Increased Susceptibility of Down's Syndrome Fibroblasts to Transformation by SV_{40}.* (31974)

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A quantitative system has recently been described using cell culture techniques(1) that permits a precise measurement of the susceptibility of human fibroblasts derived from different individuals to *in vitro* transformation by the oncogenic virus, simian virus 40 (SV40). Fibroblast cultures from patients with Fanconi's anemia, an autosomal recessive disease which is associated with a high incidence of chromosome aberrations and a greatly increased risk of neoplasia(2,3), showed a much greater susceptibility to transformation in culture by SV40 and by an adeno 7-SV40 "hybrid" virus when compared with fibroblast strains derived from normal individuals and from individuals with diseases not associated with an increased tumor incidence(1).

Down's Syndrome (trisomy 21) is another disease associated with an abnormally high incidence of tumors(4,5); the nature of the chromosome abnormality in Down's Syndrome is, however, fundamentally quite different from that of Fanconi's anemia. It was therefore of interest to know if the trisomic cells would also prove to be more susceptible to neoplastic transformation in culture. Such information would shed light on the relationship between cell genotype, chromosome abnormalities, and susceptibility to cancer. In the study reported here fibroblast cell strains derived from normal neonates, from neonatal patients with Down's Syndrome, and from one patient with trisomy 18 syndrome were compared in cell culture for their sensitivity to transformation by SV40.

Materials and methods. The trisomy 18 cell strain (GALV) was derived from post-mortem thoracic skin of a one-week-old male; all other strains were initiated from neonatal foreskins. Explant cultures were established in Waymouth's medium containing 10% newborn calf serum, penicillin (50-100 units/ml) and streptomycin (50-100 μg/ml) and were maintained in the same medium without streptomycin. They were passaged every 7-14 days with a split ratio of 1:5. All but 3 of the strains (DEYM, HOWA and AUBE) were stored in medium containing 5% glycerol at —75°C for periods of 9 to 28 months before chromosomal studies and the transformation assays were carried out. The techniques employed to examine the chromosomes were as previously described(6).

Each of the cell strains was tested in the transformation assay prior to the fifteenth cell doubling in culture. For the assay the cells were grown in 50 mm Falcon plastic Petri dishes in Dulbecco and Vogt's modification of Eagle's medium supplemented with 10% calf serum. When the Petri dish cultures were roughly one-quarter confluent ($2\text{-}5 \times 10^5$ cells/plate) the medium was removed, the cell layer was washed twice with serum free medium and the cells were exposed to an SV40 strain 776 preparation titering $10^{9.0}$ T.C.I.D./ml. After a 3-hour adsorption period the infected cells were trypsinized and transferred to new Petri dishes at cell densitites ranging from $1\text{-}5 \times 10^4$ cells per plate. The medium was supplemented with 0.5% rabbit anti-SV40 antiserum to prevent continuing cell destruction by the virus. Sixteen to eighteen days after plating, the cultures were fixed with formalin and stained with 1% hematoxylin. Discrete colonies of transformed cells were seen on the infected plates; they were recognized by their multilayered pattern of growth and the random orientation of the cells. The transformed colonies were counted under low power with a dissecting microscope. Such foci have never been seen in the cultures not exposed to SV40.

Results. Table I presents the chromosomal findings for the cells of a group of normal

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TABLE I. Results of Chromosomal Analyses on a Group of Normal and Trisomic Strains of Human Skin Fibroblasts. The estimated number of cell doublings does not include the primary culture.

		Estimated No. cell doublings	% Tetraploid	Total cells counted	Chromosomes per cell					Karyotypes
					<45	45	46	47	48	>48	
Normal	GULB	6–8	3.8	50	2	0	47	1	0	0	46 Xsomes (1 cell): normal male
											47 " (1 cell): extra E
	MART	6–8	5.4	70	6	0	63	1	0	0	46 " (1 cell): normal male
											47 " (1 cell): extra G
	DEYM	6	4.6	34	8	5	21	0	0	0	46 " (3 cells): normal male
											45 " (2 cells): loss of A & E
	TAYL	—	—	—	—	—	—	—	—	—	No karyotypes
Trisomy 21-22	HOWA	6	4.4	34	0	0	0	34	0	0	47 Xsomes (2 cells): extra G
	AUBE	4	4.2	34	0	0	0	34	0	0	47 " (2 cells): extra G
	RICE	13–17	10.6	23	1	0	0	22	0	0	47 " (3 cells): extra G
	LAVI	15	10.0	36	6	3	3	24	0	0	47 " (4 cells): extra G
											47 " (1 cell): extra D
											46 " (1 cell): missing D, extra G
	MEYE	4–8	10.0	40	8	4	1	26	0	1	47 " (2 cells): extra G
											Hypomodal cells have extra G's
Trisomy 18	GALV	4	4.4	25	0	0	0	25	0	0	47 Xsomes (3 cells): extra #18

male infants, a group of male infants with a clinical diagnosis of Down's Syndrome and a male infant with a clinical, cytogenetic (peripheral blood leukocytes) and autopsy diagnosis of trisomy 18 syndrome. The cultured skin fibroblasts from the latter also proved to be trisomic for chromosome No. 18. Trisomy of the G group (21 or 22) was found in all the fibroblast cultures from the Down's Syndrome patients. One cell strain, LAVI, showed a D trisomy; partial or complete karyotypes of the remaining 23 trisomic cells which were counted in this culture revealed only extra G group chromosomes. Cultures from one of the normal strains (TAYL) became contaminated before chromosomal studies could be carried out; the three others revealed normal diploid male cells with 2 exceptional trisomic cells (G and E), presumably the result of an *in vitro* nondisjunctional accident. Most of the cultures showed occasional hypo-modal cells due to cell breakage in the preparation of the slides. For each of the cell strains 500 mitoses were examined to determine the fraction of tetraploid mitoses. These values ranged from 3.8 to 5.4 for the normal cell strains and from 4.2 to 10.6 for the trisomy cell strains (Table I). Preliminary analysis of the incidence of abnormalities in metaphase (chromatid breaks, quadriradials, dicentrics), anaphase (bridges and lags) and interphase (Feulgen positive cytoplasmic material) have revealed no significant differences among the various strains except for a high incidence (5 of 100 metaphase plates) of probable dicentric chromosomes in the trisomy 21-22 strain LAVI.

Table II shows the results of the transformation assay. Each of the cell strains was infected on at least 2 separate occasions; in each infection the number of transformed colonies developing from at least 2×10^5 infected cells was counted. The 4 normal cell strains gave transformation frequencies of from 2.1 to 3.1 transformed colonies per 10^4 cells plated. The trisomy 21-22 cell strains had values from 6.4 to 10.6 transformed colonies per 10^4 cells. There was no overlap between the two groups; the 5 trisomy 21-22 cell strains were at least twice as susceptible and, on the average, 3 times more susceptible

TABLE II. Transformation of Normal and Trisomic Human Skin Fibroblast Cultures by SV40.

Cell strain		Transformed colonies/10^4 cells Exp 1	Exp 2	Exp 3	Exp 4	Avg	
Normal	GULB	2.6	2.2	—	—	2.4	
	MART	1.9	2.8	1.5	2.2	2.1	2.5
	DEYM	3.0	1.6	—	—	2.3	
	TAYL	—	—	3.1	3.1	3.1	
Trisomy 21-22	HOWA	7.2	9.3	—	—	8.2	
	AUBE	9.2	4.3	—	—	6.8	
	RICE	10.6	10.5	—	—	10.6	7.8
	LAVI	—	—	6.6	7.6	7.1	
	MEYE	—	—	4.6	8.3	6.4	
Trisomy 18	GALV	—	—	14.2	15.5	14.8	

to SV40 transformation as compared to the normal cell strains. The one trisomy 18 cell strain gave a transformation frequency of 14.8, roughly 6 times the mean value of the diploid cell strains.

The transformed colonies, both from the normal and from the trisomic cells, could first be recognized on the 9th and 10th day after plating. The cells in these colonies actively grew over one another and maintained a high mitotic rate under conditions where the surrounding cells were inhibited from continued cell division by cell-cell contact. The transformed colonies in the trisomic cultures were not recognized any sooner nor did they grow any faster than those in the normal cultures. Uninfected normal and trisomic cultures eventually ceased dividing after roughly 50 cell generations in culture, a property characteristic of human fibroblast cultures(7). None of the strains, normal or trisomic, spontaneously evolved into permanent cell lines.

Discussion. The results described here indicate that trisomy 21-22 and trisomy 18 cell cultures are intrinsically more susceptible to *in vitro* transformation by the oncogenic virus, SV40, than are normal cell cultures. Under the conditions described, using a virus concentration of $10^{9.0}$ infectious units per ml, 0.02 to 0.03% of the normal foreskin fibroblasts are transformed. This value is comparable to that obtained with human embryonic lung fibroblasts (unpublished experiments) but slightly lower than that reported for skin fibroblast strains derived from children and young adults(4), where the mean value was 0.037%. With the trisomic foreskin cultures, transformation is still an extremely rare event, but for each of the six strains the probability of transformation is increased.

The heightened probability of transformation of trisomic cells as compared to diploid cells results from either 1) an increased susceptibility of each of the cells in the population to viral transformation or 2) the presence in the trisomy cell populations of a greater proportion of cells that are particularly likely to respond to viral infection by becoming transformed. The first interpretation assumes that the cell populations we are studying are homogeneous with respect to sensitivity to transformation. However, it is known that human fibroblast cultures are composed of genetically distinct cells having different metabolic capabilities(8,9); these cells may also differ in their responsiveness to viral infection. One common variety of genetically distinct cells in human "diploid" fibroblast cultures are tetraploids. The percentage of tetraploid cells is generally below 5% for human fibroblasts until close to the end of their *in vitro* lifetime, when it becomes greatly elevated. In such "end stage" human cell populations transformation can be detected more rapidly(10,11). The transformed "end stage" cells, even when examined only a few days after infection are almost always found to be near-tetraploid(12). As shown in Table I, the fraction of tetraploid mitoses is unusually high in three of the five trisomy 21 strains; it is not elevated in the trisomy 18 strain, however. McKee, *et al* (13) and Ferrier and Ferrier(14) have also noted a high incidence of endo-reduplications and tetraploid cells in Down's Syndrome fibroblast

populations. A further source of variation results from the fact that a certain small fraction of cell divisions, even in a normal population, contains grossly abnormal mitoses. The fraction of cells with chromosomal aberrations is greatly elevated in Fanconi's anemia and this may well be correlated with the high transformability of Fanconi's anemia cell cultures. Ferrier and Ferrier(14) have suggested that there also may be an increased frequency of chromosomal breaks and rearrangements in Down's Sydrome cell cultures. We have, however, only been able to find a significant level of chromosome aberrations in one of the trisomy 21-22 cell cultures.

Another kind of heterogeneity in fibroblast cultures is physiologic rather than genetic in character. At the time of infection in a randomly growing culture, the cells are in different phases of their growth cycle. There is evidence that a cell is only capable of being transformed by SV40 when it is in that phase of the cell cycle when DNA is duplicated (*S* period)(15,16). Since the transformation frequency, even with high titered virus, is very low, it is possible that all the transformants are derived from a minority population of cells. Thus, an analysis of the genetic and physiologic components of heterogeneity in human cell populations is necessary for further interpretation of the results. The use of clonally isolated human cell strains, mitotically synchronized cell cultures, and more actively transforming viral preparations should further clarify this situation.

The increased transformability of trisomy 18 cultures as well as trisomy 21-22 cultures suggests that the trisomic condition itself, rather than an excess of specific genetic information, is responsible for the altered susceptibility, children with trisomy 18 syndrome rarely live beyond the first two years; consequently the relationship between this syndrome and cancer susceptibility is not known. With two genetic diseases that are known to be linked to an increased risk of neoplasia, Fanconi's anemia and Down's Syndrome, the fibroblasts from individuals with these diseases when cultivated *in vitro* show an abnormal responsiveness when challenged with the tumor virus, simian virus 40 (SV40). The parallel between *in vitro* susceptibility to a tumor virus and *in vivo* susceptibility to neoplasia with both of these diseases suggests that the assay may allow us to recognize individuals that are particularly "cancer prone." The property of tumor susceptibility is very difficult to study in man and even in laboratory animals because its expression in undoubtedly influenced by a variety of extrinsic modifying factors. That there is a genetic component in cancer is clear from numerous studies with inbred animal strains. Genetic resistance at the cellular level is known for chicken cells grown in tissue culture and exposed to avian tumor viruses(17). The results of the above experiments indicate that in man also there can be profound differences between cells of different genotypes in their responsiveness to a carcinogenic factor. SV40, and SV40-adenovirus type 7 "hybrid" virus (18) and, perhaps, Rous sarcoma virus(19) are the only agents that have, as yet, been shown to effectively transform human cells. The study of isolated human cells in culture and their interaction with known carcinogenic agents should simplify our understanding of the relationship between genetics and cancer in man.

Summary. Tissue culture cell strains of foreskin fibroblasts obtained from 5 neonates with Down's Syndrome (trisomy 21-22) were shown to be at least twice as susceptible and, the average, 3 times as susceptible to transformation by the SV40 virus as diploid foreskin fibroblasts from normal neonates. An even greater susceptibility to *in vitro* SV40 transformation was found in a strain of trisomy 18 fibroblasts. It is suggested that one reason for the increased incidence of neoplasia in Down's Syndrome is a heightened intrinsic susceptibility of the trisomic cells to carcinogenic agents.

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Effects of Prior Semen Injections and Weekly Inseminations on Hybridization of Chickens and Turkeys. (31975)

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It is now generally accepted that in attempts to cross chickens with turkeys the chicken ♂ × turkey ♀ combination produces greater fertility and embryonic viability than the reciprocal(1). Based on this, and the fact that turkey semen normally has a lower anti-chicken-erythrocyte agglutinin titre than the reciprocal, Ryle(1) postulated that serological (immunological) factors might play some part in inhibiting cross-fertilization or impairing hybrid embryonic viability.

Olsen(2) reported that the chances for successful hybridization of these species are increased when the cross is made with parental strains exhibiting a tendency to reproduce parthenogenetically. His data (unpublished) also indicated that in spite of repeated artificial inseminations hybrid fertility and embryonic development might not have persisted more than 3 weeks following the first insemination.

The data reported here are from 2 experiments performed to explore the following possibilities. 1) Hybrid fertility and embryonic development in turkey eggs might be influenced by prior intraperitoneal injections of hens with semen from the same chicken males by whom they were to be subsequently inseminated. 2) Hybridization, after initial success, might fail regardless of repeated inseminations.

Material and methods. The virgin turkey hens in these experiments were from a Beltsville Small White flock, 16-20% of whose incubated eggs exhibit parthenogenesis. Such development is limited almost exclusively to growth of undifferentiated embryonic membranes(3). The chicken males were from a nonpurebred Dark Cornish strain selected for a high incidence of parthenogenesis. All birds were housed indoors in individual laying cages and were subjected daily to approximately 14 hours of light and 10 hours of darkness. Each bird had free access to feed and water.

Throughout both experiments all eggs were placed in the incubator within 24 hours of lay. After 9-10 days incubation, the eggs were candled and those without live embryos were opened and the incidence and degree of parthenogenetic developmnt (if any had occurred) were recorded. Eggs with live embryos were returned to the incubator and were candled on each succeeding day until the embryos died. Hybrid embryos reaching 14 or

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