GENETICS OF SOMATIC MAMMALIAN CELLS*

III. Long-Term Cultivation of Euploid Cells from Human and Animal Subjects

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Plates 63 and 64

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The animal cell lines most readily cultivated by current tissue culture techniques are usually aneuploid in chromosomal constitution and often have originated in malignant tissue (1). Most workers report that normal cells from adult individuals either fail to grow *in vitro*, or grow only for brief periods, usually a few weeks or months, after which proliferation ceases (2). Application of recently devised single cell techniques (3) to genetic studies of human and animal somatic cells would be facilitated by availability of means for initiating *in vitro* cultures from normal cells of any individual. Thus, if growth could regularly be secured from tissue samples small enough to permit routine culturing of any person, cell lines could be obtained from individuals with known or suspected morphologic, biochemical, immunologic, or pathologic genetic markers. Ideally, such a culture technique should also permit long term cultivation of normal cell lines instead of the short-lived proliferation which is usually obtained (2). Finally, it is essential to minimize or eliminate the chromosomal labilization which frequently overtakes animal cell cultures (1) to the detriment of systematic genetic studies. This paper describes a methodology which in large part accomplishes these objectives, and indicates some applications of the technique.

The elongated, fibroblast-like cells which most commonly proliferate from explants obtained from practically any organ appear best suited for comparative genetic studies from different individuals. These, however, are nutritionally more exacting than the epithelial-like variety, since they will not grow like the latter in media supplemented only with adult mammalian sera, and more readily succumb to the presence of toxic agents (4). While methods for cultiva-

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tion of these cells have been described (4), the need for inclusion of embryo extract in the growth medium has made their culture precarious because of the frequency with which toxic batches of embryo extract occur, despite great care taken to standardize the preparative procedures.

With the finding that fetuin which occurs in high concentration in fetal calf serum promotes growth of the S3 cell (5 a), and because of other characteristics of embryonic serum such as its low fat and gamma globulin content, experiments were undertaken to determine whether fetal calf serum might not eliminate the need for embryo extract in the growth of fibroblast-like cells. This expectation was realized. By means of the procedures here described, it has become possible to make reliable transfer to tissue culture of cells obtained from extremely small biopsies taken from adult as well as young individuals, and to maintain long term cultivation without loss of karyotype integrity.

Materials

All of the solutions employed are described in Table I. Their constitutions have been changed in various ways over previously described formulations (4, 5). The use of embryo extract has been eliminated and embryonic (and occasionally newborn) mammalian sera have been substituted in its stead. The standard method for serum collection involved withdrawal of blood with a sterile syringe, transfer to a dry, sterile bottle, storage at room temperature for 4 to 24 hours for clot formation, then centrifugation for 30 minutes at 1500 R.P.M. in a clinical centrifuge, and storage in tubes at −40°C. Batches in which extensive hemolysis occurred were discarded.

Most of the experiments here described were carried out with fetal calf serum.[1] The age of the fetus did not appear markedly to affect the results. However, other factors, as yet not delineated, tended to make some sera unsatisfactory. The presence of toxic factors in serum is insidious in that they may not make their presence obvious in the mass cultures used in routine farming until after a long lag period. Cells so intoxicated may require weeks or months for return to healthy growth as previously described (4). However, the use of single cell plating can reveal even very low levels of serum toxicity immediately because of the greater sensitivity of the cells in the isolated state. Hence, as routine, each batch of serum was tested for its ability to produce colony formation from single euploid cells using the procedure to be described below, and all sera for which a plating efficiency less than 10 per cent was achieved were discarded. Of the sera which were accepted, appreciable variability of performance was observed, some yielding plating efficiencies as high as 70 per cent. During the summer and fall months of 1957 almost 80 per cent of the fetal calf sera collected yielded results of this kind, but in those collected during the following winter and spring, a fairly high proportion of sera had to be discarded.

In a few recent experiments, normal human cord sera, obtained during delivery, from placental blood were used. These have given results similar to that of the fetal calf, but have not yet been in use for sufficient periods to determine their long term effects.

Technique for Biopsy and Culture.—The changes in the technique which have made possible the results described were all designed to reduce still further trauma to the cells by the action of unfavorable pH, temperature, or toxic substances present during cell dispersal

[1] Supplied by Colorado Serum Co., Denver, to whom we are greatly indebted for patient and skillful cooperation in many tests of the conditions needed to obtain maximally effective serum.

or subsequent cultivation. The following procedure was devised to establish euploid cell growth from samples of human skin so small as to be readily obtainable from any individual: The skin on the ventral surface of the forearm or the back of the neck is washed in 70 per cent alcohol, and allowed to dry. Approximately 25 mg. of skin is excised with a very sharp conjunctival scissors. For greatest convenience, the skin may be lifted by grasping a hair follicle, so that the scissors can be slipped underneath. Usually almost no bleeding results and the wound, which can be covered with a bandaid, heals with little or imperceptible scarring.

The sample is immediately placed in a sterile, 60 mm. Petri dish, and subdivided with the scissors into about 8 or 10 smaller pieces, which are then covered with 2. cc. of 0.04 per cent trypsin (Table I). The Petri dish is placed on a horizontal rotary shaker in a 37°C. room and agitated for approximately 5 minutes, at which point the suspension becomes slightly turbid. The entire sample is pipetted once or twice to promote further disintegration of the tissue pieces, and then apportioned among 4 sterile Petri dishes, to each of which is added 4.0 cc. of the growth medium in Table I. The dishes are incubated at 37.0°C. in an atmosphere of water-saturated air containing CO_2 at a partial pressure of 31 mm. Hg. The medium is replaced every 72 hours until the culture appears well established, after which the interval can usually be lengthened to 4 days. Great care is maintained at all times to insure that the cellular environment does not deviate from pH 7.1 and 37.0°C. by more than 0.2 of a pH unit at any time, or more than 0.3°C. during incubation.

Within a few hours of incubation, extensive glass attachment of single cells as well as of occasional undigested tissue fragments occurs. Within several days or a week, cell proliferation is initiated around a number of these *foci*. Actively growing cultures have been obtained from persons of both sexes, varying in age from a few weeks to more than 40 years. Under the conditions here described, the cells obtained are almost invariably spindle-shaped (fibroblastic), probably because of the selective advantage enjoyed by these more migratory cells in nutritionally complete medium (4). When the cell layer becomes confluent, it is trypsinized by removal of the medium, a single wash with Solution 7 of Table I, then incubation for 5 to 8 minutes at 37°C. in 2.0 cc. of the same trypsin solution. The solution is pipetted once or twice, with the liquid stream directed against the glass, to loosen any remaining cells. The suspension is then quickly diluted with culture medium to stop the tryptic action, and subdivided, usually into larger culture bottles which then may be farmed in routine fashion (4).

EXPERIMENTAL RESULTS

In a series of such skin biopsies carried out on 16 persons, ten produced actively proliferating cultures. Biopsies from other human organs like the bone marrow, ovary, testis, endo- and myometrium, and cervix, made available as a result of various hospital procedures, were equally successful. Cells have been cultivated by this technique from patients with genetic defects like phenyl ketonuria, as well as from normal human subjects. Euploid cells obtained from lung, kidney, ovaries, and other tissues of animals like the American opossum, the Chinese hamster, and the mouse also readily initiate growth by this procedure.

Karyotype Delineation.—A rapid and precise method for visualization of the chromosome constitution of cells cultivated by the present technique has been described in the second paper of this series (6). The combination of these two methodologies makes possible convenient delineation of the somatic cell

TABLE I

Composition of the solutions employed

1. Saline F: Balanced salt + glucose solution.

	gm./liter		*gm./liter*
NaCl	7.40	KH_2PO_4	0.083
KCl	0.285	$NaHCO_3$	1.20
$MgSO_4 \cdot 7H_2O$	0.154	Glucose	1.10
$CaCl_2 \cdot 2H_2O$	0.016	Phenol Red	0.0012
$Na_2HPO_4 \cdot 7H_2O$	0.29		

2. Saline G: Balanced salt and glucose solution for use outside of a CO_2 atmosphere, identical with saline F except for the following changes:

	gm./liter
NaCl	8.0
KCl	0.4
$NaHCO_3$	0
KH_2PO_4	0.150

3. N16: Synthetic nutrient solution.

	gm./liter		*gm./liter*
L-Arginine HCl	0.0375	Glutamine	0.20
L-Histidine HCl	0.0375	L-Tyrosine	0.040
L-Lysine HCl	0.080	L-Cystine	0.0075
L-Tryptophane	0.020	Hypoxanthine	0.025
β-Phenyl-L-alanine	0.025	Thiamine HCl	0.0050
L-Methionine	0.025	Riboflavin	0.00050
L-Threonine	0.0375	Pyridoxin HCl	0.00050
L-Leucine	0.025	Folic acid	0.00010
DL-Isoleucine	0.025	Biotin	0.00010
DL-Valine	0.050	Choline	0.0030
L-Glutamic	0.075	Ca pantothenate	0.0030
L-Aspartic	0.0300	Niacinamide	0.0030
L-Proline	0.025	i-Inositol	0.0010
Glycine	0.100		

These constituents are dissolved in Saline F as described above.

4. Antibiotics solution (made up in water or Saline F).

	gm./liter
Crystalline peniclin	20
Crystalline streptomycin	12
Crystalline terramycin	0.25

5. Complete growth medium.

	per cent		*per cent*
N16	40	Solution NCTC 109 (11) (obtained from Microbiological Associates, Bethesda)	4
Fetal calf serum (obtained and tested as explained in text)	15		
Antibiotics solution	1	Saline F	to 100

The composition of these nutrients is obviously redundant to some extent, and contains materials suggested by several laboratories, as acknowledged earlier (3, 4, 8).

TABLE I—*Continued*

6. N15: Dilution medium for manipulation of cell suspensions outside of a CO_2 atmosphere. This solution has all the organic components of N16 in the same concentration, but dissolved in saline G instead of saline F. For use, 40 cc. is then diluted further with 60 cc. of saline G.

7. Trypsin solution for cell dispersal from tissue biopsy, and preparation of cell suspensions from sheets of cells grown on glass surfaces. Trypsin (1-300-Nutritional Biochemicals). 0.04 per cent solution made up in N15.

chromosome constitution of any individual. If the cells resulting from trypsinization of the original biopsy are plated directly on microscope cover glasses in the complete growth medium of Table I, chromosomal delineation can often be accomplished within a week of the original biopsy. Thus, large scale surveys of human chromosomal constitution can readily be carried out. Such a survey is now in progress, a report of the first group of results having appeared in the previous publication (6).

Long-term Cultivation.—

All of the cell lines so obtained have demonstrated the same characteristic, elongated morphology, as described and shown in photographs published previously (4). These cell lines have reproduced with a generation time of 16 to 20 hours, although cells cultivated for more than 6 months have sometimes tended to exhibit reduced mitotic rates. Chromosome determinations on all human cell lines have uniformly yielded the expected value of 2 n = 46, as described elsewhere (6). Two human cell strains, from skin samples of a male and female subject, respectively, and one arising from the ovary of a Chinese hamster, were selected for long term cultivation in the complete growth medium of Table I. These have now been carried for more than 9 months during which they have undergone a minimum of two generations per week, or a total of 78 generations, equivalent to 10^{23} progeny. Approximately 5 other human cells have been similarly established and maintained in high multiplication rates for 3 to 5 months. No alteration in chromosome number has been found in any of these cultures. A typical chromosomal complement from a human cell culture cultivated for more than 5 months is shown in Fig. 1. The incidence of polyploidy in these cultures is approximately 2 to 3 per cent, a value comparable to those observed for multiplying somatic cells *in vivo* (7). In contrast to this behavior, a culture of human lung cells grown in the presence of embryo extract by the procedure described earlier (4) was found after two months of cultivation to be highly aneuploid, with chromosome numbers ranging around 60 to 70.

Occasionally bottles in which these cells have grown for long periods have decreased their reproductive rate and in other ways given evidence of entering the refractory state previously described as a characteristic tendency of the fibroblast-like cells (4). Of the two long-term euploid human cultures, one was lost after approximately 10 months. The other is still growing after a year of continuous culture, but at present writing its growth rate is greatly decreased. Study of these non-multiplying episodes (4) which affect euploid cells is continuing.

It is our impression that this non-multiplying state of fibroblastic cells is a response to the presence of inhibitory substances occasionally present even in fetal serum, because cultures exhibiting a decreased growth rate have often been restored to active proliferation when a better batch of fetal calf serum was substituted in the growth medium. While the cultivation of these cells may not achieve absolute reliability until a defined medium like that now available for epithelial-like cells (8) is at hand, there is no question that the methodology described here permits more reliable results than any other of our experience.

In a limited number of trials bottles containing confluent monolayers of such cells have been placed in a deep freeze at −50°C., immediately after cooling to room temperature and addition of glycerol in a final concentration of 5 to 10 per cent to the supernatant medium. After a month of storage, such bottles have been thawed at room temperature and fresh medium replaced. On reincubation, an actively growing culture again resulted. Chromosome studies on such frozen cultures are in progress.

Cells from the Chinese hamster[2] and the American opossum[3] are of interest for genetic studies because of their low chromosome number of 2 n = 22. The procedure here described has successfully grown cells of both animals, those of the Chinese hamster being particularly hardy and reliable. Cultures from the lung, kidney, spleen, and ovary of this animal have grown excellently and that from the ovary has been in continuous cultivation for more than 10 months with no diminution in growth rate or change in cellular or colonial morphology. The chromosomal constitution of these cells has been determined and described elsewhere (6). No change in karyotype has been observed to date, 10 months after the original biopsy was transferred to growth *in vitro*.

Growth of Colonies from Single Cells.—Monodisperse suspensions of these euploid cultures can readily be plated so as to yield clonal colonies, by the procedure previously described (3 (*a*), 4), but modified by substitution of the appropriate media from Table I. The plating efficiency is usually lower than that achieved as routine with epithelial-like cells, and varies between 10 and 70 per cent, this lower yield probably reflecting the greater sensitivity of this cell type (4) to toxic factors or traumatic handling. As previously noted, greater care must be taken to insure that the culture is in optimal physiologic state at the time of plating, and exposure times to trypsin, and to pH or temperature different from optimal must be minimized. The colonies develop the typical, rough edges which characterize the clonal growth of fibroblast-like cells in other media (4). A typical plating is illustrated in Fig. 3. It is of interest that the elongated cells of the Chinese hamster tend particularly to pack tightly in par-

[2] The animal was supplied through the courtesy of Dr. George Yerganian, Children's Cancer Research Foundation, Boston.

[3] The animal was supplied through the courtesy of Dr. Matthew Block, Department of Medicine, University of Colorado Medical Center, Denver.

allel fashion, forming colonies shaped something like sheaves of grain. While the plating efficiency and growth rate of one of the euploid human skin cells has, after almost 10 months of cultivation, fallen to about one-tenth of its earlier value, those of the Chinese hamster have remained close to their maximal values, presumably a reflection of the greater resistance on the part of these cells to toxic conditions present even in occasional batches of the media here employed.

In the preceding report of this series the great difference in integrity of chromosome numbers in cultured cells from different animals was described (6). Thus in analysis of more than 2000 human mitoses no chromosome number other than 46 was found (except for a small per cent of polyploids). Similarly in analysis of 112 cells of the male opossum only the numbers 22 (or 44) were found. In contrast, cultures from various organs of the Chinese hamster, while exhibiting a stemline chromosome number of 22, also possessed a significant proportion of cells with 21 or 23 chromosomes. In study of the parameters

TABLE II

Distribution of Chromosome Numbers in Cultured Cells of the Chinese Hamster Ovary R2 Clone, Developed from a Population Which Had Survived 75 r of X-Irradiation

Counts were made on 87 mitoses. Only well-isolated mitotic figures were counted.

Chromosome No.	21	22	23	24	25	26
Per cent of cells	0	17	75	8	0	0

affecting chromosome constancy or change, in culture in the presence or absence of mutagenic agents, clonal isolations have been carried out from irradiated and unirradiated human and Chinese hamster cultures. While these experiments will be reported in detail later, it can be stated that clonal Chinese hamster lines have been produced with different stemline numbers. The stemline number 23 is represented in the distribution of chromosome numbers in such a clonal strain which was picked from the survivors of irradiation with 75 r and subsequently cultured for more than 15 generations as shown in Table II. Fig. 3 presents a photograph of a typical mitotic figure from this culture. Clonal stocks with such cytogenetic markers make possible a variety of genetic experiments.

In the previous paper of this series, the Y chromosome of man was identified (6) so that ready determination of the chromosomal sex of cell cultures like those here described became possible. Experiments are now in progress testing the chromosomal sex of patients with varying degrees of clinical hermaphroditism and comparing the results so obtained with the sex-chromatin procedure of Barr and his associates (9). It is of interest that while the chromosomal differentiation of male and female cells has continued to be unequivocal throughout months of active cultivation *in vitro*, cultured female cells (though

not the original buccal smears) have often given uncertain results with the sex-chromatin test of Barr.

DISCUSSION

Availability of techniques for large scale growth of euploid cells for fairly long periods has immediate application to a variety of studies. Biochemical and immunologic differences in single cell lines taken from different individuals may now be investigated with greater confidence about the meaningfulness of the results. Clonal lines can be isolated from cultures exposed to high energy radiation or other mutagenic agents, and examined for evidence of chromosomal or gene mutation. The frequency of such radiation-induced events can now be quantitated, and a preliminary survey of this kind has established that the mean dose needed to produce one chromosome break per cell, on the average, in euploid human cells is less than 50 r (10). It now also is possible to compare directly patterns and frequencies of radiation-induced cellular abnormalities when the radiation is administered directly to the animal or to the cells *in vitro*. Genetic mechanisms shown to be operative in other kinds of cell systems, such as direct sexual exchange of genetic components, virus transduction, DNA transformation, and mitotic crossing-over may now be conveniently searched for in euploid mammalian cells. Cells from normal and cancerous tissues from the same individual may be cultivated reliably *in vitro*, with greater hope of elucidating significant biochemical and genetic differences in their metabolism. Studies along some of these lines are now in progress in this laboratory.

The differences in the procedures here described over those previously employed by us and other workers involve only matters of detail. In this case, however, detail is all important. The mammalian euploid cell apparently can tolerate only very limited departures from certain optimal physical and molecular environmental conditions—a characteristic which sharply sets it off from microorganisms like *Escherichia coli* and *Neurospora*.

The fact that when the physical conditions of growth are carefully regulated and the nutritional medium carefully screened, to minimize growth inhibition, the usual pattern of chromosomal aneuploidy in tissue culture fails to develop, appears to be of fundamental significance. The obvious interpretation would involve minimization in our procedures of physical and chemical inhibitors which prevent completion of mitosis after doubling of the chromosomes. The resulting polyploid cells in subsequent mitoses are known to be capable of producing aneuploid progeny by nondisjunction or development of aberrent multipolar mitoses. Any such forms with more favorable growth potential could outgrow the euploid cells in the culture. The present data demonstrate the need for maintaining maximally favorable growth conditions of the euploid forms, to prevent both karyotype distortion and overgrowth of abnormal forms. Other considerations governing maintenance of genetic stability of animal cells in culture have been discussed elsewhere (6, 8). The single cell

screening procedure would appear to be the most important step of the technique here described for securing long-term cultivation of euploid cells. It becomes extremely important to define the parameters which influence the integrity of the genetic constitution of these cells *in vitro*.

All of the euploid cells here described have grown as elongated structures tending to align in parallel fashion to form colonies with rough edges. We have previously (4) described such behavior as "fibroblast-like" in contrast to the "epithelial-like" cells which form almost regular polygons which pack tightly on glass. We described isolation of stable clonal populations from the first harvest of cells cultivated from a normal human lung. Subsequent study has shown that the cells which grew stably as "epithelial-like" colonies were aneuploid. In our experience so far all cells with euploid karyotype adopt the elongated configuration when grown for sufficiently long periods in culture. The significance of this phenomenon, and the role of cell "stretching-factors" (3 *a*, 5 *a*) in affecting cell morphology will be discussed elsewhere.

The greater growth potential of mammalian serum from newborn, as opposed to adult, individuals has been recognized by previous workers (11). Among the possibilities which may account for the better performance of fetal over adult mammalian serum are its high concentration of fetuin (5) and its deficiency in γ-globulin content. Preliminary experiments which are continuing have demonstrated that factors toxic to cell growth *in vitro* can be demonstrated in the γ-globulin fraction of adult human serum.

Experiments are also in progress to define chemically the medium constituents which will support indefinite growth of single euploid cells *in vitro*, as has already been accomplished with the S3 HeLa clonal strain (8). It is probable that completely satisfactory control of the growth of such cells *in vitro* will not be achieved until this has been accomplished. The great sensitivity of these euploid, spindle-shaped cells to toxic factors present in some animal and human sera makes such cell lines particularly suited as a system for study of growth inhibitors and their distribution in the blood of individuals of different age groups and metabolic condition. Such studies are now in progress.

The ability to grow cells as routine from minute biopsies of almost any individual makes less remote the possibility that cell growth techniques from individual patients may find application as diagnostic tools in medicine. The present demonstration that enormous cell proliferation can be obtained *in vitro* without development of appreciable aneuploidy also increases the feasibility of the eventual establishment of banks of cells multiplying *in vitro* for use in therapeutic procedures.

SUMMARY

A methodology designed to eliminate mitotic inhibitor action and involving use of pretested fetal calf serum and careful pH and temperature control has been described by which cells from normal human and animal tissue can be

maintained in active growth for long periods *in vitro* without development of aneuploidy.

By means of this procedure, it is possible reliably to establish stable cell cultures from minute skin biopsies which can be taken from any individual.

Clones of mammalian cells with chromosomal markers have been isolated by this means from x-irradiated and non-irradiated cell cultures.

Applications of these techniques to chromosome delineation in large numbers of human subjects; determination of chromosomal sex in patients; spontaneous and induced genetic changes in somatic mammalian cells *in vivo* and *in vitro*; comparison of metabolic differences between normal and cancerous cells and other problems have been indicated.

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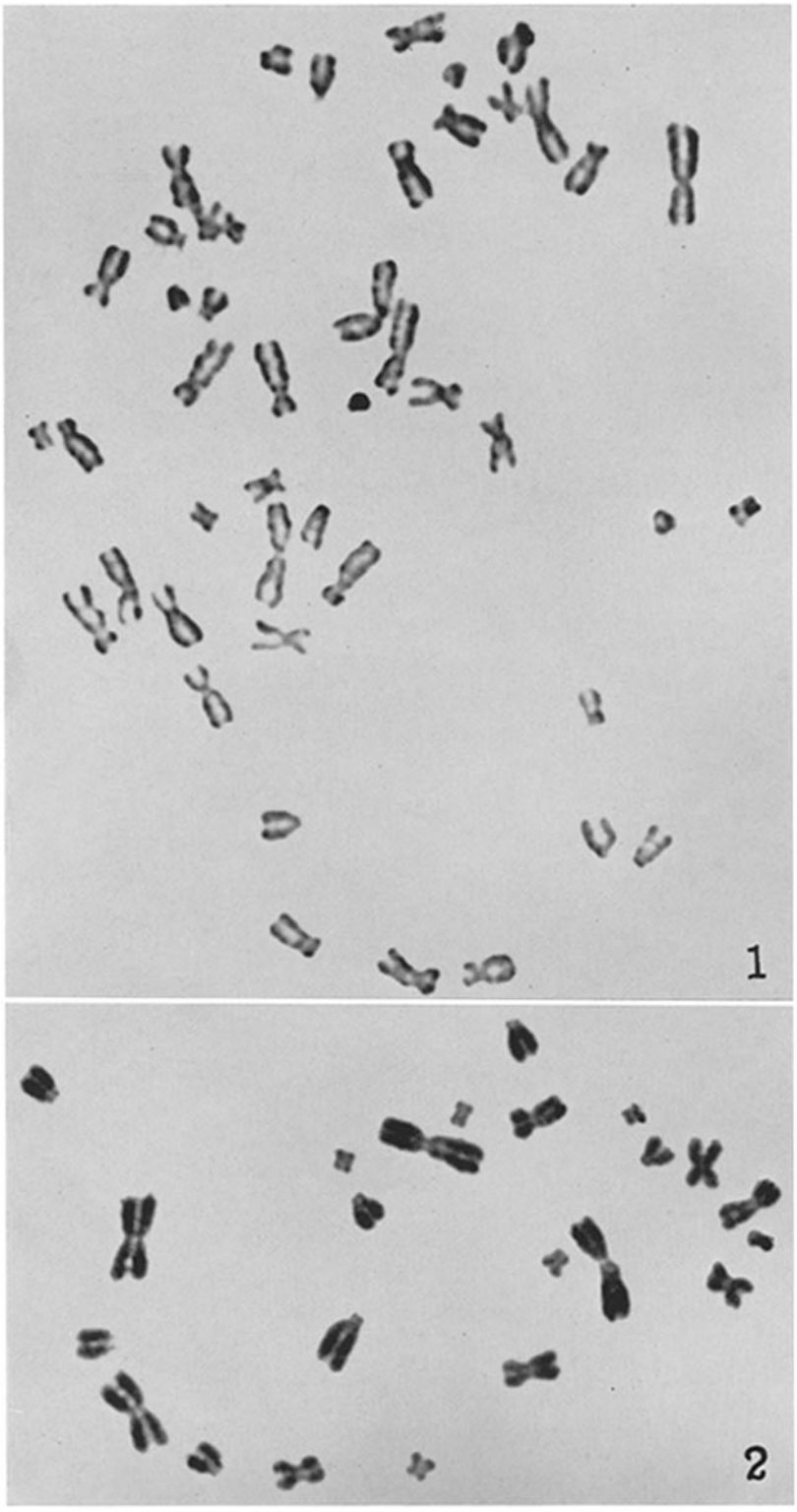
EXPLANATION OF PLATES

PLATE 63

FIG. 1. Typical set of 46 chromosomes obtained by the technique previously described (6) from human cells cultivated as here described. The particular culture from which this picture was taken arose from a skin biopsy on a 42 year old male, and had grown *in vitro* for approximately 5 months during which reproduction had taken place equivalent to more than 2^{40} progeny. Orcein stain. × 1800.

FIG. 2. Typical set of 23 chromosomes from cell of the R2 clone of Chinese hamster ovary. The original culture had a stemline of 22 chromosomes with a small proportion 21- and 23-chromosome karyotypes. Orcein stain. × 1800.



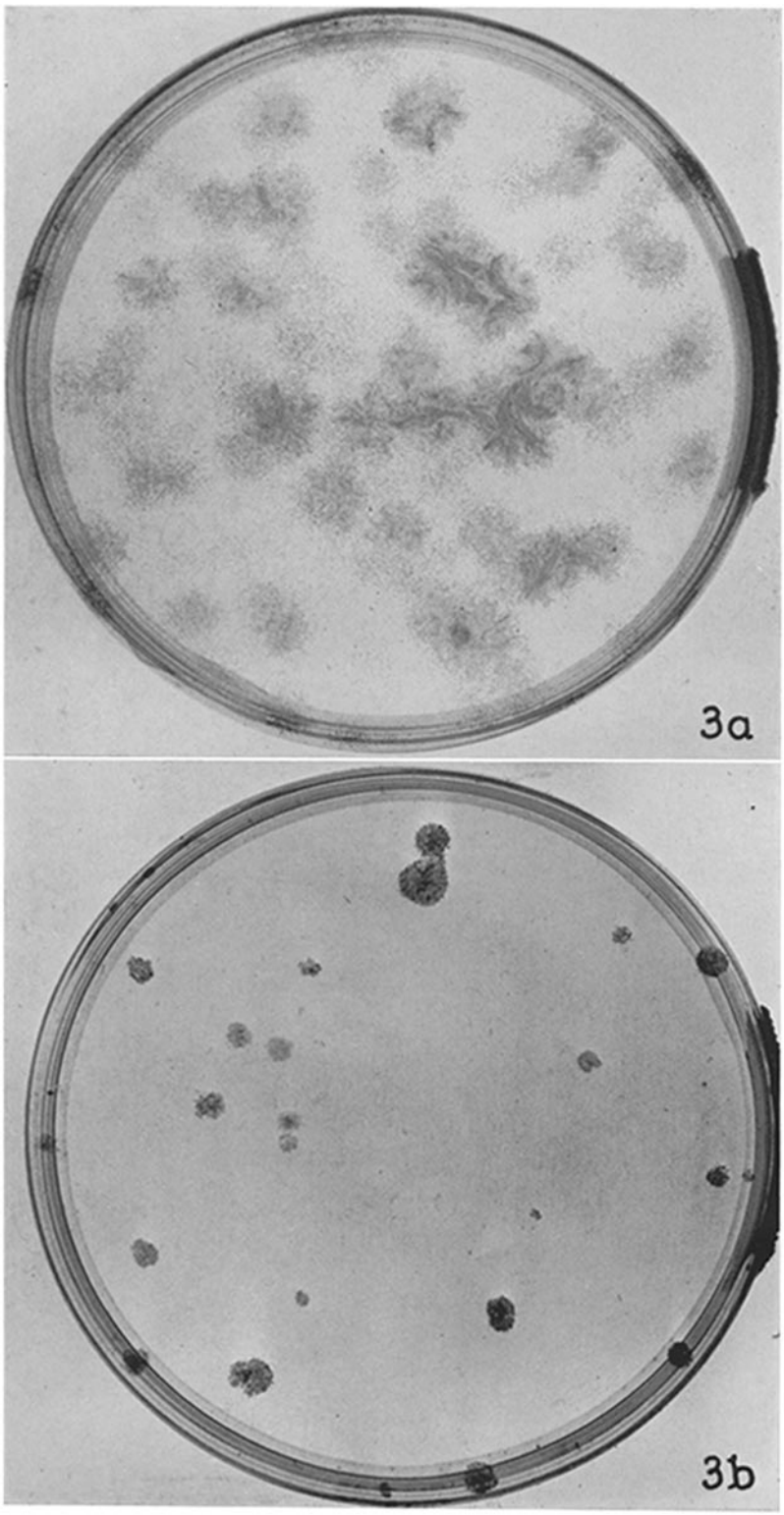
(Puck *et al.*: Genetics of somatic mammalian cells. III)

PLATE 64

FIG. 3. Representative single cell platings from the euploid fibroblast-like cultures here described.

FIG. 3 *a*. Colonies developing from cultured human male skin cells, whose chromosomes are shown in Fig. 1. The colonies were permitted to become sufficiently large to display the characteristic whorls formed by these elongated cells. × 1800.

FIG. 3 *b*. Colonies developing from single fibroblast-like cells of the Chinese hamster ovary. These cells grow in a more densely packed array than the human cells. × 1800.



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