# Human Nasal Cells in Continuous Culture.* I. Establishment of Two Lines of Epithelial-like Cells. (22638)

WILLIAM S. JORDAN, JR.†

*Departments of Preventive Medicine and Medicine, School of Medicine, Western Reserve University, and the University Hospitals, Cleveland, O.*

The widespread application of tissue culture techniques to the study of the behavior of both malignant(1-3) and normal(4,5) human cells, and the use of such cells for the propagation of viruses(6-8) has resulted in the establishment of a number of strains of epithelial or epithelial-like cells in continuous culture. The present report describes the establishment of 2 lines of such cells from normal nasal mucosa in the search for tissue capable of supporting the growth of common cold viruses.

*Material and Methods.* The cultivation procedures were similar to those used by Chang(4) and to those used in this laboratory for the growth of HeLa cells(9). Nasal mucosa obtained at the time of elective surgery was minced and embedded in plasma clots in 18 × 150 mm roller tubes. *Media.* Homologous serum obtained from each donor was used to make growth fluid, as follows: 35% human serum, 5% chick embryo extract, and 60% Hanks' balanced salt solution. Final processing of the chick embryo extract included freezing and thawing twice followed by centrifugation at 18,000 rpm for 30 minutes. It had been stored at −20°C for 5 months prior to use. Penicillin and streptomycin were added to the growth fluid to give concentrations of 100 units and 0.1 mg per ml, respectively. Later, the media consisted of 40% of pooled human serum and 60% Hanks' solution with 5 units of polymixin B plus penicillin and streptomycin. For subcultivation, 0.1% trypsin was initially used to make cell suspensions, and 0.1 ml of 1% soya bean trypsin inhibitor was added per ml of medium. Following the establishment of sheets of epithelial-like cells in stationary flasks and bottles, 0.5% versene was substituted for the trypsin(10), and the inhibitor omitted. To prepare the versene 2.5 g of ethylene diamine tetra acetic acid was added to 500 ml of NaCl, the solution alkalinized with 5% $NaHCO_3$, and autoclaved. *Measurements.* Certain characteristics of the cells were measured as follows: Size was determined using stained preparations and living sheets in 24-hour-old tubes; the dimension of 100 cells in the plane of the scale was measured with an ocular screw micrometer. Growth was measured by adding 25,000 cells to each of 30 tubes and counting the number of cells in 4 of these tubes at 24 hour intervals. Details of the comparison of effect of trypsin and versene on growth rates constitute part of another study(10).

Luxuriant outgrowth was obtained with tissues from 2 of 4 donors, a 17-year-old female (DMB) and a 32-year-old male (DHov), and the subsequent behavior of these outgrowths was quite similar.

*Experimental. DMB strain (Fig. 1).* The cilia on the mucosal explants continued to beat actively for 7 days, but no cilia were observed on the outgrowth of new cells that became visible in 4 days. Nearly all of the cells that appeared and spread over the surface of the tube resembled fibroblasts, although occasional round cells and flat cells were observed in the cultures. After 24 days, the sheet of cells was trypsinized and new roller tubes and some small stationary tubes were made. The small tubes were used for virus studies(11), and the large tubes subcultured after 8 days. Again, the cells of the sheets which formed resembled fibroblasts.

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† Markle Scholar in Medical Science.

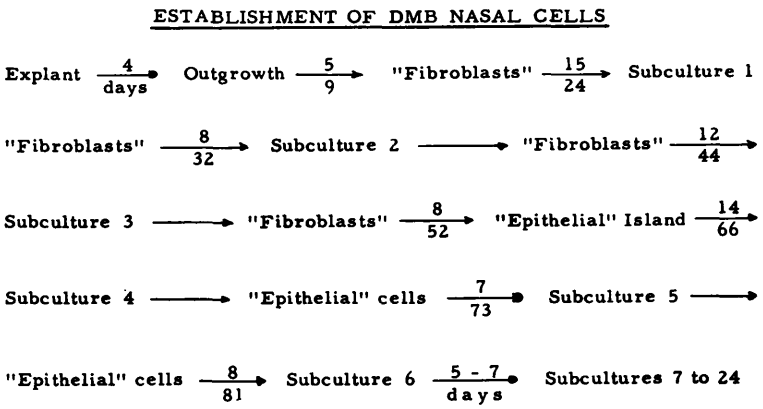


FIG. 1.

Figs. 1 and 3. Number above the arrow indicates number of days between events; number below the arrow indicates cumulative number of days since tissue was explanted.

However, 8 days following the third subculture and 52 days from the date of the original explant (Fig. 1) an island of epithelial-like cells was noted among the fibroblasts (Fig. 2). The cells of this island filled the space between the fibroblasts in an additional 14 days, producing a macroscopically visible mass of tissue. The island was then dissected from

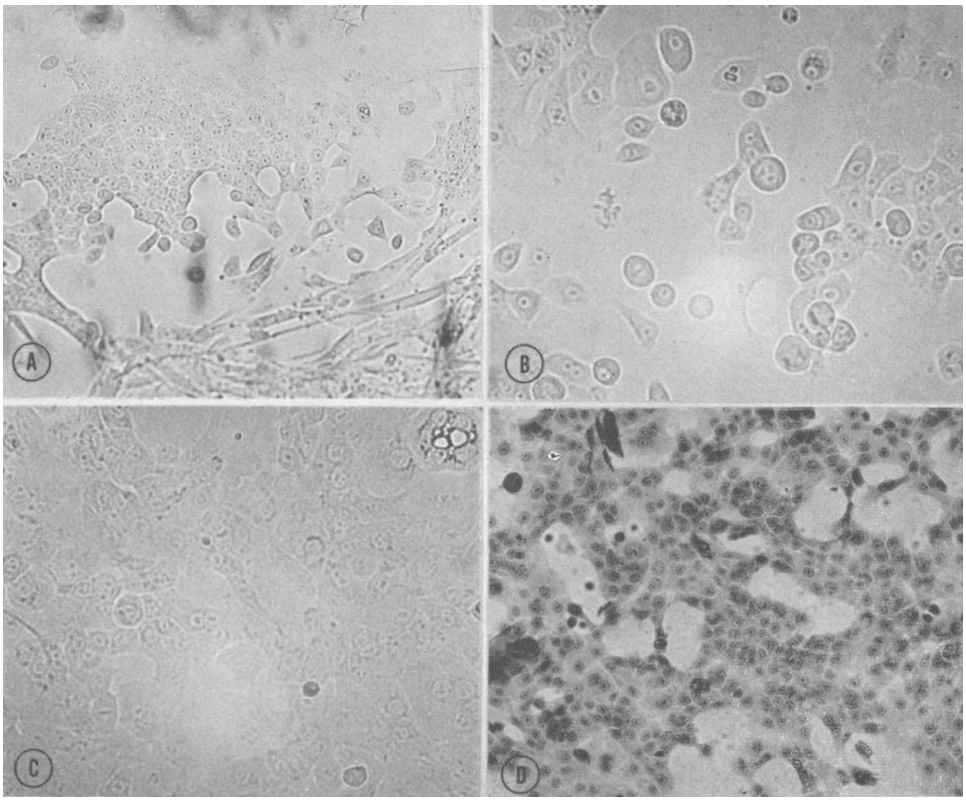


FIG. 2. A. Island of DMB cells which appeared 52 days after original explant. Photographed on 56th day. B. 24 hr culture of DMB cells; × 100. C. 4 day culture of DMB cells; × 100. D. DMB cells stained with $EA_{50}$; × 84.

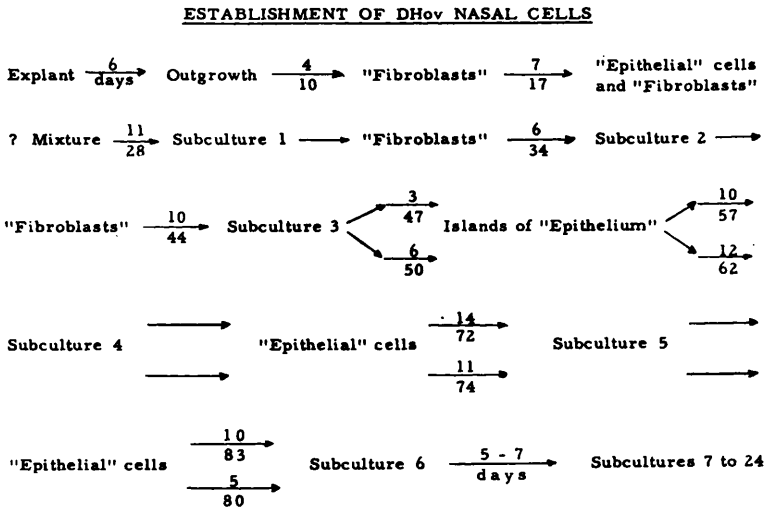


FIG. 3.

the sheet in the roller tubes, trypsinized, and the cells resuspended in growth fluid containing chick embryo extract. The sheet of epithelial-like cells which formed within a week was removed to make 2 roller tubes. Subsequently, cells from these tubes were transferred to $T_{15}$ flasks, then to $T_{60}$ flasks and small prescription bottles. The DMB strain has been maintained through more than 50 subcultures for over a year.

*DHov strain* (*Fig. 3*). The outgrowth from these explants also consisted predominantly of fibroblasts, but more round and polygonal cells were evident in among the elongated fibroblasts. In 1 of 6 original tubes, an area, not a discrete island, of the cellular sheet seemed to consist predominantly of epithelial cells. After 23 days, an attempt was made to remove this area, but the resulting subculture again consisted predominantly of fibroblasts. However, following a third subculture in a different series, islands of epithelial-like cells (Fig. 4) were noted in 2 different tubes 47 and 50 days from the beginning. These islands filled the gaps between the fibroblasts in 10 and 12 more days, and were then removed, trypsinized and resuspended in growth fluid containing embryo extract. Sheets of epithelial-like cells formed in 11 and 14 days. Cells of this strain have also been maintained through more than 50 subcultures for over a year.

*Characteristics of cells.* As cultivation of the DMB and DHov strains continued, the interval between subcultures could be shortened because of the rapid formation of complete sheets. Chick embryo extract was omitted from the feeding fluid, but rapid growth rates continued, and were similar to those for HeLa and Detroit 6 cells in the same medium (10). At 2 days, the number of cells had increased 2X; at 3 days, 4X; at 4 days, 6X; at 6 days, 10X. As with the cells of malignant origin, tubes ready for use in 24 hours can be produced by adding 60,000 cells per tube. Both strains are quite active metabolically, and require daily growth fluid changes. Mechanical disruption alone, satisfactory in the case of HeLa cells, did not produce good cell suspensions because of the tendency of the nasal cells, particularly the DMB strain, to clump. A 5 minute exposure to versene effectively removed the sheets and separated the cells(10). With versene, 98% of the cells observed in the hemocytometer were single cells. A maintenance fluid(12) used for HeLa cells has been satisfactory for the propagation of viruses in these nasal cells. The DMB strain holds better than the DHov strain and even better than HeLa cells. No truly satisfactory maintenance medium for DHov cells has been found. The titers of viruses, including poliomyelitis viruses, grown in HeLa cells are essentially the same in DMB, DHov, and

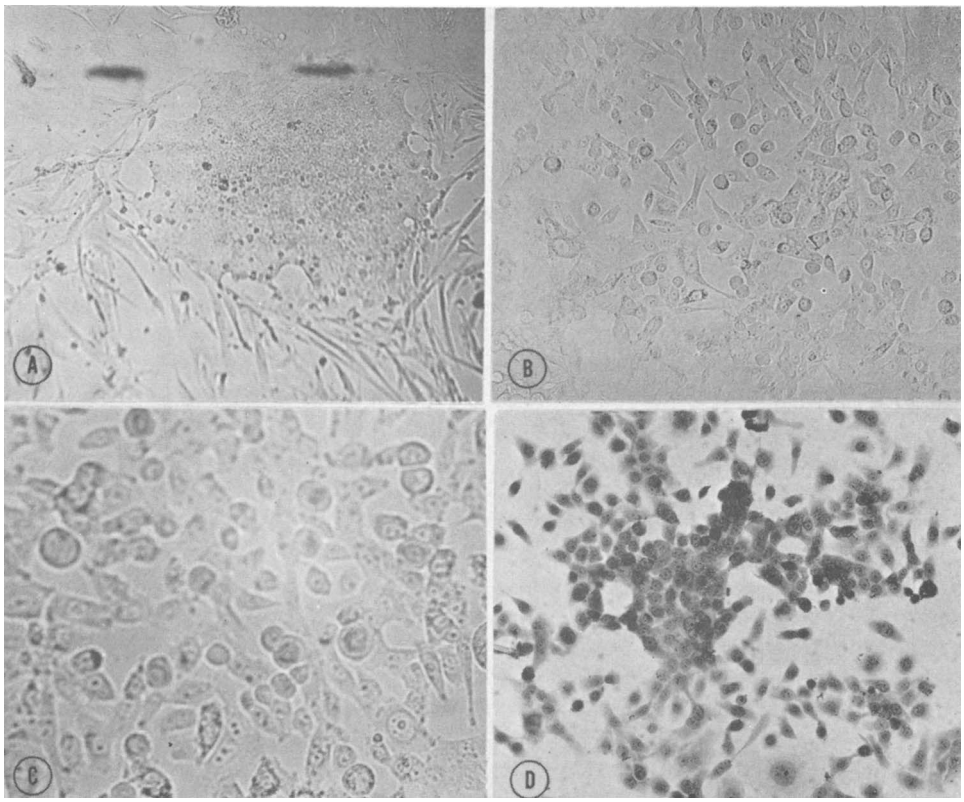


FIG. 4. A. Island of DHov cells which appeared 50 days after original explant. B. 24 hr cultures of DHov cells; × 50. C. 4 day culture of DHov cells; × 100. D. DHov cells stained with $EA_{50}$; × 84.

HeLa cells(11).

Although both strains were derived from nasal mucosa, the cells of the two lines differ morphologically. DMB cells (Fig. 2) are broad, flat, pavement-like cells. DHov cells (Fig. 4) are narrower, more elongated, angular and pointed, and a number of small round cells are persistently present. These differences in shape were readily apparent on inspection of the cultures although the cell sizes were similar. DMB cells measured as follows: Stained, 16 to 105 $\mu$ with average diameter of 36 $\mu$; living, 16 to 88 $\mu$ with average diameter of 44 $\mu$. DHov cells measured: stained, 13 to 101 $\mu$ with average diameter of 36 $\mu$; living, 16 to 135 $\mu$ with average of 41 $\mu$. As would be expected from the growth rates, many mitotic figures are seen in active cultures.

Cover slip preparations stained with $EA_{50}$, a polychrome stain used to bring out nuclear detail, were examined by Dr. James W. Reagan,‡ a cytologist who has studied the morphology of malignant cells for a number of years. He reports that both DMB and DHov cells exhibit the following characteristics: cellular pleomorphism and gigantism, multinucleated cells, nuclear enlargement, variation in nuclear size and shape, altered chromatin patterns, large chromocenters, multiple nucleoli, macronucleoli, variation in nucleolar shapes, and abnormal mitoses. Dr. Reagan states that these are the *morphologic* features of malignant cells.

Cell suspensions therefore were injected into the anterior chamber of guinea pig eyes(13) and into the cheek pouches of cortisonized hamsters(14,15). No growth of tumor occurred in the guinea pig eyes, but since HeLa

‡ Associate Professor of Pathology, Western Reserve University.

cells similarly inoculated failed to grow, these results are of no significance. The hamsters have been followed for 7 weeks without the appearance of tumor nodules. No human transplantation studies have been done.

*Discussion.* The delayed appearances and subsequent rapid growth of epithelial-like cells in roller tube cultures of nasal mucosa have also been observed by Feller(16). The most logical explanation would seem to be that these cells were always present in the cultures, and eventually, for reasons unknown, were able to outgrow the fibroblasts and establish themselves. Subcultivation of the fibroblasts was continued in the same 40% human serum medium, but the fibroblasts of both lines failed rapidly after 60 days, and could not be maintained in continuous cultures. Chang(4) has discussed the possibility of chick embryo extract being the source of epithelial-like cells. The 2 cycles of freezing and thawing followed by ultra-centrifugation and storage make the presence of viable cells in the extract extremely unlikely. Further, as pointed out by Chang(4), the susceptibility of the cells to poliomyelitis virus indicated that they are of human and not of chick origin.

The emergence from normal tissue of cells with the cytologic characteristics of malignant cells has been reported in the past, and the implications of such an observation are many. Since normal mouse or rat fibroblasts can be transformed in tissue culture into malignant cells capable of producing sarcomas in the animals of origin(17-19), it is reasonable to assume that cells of human origin are capable of such transformation. Indeed, a recent report describes the transformation of normal human fibroblasts into histologically malignant tissue *in vitro*(20). It is believed that the DMB and DHov strains are epithelial, or certainly epithelial-like, cells, but at present the only evidence that these cells are malignant is their morphologic characteristics. The behavior of other epithelial cells(4,5) derived from normal tissue suggests that they may have similar properties. If malignant transformation of these and other strains can be demonstrated—and this will require the establishment of new criteria for malignancy—tissue culture will become an increasingly important tool for studying the origin and metabolism of cancer cells(21,22).

*Summary.* Two strains of epithelial-like cells derived from normal nasal mucosa have been maintained through more than 50 continuous subcultures for over a year. Both of these strains now have rapid growth rates, and exhibit the morphologic characteristics of malignant cells.

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1. Gey, G. O., Bang, F. B., and Gey, M. K., *Ann. N. Y. Acad. Sci.,* 1954, v58, 976.

2. Berman, L., Stulberg, C. S., and Ruddle, F. H., *Blood,* 1955, v10, 896.

3. Eagle, H., PROC. SOC. EXP. BIOL. AND MED., 1955, v89, 362.

4. Chang, R. S., *ibid.,* 1954, v87, 440.

5. Perry, V. P., Evans, V. J., Earle, W. R., Hyatt, G. W., and Bedell, W. C., *Am. J. Hyg.,* 1956, v63, 52.

6. Scherer, W. F., and Syverton, J. T., *Am. J. Path.,* 1954, v30, 1075.

7. Stulberg, C. S., Berman, L., and Ruddle, F. H., PROC. SOC. EXP. BIOL. AND MED., 1955, v89, 438.

8. Eagle, H., Habel, K., Rowe, W. P., and Huebner, R. J., *ibid.,* 1956, v91, 361.

9. Ginsberg, H. S., Badger, G. F., Dingle, J. H., Jordan, W. S., Jr., and Katz, S., *J. Clin. Invest.,* 1955, v34, 820.

10. Gold, E., *et al.,* unpublished data.

11. Jordan, W. S., Jr., companion paper.

12. Ginsberg, H. S., Gold, E., and Jordan, W. S., Jr., PROC. SOC. EXP. BIOL. AND MED., 1955, v89, 66.

13. Greene, H. S. N., *J. Exp. Med.,* 1941, v73, 475.

14. Lutz, B. R., Fulton, G. P., Pott, D. I., Handler, A. H., and Stevens, D. F., *Cancer Res.,* 1951, v11, 64.

15. Handler, A. H., and Foley, G. E., PROC. SOC. EXP. BIOL. AND MED., 1956, v91, 237.

16. Feller, A. E., personal communication.

17. Earle, W. R., *J. Nat. Cancer Inst.,* 1943, v4, 165.

18. Gey, G. O., Gey, M. K., Firor, W. M., and Self, W. O., *Acta, Unio. Intern. contra cancrum,* 1949, v6, 706.

19. Goldblatt, H., and Cameron, G., *J. Exp. Med.,* 1953, v97, 525.

20. Leighton, J., Kline, I., and Orr, H. C., *Science,* 1956, v123, 502.

21. Gey, G. O., Coffman, W. D., and Kubicek, M. T., *Cancer Res.,* 1952, v12, 264.

22. Warburg, O., *Science,* 1956, v123, 309.

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