properties may, or may not, block the periodic acid-Schiff reaction, and that for these compounds, no correlation exists between their aldehyde blocking capacity and their ability to produce lathyrism in tadpoles. The mechanism by which those compounds produce lathyrism therefore continues to be obscure.

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"Epithelial Transformation" of Human Synovial Connective Tissue Cells: Cytologic and Biochemical Consequences. (27001)

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While it is generally recognized that cells cultured *in vitro* may undergo "alteration" during the course of serial propagation, the precise nature of the observed changes remains obscure. One distinctive pattern of alteration is well illustrated by the 8 Detroit strains of human epithelial-like cells(1). In these cell lines, cultures of fibroblast-like cells derived from bone marrow, pleural and ascitic fluid, developed plaques of cells characterized by an epithelial growth pattern, rapid multiplication, giant nucleoli, and polyploidy. Since cell lines which accomplish this "transformation" are sometimes capable of extended propagation *in vitro*, they provide a system which can be studied on a mass scale over a prolonged interval. Under these circumstances, the cellular system might be used to study "retained" structural and biochemical characteristics on the one hand, while on the other hand this system may unfold for critical scrutiny the biochemical alterations and related changes in morphology that provide the new cell with an improved outlook for survival in the abnormal environment of the culture flask.

The present report describes the cytologic and biochemical consequences of a fortuitous "epithelial transformation" of a cell population derived from human synovial tissue. In previous investigations, microscopic examination of primary outgrowth from normal and malignant synovial tissue revealed cells with fibroblast-like morphology(2,3), while functional studies have demonstrated the capacity of these cells to form mucopolysaccharides(4, 5,6,7,8,9). In view of the biochemical changes documented in this communication, one might hope that understanding of such "transformations" would be advanced by systematic study of biochemical parameters in addition to the usual microscopic observations.

Materials and Methods. Synovial tissue was obtained by needle biopsy from the knee-joint of a 50 year-old man with rheumatoid arthritis of 6 months duration. This diagnosis was supported by clinical examination, x-ray evidence, a Latex fixation test for rheumatoid factor positive in a titer of 1/5120, and by the pathologic interpretation of the biopsy itself. At another hospital the patient had had a 10-day course of treatment with triethylenemelamine. Explantation of tissue fragments was carried out in T-flask under perforated cellophane as described earlier(9). Establishment of monolayer cultures, feeding schedules, and harvesting techniques were carried out as in previous studies(9).

Cell enumeration and volume measurements were carried out with a model B Coulter Electronic Cell Counter and Cell Size Analyzer. Hyaluronic acid was measured in culture medium as uronic acid by the method of Bollet as well as by a method developed in this laboratory(10,11). Hexose was determined by

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the anthrone method(12), while cellular protein was estimated by a modification of the procedure described by Lowry(13). RNA and DNA were measured by the orcinol and diphenylamine reactions for their respective carbohydrate moieties(14). These latter 2 analyses were carried out on the cellular fraction extracted by 10% trichloroacetic acid at 90°C in 15 minutes. In an effort to detect the presence of the oligosaccharides of hyaluronic acid in culture medium, ethanol fractionated medium was subjected to the charcoal column procedure described by Rapport, Meyer and Linker(15).

Media used in this study included CMRL 1066(16), Eagle's basal medium(17), and "Yela medium." The latter is an undefined medium consisting of a mixture of 0.1% yeast extract and 0.5% lactalbumin hydrolysate in Hanks' balanced salt solution. Experiments with agitated suspension cultures were carried out on a New Brunswick Model G10 rotary shaker at speeds of 120-130 rpm. Cytologic studies on cover-slip preparations were performed with the aid of the May-Grunwald Giemsa and Wright's stains. Chromosome enumeration was carried out on acetic orcein stained specimens prepared by the method of Tjio and Puck(18).

Results. History of culture through period of "transformation": Biopsy fragments were explanted in a T-15 flask under perforated cellophane and nourished by a medium containing 80% CMRL 1066 and 20% human serum, supplemented with L-glutamine (2mM), penicillin (200 μg/ml) and streptomycin (200 μg/ml). Radial outgrowth appeared in 3 days and was sufficient in 17 days to permit trypsin dispersion and subculturing as a monolayer culture. The culture grew relatively rapidly with an average culture generation time varying between 36.5 hours and 80.2 hours. At the second subculture the supernatant medium gave a positive mucin clot test with acetic acid, (0.13 ml 7.0 N acetic acid added to 5.0 ml culture medium). The mucin clot test on culture medium was variable during the first 3 months *in vitro,* and eventually disappeared altogether. About 85 days after explantation, at the tenth subculture, the culture growth rate slowed strikingly and generation time increased to the range of 80 to 130 hours. During this phase of slow growth, epithelial-like plaques appeared in numerous places in the culture vessel. These epithelial-like cells rapidly overgrew the ordinary fibroblastic cells, and soon became the representative cell form. Once this "transformation" had taken place, the cells returned to their previous rate of growth, although permanently losing the capacity to induce mucin clot formation in the supernatant medium.

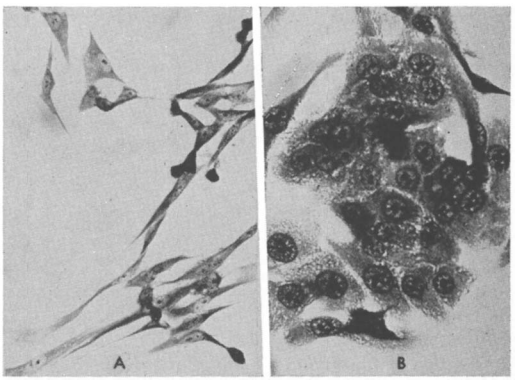

FIG. 1. Photomicrographs of synovial cells before and after transformation. X65. (A) Spindle-shaped cells prior to transformation, (B) polygonal cells after transformation. (Relative measurements on original preparations confirm equality of magnification of (A) and (B).)

Cytology. Stained cover slip preparations illustrate the striking alteration in morphology (Fig. 1). The pre-transformation fibroblastic cells are spindle-shaped and grow in a reticular pattern, in contrast to the post-transformation colonies of polygonal cells growing with contiguous cell borders. The photomicrographs, made at the same magnification, demonstrate the nuclear and nucleolar enlargement in the transformed cells. The nucleolar diameter of the transformed cells is frequently 3 times that seen in the unaltered cells.

Chromosome counts prior to the "transformation" indicated that the normal human complement was numerically dominant in the cultures. On the other hand, the post-transformation cultures revealed a markedly abnormal chromosome make-up, with virtually no cells showing the normal number of chromosomes. The magnitude of this shift in chromosomal complement is revealed in Table 1.

Culture behavior. Although the epithelial shape and growth pattern first drew attention

TABLE I. Exaggerated heteroploidy following "epithelial transformation."

"C. W." Rheumatoid Synovium	NO. OF CHROMOSOMES PER CELL					
	40-45	46	47-80	80-90	"Near Tetraploid" 91-94	100+
Pre-transformation 3rd Subculture (28 Cells Counted)	35%	61%	—	4%	—	—
Pre-transformation 5th Subculture (9 Cells Counted)	56%	44%	—	—	—	—
Post-transformation 13th Subculture (25 Cells Counted)		4%	24%	8%	40%	24%

to the cellular alteration, several other peculiarities of the new cell were soon evident. The transformed cells had a reduced affinity for glass surfaces and frequently a newly inoculated suspension of cells required 2 or more days to attach to the surface of a "T-flask." This reduced "adhesiveness" of cells for glass was in large part corrected by incorporating 5 to 10% fetal calf serum in the nutrient medium, and to a lesser extent by treating the "T-flasks" with 0.2 N NaOH prior to final rinsing and sterilization. The transformed cell cultures rapidly acidified the medium, occasionally requiring neutralization twice daily. When the medium pH fell to approximately 7.0, a large portion of the cells detached from the glass and floated freely in the supernatant medium. Cells could be promptly removed from a glass surface with 0.25% trypsin, and in addition by 0.01 M EDTA. Limited previous experience indicated that EDTA was an ineffective agent for separating unaltered primary synovial cultures from glass.

While previous experience with primary synovial culture suggested that synthetic medium CMRL 1066 was superior to Eagle's basal medium, the transformed cells thrived in flask cultures nourished by 80% Eagle's basal medium and 20% human serum. Further, they grew reasonably well in an undefined medium where 20% serum was supplemented by 80% Yela medium.

While previous attempts to grow unaltered synovial cells in agitated suspension culture have failed, in our hands, to realize a net increase in cell numbers, the transformed cells were capable of moderate growth under these circumstances. In one experiment with agitated suspension cultures, 1.3×10^7 cells were suspended in 108 ml of culture medium composed of 80 ml Yela medium, 20 ml human serum, 1.0 ml 200 mM L-glutamine, 1.0 ml 100× penicillin-streptomycin solution, 2.0 ml 0.05 M EDTA, and 4.0 ml 4% methylcellulose. The cell suspension was placed in a 250 ml Erlenmeyer flask and agitated at 130 rpm on a rotary shaker in a 37°C incubator room. At intervals during the 16-day culture period, 1.0 ml volumes of 100× glucose, amino acids*, and vitamin stock solutions* were added (Fig. 2).

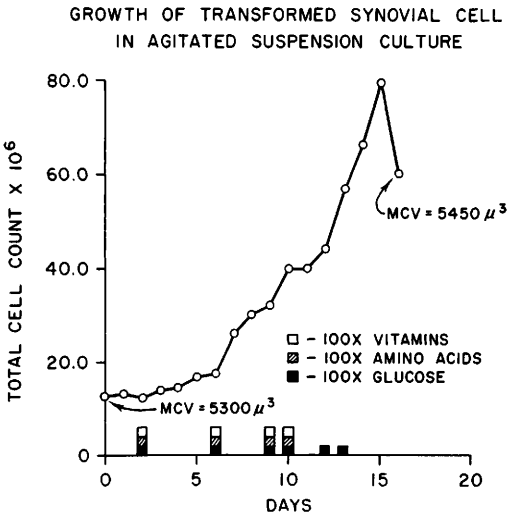

FIG. 2 A 5-fold increase in population occurred during the second week of agitated culture.

* These stock solutions are those used in preparing Eagle's basal medium.

TABLE II. Comparison of chemical parameters of "transformed" cells with those of "normal" and "malignant" cells.

PARAMETER MEASURED	"Normal" Synovial Cells		"C.W." Cells Pre-Transformation		"C.W." Cells Post-Transformation		J-111 "Malignant Monocytes"	
	No. Obs.	Mean ± S.E.*	No. Obs.	Mean ± S.E.	No. Obs.	Mean ± S.E.	No. Obs.	Mean ± S.E.
Mean Cell Vol-μ^3	90	7421 ± 265	—	—	16	5669 ± 248	9	4987 ± 289
Cell Protein, 10^{-12} g/cell	50	380 ± 19	—	—	9	1070 ± 75	7	794 ± 65
μ g DNA/mg Cell Protein	4	32 ± 6.6	4	40.0 ± 8.5	4	40.0 ± 3.2	7	58 ± 4
RNA/DNA	4	3.33 ± .71	4	3.43 ± .70	4	1.32 ± .48	7	1.40 ± .22
Hexose Uptake mg/mg cell protein/day	33	4.04 ± .49	—	—	7	1.26 ± .07	5	1.62 ± .23
Hyaluronic Acid Production 10^{-12} g/cell/day	76	19.6 ± 1.8	1	29.6	5	1.64 ± .59	—	—

* Standard error of mean.

A 5-fold increase in cell numbers took place after a prolonged lag period (Fig. 2). Non-dialyzable acid mucopolysaccharide was present in the medium in concentrations of 3.7 μg/ml, (calculated as hyaluronic acid) a value 2 to 6 times greater than that found in static cultures. Calculation of the comparative rates of synthesis, taking into account the prolonged contact of cells with the medium in the agitated cultures, indicate that the cells in the suspension culture form hyaluronate at a rate similar to that found in static cultures.

Structural and functional characteristics. Data concerning the size, protein, and nucleic acid content, as well as hexose consumption and hyaluronate synthesis of the altered cell line (grown in static cultures) are summarized in Table 2. The transformed cell, although slightly smaller than the average "normal" synovial cell, appeared to contain over twice as much protein. Prior to "transformation" the cells in question exhibited DNA/protein and RNA/DNA ratios which closely resembled the values commonly seen in unaltered cells derived from human synovium. Following the alteration in morphology, the RNA/DNA ratio decreased, without evidence of change in the DNA/protein ratio. The quantity of RNA per cell appears to be unchanged in the face of over 2-fold increases in cellular protein and DNA.

Measurements of hexose uptake indicated that the transformed cell removed glucose from the medium at less than one-half the rate common to healthy proliferating synovial derivatives, when cell protein measurements were used as a basis for comparing rates. The decreased rate of hexose uptake (per unit of cell protein) is nearly the reciprocal of the increase in protein per cell, suggesting that the actual uptake of glucose per cell is virtually the same in the normal and transformed cells.

Hyaluronic acid synthesis fell off drastically following the transformation. Loss of the positive mucin clot reaction in the culture medium, as well as a profound reduction in chemically measurable uronic acid-containing acid mucopolysaccharide, both point to decreased elaboration of high molecular weight hyaluronate. Since the procedures used above do not distinguish between defective polymerization of hyaluronate and decreased elaboration of the disaccharide repeating unit, (or larger oligosaccharides), we undertook the identification of oligosaccharides in used culture medium. No trace of uronic acid-containing material was found in the medium from transformed cultures, although disaccharide and tetrasaccharide added to unused medium could be recovered in the pyridine eluate from a charcoal column.

Discussion. The dramatic appearance of colonies composed of epithelial-like cells in the midst of fibroblast cultures quite properly raises the question of contamination by one of the established epithelial cell lines. Fortunately, we have not carried epithelial cell lines and the only stable cell line of non-synovial origin present in the laboratory at time of the transformation was the J-111 line derived

from the peripheral blood of a woman with acute monocytic leukemia(19). Morphologic and chemical data do not support the possibility that the transformed cells were contaminants from the monocyte line. It is unlikely that skin epithelium was carried into the original biopsy material, since the procedure is carried out through a trocar after a skin incision. Available cytologic and chemical data suggest that the pre-transformation cell was similar to material examined in our previous experience with normal synovial connective tissue cell derivatives. If one uses the cellular protein values from the collected normal synovial cultures as a base line, the major compositional changes in the transformed cell would appear to be 2-fold increases in cellular protein and DNA.

While the data do not suggest a quantitative alteration in cellular RNA, a qualitative change would not be detected by the methods employed. Cytologic measurements of nucleoli, the locus of nuclear RNA, indicate that the volume of this structure increased 8-fold or more in the transformed cell. lending some support to the notion that at least a shift in the proportions of the various RNA fractions may have taken place. Such an alteration in the enzyme forming systems of the cell provides a reasonable explanation for the decreased hyaluronate synthesis observed. The absence of significant amounts of oligosaccharide in the medium suggests that the decreased synthesis of high molecular weight hyaluronate largely resulted from reduced synthesis of the low molecular weight repeating units, rather than failure of polymerization.

Changes in the form and growth pattern exhibited by the transformed cell may reflect alteration in the character of the plasma membrane. Acquired resistance to the battering effects of agitated suspension culture could be explained in the same way. Decreased adhesive properties of the cell as manifested by increased ease of separation from glass with trypsin, versene, or fall in medium pH, all suggest that the negative charge density of the plasma membrane was reduced. While these changes in culture behavior may all be construed to reflect some modification of the plasma membrane, there is no reason to believe that a single change in the chemical structure of the membrane is responsible for the diverse effects.

Summary. Primary cultures of fibroblast-like cells from rheumatoid synovial tissue changed *in vitro* to a cell population exhibiting epithelial morphology, heteroploidy, altered growth characteristics, and decreased capacity to synthesize hyaluronic acid. Quantitative changes in the protein and nucleic acid composition of the cells were also demonstrated.

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