*ANEUPLOIDY IN THE DEGENERATIVE PHASE OF SERIAL CULTIVATION OF HUMAN CELL STRAINS**

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The factors affecting chromosomal stability of mammalian cells in long-term culture are not well understood, aside from the effects of experimentally applied agents such as irradiation and viruses. Species differences[1-3] and perhaps cell types appear to be important with respect to the occurrence of spontaneous alteration or heteroploid transformation.[4, 5] Cells cultured from mouse tissues seem to be extremely labile with regard to chromosome changes and associated morphologic and growth alterations.[1, 6, 7] On the other hand, in metaphase studies to date, cultured human cells of the fibroblastic type remain diploid and show no tendency to undergo spontaneous transformation.[8-11]

Interest in spontaneous occurrences of *in vitro* transformation of mammalian cells has been based upon hope for its confirmation as a model of the *in vivo* process of malignant change. The two processes have many features in common,[5, 12] and early proposals that viruses present in the original tissue or serum might be causative agents have been strengthened by the numerous studies showing that certain viruses do produce transformation *in vitro*. Experiments with SV40 virus and human cells have provided another reproducible cell-virus system for the study of transformation throughout its course.[13, 14] Furthermore, the discovery of chromosome lesions in human cells in association with measles infection[15] has increased our interest in the nature and extent of chromosomal changes which occur *in vitro* in the absence of any known agent.

This report is concerned with a study of metaphase chromosomes in two human cell strains during their total period of *in vitro* cultivation. In a previous less extensive study no aberrations were seen in various human diploid cell strains in material from the 9th to the 40th subcultivation *in vitro*.[10] In the present study spontaneous chromosome changes were encountered, but only in those cultures which had been subcultivated for 40 or more times, i.e., during the degenerative phase of their characteristically limited *in vitro* life. Although this aneuploidy seems to be associated with the degenerative period in long-term cultivation, on no occasion did transformation in terms of altered morphology, growth rate, or capacity for indefinite cultivation occur among many parallel cultures of these two strains. In long-term cultures of human fibroblasts Sax and Passano [16] have previously shown an association between "age *in vitro*" and increase in spontaneous rate of anaphase aberrations.

Materials and Methods.—Cell strains: Cell strains WI-26 and WI-38, male and female, respectively, were derived from fetal lung tissue by L. Hayflick according to procedures previously described.[10] In essence, cultures were grown as monolayers of fibroblastic-like cells in Eagle's basal medium with 10% calf serum and 50 μg aureomycin per ml and subcultivated by trypsinization with 0.25% trypsin (Difco 250:1). Subcultivation from one to two milk-dilution bottles was performed twice weekly, i.e., when the monolayer had become confluent. Confluency was achieved every 3–4 days during the major period (4–5 months) of total *in vitro* cultivation. After approximately 35 total passages *in vitro*, cultures required longer periods (5–8 days) to achieve confluency;

eventually (50 ± 10 passages), all cultures of these diploid cell strains failed to replicate sufficiently to permit any further subcultivation. It is recognized that each subcultivation or "passage generation" is only an approximation to cell population doubling, and the total number of passages has no significance except as a crude measure of the *in vitro* stage any strain has reached. The terminal portion of the finite period of serial subcultivation is referred to as Phase III[10] or as the degenerative phase, and in the absence of heteroploid transformation this limitation on cultivation has long been recognized. Ampoules containing 2×10^6 cells were frozen and stored in liquid nitrogen by procedures described previously.[10] Substrain designations in Tables 1 and 2 refer to cultures reconstituted from such ampoules and carried independently.

Chromosome preparations: Chromosome studies were made from permanent mounts of Giemsa stained air-dried metaphase preparations.[17] Ordinarily cells were harvested with trypsin on the second day after subcultivation, following 3–5 hr of treatment with Colcemid (CIBA), 0.05 μg per ml of medium. The cell suspension was concentrated in 1/2 ml of trypsin solution, and a 4-fold volume of distilled water was added to swell the metaphase cells hypotonically for 8–10 min. Fixation was made with 3:1 methanol:acetic acid. Spreading was done by ignition of a drop of fixative, containing cells in suspension, immediately after its application to the surface of a clean wet slide. Suitable metaphases were selected under low power (150×) observation, and those judged to be free from excessive spreading were then studied under oil immersion optics. In all cells so selected, the chromosomes were counted, and 25–40% of the metaphases of each sample were subjected to detailed karyotypic analysis, involving the identification of individual chromosome pairs or groups of the human karyotype according to the Denver convention: Nos. 1, 2, 3, 4–5, 6–X-12, 13–15, 16, 17–18, 19–20, 21–22-Y. Metaphase counts considered to be artifacts because of scattering of some chromosomes or because of accidental contamination of one metaphase with chromosomes of another were not excluded from the data on the various samples studied. For each determination of the level of tetraploidy existent in the dividing cell population 250–300 unselected metaphases were examined and roughly estimated as being diploid or tetraploid.

Observations: WI-26: Chromosome counts, numbers of cells analyzed, and other karyologic observations from seven different cultures of strain WI-26 are presented in Table 1.

Chromosome preparations from diploid cell strain WI-26 were first made at its 19th subcultivation passage. In a sample of 100 cells the exact chromsome number was determined, and 36 cells were analyzed in detail. It could be seen that most of the cells had a normal male chromosome complement, and in the hypodiploid cells no pattern concerning the missing chromosomes could be deduced. Of the dividing cell population 3.1% were tetraploid, and no abnormal chromosomes were found.

Between the 28th and 37th *in vitro* passages, 106 cells were examined, and of these 28 were analyzed in detail. A normal distribution of chromosome numbers, skewed toward hypodiploid counts, was observed. The analysis of each of the 9 hypodiploid cells of the sample did not reveal any consistency as to the chromosomes which were missing, and these counts are presumed to be artifacts. A somewhat higher value of tetraploidy was recorded at the 28th passage, and again at the 37th passage, 4.5 and 4.4%, respectively. In the sample from the 32nd passage, 1.6% of the dividing cells were tetraploid. An acentric fragment was observed in one of 45 cells examined at the 37th passage. It was found in a cell with 46 (excluding the fragment) chromosomes, which was lacking a No. 4–5 chromosome and contained an extra chromosome in the 6–12 group. It is possible that the fragment resulted from breakage in the long arm of a 4–5 chromosome; this deficient chromosome would then be indistinguishable from members of the 6–12 group. Thus, from a total of over 200 cells counted in cultures of the 19th, 28th, 32nd, and 37th passages, of which 64 were studied in detail, only one cell was found which had an abnormality.

Between the 41st and 54th *in vitro* passage, 58 cells of WI-26 were karyologically examined. Six of 25 metaphases at the 41st passage contained abnormal dicentric chromosomes, while 3 out of 20 did so at the 54th passage. A small sample of 13 cells from 43rd passage material revealed no abnormalities, and the extremely low frequency of mitosis prevented extension of this sample. At the 41st passage, 3.6% of the cells were tetraploid, and the very high value of 16% tetraploidy occurred in the 54th passage material.

Substrains of WI-26 (X, XI, XIII, XIX, and XXIII) could not be subcultivated more than 40–49 total passages; XXVI survived for 56 passages *in vitro*.

TABLE 1

CHROMOSOME COUNT DISTRIBUTION AND KARYOLOGIC DATA FROM HUMAN DIPLOID CELL STRAIN WI-26

In vitro passages	WI-26 substrain designation	Chromosome Counts: <	43	44	45	46	47	49	72	76	91	92	Tetraploidy %	Identity of "Missing" Chromosomes: 1–5	6-x-12	13–15	16	17–18	19–20	21–22-Y	Total cells	Analyzed cells	Types of Abnormal Chromosomes: D	f	m	other
19th	X	1	1	2	5	88			1			2	3.1	1	2	1		1		2	100	36				
28th	XI	1	1		2	16							4.5	1	2		1	1		1	20	4				
32nd	XIX			1	1	38	1						1.6	1	2						41	12				
37th	XXIII				3	42							4.4	1		1	1				45	12		1		
41st	XXIII	1		1	4	19							3.6	1	4	2			2		25	12	6			
43rd	XIII		1	1	1	10														1	13	5				
54th	XXVI			1	5*	12†		1		1	1		16.0		2			2	1		21	6	4			

* Cell presumed 13/21 type translocation.
† Pseudodiploid cell; monosomic and trisomic.
D = Dicentric; f = acentric fragment; m = minute.

TABLE 2

CHROMOSOME COUNT DISTRIBUTION AND KARYOLOGIC DATA FROM HUMAN DIPLOID CELL STRAIN WI-38

In vitro passages	WI-38 substrain designation	Chromosome Counts: <	43	44	45	46	47	53	64	92	Tetraploidy %	Identity of "Missing" Chromosomes: 1–5	6-x-12	13–15	16	17–18	19–20	21–22	Total cells	Analyzed cells	Types of Abnormal Chromosomes: D	f	m	other
4th	P	2	1	3	7	183	3‡		1		1.0		8	1	1	3	4	3	200	84				
14th	II					19				1	0.8								20	9				
21st	P				1	21							1						22	10				
31st	IV			1	2	47					1.0	1		1			1		50	22				
33rd	V			2	3	46†					0.4	2	2	1				1	51	19				
35th	V				2	28													30	10				
37th	IV			1	5	43				1	3.8	1	2	2				1	50	18				
41st	P	1	2	4	9*	87†	1				2.7	8	4	5	2	4	3	3	104	51	3	1		
41st	IV		1	1	6*	30†		1		1	5.1	5	2	5	1	1		4	40	18	2	1	1	2
46th	II	1	1		7	11						4	2	3	2	1	1	3	21	10	6			

* Cell with presumed 13/21 type translocation.
† Pseudodiploid cell; monosomic and trisomic.
‡ Artifact due to contaminating chromosome from another cell, two cases.
P = parental culture; D = dicentric; f = acentric fragment; m = minute.

WI-38: At the fourth subcultivation passage 200 WI-38 cells were examined and, of these, 84 were subjected to detailed karyotypic analysis. The distribution of the exact chromosome counts obtained is given in Table 2. Among 14 hypodiploid cells no consistent pattern as to the identity of the missing chromosomes could be determined. Three cells with 47 chromosomes were observed, but in two of these contamination from another metaphase was definitely indicated by a differential state of condensation in the extra chromosome. In all other cells examined, no deviation from the normal female karyotype was observed. At this early stage *in vitro*, the frequency of tetraploid cells in the dividing population was 1.0%.

Essentially similar findings were obtained concerning the chromosomal constitution of various WI-38 substrains from the 14th to the 37th passage; 223 cells were examined and 98 of these karyotypically analyzed. A single pseudodiploid cell was observed in the 33rd passage sample. The level of tetraploidy remained low, except for one slightly higher value at the 37th passage, 3.8%.

From WI-38 cultures between the 41st and 46th *in vitro* passages, the chromosomes of 165 cells were counted, and 79 of these were karyotypically analyzed. Aneuploid changes and a marked increase in frequency of hypodiploid cells are evident in all of these late passage samples. Although no obvious pattern as to the missing chromosomes can be observed (Table 2), the fact that 2% (41st passage) to 30% (46th passage) of the cells exhibited obviously abnormal chromosomes would imply that perhaps many of the hypodiploids were not artifacts of technique. The abnormalities observed were mainly dicentrics formed by translocations between two chromosomes of the complement, in some cases identifiable chromosomes. Less frequently (Table 2), fragments, minute chromosomes, and abnormal monocentric chromosomes were observed. In this late passage WI-38 material, the frequency of tetraploid cells (2.7–5.1%) was elevated above values seen in lower passage levels (Table 2). The original or parent strain of WI-38 was cultivated for a total of 48 passages, and none of the three substrains (II, IV, V) could be subcultivated more than 43 to 48 passages *in vitro*.

Discussion.—Aneuploid changes appeared in different substrains of each strain studied following a long period of apparent chromosome stability. The earliest chromosome aberrations were coincident, with the beginning of the decline of each strain during its serial subcultivation. Setting aside the question of criteria for chromosomal or karyotypic "normality," we must consider whether these observations are generally applicable to human fibroblast strains in long-term culture. Since strains WI-26 and WI-38 were selected for extended investigation for the sole reason that one is male and the other female, it seems warranted to regard them as representative of such serially propagated strains, at least for those of embryonic origin. Further, two other human fibroblast strains cursorily examined also showed some aneuploid cells at high passage levels of cultivation.

That our previous study[10] failed to reveal aneuploid changes with increased "culture age" is attributed to the fact that only two of the 16 samples studied from 13 strains were from cultures approaching or in the period of decline, i.e., above the level of the 35th passage. At its 40th passage, WI-12 revealed no abnormalities in a sample of 33 metaphases, and only 17 cells were available from the 39th passage of WI-1 because of the low growth rate in its declining phase.

No aneuploidy was observed in earlier studies on long-term cultivated human fibroblasts. Among cultures from individuals 1–41 years of age, Tjio and Puck[8] reported "no variation in chromosome number and morphology." Their cultures were studied over periods comparable to those reported in the present paper. In a later paper[9] these authors remarked upon the constancy of the human karyotype, finding no change in number or morphology over "20 successive harvests (transfers). . . involving more than 40 generations." Makino and co-workers[11] reported the maintenance of a "normal complement of 46 chromosomes" in cells obtained

from the 2nd to the 44th subculture; however, they also state, "In comparison with the results of chromosome counts in the primary cultures, cells with hypo- or hyperdiploid chromosome numbers occurred at a higher incidence in the subcultured specimens."

These authors did not present details concerning the proportion of their large total sample which was derived from the late-passage cultures mentioned. On the other hand, in this study we deliberately sought information during the period when the cultures' growth rates had begun to diminish. This fact may account for our observing aneuploidy, whereas they reported none. Our results, in respect to samples taken prior to about the 40th subculture, are in accord with the other studies. The constancy of the human karyotype in such material is impressive when compared to continuously cultivatable cell lines from mammalian tissue which are near-diploid, i.e., containing a large number of apparently euploid cells but with some pseudo-, hypo-, and hyper-diploid cells also detectable.[2]

For *a priori* reasons, criteria for the "normal" human karyotype should be based upon *in vivo* dividing populations (such as direct preparations from bone marrow) or upon primary or very early tissue cultures. This must be determined against a background of a certain amount of hypodiploid counts which, for technical reasons, are spurious. Within these limitations of technique and of the experience of the investigators, prior to the 40th passage level, both strains WI-26 and WI-38 may be regarded as normal or classic diploid. A minor reservation may be made with respect to strain WI-26, however, as the tetraploid percentages observed at the 28th and 37th passages were slightly above values usually observed. A number of studies[8, 10, 11] have shown that the tetraploidy level in presumably normal cultures of human fibroblast cells is seldom greater than 3 per cent. An increase in proportion of tetraploids in the metaphase population is a common feature of SV40 transformation of human fibroblasts.[18, 19] We have observed a similar association between tetraploidy increase and subsequent spontaneous heteroploid transformation in serially cultivated cells of the rhesus monkey.

Considerations as to the tissue's species of origin and the possible presence of inapparent viruses may explain the fact that adult rhesus monkey kidney tissue cultures ordinarily degenerate within a few weeks, surviving no more than 3 or 4 subcultivations. These monkey kidney subcultures may show an extremely high frequency of mitotic aberrations (16–40 per cent) even before the second week of *in vitro* cultivation.[20] That human cell types other than fibroblasts may show entirely different patterns with respect to their fate *in vitro* is well known. For example, extra-embryonic amnion can usually be cultivated for only a few subdivisions before it degenerates or, on occasion, transforms. It is possible that the inherently limited *in vivo* growth potential of amnion, a kind of "tissue age," may be expressed even *in vitro*.

The three WI-26 substrains (XIII, XXIII, XXVI) which revealed aneuploidy survived as serial cultures no longer than various parallel subcultures from this strain which were not examined for chromosome changes. The same applies to the particular substrains (Parental, II and IV) comprising the post-40th passage material in which aneuploid changes were observed in the WI-38 strain. Among the rearrangements and chromosome breaks observed, there was no consistency as to the particular chromosomes affected.

We cannot exclude the possibility that the two particular strains, WI-26 and

WI-38, were in some way predisposed to undergo aneuploid changes or that some unknown effect of the medium used has induced an instability. However, aneuploid changes were noted in different substrains, even though they were carried on medium from independent commercial sources. It therefore appears more likely that our findings may apply generally to such cells and confirm the study by Sax and Passano[16] in which anaphase bridges, lagging chromosomes, rod and dot deletions were found to increase approximately 3-fold during six months of serial cultivation of human fibroblast strains. Interphase nuclei with abnormal sizes and shapes are seen in daughter cells in later passage material[10] and are presumably the products of abnormal division.

The conditions in these human fibroblast cultures, after loss of their proliferative capacity, are superficially identical to those reported in cultures of mouse cells just prior to the usual reversal of the latter's declining growth rate.[1, 7] This increase in growth rate then leads to establishment of the mouse cell culture as a continuously propagated and usually heteroploid cell line. In spite of the degenerate state of late passage cultures of strains WI-26 and WI-38, with chromosome aberrations and reduced mitotic activity, none has undergone a spontaneous transformation, although numerous cultures have been observed 1–3 months after cessation of growth.

Note added in proof: It has come to our attention that M. C. Yoshida and S. Makino have reported quite similar findings in the *Japan. J. Human Genetics*, **5**, 39 (1963). These independent observations strengthen our conclusion that the presence of aberrations associated with *in vitro* decline of such cell strains is a general phenomenon.

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