Cultivation and Survival Studies of *Neisseria gonorrhoeae* in a Human Diploid Cell Strain

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*Neisseria gonorrhoeae* was cultivated in a human diploid cell strain (WI-38). Eighty percent of the cultures contained viable gonococci for at least 4 months at 36°C, as evidenced by subculture to brain heart infusion broth. Monthly subcultures of bacteria could be made to fresh WI-38 cultures for at least 11 monthly passages with a 69% survival rate. The identity of gonococci was confirmed by morphology, gram staining, oxidase testing and fermentation reactions. Viability in brain heart infusion broth, minimum essential medium (Eagle), and WI-38 spent fluid was of much shorter duration. The organisms grown in WI-38 cultures appeared to orient largely in the vicinity of the WI-38 cells as well as within the cytoplasm of the cells.

The importance of *Neisseria gonorrhoeae* in human infections has increased considerably over the past decade. This organism has been grown in the presence of living cells such as chick embryo (1), in tissue culture using KB cells (3), and in mouse and monkey cell lines (7). However, *in vitro* studies with it have been somewhat restricted because its primary cultivation on laboratory media is difficult and because it is exceedingly susceptible to the toxic effects of substances commonly present in ordinary media. Although *N. gonorrhoeae* has been grown on normal human amnion cells for testing the role of pili in attachment to tissue culture cells, these cells were not utilized as a continuous passage medium (6). In addition, human amnion as primary cells might have certain disadvantages for continuous cultivation of bacteria since each passage would represent a separate substrate with possible unknown extraneous agents in each passage. The objective of this study was to determine whether a normal human cell line (WI-38) could serve as a substrate for the propagation of *N. gonorrhoeae* and to investigate the viability of growth characteristics of the organism under these conditions. Studies of these cultures by electron microscopy have revealed that the gonococci may be found intracellularly.

**MATERIALS AND METHODS**

WI-38 cells were obtained from Dr. Leonard Hayflick of Stanford University Medical School at an early generation and passaged to the 16th generation. One milliliter aliquots of the 16th generation, containing 1 to $2 \times 10^6$ cells, were sealed and frozen in ampoules and stored in a liquid nitrogen refrigerator. Am-
poules were removed as required for passage and cells were grown routinely in 75 cm² plastic flasks or on cover slips in Leighton tubes. Cell growth medium consisted of Eagle's Minimum Essential Medium (MEM) in Hanks B.S.S. supplemented with 5% fetal bovine serum using penicillin and streptomycin as antibiotics.

Two separate isolates of *N. gonorrhoeae* on Thayer-Martin agar plates were obtained from Dr. Henry R. Beilstein of the Philadelphia Department of Public Health. The first isolate (GC-4) was subcultured for three further passages in WI-38 and brain heart infusion broth (BHIB) prior to the survival study. The second isolate (GC-5) was used for the survival study in its seventh passage. The gonococcus was identified at each passage by its gram stain and morphology and by the oxidase test. Sugar fermentation was also used at an advanced passage stage to confirm the identification. Passages of each culture were made by transfer of 0.1 ml of infected fluid to a fresh flask.

Confluent sheets of WI-38 cells were washed three times with Hanks B.S.S. and refed with 25–30 ml of antibiotic-free MEM containing 0.5% agamma calf serum. Subsequently 0.1 ml of *N. gonorrhoeae* infected fluid was added to each flask and cultures were incubated at 36°C.

In the early passages of gonococcus occasionally a passage of previously inoculated WI-38 was necessary to stimulate the growth of the gonococcus. In such cases the cell sheet was again trypsinized and passed in the usual manner, with the exception that antibiotics were omitted from the growth medium.

Passages in BHIB, MEM and spent tissue culture growth medium were made by inoculating 0.1 ml of the culture into tubes containing 5 ml of the medium.

To prepare the cultures for electron microscope examination the following procedure was used: Infected WI-38 cell sheets were trypsinized 2 days after inoculation and washed twice. Cell pellets were fixed in 2.5% glutaraldehyde and 1.0% osmium tetroxide, both phosphate-buffered at pH 7.4. The cells were dehydrated in graded dilutions of ethanol and 100% acetone and embedded in Epon-Araldite (4). Thin sections, stained with uranyl acetate and lead citrate, were examined in an RCA EMU-3H electron microscope.

**RESULTS**

The turbidity of both the WI-38 and BHIB cultures varied considerably, from clear to definite cloudiness. The BHIB cultures when clear indicated no growth of microorganisms, but in most instances a clear inoculated WI-38 substrate revealed definite growth of the bacteria by subculture to BHIB.

The changes produced on WI-38 cells were similar to the effects of other bacteria on cell cultures. The cell sheet initially remained intact, although the cells developed an overall granular appearance. As the infection progressed many of the cells degenerated, resulting in the appearance of a shrinking cell sheet with much floating debris. Generally within 1–2 wk the entire sheet was disrupted, although in many instances cells were still to be seen clinging to the surface several months after inoculation (Fig. 1).

The two gonococcal isolates (GC-4 and GC-5) were divided into 8 cultures each by passage into separate flasks of WI-38 cells and by repeated weekly and then monthly subcultures maintained at 36°C. The results of this study are shown in Table 1. Three of the cultures were not viable on the first passage. There were
no further non-viable gonococcal cultures among the remaining 13 strains until the seventh monthly passage, at which point 1 further strain failed to grow. In the interim three other series had become contaminated with an extraneous microorganism, leaving intact nine of the original 16 passage strains from the seventh month to the current time at 11 months, giving an overall survival rate of over 56%. However, since three cultures were excluded because of contamination rather than loss of viability, nine out of 13 legitimate passage strains survived for months, giving a true survival rate of 69%.

The same 16 gonococcal strains (or certain selected cultures of the original 16) were passed in other media on a weekly or monthly basis. The media tested consisted of brain heart infusion broth (BHIB), minimal essential medium (MEM) plus 0.5% agamma calf serum (ACS), which was the medium used in the maintenance of the WI-38 cells, and spent fluid from the growth medium for WI-38 cells. BHIB was a relatively poor medium for the maintenance of gonococcal viability. Since only 50% of the cultures were viable after the second week, 19% after the third and fourth weeks, and about 6% after the fifth week. Two series of monthly passages were made with nine strains of gonococcus using MEM as the substrate. In the first series the medium retained 44% viability on the second and third passages, 22% on the fourth passage, and 0% on the fifth passage. The second series showed a lower survival rate with 56, 11, 11 and 0% viability for
TABLE I

Viability of Neisseria gonorrhoeae in Various Substrates

| Culture no. | WI-38 | MEM | MEM repeat | Spent growth medium | BHIB |
|-------------|-------|-----|------------|---------------------|------|
| 1           | 11    | 1   | 1          | 3                   | 1    |
| 2           | 11    | 2   | <1         | 1                   | 1    |
| 3           | 6     | 4   | <1         | <1                  | <1   |
| 4           | 11    | ND  | ND         | ND                  | 1    |
| 5           | 5º    | ND  | ND         | ND                  | <1   |
| 6           | <1    | ND  | ND         | ND                  | <1   |
| 7           | 11    | 3   | <1         | 3                   | <1   |
| 8           | 11    | 3   | <1         | 1                   | <1   |
| 9           | <1    | ND  | ND         | ND                  | <1   |
| 10          | 11    | 4   | 1          | <1                  | <1   |
| 11          | 11    | 1   | 1          | 3                   | <1   |
| 12          | <1    | <1  | ND         | ND                  | <1   |
| 13          | 5º    | <1  | ND         | ND                  | <1   |
| 14          | 11    | 1   | 1          | 1                   | <1   |
| 15          | 11    | 1   | 3          | 4                   | <1   |
| 16          | 5º    | <1  | ND         | ND                  | <1   |

º Contamination developed after month indicated.
ND = not done.

TABLE 2

Survival of Neisseria gonorrhoeae Stored at 36°C in Six WI-38 Cell Cultures

| Culture no. | Months of storage at 36°C |
|-------------|---------------------------|
|             | 2 | 3 | 4 | 5 | 6 |
| 1           | +º | + | 0 | 0 | 0 |
| 2           | 0 | + | + | + | 0 |
| 4           | + | + | + | + | + |
| 7           | + | + | + | + | + |
| 8           | + | + | + | 0 | 0 |
| 11          | + | + | + | + | + |

º Positive cultures were confirmed as gonococcus by passage to brain heart infusion broth followed by gram stain and oxidase test.

the first, second, third and fourth monthly passages, respectively. Viability was not significantly improved by utilizing the spent growth medium (the fluid removed following the growth phase of the WI-38). The survival rates for the first, second, third and fourth monthly passages were 77, 44, 44 and 11%, respectively (Table 1).

Six samples of gonococcus passed in WI-38 cells were tested for viability after several monthly periods of storage at 36°C in the presence of their original WI-38 cultures. Viable N. gonorrhoeae were isolated from 83%, 100%, 83%, 67% and 50% of the cultures at 2, 3, 4, 5 and 6 months, respectively, even though the WI-38 cell sheet was no longer intact (Table 2). Isolated cells and strands of cells could be observed by microscopic examination of the tissue.

Neisseria gonorrhoeae was inoculated onto tissue culture in Leighton tubes containing cover slips on which the WI-38 cells were grown. Twenty-four hr after
inoculation the cover slips were removed, fixed, and stained with gram’s stain. Examination showed that the bacteria tended to concentrate in the vicinity of the WI-38 cells, with relatively few microorganisms in the clear areas (Fig. 2).

Typical *N. gonorrhoeae* particles (2, 5) were observed by electron microscope within the cytoplasm of WI-38 cells (Fig. 3A, B). Occasionally the bacteria were enclosed within myelin-like inclusions. Extracellular bacteria, possibly released from damaged WI-38 cells, were observed as well (Fig. 3C).

**DISCUSSION**

The present results seem to indicate that WI-38 is an excellent substrate for the propagation of *N. gonorrhoeae*. It was further shown that the organism survives for periods up to six months without passage in this cell culture and that propagation of *N. gonorrhoeae* may occur intracellularly. Electron microscope observations as well as the inability to elute the organisms on repeated washings and centrifugation would appear to substantiate this conclusion. Although practical applications of the WI-38 diploid strain might include screening for antigenococcal agents under a more closely related *in vivo* environment or as a substrate for the production of a gonococcal vaccine in the presence of normal human cells, the paramount question is whether a type reversal (from I to IV) occurs in WI-38. Studies of the colonial types used for inocula and subsequently isolated from the cultures should help clarify this point.
Fig. 3. *Neisseria gonorrhoeae* particles in thin sections of infected WI-38 cell cultures. (A) Two intracytoplasmic bacteria, one within a myelin-like inclusion. (X22,100). (B) Higher magnification of an intracytoplasmic bacterium (X35,200). (C) Two extracellular bacteria (X35,200). Bars = 0.5 μm.

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