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Cells in Culture

Edited by

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CHARLES MARC POMERAT

This symposium was one of the last scientific activities of the late Dr. Charles M. Pomerat, who was its co-organizer and host at Pasadena, California. The death of this distinguished scientist on June 17, 1964 ended a brilliant career and saddened his many friends and colleagues throughout the world.

Dr. Pomerat, in addition to distinguishing himself as a biologist and teacher, was a talented artist of professional ability, a student of the history of architecture, a linguist, a world traveler, and a brilliant lecturer and raconteur.

All of us who had the pleasure of participating in this symposium and whose lives were so enriched by knowing him dedicate this volume to the memory of Dr. Charles M. Pomerat.

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PREFACE

Cytogenetics, as an offshoot of genetics, has, in the past several years, moved from a relatively minor role to one occupying a major influence in shaping modern biological thought. No longer can this approach be considered in a purely static sense, useful only for the systematic recording of chromosomal aberrations. The presence of trained cytogeneticists on the staffs of numerous medical schools and hospitals is indicative of the newer applications of this discipline. Modern tissue culture techniques, combined with autoradiographic advances and well-developed methods of preparing and analyzing chromosome populations, have joined hands in the DNA era to provide the means for a functional understanding of genetic behavior at the chromosomal level.

It was the goal of this symposium to not only establish guideposts to the direction of the most recent progress in this rapidly moving field, but to cross-pollinate, in the best Mendelian tradition, the thinking of widely recognized leaders in this field with the current ideas of their colleagues, within the intimacy of this small meeting. Tangible evidence of profitable interchange was observed, judging from the many stimulating discussions which ensued during the 3-day gathering. It is hoped that the products of such cross-fertilization will stimulate increased vigor and proliferation of research by the participants. It is further hoped that the publication of this volume will infect the reader with new concepts and ideas which will eventually broaden our views of cytogenetics.

The contributors to this symposium were carefully selected by the Advisory Committee of the International Society for Cell Biology from laboratories around the world because of their courage to explore new frontiers of cytogenetics with the confidence gained from extensive experience in the field. Although there was no pretext of surveying the entire scope of cytogenetics within the framework of this meeting, the contributors emphasized (1) new techniques to detect patterns of DNA replication, to better understand cellular control mechanisms, and to perform chromosome analyses with automated instrumentation; (2) the effects of cell hybridization and of noxious substances such as radiation, chemical agents, and viruses on chromosomal patterns; and (3) the influence of altered chromosomal complement on the survival of cells to long-term tissue culture or to ionizing radiation treatment. It was comforting that two participants observed that naturally occurring chromosomal abnormalities in mammalian populations were relatively rare and were not necessarily associated with physical defects or disease.

The organizers of this symposium would like to acknowledge the vital roles played by the National Aeronautics and Space Administration in providing operating expenses and by the Associates and the Board of Directors of the Pasadena Foundation for Medical Research in arranging the social aspects of the program. A special tribute should be paid to the late Dr. C. M. Pomerat who was the driving and stimulating force which made the program so successful.

November 1964

DONALD E. ROUNDS

FREDERICK H. KASTEN

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CYTOGENETIC ALTERATIONS IN MIXED CULTURES OF MAMMALIAN SOMATIC CELLS *IN VITRO*

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INTRODUCTION

Carrel [13] was the first to make a permanent *in vitro* culture of a cell line. This was his famous chick embryo heart strain maintained *in vitro* for 34 years of uninterrupted passages by the simple, though tedious, technique of plasma-embedded cultures. So far, this record has never been surpassed.

Unfortunately, we have very little information concerning the phenotypic and genetic properties of Carrel's chick heart cells, especially the modifications of histiotypic, metabolic, chromosomal, or other characteristics of this unique cell line during its long life *in vitro*.

Later, in the 1940's and early 1950's, new techniques of tissue culture, improved media, and the use of antibiotics greatly facilitated long-term cultivation of tissue cells and the development of permanent *in vitro* cell lines.

Nevertheless, only during recent years have we learned to study and define in a more rigorous way characteristics of *in vitro* cultivated cells such as movement, surface properties, protein and nucleic acid content, enzymatic activity, and karyotype. Another important achievement has been the development of techniques of cell cloning [35, 31]. This remarkable progress in general tissue culture procedures was a prerequisite for any precise study of somatic cell genetics in *in vitro* cultures.

It does not seem necessary to dwell extensively on the phenomena observed following explantation of tissue cells *in vitro*, phenomena involving, on the one hand, modifications in identifiable hereditary characteristics and, on the other, chromosomal changes which usually take place during adaptation of somatic cells to *in vitro* life. Nevertheless, it seems worth while to recall and reformulate certain essential points.

(1) We must bear in mind that explanted tissues, either in fragments or in suspensions, are practically always, genetically speaking, mixtures of many types of cells characterized by different degrees of more or less irreversible histological differentiation.

(2) However, independent of this histological or species origin of

the explanted tissue cells, their *in vitro* evolution essentially follows the same pathways. The stages usually are, first, primary outbursts of cell multiplication, then stagnation, and, eventually, progressive or abrupt reversal of the situation designated as "adaptation" or "transformation," followed by resumption of growth. This evolution produces a cell population apparently different in its morphological and functional properties, and frequently in its karyotype, from *any* cell component of the initially explanted normal tissue cells.

The important questions in this connection are: What is the nature of these modifications and what is their mechanism and their extent? In other words: How far and in what way can the *in vitro* cultivated cells depart from their original characteristics?

Two general trends of cell modification during prolonged culture *in vitro* are generally recognized: (1) a tendency toward cell dedifferentiation corresponding to loss of certain morphological and functional attributes; (2) changes in cell karyotype with concurrent shifts of chromosomal numbers to aneuploid values, and the appearance of new, unusual chromosomes.

Unfortunately, during the last few years, data concerning these striking, but readily occurring, modifications have been full of experimental errors due to unrecognized cell-cross contaminations or mislabeling of cultures [14, 33]. In some cases, these errors led to misinterpretations concerning the extent of cell transformation *in vitro*.

However, when we select for consideration only well-verified experimental data, it becomes quite clear that even adapted *in vitro* cells do not proceed beyond certain limits in their evolution. For example, two cell lines obtained from rabbit and human Fallopian tubes, which we studied in our laboratory [8], maintained their proper sensitivity to corresponding species-specific antisera after years of cultivation *in vitro*. They also preserved their characteristic range of sensitivity to virus infections and kept, in spite of important deviations and numerical shifts, recognizable species-specific karyotypes. Similar observations have been reported by others [12, 16].

Long-term maintenance of original antigenic structure can be even more expressive. Mouse cell lines, originating from C3H mice and cultivated for many years in Dr. Sanford's laboratory [35] and then in our Institute [10], as well as C57BL mouse pulmonary cell lines we developed in Villejuif [3], maintained their strain specificity for many years, as evidenced by transplantation tests and also by sensitivity to corresponding homologous anti-C3H and anti-C57BL sera.

The conclusions drawn from these and other observations which bear heavily on any genetic experiments on somatic cells *in vitro* are:

(1) The transformation undergone by these cells during culture is not limitless, and, in fact, obeys certain rules even if these rules do not appear very clear to us at the moment.

(2) *In vitro* cell strains, after having passed through a rather stormy period of adaptation, attain a relative stability in their essential characteristics.

The phenomenon of relative stability of established cell strains *in vitro* can be illustrated by many examples.

Earle's C3H mouse L strain [17], cultivated for years in many laboratories throughout the world, preserved its essential morphological, chromosomal, and biological characteristics [25], particularly, its low receptivity to polyoma virus [6]. Incidentally, chromosomal metacentric markers of this line greatly facilitated discovery of cell contaminations by this line in many "transformed" homologous and heterologous cell cultures [14, 16].

The TG cell line, developed in our laboratory [8] from a normal human oviduct and cultivated since 1960, shows a constant characteristic epithelioid morphology and a fairly stable karyotype containing a modal number of 59 chromosomes with permanently present markers [4]. Satisfactory chromosomal and phenotypic stability has also been demonstrated by Harris and Ruddle in their pig kidney cell strains [23]. Other illustrations of this kind of *in vitro* stability are the mouse lymphosarcoma MB I, MB VI, MB II, and MB III variant strains cultivated since 1935 by De Bruyn [15].

However, while considering the problem of stability or modification of cells in culture, we must be conscious of the fact that, in spite of recent progress of the identification of cellular morphological and functional characteristics *in vitro*, we are still handicapped in this field due to deficiencies in available cytological and cytochemical methods. It is obvious that any extension of these methods immediately offers means of studying more efficiently hereditary phenomena in the unique system represented by the cultures of metazoan cells *in vitro*.

The present limitations simply illustrate how much less precisely defined is the system of animal somatic cells than similar systems of unicellular organisms. The difference primarily concerns the more satisfactory correlation, on one hand, between structure and known topography of genetic material and, on the other, expressed hereditary characters.

In spite of these restrictions, recent progress in defining animal cell properties *in vitro* has provided a new approach to the problem of transfer of genetic information between animal cells cultivated *in vitro*.

Experimental attempts to achieve transfer of genetic material with

the aid of subcellular fractions, especially with preparations of nucleic acids or nucleoproteins have been reported by several authors [11, 37]. Initially, we used this approach when we began our work on secondary differentiated *in vitro* NCTC low and high cancer cell lines developed by Earle, Sanford, and their co-workers [34, 35]. Subsequently, we adopted, as an experimental procedure, long-term mixed cultures of two cell lines each having well-defined chromosomal and biological markers. The theoretical premises for these experiments were the observations of many authors concerning either cytoplasmic fusion between cells, especially cells of connective tissue origin [24, 29, 39] and intercellular transfers of cell organelles such as mitochondria, or Golgi apparatus [32].

Before reporting and discussing our results, it should be pointed out that there is abundant evidence that, in normal metazoan tissues *in vivo*, the dominating and natural trend is toward a strict maintenance of histological identity of differentiated cells. One can suppose that some special homeostatic mechanisms are at work that contribute to the maintenance of genetic stability and identity of tissue cells, in spite of the intimate contact between different body cells in tissue sites and in tissue fluids.

The same may also be true, at least as a dominating tendency, for malignant cells, as was recently demonstrated by Wakonig-Vaartaja [38] on mouse leukemic cells which happened to have distinct chromosomal markers.

It can be admitted that *in vitro* rapidly multiplying cell cultures the situation is different. The frequency of abnormally occurring mitoses and of cytoplasmic fusions indicate that, in the absence of homeostatic mechanisms which operate *in vivo*, we can expect certain special phenomena to occur *in vitro* which are precluded *in vivo*.

In our laboratory in 1959, with these ideas in mind, we began experiments on mixed cultures *in vitro*. Our choice, which was probably a lucky one, was fixed on two isologous clonal cell lines of C3H mouse origin developed since 1956 by Sanford in her laboratory in Bethesda [35]. The essential and encouraging point was that these cell lines, designated as NI high cancer and N2 low cancer strains, when checked previously in our Institute [10], showed a fair degree of stability in their essential characteristics. This situation, in spite of some shifts and deviations, is grossly similar at the present time; the NI cells were, and still are, composed of cells appearing as typical spindlelike fibroblasts with very ramified cytoplasm forming an intermingled network in dense cultures. The N2 cells display predominantly circular cytoplasmic membranes, and, in more dense cultures, show a mosaiclike arrangement, with no cell overlapping. The karyotype of the two cell lines is strikingly differ-

ent. Every mitosis can easily be identified as belonging to the NI or to the N2 family.

The NI type cells, either the original Sanford clone or the secondary clones developed in our laboratory, had a modal number of 55 chromosomes with only 0-2 metacentrics and, frequently, an extra-long telocentric marker chromosome. More recently, we obtained an NI clone having nearly 80 chromosomes, but always characterized by the same low number of metacentrics and high malignancy rate for the isologous C3H mice.

The N2 cell clones have a modal number of 62-65 chromosomes with nearly 13 metacentrics. This karyotype remained remarkably stable for many years as did the characteristic morphology and the very low tumor-producing capacity of 2-3 per cent of very slowly growing tumors in isologous mice inoculated with 2-3 million cells.

As pointed out previously [7, 9], in combined cultures of these two cell lines, we repeatedly observed profound changes in the composition of the mixed population and the appearance of a new type of cell, designated as the M cell. This cell exhibited a cumulation of chromosomal and biological characteristics of the parental cells and was, morphologically, of intermediate type between NI and N2. It is worth while to emphasize the fact that this kind of cell was unknown in our laboratory before we started these experiments, but it appeared repeatedly in several experiments in associated NI + N2 cultures.

More detailed analysis of the characteristics of M cells, in comparison with the parental NI and N2 cells, was performed on M-type clones isolated either directly from NI + N2 cultures *in vitro* or from tumors produced by inoculation of C3H mice with mixed cell populations [1, 5]. Karyotypes and biological behavior were very similar for the 15 clonal M cell lines isolated from both origins.

As can be seen in Table I taken from our previous publication [5], some morphological and biological traits were common to the M and the low cancer N2 cells; some other traits (especially malignancy as checked by proportion of takes and rapidity of tumor development in isologous C3H mice) approximated those of NI cells.

Some of the M cell clones (clone MI and clone M6) were maintained in proliferating cultures in our laboratory for more than 3 years. During this period of time, they preserved entirely their "hybrid" cell morphology, which was intermediate between the rounded N2 and spindle form NI cell shapes. They preserved similarly their high malignancy rate for isologous C3H mice. However, their karyotypes tended progressively toward decreasing chromosome numbers, stabilizing around modal values of 95-100, with 8-10 metacentrics. Supposedly, nondisjunction accidents, frequent during divisions of the primary hybrid cells, supplied chromo-

somal variants for further selection. It can be assumed that the segregation operated in favor of cells having some chromosomes deleted from their complete initial hybrid karyotype.

TABLE I. *Comparison of Characteristics of M Cell with N1 and N2 Cells*

M Characteristics common with:	N1	N2
Cell morphology		
Tendency to form "circular" cytoplasmic membranes		±
Extensive cytoplasmic ramifications	+	
Cell agglomerations and network of intercellular ramifications in dense cultures	+	
Tendency to form polygonal, mosaiclke arrangements		+
Formation of largely spread giant cells		+
Chromosomes		
Telocentric chromosomes more than 90 per cent	+	
Extra-long telocentric chromosome	Sometimes	
Metacentric chromosomes regularly present		+
Production of tumors		
Short lag period after subcutaneous inoculation (less than 6 days)	+	
Close to 100 per cent of takes with 1 million cells	+	
High rapidity of growth	+	
100 per cent mortality in less than 2 months	+	
Growth on chorioallantoic membrane		+
Production of latent infection with polyoma virus		+

Since our experiments on N1 and N2 mixed cultures have been completed, the phenomenon of hybridization between these two cell strains has been confirmed by karyological observations in Ephrussi's laboratory. This author later described similar events in other homologous cell mixtures [18]. The appearance of hybrid cells in associated cultures of two homologous mouse cell strains of C3H and Swiss origin was also recently reported by Gershon and Sachs [20].

We wish to report some recent observations made in our laboratory on phenomena occurring in homologous combined cultures of the nonmalignant N2 line of C3H origin and the PTT 12 high cancer line we obtained following malignant transformation *in vitro* of C57BL lung tissue [3], a line which has remained, so far, rigorously isotransplantable.

The constant phenomenon which occurred in these cultures, according to morphological and chromosomal observations, was an overgrowth of the mixed cultures by the nonmalignant C3H N2 line. No apparent reason for this repeatedly observed overgrowth could be found since the PTT 12 cells grown separately had a replication rate at least as high as the N2 cells. It may be noted that the overgrowth of one cell strain

by another in mixed cultures *in vitro* is a frequent phenomenon and has been observed by many authors [26, 30]. This overgrowth may also occur *in vivo* when two ascites tumor strains are inoculated in the same animal [36]. We observed, in many instances, that in mixed cultures of C3H and C57BL cell strains *in vitro*, the C3H cells suppressed the C57BL component independent of the rate of growth of the associated cell strains. To overcome this difficulty in the N2 + PTT 12 cultures, the PTT 12 cells were added repeatedly to the mixture. No perceptible karyological proof of hybridization could be found in these combined cultures by repeated checking of mitoses for more than 5 months. However, cross-grafting analysis suggested that some changes could occur in the mixed cell populations.

The combined cultures were inoculated, starting from the fourth month after N2 + PTT 12 association, in normal C3H, C57BL, and (C3H $\times$ C57BL) F1 mice. The result was negative in C3H mice, as expected, due to the non-malignant character of the N2 cells for isologous mice. No tumor growth appeared in the C57BL mice, as could be predicted following the disappearance of PTT 12 cells from the mixed cultures. However, the striking fact was the constant production of tumors in the F1 hybrids. These tumors grew progressively, and killed the inoculated mice in 3–5 months. They were easily transplantable, directly or after culture *in vitro*, but only in F1 hybrids. They were definitely rejected by the parental C3H or C57BL mice. In other words, by their graft histocompatibility, these tumors behaved as if they were composed of hybrid cells.

Thorough karyological analysis of cells of the tumors produced in this way in F1 mice disclosed mitoses practically indistinguishable from the parental N2 cells. Their chromosomal modal number was around 62, and the modal number of metacentrics was 13. This fact practically negated the idea of their originating from tissues of the inoculated F1 hybrids, and confirmed that tumors growing exclusively in F1 mice were derived from the inoculated cells.

Further work, now in progress, using clonal isolates and serological tests with homologous antisera, tends to clarify the exact nature of these cells.

It must be mentioned that in the control experiments the N2 cells, which usually did not grow in the parental C3H strain, produced, for reasons which are not well understood, transplantable nodules much more frequently in the F1 hybrids. This phenomenon is now under study.

These data, together with those of Ephrussi and his group,¹ and the recent observations of Gershon and Sachs [20], suggest that phenomena

¹ See paper by Ephrussi *et al.* in this Symposium.

of intercellular transfer of genetic material in *in vitro* cultures are not necessarily restricted to isologous cells, and can be expected in homologous cell associations.

It can also be supposed, although definite proof is still missing, that in combined cultures of two distinctly different cell components we can expect not only complete association of the two karyotypes but also partial genetic transfers, which may imply biological, antigenic, or biochemical consequences associated with very subtle or even indistinguishable karyological modifications.

CONCLUSIONS AND SUMMARY

(1) Interactions of somatic metazoan cells *in vitro*, implying genetic changes, have to be envisaged as a real possibility.

(2) Allopolyploidization, as a consequence of the addition of chromosomes from two different karyotypes into one viable cell, capable of replication in mixed cultures, was first demonstrated in an isologous system of N1 and N2 cell strains derived from C3H mice. Later, other karyological proofs of somatic hybridization were reported, in quite a convincing way, in the same system, as well as in other mixed cell cultures including homologous cell combinations [18, 20].

However, it is basically important that these observations, at least in the case of N1-N2 hybridization, have been corroborated by parallel analysis of distribution of hereditary characteristics in clones developed from individually isolated hybrid M cells [1, 5]. From this analysis, it appeared that the M cells inherited from their N2 parents, along with easily identifiable marker metacentric chromosomes, some morphological and biological traits, such as a tendency to form rounded cytoplasmic membranes and large extended giant cells, and in a quite different scope, showed a particular type of reaction to polyoma virus infection [6]. On the other hand, an extra-long telocentric marker chromosome frequently present, some properties of the cell membrane, and, above all, a persistent high malignancy rate related the M cells to their N1 parent.

Though these observations are only tentative, they constitute the first experimental confirmation of the fact that an intercellular transfer of genetic material, visualized by cumulation of chromosomes from two different somatic cells into a new cell, had its biological consequences in terms of heredity of some characters. This statement is valid even though we have no precise data concerning the specific interrelationship between particular chromosomes and heredity of identifiable characters in our system.