

Scanning Electron Microscope Study of *Neisseria gonorrhoeae*

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Morphological studies utilizing various microscopy techniques have aided in our understanding of the gonococcus and gonorrhea. In this study scanning electron microscopy was used to study differences in virulent (colony types 1 and 2) and avirulent (colony types 3 and 4) gonococci relative to colony appearance, patterns of growth in liquid media, and surface features of individual cocci. Colony types of virulent gonococci are smaller in diameter but have a higher evaluation than those of avirulent mutants. Colony type 2 has a convex undersurface that is associated with surface pitting of solid media. When the colonies are grown in liquid media, various degrees of autoagglutination are observed; this is most pronounced with type 2 and least evident with type 4. Although pili may be involved in this phenomena, other mechanisms must be employed, since type 3 gonococci that lack pili autoagglutinate. Pili are seen on types 1 and 2 and are absent from types 3 and 4. They appear as individual threads radiating from the bacteria or as bundles of pili attaching adjacent cocci. Another extracellular structure consists of small spherical bodies that can coat the bacteria surface, attach to pili, or exist free from other bacterial components. These spheres are least evident with type 4. The gonococcal surface is pebbly with multiple sulci.

Morphological studies with various microscopy techniques and instruments have aided our understanding of the gonococcus and gonorrhea. For instance, light microscopy studies of colony types on solid media have shown that virulent gonococci grow as small colonies, and avirulent variants grow as much larger colonies (6, 7). Transmission electron microscopy (TEM) revealed that the gonococci within small colonies possess extracellular structures known as pili or fimbriae and that these are absent on gonococci from the large avirulent colonies (5, 8). TEM, aided by cultural methods, has also disproven the hypothesis that gonococci survive and multiply after phagocytosis by polymorphonuclear leukocytes (10).

Scanning electron microscopy (SEM) is a relatively new technique that provides greater depth of field and permits easier interpretation of topography and slopes of surfaces. Specimens to be studied require either no sample preparation or at most a thin metal coating to provide conductivity over a relatively insulated surface. Such a simple procedure reduces artifacts that may be introduced by other preparative techniques. An advantage of SEM over replicate techniques is that the latter cannot be used easily over very rough, fragile, or multiple interconnected surfaces.

The advantages of SEM have been applied to a study of *Neisseria gonorrhoeae* in the hopes that it could further our understanding of the gonococcus. It was felt that the great depth of field of SEM could be of value in studies of colony morphologies and patterns of growth in liquid media. Observation of fragile extracellular structures also seemed to be within the potential of SEM.

MATERIALS AND METHODS

***N. gonorrhoeae* isolates.** A fresh clinical isolate was obtained from a male patient with gonococcal urethritis and cultured on selective media (9). This was further identified as being a gram-negative diplococci that produced a positive oxidase reaction with a 1% solution of *N,N*-dimethyl-*p*-phenylenediaminemonohydrochloride and fermented glucose but not maltose, lactose, or sucrose. Cystine tryptic agar (BBL) containing 1% of the test sugar was used for fermentations. Two passages were performed to isolate the four colonial variants, which were then preserved by freezing in a medium containing 10% glycerol in phosphate-buffered saline, pH 7.2. A second isolate, CDC no. 2686, was supplied by D. Kellogg, and the four colony types have been consecutively passed in our laboratory for over 2 years.

Growth of the organisms: (i) Solid medium. Gonococci were plated on GC agar base supplemented with Iso-Vitalex (BBL) and growth for 20 h at 35.5 C in a candle-extinction jar.

(ii) **Liquid medium.** Organisms were grown in Frantz medium (4) supplemented with 0.4% dialyzed yeast extract. Organisms from solid medium were removed with sterile cotton swabs, suspended in 100 ml of liquid medium contained in a 500-ml Erlenmeyer flask, and incubated at 35.5 C for 5 h on a rotary platform shaker revolving at 125 rpm.

Colonial morphology. The organisms were grown on solid medium as described before. The colony typing system was that of Kellogg et al. (7), in which a stereoscopic microscope was used with light transmitted through the medium from below.

Electron microscopy. Colonies on solid medium were fixed by the addition of 1% glutaraldehyde in phosphate-buffered saline, pH 7.2. The colonies were gently floated off the agar surface and onto glass cover slips. The samples were then flooded with distilled water, and the wash was removed by touching bibulous paper to the cover-slip surface.

Gonococci grown in liquid medium were fixed by adding a 1/20 volume of a 2% glutaraldehyde solution and then washed six times with distilled water. Types 1, 2, and 3 showed spontaneous sedimentation, and the washing was done without centrifugation. Sedimentation of type 4 required centrifugation at $4,000 \times g$ for 5 min. A drop of the final aqueous suspension was applied to a glass cover slip.

The cover slips containing the specimens were placed in a vacuum chamber and coated with a thin layer of carbon and an outer coating of gold-palladium. These specimens were examined and photographed with a Cambridge scanning electron microscope.

RESULTS

SEM micrographs of the four colony types are presented in Fig. 1 to 4. Included are three different views of each colony type: a view looking straight down on the top of the colony (0° angle), a tangential view (85° angle), and a view at an intermediate angle (60 to 70° angle).

A typical type 1 colony is shown in Fig. 1. Its minimal diameter measures $125 \mu\text{m}$, and its maximal diameter is $135 \mu\text{m}$. The colony gradually meets the agar surface at its periphery, and from here it slowly rises to its summit at the center of the colony where it measures $28.1 \mu\text{m}$ in height. The T2 colony pictured in Fig. 2 is slightly larger than the type 1 colony in that its diameter varies from 172 to $191 \mu\text{m}$. In contrast to the type 1 colony, there is a sharp increase in height at the periphery of the colony and then it levels off at a height of $36.9 \mu\text{m}$. Since type 2 colonies have a convex undersurface, they differ from the other three colony types.

The largest colony is pictured in Fig. 3 (colony type 3). Its diameter ranges from 544 to $628 \mu\text{m}$. The largest colony is also the flattest and has an elevation of only $2.8 \mu\text{m}$. The surface granularity of T3 seen with stereoscopic microscopy is also evident on SEM. The type 4 colony

shown in Fig. 4 has a maximal diameter of $480 \mu\text{m}$ and a minimal diameter of $405 \mu\text{m}$. The elevation of type 4 is $9.2 \mu\text{m}$.

The colonies depicted in this study are representative of the four colony types of a typical *N. gonorrhoeae* isolate. It must be appreciated, however, that different *N. gonorrhoeae* isolates vary somewhat in their rate of growth and therefore in the colony dimensions at a given time interval. Rare gonococcal isolates grow exceedingly slowly and were purposefully excluded from this study of typical isolates of *N. gonorrhoeae*. No differences were detected between the SEM appearance of the fresh isolate and laboratory stain no. 2686.

The clumped growth of certain gonococci in liquid medium was readily evident on SEM. The clumping was most evident with type 2 gonococci where the aggregates exceeded $60 \mu\text{m}$ in diameter (Fig. 5B). Types 1 and 3 exhibit an intermediate degree of clumping (Fig. 5A and C), and type 4 demonstrates only rare clumps, with most gonococci existing as individual cells or diplococci (Fig. 5D).

The classic diplococcal arrangement of *N. gonorrhoeae* is seen in Fig. 6D. The adjacent sides of the diplococcal pair are flattened so that the individual coccus is not spherical but has a diameter that varies from $1.1 \mu\text{m}$ on its long axis to $0.9 \mu\text{m}$ on the short axis. The outer surface of the gonococcus consists of multiple fissures, and it has a pebbly appearance. Pili were absent in this type 4 gonococcus as well as in type 3 gonococci.

Pili were found on type 1 and type 2 colonies and appeared as threads extending free from the gonococcus (Fig. 6B and C) or as parallel aggregates joining adjacent cocci (Fig. 6C). A second extracellular structure consists of small spherical structures that range in diameter from 0.2 to $0.6 \mu\text{m}$. These spheres could be found with all four colony types but appeared to be least numerous with type 4 gonococci. They were associated with bacterial surfaces (Fig. 6A and B), on pili (Fig. 6B), or completely free of other structures (Fig. 6B and D).

DISCUSSION

In vitro cultivation of *N. gonorrhoeae* results in a multitude of growth patterns. This pleomorphism occurs with growth on solid media (1, 3, 7, 12) as well as in liquid media (8). A frequently used system for classification of the various colonial morphologies as seen on solid media is that of Kellogg et al., who described four colonial types (7). Although gross differences in colony size are evident to the unaided eye, the fine points of the colony type differen-

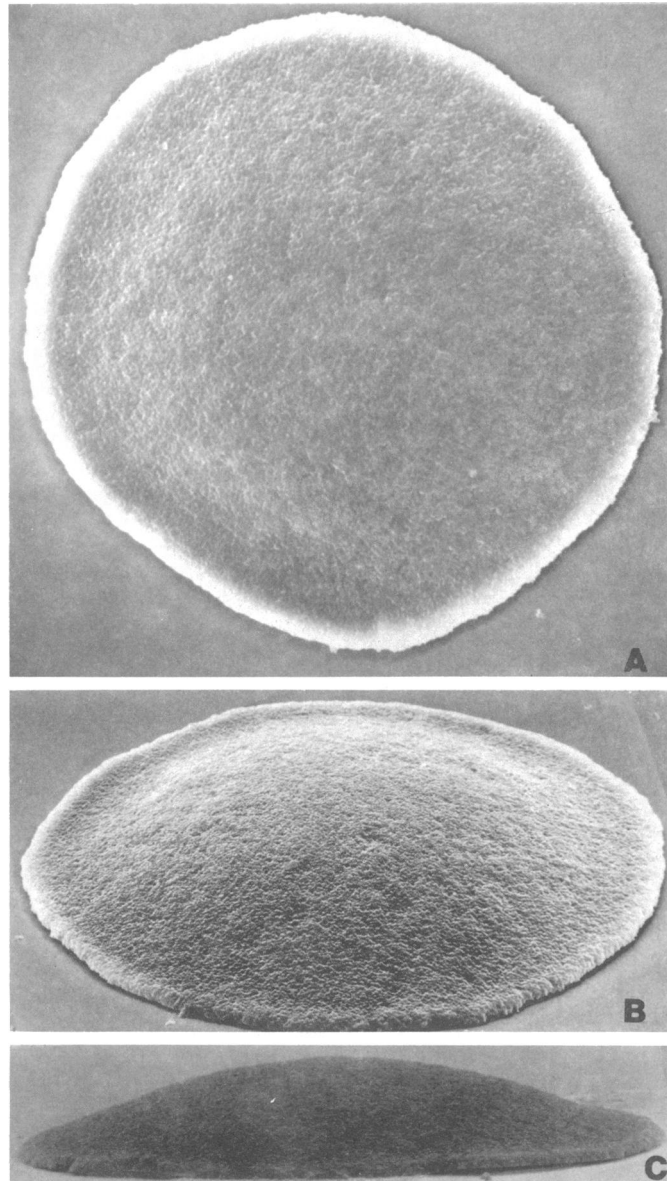


FIG. 1. Colony type 1 *N. gonorrhoeae*. (A) 0° angle and $\times 610$. (B) 60° angle and $\times 605$. (C) 85° angle and $\times 570$.

tial are observed with a stereoscopic microscope with light transmitted through a translucent medium. Colony types 1 and 2 are small in contrast to the larger colony types 3 and 4. Type 2 colonies differ from type 1 in that they have a well-defined peripheral margin that appears as a thin dark line circumscribing the colony. Type 3 colonies differ from type 4 in that they have a light brown color and a somewhat granular surface. SEM has allowed a quantitative com-

parison of the definitions and topography of the various colony types.

SEM has also shown that type 2 colonies can have a convex undersurface, whereas the other colony types have a flat undersurface. This is in agreement with our observation that type 2 colonies grow into an agar surface (E. Cowart and S. J. Kraus, personal communication). The latter results in pitting of the agar, which is evident when the colonies are carefully removed

from the surface. TEM studies have shown that type 2 colonies are the most heavily piliated. Heaviest piliation therefore is associated with agar pitting by gonococci, although this may not be a cause-effect relationship. A similar association between pili, convex colony under-surface, and agar pitting is known to occur with *Moraxella* (2).

When gonococci are grown in liquid media, several different patterns of growth are observed, and these are associated with growth patterns on solid media (8). Type 2 gonococci grow as large clumps, and type 4 grow as a smooth suspension. Types 1 and 3 are intermediate in their degree of clumped growth in liquid media. SEM shows that the marked clumping

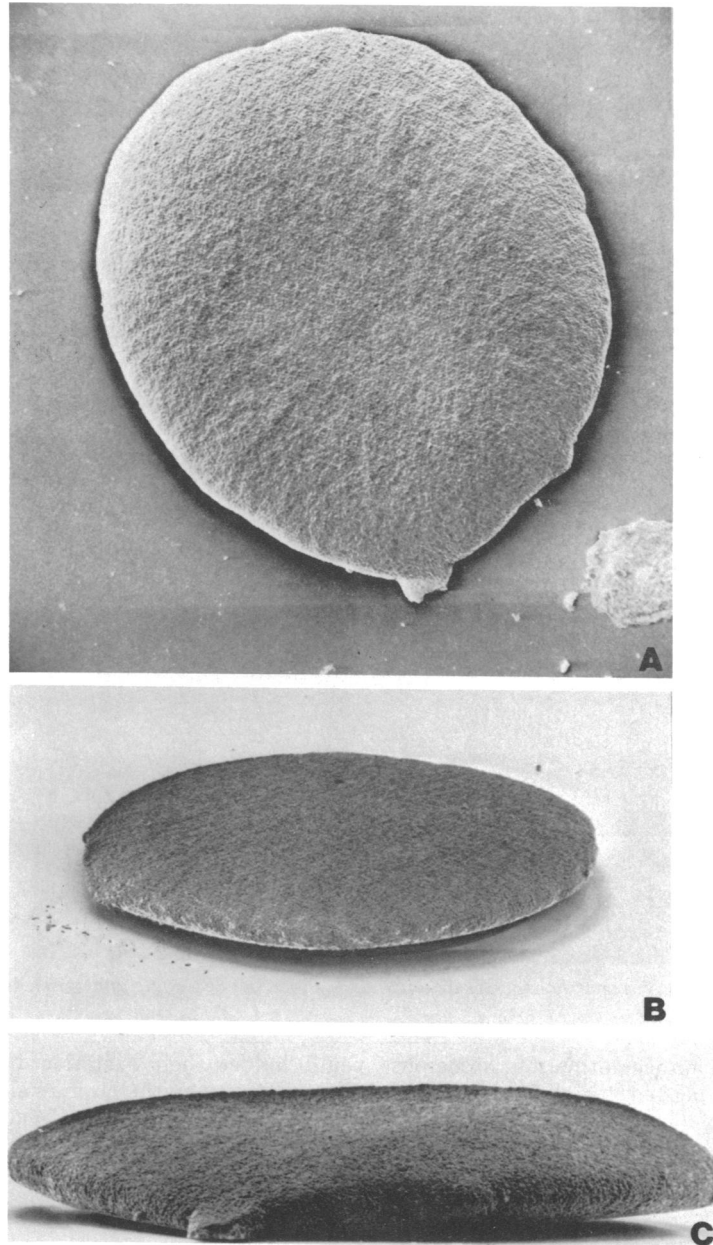


FIG. 2. Colony type 2 *N. gonorrhoeae*. (A) 0° angle and $\times 372$. (B) 70° angle and $\times 376$. (C) 85° angle and $\times 515$.

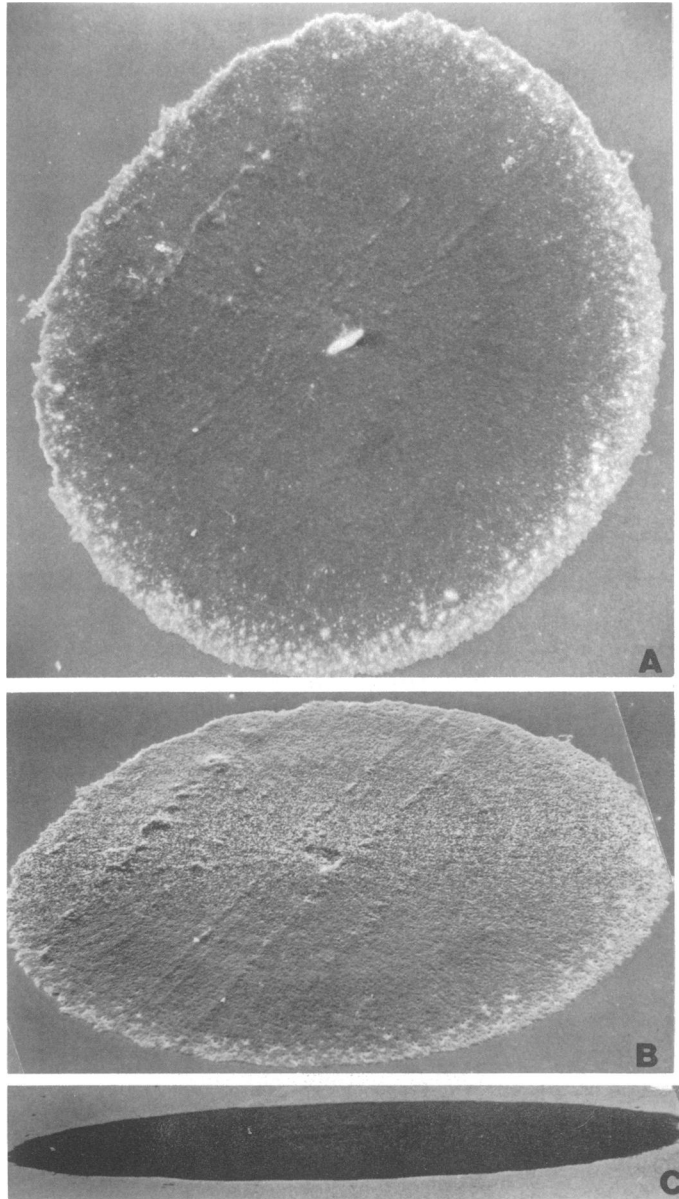


FIG. 3. Colony type 3 *N. gonorrhoeae*. (A) 0° angle and $\times 140$. (B) 60° angle and $\times 153$. (C) 85° angle and $\times 178$.

of type 2 is due to autoagglutination; numerous cells are tightly packed together to produce large aggregates. The smooth homogeneous growth of type 4 shows little autoagglutination, with most gonococci present as single cocci, diplococci, or tetrads. The adherent properties of gonococci have been postulated to play some role in their attachment to urethral epithelial cells and mucosal cells. Such an attachment

could hinder their excretion by micturition. Attachment to epithelial may also precede the intracellular localization of the gonococcus (11). Certain types of pili are known to have adherent properties, and indeed the most heavily pilated type (type 2) is the most autoagglutinated. Yet pili are not the sole cause of gonorrhea adherence, since type 3 gonococci lack pili but exhibit a degree of autoagglutination.

N. gonorrhoeae has at least two extracellular structures. One of them is a hairlike structure known as a pilus or fimbria. Pili were found by SEM on types 1 and 2 gonococci, and they appeared either as bundles of fibers joining adjacent cocci or radiating free from the gonococcus. The second extracellular structure consists of small spherical bodies. Prior TEM studies have shown these to be in close proximity to the outer layer of the cell wall. SEM also shows these on the bacterial cell surface, but in addition they are associated with pili as

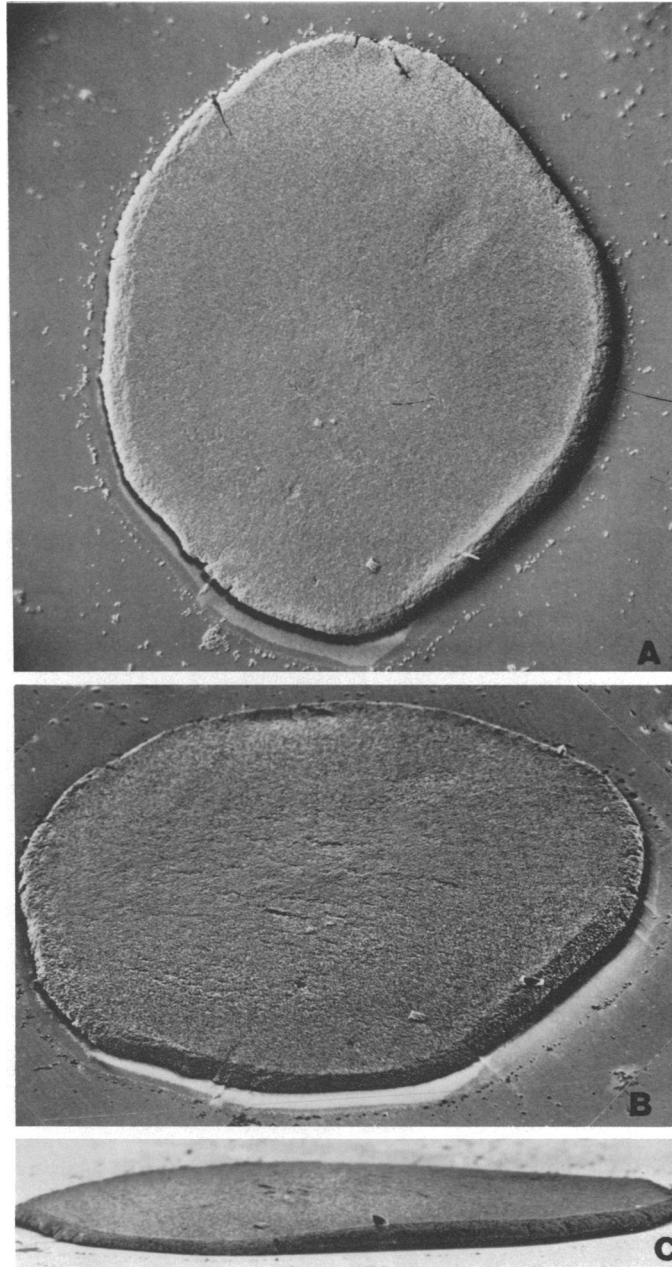


FIG. 4. Colony type 4 *N. gonorrhoeae*. (A) 0° angle and $\times 160$. (B) 60° angle and $\times 215$. (C) 85° angle and $\times 218$.

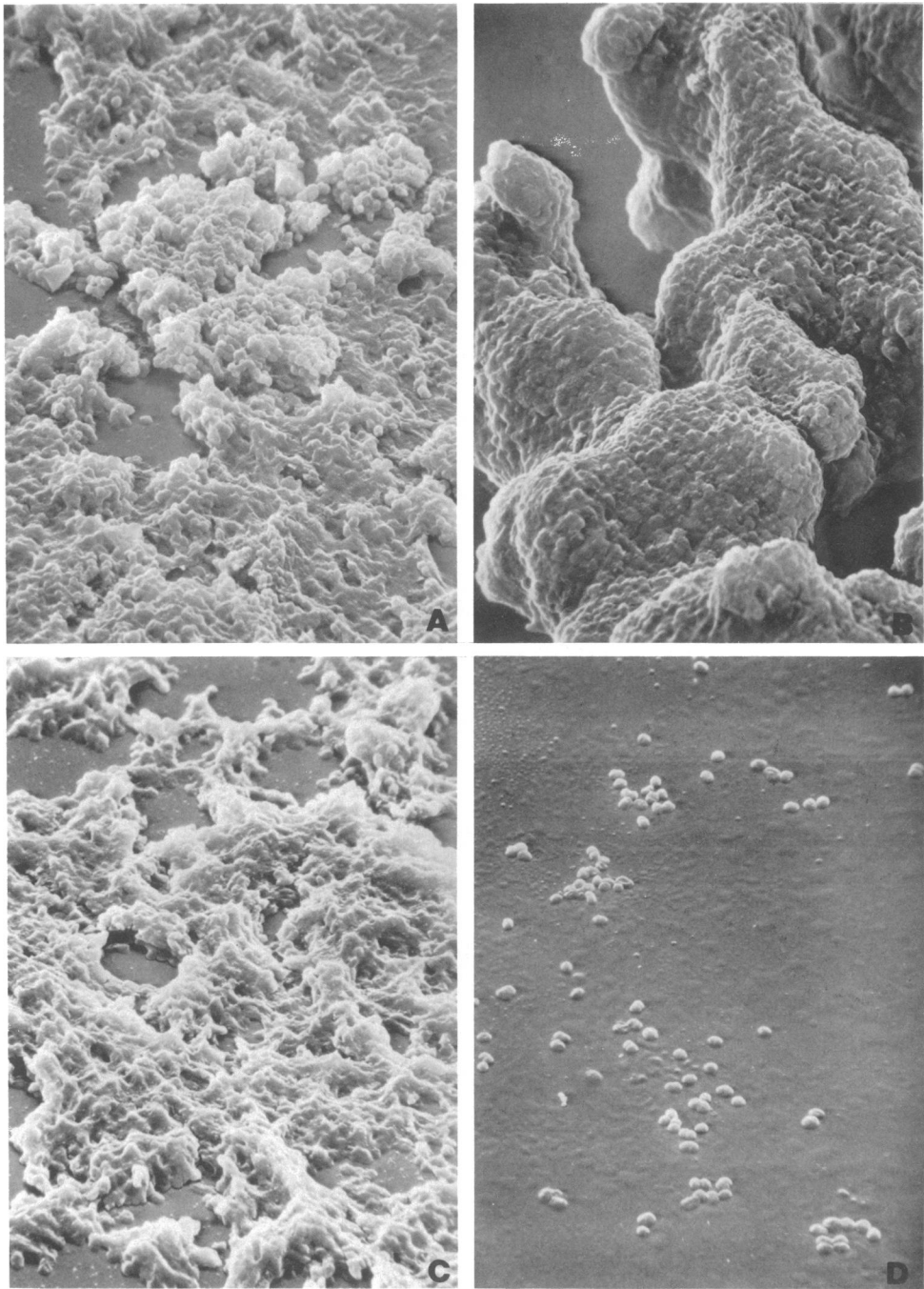


FIG. 5. *N. gonorrhoeae* grown in liquid medium. (A) Colony type 1, (B) colony type 2, (C) colony type 3, and (D) colony type 4. $\times 1,550$.

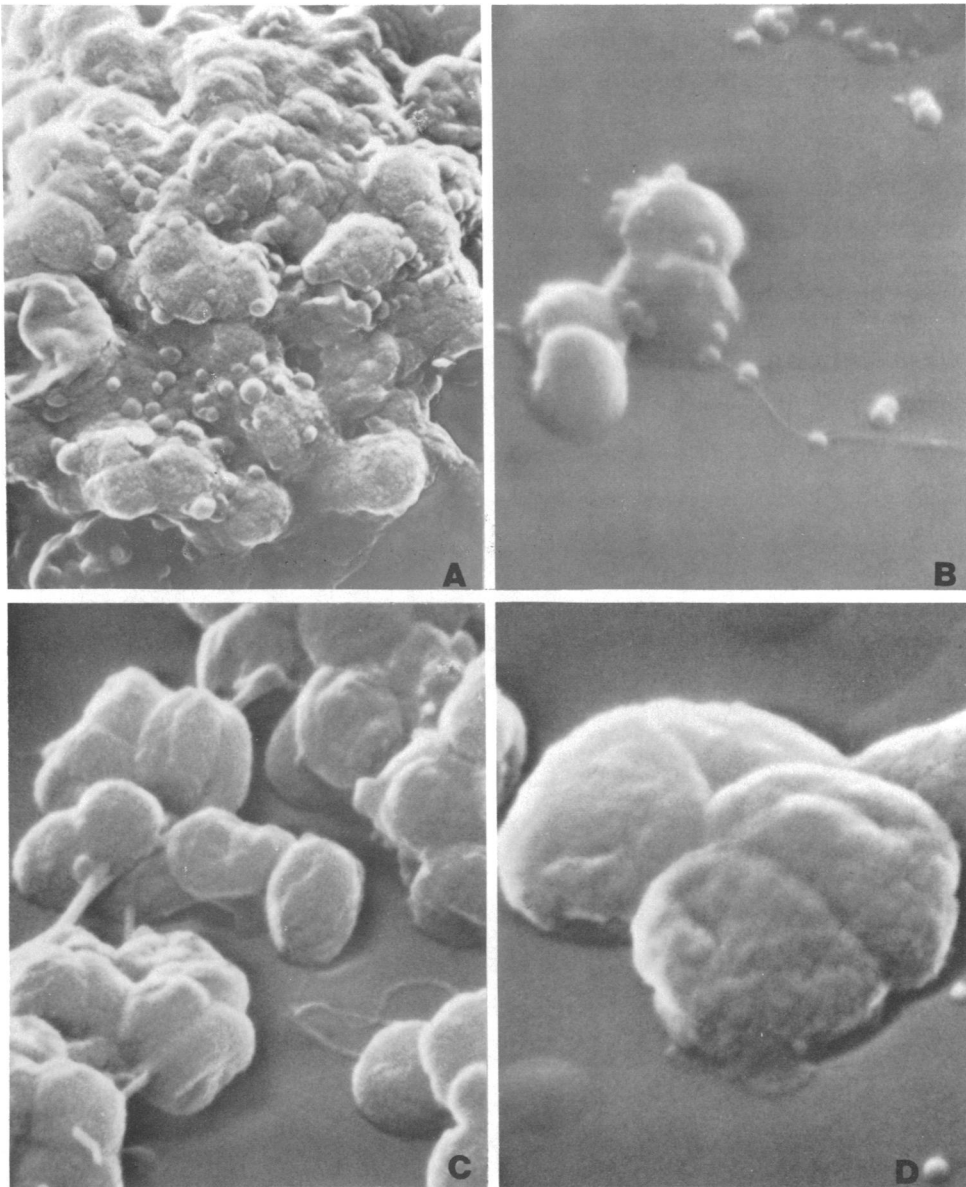


FIG. 6. *N. gonorrhoeae* grown in liquid medium. (A) Colony type 2, $\times 12,700$. (B) Colony type 1, $\times 17,800$. (C) Colony type 1, $\times 18,700$. (D) Colony type 4, $\times 32,300$.

well as free of other gonococcal structures. These spherical bodies were seen in all four colony types, although they were less numerous on type 4 colonies.

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