was taken as the highest dilution in which the cells remained agglutinated after the tube was given a gentle flick with the finger. A magnifying glass was helpful under these conditions.

Results

Discovery of colonial Type 1^1

Difficulties were encountered in the classification of colonies as Type 1 or Type 2 according to the criteria of Kellogg and others (1963) because of the appearance of an additional small and shiny colony. This type appeared to be too granular and its edge was too



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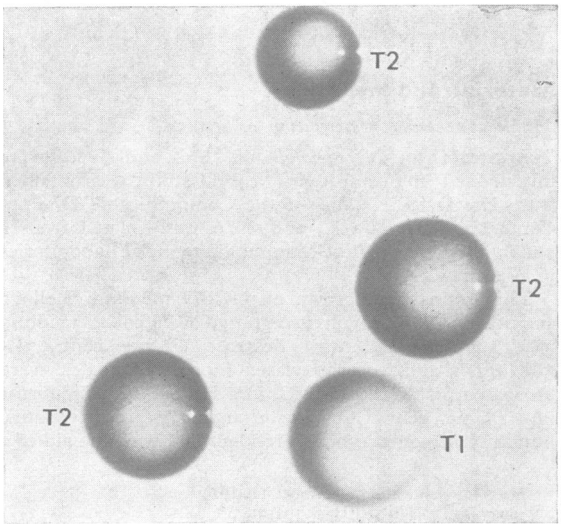

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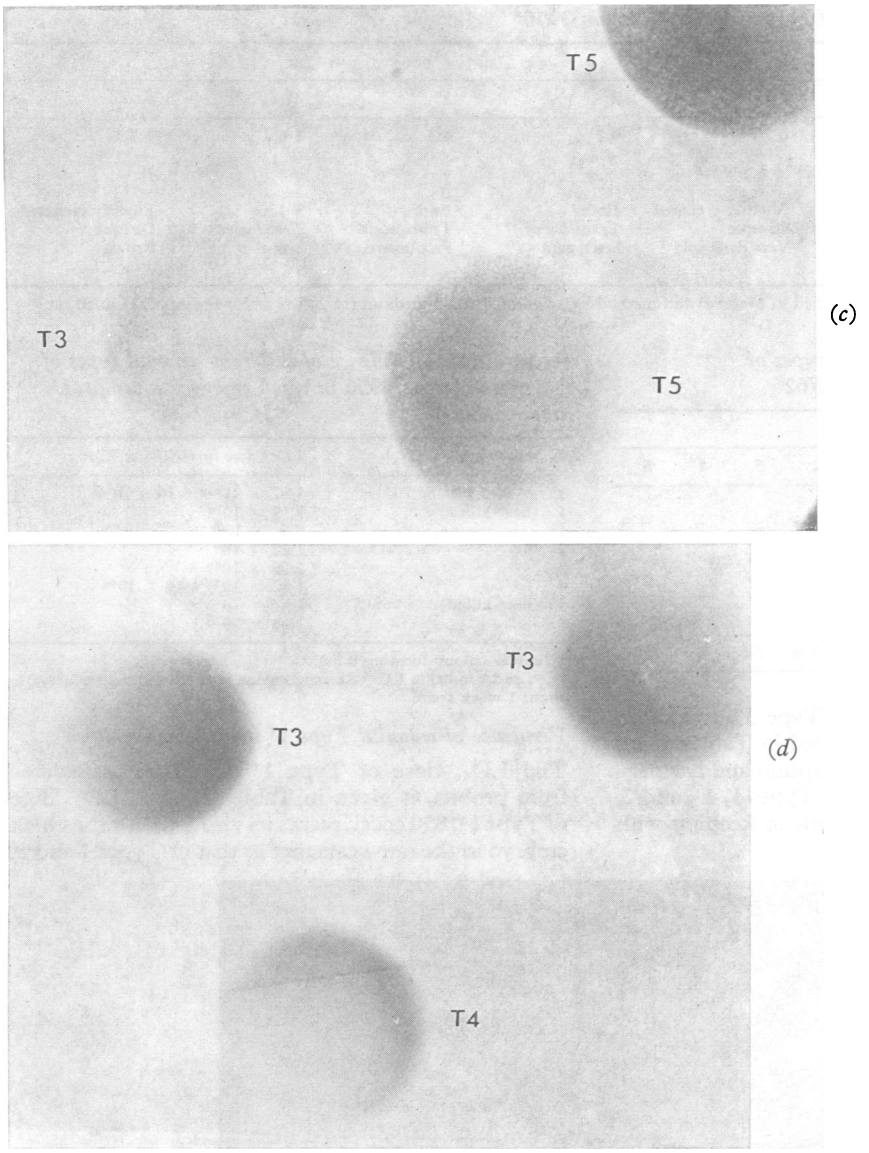


FIG. 1 *Colonial types of* N. gonorrhoeae. *The oblique light source is responsible for a reflection on one side of the colonies.* (*a*) *Types* 1 *and* 1^1; (*b*) *Types* 1 *and* 2; (*c*) *Types* 3 *and* 5; (*d*) *Types* 3 *and* 4

irregular to fit Kellogg's description of Types 1 or 2. We have designated this new colony Type 1^1, in view of the fact that it is darker and more granular than Types 1 and 2 (Fig. 1). However, it is about the same size as these types. Type 1^1 is a stable colonial form as we have been able to carry it in the laboratory for 6 months. Both F62 and 3956 strains yielded colonies of the new type.

In addition to Type 1^1, Types 1, 2, 3, and 4 (Kellogg and others, 1963) and Type 5 (Jephcott and Reyn, 1971) were also identified in the two strains, as shown in Fig. 1 for strain 3956. Morphological features of the types were identical in both strains except that in strain 3956 all colonies were very slightly larger than those of F62.

Conversion from one colonial type to another was observed initially. However, after about sixty selective subcultures, the colonial types stabilized and further transfers yielded 99 per cent. of the original type. The conversions usually noted were as follows:

Type 1→4;
Type 2→3;
Type 3→4;
Type 1^1→5.

TABLE I *Colonial morphology of* N. gonorrhoeae *strain* 3956*

Character	*Colonial type*					
	1	1^1	2	3	4	5
Average diameter (mm.)	0·7	0·7	0·7	1·4	1·4	1·4
Elevation (convexity)	++	++	++	+	+	+
Reflection of light	+	++	++	−	−	+
Surface granularity	−	++	+	+	−	++
Edge	Entire	Slightly crenated	Entire	Entire	Entire	Slightly crenated
Opacity	Translucent	Opaque	Translucent	Translucent	Translucent	Opaque
Colouring	Grey-gold	Very dark gold	Dark gold	Pale brown	None	Brown

*Observed under the light conditions described in Material and methods. Grown on GCBD medium for 20 hrs in 5 per cent. CO_2 in air, at 37°C.

TABLE II *Properties of colonial types of* N. gonorrhoeae *strains* 3956 *and F*62

Property	*Colonial types*					
	1	1^1	2	3	4	5
Auto-agglutinability in saline	+	++	+	−	−	++
Viscidity	++	−	+	++	++	−
Haemadsorption	+	+	+	−	−	−
Haemagglutination:						
3956 + rabbit red cells	±	±	+	−	−	+
3956 + sheep red cells	++	+	++	−	−	+
F62 + sheep red cells	±	++	+	−	−	−
Pili present	Yes	Yes	Yes	No	No	No

Differences between the new Type 1^1 and other morphological types are summarized in Tables I and II. Table II shows that haemadsorption and haemagglutination are largely confined to Types 1, 1^1, and 2. Type 1^1 was shown to possess pili in keeping with virulent types (Fig. 2).

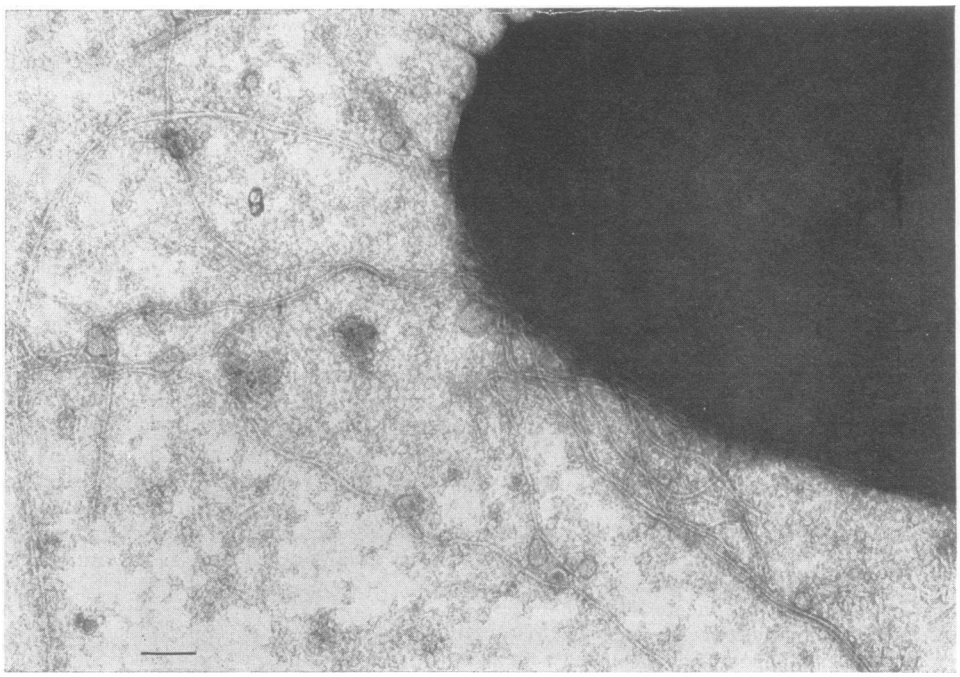
TABLE III *Virulence of different colonial types of* N. gonorrhoeae 3956 *in chick embryos inoculated intravenously*

Colonial type	*LD^{50} dose in CFU† at 72 hrs*
1	$1{\cdot}82 \times 10^{4a}$* $6{\cdot}10 \times 10^{3c}$
1 (heat-killed)	$5{\cdot}19 \times 10^{7a}$
1^1	$8{\cdot}5 \times 10^{2b}$
2	$1{\cdot}22 \times 10^{b}$
3	$2{\cdot}99 \times 10^{6b}$
4	$1{\cdot}82 \times 10^{7a}$ $4{\cdot}44 \times 10^{6c}$
4 (heat-killed)	$4{\cdot}13 \times 10^{6a}$
5	$1{\cdot}16 \times 10^{7b}$

†Denotes colony-forming units
*[a], [b], and [c] indicate LD^{50} determinations at three different times, about 1 week apart

Virulence of colonial Type 1^1 *in the chick embryo*

The LD_{50} dose of Type 1^1 organisms, calculated from probits, is given in Table III. The LD_{50} dose of Type 1^1, 850 cocci, places its virulence for the chick embryo in the same category as that of Types 1 and 2.



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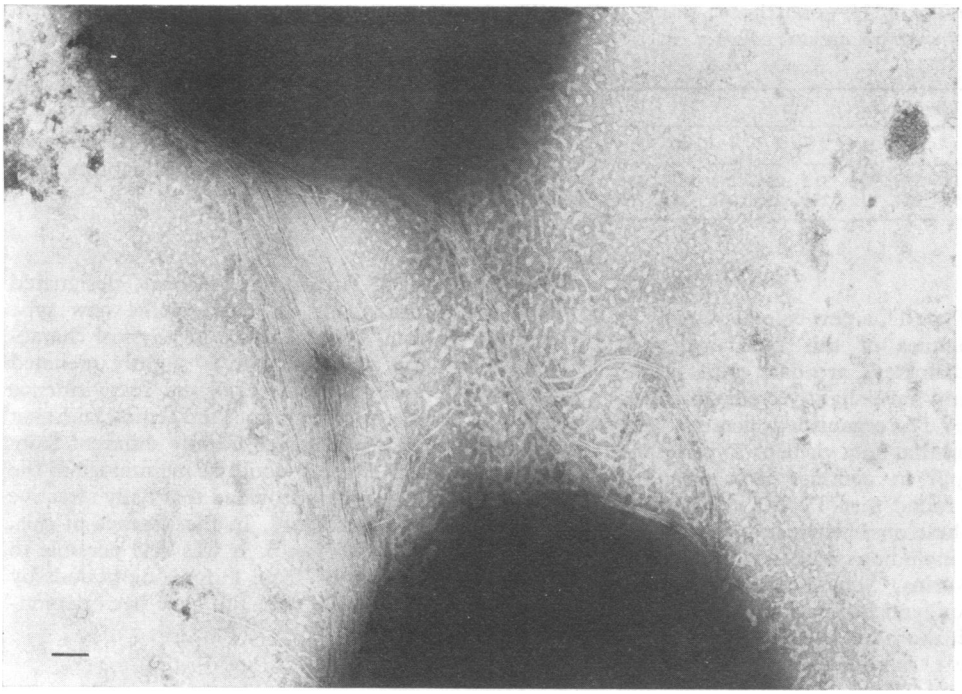

(*b*)

FIG. 2 *Pili of* N. gonorrhoeae. *Visualized by negative staining with uranyl acetate. The bar in the photographs represents* 0·1 μ. (*a*) *Type* 1[1]; (*b*) *Type* 2

The LD_{50} doses of Types 3 and 4 are much higher and are similar to that of Type 5 organisms. Considerable differences were also noted between heat-killed Type 1 and untreated Type 1 cells.

For technical reasons, it was not possible to compare all eight preparations set out in Table III simultaneously. Changes in LD_{50} doses of Types 1 and 4 were investigated with respect to time, and it was found that the values varied as indicated in the Table. Probit analysis of relative virulence in two separate experiments, as shown in Fig. 3, indicates that the difference between Types 1 and 4 remains essentially unchanged, despite the variation of LD_{50} doses determined at different times. Thus, the difference in lethality between virulent and avirulent types of gonococci is real and reproducible.

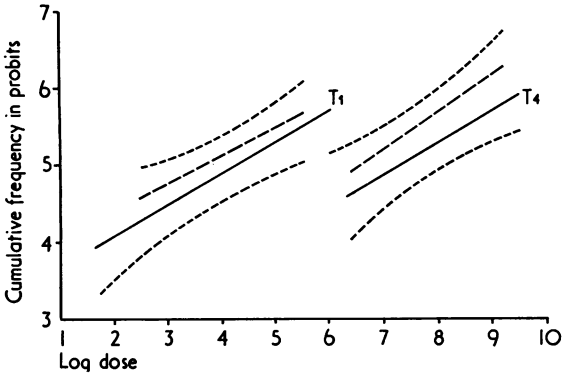

FIG. 3 *Probit analysis of relative virulence of* N. gonorrhoeae *strain* 3956 *Types* 1 *and* 4 *in the chick embryo. The measurements were made about* 1 *week apart. Confidence limits (dotted lines) are placed above and below and the* LD_{50} *dose corresponds to a probit value of* 5.
Solid line data obtained at the same time
Broken line data obtained 1 *week later*

Blood samples removed from dead embryos inoculated with Types 1 and 4 gonococci were cultured. In embryos which succumbed to large doses of Type 4 cells, only Type 4 cells were recovered within one day of inoculation. Where embryos survived 2 to 4 days, Type 1 cells were frequently recovered or a mixture of types was observed. In embryos inoculated with Type 1 cells which survived no longer than 2 days, only Type 1 cells were recovered. If the embryos survived longer, some Type 4 organisms occasionally appeared.

Agglutination titrations

Antisera prepared against colonial Types 1 and 4 agglutinated gonococci of all types including Types 1[1] and 5 (Table IV). This observation confirms the close relationship of these two types to Types 1, 2, 3, and 4 of Kellogg.

TABLE IV *Agglutination of* N. gonorrhoeae *strain* 3956 *in the presence of antisera against colonial Types* 1 *and* 4

Antiserum against 3956 *types*	*Colonial types*							
	1	1[a]	1[a]	2	3	4	4*	5
1	512	256	512	512	256	256	128	256
4	256	128	512	256	256	256	128	256

[a]F62 strain

Discussion

We have described Type 1^1, a new colonial variety of *N. gonorrhoeae*. Colonies of this type are small (about 0·7 mm. in diameter), are dark gold, reflect light, are granular, and have slightly crenated edges. Furthermore, Type 1^1 organisms possess pili. Swanson (1971) found that only virulent Types 1 and 2 gonococci have pili in contrast with avirulent Types 3 and 4. We found that Type 5 also had no pili. In addition to these observations, other properties of Type 1^1, including tendency to clump (Kellogg, Cohen, Norins, Schroeter, and Reising, 1968), haemadsorption, and haemagglutination, were most in keeping with those of Types 1 and 2. Thus it appears that Type 1^1 is a virulent variant. Brown and Kraus (1974) and Diena, Ryan, Ashton, and Wallace (1974) also noted some intrastrain variation of Type 1 colonies. The colony which Brown and Kraus (1974) referred to as a Type 1 variant was about the size of Types 1 and 2 but was less coloured. They did not comment on any other properties of the Type 1 variant.

Experiments with 11-day chick embryos showed that Types 1, 1^1, and 2 were significantly more virulent than Types 3, 4, and 5. Heat-killed Types 1 and 4 cells had essentially the same degree of toxicity as living Type 4 cells. Thus, differences observed between the two groups of gonococci are a manifestation of the living cells. Buchanan and Gotschlich (1973) have also noted that Types 1 and 2 gonococci are more virulent for the chick embryo than Types 3 and 4. Their work was based on infection of the chorioallantoic membrane. Bumgarner and Finkelstein (1973) inoculated embryos intravenously with Types 1 and 2, finding that they were considerably more virulent than Types 3 and 4. In this study there is evidence that incubation of gonococci in the chick embryo favours the emergence of virulent Type 1 organisms. Walsh, Brown, Brown, and Pirkle (1963) have also found that the chick embryo acts as a selective medium for virulent types, permitting their growth and suppressing multiplication of avirulent cells. Their studies were based on inoculation of the allantoic cavity.

The agglutination of erythrocytes by virulent gonococci has been attributed by Punsalang and Sawyer (1973) to the presence of pili. Since Type 5 organisms have no pili, other surface properties must be responsible for this phenomenon. Type 5 gonococci also clumped in saline, which suggests that the apparent correlation of agglutinability with virulence is perhaps coincidental.

Summary

A new variant of *Neisseria gonorrhoeae*, designated Type 1^1, is described. Colonies of the new type resemble those of Types 1 and 2 in physical characteristics but are granular with a slightly crenated edge and are a deeper gold in colour. The virulence of Type 1^1 in the chick embryo is in keeping with that of Types 1 and 2 but is significantly different from Types 3, 4, and 5. Type 1^1 could be maintained in the laboratory for 6 months, provided that daily selective subcultures were performed. In the absence of this, Type 1^1 reverted to Type 5. It was also possible to preserve the stability of Type 1^1 for long periods by immersion in liquid nitrogen. Pili have been demonstrated on the new type.

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