NEISSERIA GONORRHOEAE

I. VIRULENCE GENETICALLY LINKED TO CLONAL VARIATION

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ABSTRACT

KELLOGG, DOUGLAS S., JR. (Communicable Disease Center, Atlanta, Ga.), WILLIAM L. PEACOCK, JR., W. E. DEACON, L. BROWN, AND CARL I. PIRKLE. *Neisseria gonorrhoeae*. I. Virulence genetically linked to clonal variation. J. Bacteriol. **85**:1274–1279. 1963.—One type, obtained from the purulent exudate of acute gonorrhea was maintained by 69 selective in vitro passages, at which point the organisms produced infections in human volunteers. A predominance of clonal types found in laboratory strains and a lack of ability to infect human volunteers resulted from 69 nonselective in vitro passages. Physiological and serological characteristics of the clonal types are compared. We are now in a position to study *Neisseria gonorrhoeae* organisms in their virulent form.

Although Leistikow and Loeffler initially cultivated *Neisseria gonorrhoeae* in 1882, few facts are presently available which indicate that colonial differences can be correlated with virulence, serological, or physiological characteristics (Scherp, 1955). In contrast, researchers on other pathogenic bacteria have shown definite relationships to exist between colonial morphology and antigenic mosaics, biochemical capabilities, pathogenicity, and bacteriophage host ranges.

Preliminary experiments in this laboratory have indicated an association between K antigen of *N. gonorrhoeae* and virulence (Deacon et al., 1959). More recently, results obtained in human volunteer infection studies have suggested a possible correlation between clonal morphology, virulence, and length of in vitro cultivation.

The following presentation describes several clonal types of *N. gonorrhoeae* detectable in fresh isolates from purulent exudates of acute gonorrhea and in laboratory strains. Certain clonal types are identified with acute infections.

MATERIALS AND METHODS

The *N. gonorrhoeae* laboratory strains used in these studies were originally isolated by this laboratory. Primary isolates were obtained at the Fulton County Health Department, Atlanta, Ga., through the assistance and cooperation of John H. Tiedemann.

Laboratory and primary isolate strains were passaged by loop transfer on Chocolate Agar medium, Difco G C Medium Base (GCB) plus hemoglobin with 1% Difco Supplement B (GCBBH), GCBBH minus hemoglobin (GCBB), or GCB with 2% of a defined supplement (GCB-2DS; Lankford, 1950). The defined supplement was composed of cocarboxylase, 0.001 g; glutamine, 0.5 g; and dextrose, 20.0 g; dissolved in 100 ml of distilled water and sterilized by filtration (Seitz EK). Both the Supplement B and the defined supplement were added to the base medium at 43 to 45 C and the medium poured immediately. All plates were dried at 36 C to remove surface moisture, then stored in sealed containers at 24 to 27 C.

Fermentation reactions were tested in a medium containing (per liter of distilled water): Proteose Peptone No. 3, 10.0 g; sodium chloride, 5.0 g; agar, 15.0 g; and phenol red, 10.0 ml of a 0.2% aqueous solution. Carbohydrates were added from sterile 20% solutions at 0.125 ml per 5.0 ml of medium, except for mannitol in which 0.25 ml of a 10% solution was added per 5.0 ml of medium. All incubations, unless otherwise specified, were done at approximately 36 C.

Tests for deoxyribonuclease utilized Deoxyribonuclease Medium (BBL) with added 1% Supplement B (Difco). Other test procedures were performed according to the *Manual of Micro-*

biological Methods (Society of American Bacteriologists, 1957).

Antisera used for agglutination studies or conjugated for fluorescent antibody studies were prepared against GCF-RD_1 (100% T4) by conventional immunological procedures (Deacon et al., 1959). β-Propriolactone-treated cells (0.3% β-propiolactone in saline-bicarbonate buffer for 2 hr at 37 C) were used where roughness of formalinized cells in saline interfered with agglutinations.

Human volunteers were obtained for urethral inoculations. Each volunteer received an inoculum of one standard 2-mm loopful of organisms from a 20- to 24-hr growth plate of well-isolated clones. Clonal types other than a desired type were marked with dye and avoided when filling the loop. Organisms from volunteers' urethral exudates were identified by direct and indirect fluorescent antibody techniques, gram-stained direct smears, clonal analysis, oxidase reactivity, and fermentative ability.

RESULTS

Four morphologically distinct clonal types were observed in *N. gonorrhoeae* cultures. The clonal characteristics of these types are listed in Table 1.

Figure 1A shows the four types at 24 hr from an artificial mixture. Figure 1B shows a type 3 strain with type 2 and type 4 variant clones.

Type distinction was found to be dependent upon the technique of observation, clonal age, surface characteristics of the medium, and the composition of the medium. Optimal definition of clonal characteristics was best obtained with diffuse, angled, transmitted light. Top or oblique lighting, alone or together, did not allow simple distinctions owing to interference by reflected surface light and shadow formation. The distinctive type characteristics were best observed in clones after 18 to 24 hr of development. Younger clones were too small, and older clones usually exhibited irregularities of outline and texture associated with the presence of developing growth of the other types. Figures 1C and 1D demonstrate types 1 and 2 after 48 hr of growth. Each clone exhibited one or more outgrowths which are beginning to obscure the characteristic clonal edges. Excessive moisture in or on the surface of the medium caused all four types to become granular in texture and outline and indistinguishable from one another. A similar effect was obtained upon inferior media.

In primary isolates from acute gonorrhea of males, 90% of the clones consisted of type 1 and 10% were types 2 and 3. There was a shift to a predominance of types 3 and 4 if the growth was transferred without selection. Type 1 was lost from the population in three to four unselected transfers, and did not reappear during subsequent transfers. Type 2 made a rare to an occasional appearance for months after primary isolation. Type 3 became a minority of the population composed of type 4. Type 4 was either the predominant or the only type present in the usual laboratory cultures (Table 2). A comparison of the isolation dates and type frequencies found in Table 2 indicated that length of in vitro cultivation was not the only factor influencing clonal variation. Another factor was the observed difference between primary isolate strains in their rate of clonal variation. This difference was probably a reflection of variations in their genetic constitutions.

The four clone types were readily maintained in vitro as separate entities. Type 1 was of the most interest, since it was found only in the puru-

TABLE 1. *Characteristics of four clonal types observed in Neisseria gonorrhoeae cultures on GCBB or GCB2DS medium*

Type	Size	Color density	Elevation	Edges	Opacity	Structure	Consistency
	mm						
1	0.5	Dark gold	Convex	Entire	Translucent	Amorphous	Slightly viscid
2	0.5	Dark gold	Convex	Defined, crenated, or both	Translucent	Amorphous	Friable
3	1	Light brown	Low convex	Entire	Translucent	Granular	Viscid
4	1	Colorless	Low convex	Entire	Transparent	Amorphous	Viscid

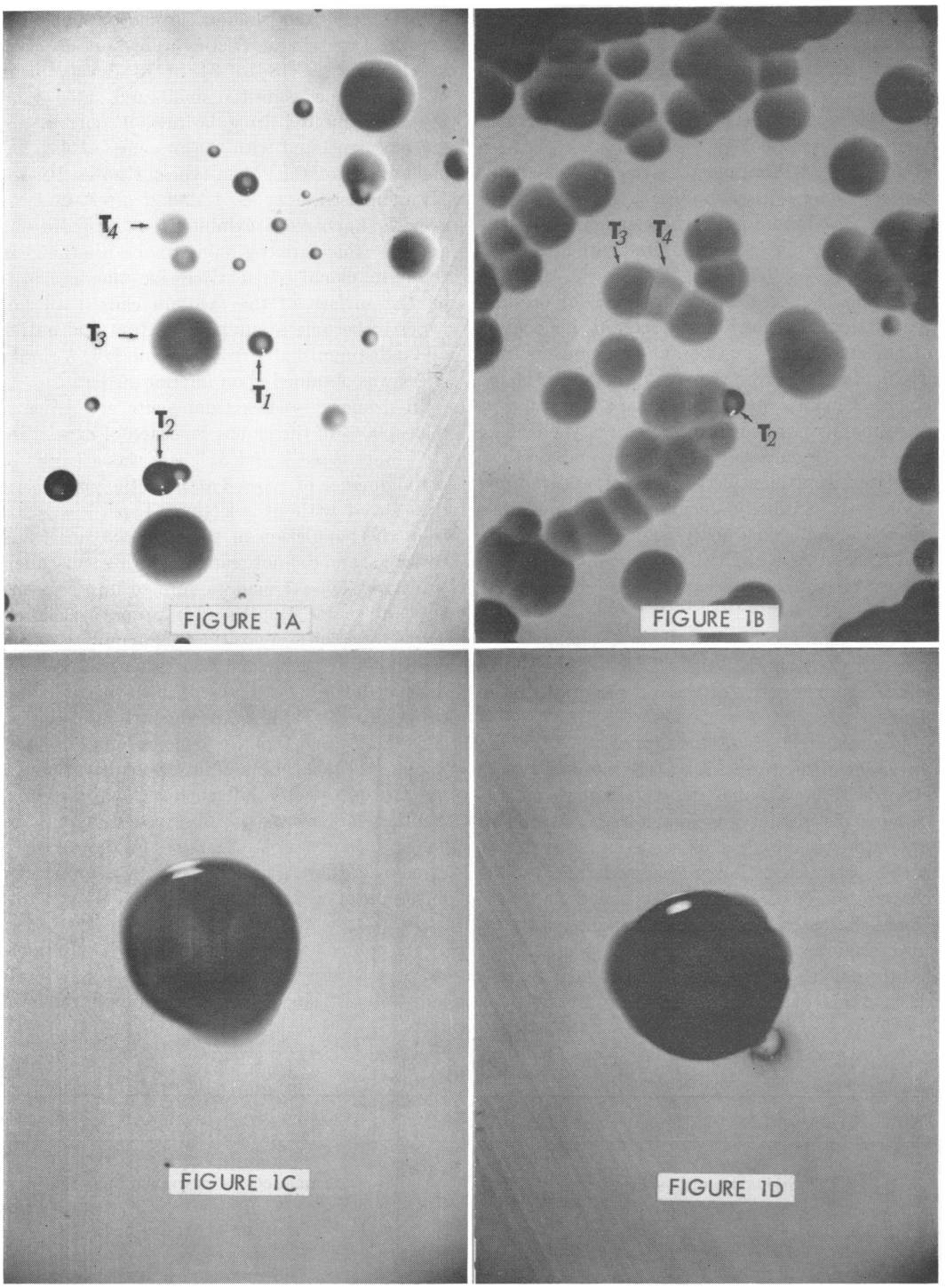

FIG. 1. *Four clonal types in Neisseria gonorrhoeae cultures. (1A) Types 1, 2, 3, and 4 at 24 hr from an artificial mixture. (1B) A type 3 strain with type 2 and type 4 variant clones. (1C and 1D) Types 1 and 2 after 48 hr of growth.*

lent exudate from acute gonorrhea of the male, and, owing to its rapid loss from an unselected population, has not been studied for its specific characteristics. During cultivation in vitro, any one or more of the other three types appeared on plates streaked from well-isolated clones of type 1. This occurred at every transfer of type 1 and could have been the result either of carrying a mixture of types or of a genetic instability of type 1 cells in vitro. Since a series of 80 selective transfers had not altered the situation, it seemed that the latter explanation had more merit. Outgrowths became evident on type 1 clones after 30 to 36 hr of continuous incubation. Such outgrowths consisted of 10 to 15% T3 and T4 as compared with the usual <1% of these types found with selected "non-outgrowth" type 1 clones. A similar situation occurred with type 2, 3, and 4 clones. Cultivation of the four types at 32 to 33 C retarded clonal growth and reduced the percentage of clones showing outgrowths. None of the four types would grow at 32 to 33 C on unsupplemented medium (GCB), though growth did occur at 36 C on such a medium. At 36 C on GCB medium, there was no size differential between the clones of types 1 and 2 and types 3 and 4. The addition of defined supplement to GCB medium allowed the types to grow at 32 to 33 C with their usual clonal morphology, indicating that the supplement had a greater effect on types 3 and 4 than on types 1 and 2. In general, types 1 and 2 were sensitive to alterations in the physical and chemical environment which had little or no effect upon types 3 and 4.

Considering the ecology of the types, it appeared that types 3 and 4 had a selective advantage over types 1 and 2 in vitro. In terms of maintaining a type or types more closely associated with a disease state, several kinds of media were tested for their ability to stabilize types 1 and 2. These included the media described in Materials and Methods as well as GCBB plus 5% normal rabbit blood, Eugonagar (BBL), GCB, GCB plus 1% rabbit antiserum to formalinized type 4 cells, GCB plus 1% rabbit antiserum to autoclaved type 4 cells, and GCB plus 1% normal rabbit serum. There was no appreciable effect by any of these media upon the relative stability of the four clonal types. Since type 1 cells produced acid at a slower rate than the other three types, the effect of acidity upon type stabilization was studied either by adding additional buffer capacity in the form of tris(hydroxymethyl)aminomethane buffer or by a variation of plate medium thickness. Acidity as such did not affect type stability. Carbon dioxide tended to promote the stability of types 1 and 2 (Fig. 2).

TABLE 2. *Per cent type frequency in laboratory strains*

Strains	T2	T3	T4	Isolation date
18	0.01	50	50	Apr. 1960
19	0	0.01	99+	Apr. 1960
21	0	50	50	Apr. 1960
26	0	0	100	Apr. 1960
28	0	30	70	Apr. 1960
49	0	10	90	Aug. 1960
1A	0	10	90	Unknown
RD_5	0	0	100	Aug. 1957
Rachel	0.1	0.1	99+	Sept. 1961

* Type 1 was not present in laboratory cultures.

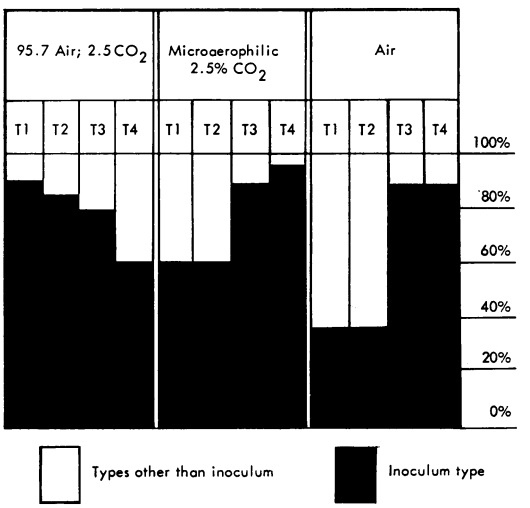

FIG. 2. *Effects of gaseous environments upon the percentages of clonal types developing from an inoculum of each clonal type. Each column represents results from inoculation of the indicated type.*

Physiologically, the cells of the four clonal types were more alike than different. The individual cells of each clonal type were alike in morphology and size. Types 2, 3, and 4 fermented glucose with acid but no gas production in 24 hr. Type 1 fermentation was indecisive in 24 hr but strong by 48 hr. All four clonal types had the same rate and final density of color production with oxidase reagent. Catalase production was generally weak and variable except after microaerophilic cultivation, in which case all four were

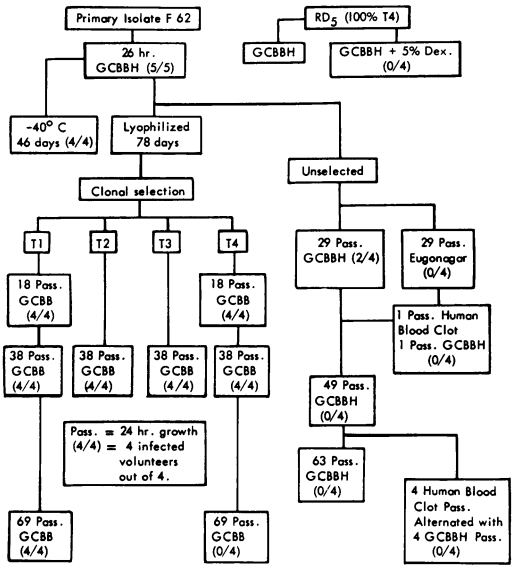

FIG. 3. *Virulence and in vitro cultivation.*

strongly positive. All four types were negative for nitrate reduction and nitrite production. None of the types were hemolytic on either human or rabbit blood at 24 or 48 hr. All four types were positive for deoxyribonuclease production. The four types derived from a single primary strain were equally sensitive to penicillin (0.05 μg/ml). The elimination of glucose from the defined supplement with or without the addition of glutamic acid had no effect upon the characteristic clonal morphology. The elimination of hemoglobin from Chocolate Agar medium did not materially affect clonal growth nor morphology.

Substitution of defined supplement for Supplement B resulted in a loss of the dark-gold color density characteristic of types 1 and 2. The addition of heme or ferric nitrate (0.005% level) to the defined supplement medium resulted in clones of types 1 and 2 which had comparable color density to those on GCBB. Increasing the percentage of ferric nitrate to 0.04% caused all four clone types to become granular in appearance. Besides the effect of ferric nitrate upon clone coloration in types 1 and 2, there was a stimulation of the growth rates of all four types. This was most pronounced with clonal types 1 and 2, which became two to three times the size attained without ferric nitrate. GCBDS with 1 mg/100 ml of nile blue sulfate would not support growth unless ferric nitrate was added to the medium. In the presence of ferric nitrate, all four types accumulated nile blue to the same extent.

Serologically, employing antisera against a laboratory strain (type 4), all four types gave approximately equivalent fluorescent-antibody pictures and agglutination patterns. Types 1 and 2 formalinized were rough in saline, whereas types 3 and 4 were smooth. This accorded well with past experience in this laboratory in which primary isolates were found to be rough; however, after several in vitro passages, the strains were found to become smooth. According to present information, this would have paralleled the alteration of predominant clonal types in the population from types 1 and 2 to types 3 and 4.

Figure 3 is a flow sheet of the progress and conditions of the in vitro cultivation of *N. gonorrhoeae* organisms with the results of periodic virulence testing in human volunteers.

A laboratory strain of *N. gonorrhoeae* (RD_5; 100% T4) did not produce infections even when stimulated to produce maximal amounts of K antigen (GCBBH + 5% dextrose). The effects upon virulence of unselected passage of primary isolate F 62 illustrated both the selection toward avirulence obtained in vitro and the additive effects of an inferior medium such as Eugonagar upon such selection. The capability of blood clots to select virulent organisms by elimination of avirulent organisms was not demonstrated with *N. gonorrhoeae.*

Rapid selective in vitro passaging of clonal types has shown that type 1 retains virulence, whereas type 4 loses virulence after 69 passages. Types 2 and 3 retained virulence after 38 passages, but were not virulence-tested at 69 passages owing to insufficient time and patient space. Regardless of the clonal type used as the inoculum, the clonal type isolated from the urethral exudates of the infected volunteers was type 1.

DISCUSSION

Our results show that there are several morphologically distinct clonal types in *N. gonorrhoeae* cultures. There is ecological significance to these clonal types, since type 1 is found only in the purulent exudate from acute gonorrhea of males, and type 4 is associated only with laboratory cultures. Characteristics of *N. gonorrhoeae* which have been studied with cultures even a few days or transfers from the patient may have been those of a predominantly type 4 laboratory culture. In a

real sense for the first time, it now appears possible to study the factors responsible for virulence in *N. gonorrhoeae* cells.

Physiologically, type 1 ferments glucose more slowly than the other three clonal types, which may account for the reports in the literature of initial lack of glucose fermentative ability in primary isolates of *N. gonorrhoeae* (Morton and Shoemaker, 1945). Growth of types 1 and 2 is stimulated by adding ferric nitrate to a defined supplement. Since nitrate ions are not utilized as such, the ferric ion may be a specific requirement of these two clonal types. Such a requirement could be useful in the formulation of an enrichment medium for *N. gonorrhoeae*. Also, ferric ions are associated with cytochrome systems of which the oxidase enzyme is a component. It would be interesting if a correlation could be found between the oxidase enzyme, the ferric ion requirement, and the propensity to autolysis of *N. gonorrhoeae* cells.

Serologically, type 1 cells treated with type 4 antiserum are not distinctive. Since the alteration in vitro of type 1 to types 2, 3, and 4 seems to be a loss variation, it is reasonable that an antigenic grouping specific to type 1 cells could be lost as well. The presence or absence of this antigenic grouping in the test system may be the reason for the limited and variable success enjoyed by serological procedures designed to ascertain the presence of circulating antibodies in gonorrheal infections.

An important result of our studies was the maintenance of virulence in association with a particular heritable clonal type of *N. gonorrhoeae* (type 1) during in vitro passage. This clonal type was recovered from infections resulting from inoculation with the other clonal types of *N. gonorrhoeae*. Since our inocula of these types were purposefully composed of billions of organisms, probably far in excess of the minimal infective dose, our results with types 2, 3, and 4 may be indicative of a progressive decline in the percentage of type 1 cells within these population types. Establishment of the minimal infective dose of type 1 cells will allow proper evaluation of virulence and clonal morphology. Future work will attempt to establish the factors responsible for virulence and to uncover associated unknown antigenic and biochemical characters of diagnostic value.

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