Chromosomal Alteration with Low Temperature Preservation of Tissue Fragments for Cultures.* (31525)

MARY E. MCKEE, SANDRA E. HARRIS, AND HAYATO KIHARA
(Introduced by E. L. R. Stokstad)
Research Department, Pacific State Hospital, Pomona, Calif.

Previous observations in our laboratory have suggested that tissue fragments might be frozen directly and explanted for tissue culture at a later date. To evaluate these observations further, foreskin from newborn infants was frozen and checked for viability and for chromosome complement.

Methods. Foreskins from 3 newborn infants were washed in several changes of Hanks' balanced salt solution supplemented with antibiotics at twice the concentration present in the growth medium. They were freed of visible capillaries, and minced with scissors into pieces 1 to 2 mm in diameter. Some of the tissue fragments were removed for direct culture (control series) and the rest placed in culture medium containing 10% dimethylsulfoxide as a protective agent(1). The dimethylsulfoxide was not sterilized. The culture medium consisted of Medium 199 without bicarbonate, 79%; NCTC 109, 5%; L-glutamine, 0.01%; sodium bicarbonate, 0.22%; penicillin, 200 units/ml; streptomycin, 0.2 mg/ml; fetal calf serum, 15%.† One ml aliquots of the tissue suspension in medium with dimethylsulfoxide were transferred to 3 ml ampules, sealed, allowed to stabilize for about one hour at room temperature, and frozen in a mechanically refrigerated bath.‡ The bath, when filled with isopropyl alcohol and stirred mechanically, cooled at a rate of 1°C per minute. The ampules were cooled to −30°C and stored in a deep freeze§ at −90°C.

The frozen tissue was thawed rapidly in a 37°C water bath, transferred aseptically to a conical 12 ml tube, and centrifuged for 10 minutes at 800 × *g*. The supernatant fluid was discarded and cultures initiated from the tissue fragments.

Primary cultures were initiated by distributing 6 to 8 pieces of tissue fragments on the bottom of a plastic T-30 flask‖ and allowing them to adhere by standing at room temperature for 15 minutes. They were overlaid with medium and incubated at 37°C in an atmosphere of 5% CO_2 in air. After sufficient outgrowth was obtained (16 to 25 days), the cells were removed by trypsinization (10 to 15 minutes at 37°C with 0.025% trypsin in saline-citrate buffer) and subcultured in two T-30 flasks. Colchicine, 10^{-7} M in culture medium, was added to one flask on the third day and left for 15 to 16 hours. Slides were prepared for chromosome analysis by the methods described by Moorhead *et al*(2) and de Grouchy *et al*(3). The other flask was carried until a confluent monolayer formed and the subculturing procedure was repeated.

Results. Outgrowths were obtained from explanted control and frozen tissue fragments in all cases. The present work indicates that tissue fragments can be frozen for at least 15 months and remain "viable" in terms of growth.

An examination of the chromosomes of the metaphase plates revealed that the majority of the cells were diploid and did indeed have a normal chromosome complement (Table I). There was, however, a significant number of cells with less than 46 chromosomes, particularly in the control series. It is believed that this was due to a technical lapse that was overcome in subsequent preparations. There was also a significant number of cells with more than the normal complement of chromosomes, and the majority of polyploid cells observed were tetraploids. Moreover, about 30% of the latter appeared to be endoredupli-

* Supported by Research Grant 14-33 from California State Department of Mental Hygiene.

† Media components from Microbiological Associates, Inc., Bethesda, Md.

‡ Model LTBC-A, Thermovac Industries Corp, Copiague, L. I.

§ Model SZC-859, Revco Inc., Industrial Products Division, Deerfield, Mich.

‖ Falcon Plastics, Division of B-D Laboratories, Inc., Los Angeles, Calif.

TABLE I. Chromosome Counts of Cultured Cells from Control and Frozen Tissue.

Tissue	Subculture	Modal number 40–45	46	47–90	92	Total plates counted
Tissue A						
Control	1	17	83	1	1 (1)*	102
Frozen 3 mo	1	7	88	4	1	100
4 "	5	9	80	2	9 (7)	100
6 "	2	0	37	0	5	42
	3	5	92	0	3	100
	4	5	48	1	9 (1)	63
Tissue B						
Control	1	16	80	4	0	100
Frozen 3 mo	1	8	90	2	0	100
4 "	2	3	96	1	0	100
	5	2	95	1	2	100
6 "	2	3	77	1	19 (7)	100
	3	5	92	0	3 (1)	100
	4	8	75	1	16 (4)	100
Tissue C						
Control	1	17	80	2	2 (1)	101
Frozen 3 mo	1	3	97	0	0	100
4 "	2	6	93	0	1 (1)	100
	5	4	91	0	5 (1)	100
6 "	2	1	89	0	10 (1)	100
	3	5	92	2	1	100
	4	2	83	1	14 (5)	100

* Figures in parentheses are number of tetraploids which were endoreduplicated.

cated. Several cells (not included in the Table) were observed as octaploids. The frequencies noted in Table I are biased by the scoring of only those plates yielding unequivocal counts. Therefore, a larger number of cells were scanned under reduced magnification and visually judged for those which were diploids, tetraploids and endoreduplications. These results are given in Table II. The frequency of tetraploids was indeed higher than previously observed, and a significant number of cells were endoreduplicated. Although the results are not clear-cut, there appeared to be an increase in tetraploidy and endoreduplication with length of storage.

Discussion. Freezing and low temperature storage of tissue culture cells is a procedure commonly practiced in many laboratories (*cf.* reviews, 4,5). These techniques were originally assessed by various criteria depending on the particular interest of the investigator, and have included retention of cytological markers, antigenicity, viral susceptibility, tumorigenicity, growth rate, plating efficiency, dye exclusion, and per cent survivors. In general, recoveries were satisfactory; however, we are not aware of any study in which the retention of normal ploidy was used as a criterion. In one instance, the proportion of lactic dehydrogenase isozymes in frozen monkey kidney cells was altered after freezing and storage, but the change was not permanent(5). The progeny of the frozen cells displayed patterns like the original culture.¶ In a report which became available during the latter phase of this study, fetal tissue fragments, rather than cell suspensions (as in the earlier work cited above),

¶ LDH isozymes, which are tetramers of 1 and/or 2 subunits, dissociate and recombine randomly when solutions of the enzymes in 1 M NaCl are frozen and thawed(6). We believe that when monkey kidney cells were frozen in culture media(5) similar dissociation and recombination of the preformed LDH occurred. The ratios observed did not agree with the theoretical ratios calculated from the isozyme content of the control cells, but this is not a necessary deterrent because (a) culture media may not render complete dissociation like 1 M NaCl, and (b) *in situ* dissociation need not result in complete randomization during recombination. Therefore, even though there appeared to be difference in the apparent phenotypic expression, the genotype was not altered.

TABLE II. Percentages of Tetraploids and Endoreduplicated Cells Counted and Scanned Under Low Power Magnification.

Tissue	Subculture	Counted*		Scanned		
		Tetra†	Redup‡	Tetra†	Redup‡	Total
Tissue A						
Control	1	1.0	1.0	3.0	.2	1006
Frozen 3 mo	1	1.0	0	7.8	.9	1021
4 "	5	9.0	7.0	27.8	6.2	403
6 "	2	11.9	0	14.5	5.8	69
	3	3.0	0	10.0	.3	667
	4	14.3	1.6	15.3	3.6	732
15 "	1			5.9	.8	387
	2			4.4	0	482
	3			8.4	2.8	430
Tissue B						
Control	1	0	0	4.1	.1	1085
Frozen 3 mo	1	0	0	6.9	.5	1042
4 "	2	0	0	10.2	.3	957
	5	2.0	0	23.1	.9	216
6 "	2	19.0	7.0	28.9	4.8	166
	3	3.0	1.0	6.0	2.1	532
	4	16.0	4.0	11.5	3.0	775
15 "	1			3.9	.4	2221
	2			6.2	1.0	420
	3			12.3	2.3	351
Tissue C						
Control	1	2.0	1.0	2.6	0	1000
Frozen 3 mo	1	0	0	4.7	.4	1002
4 "	2	1.0	1.0	7.4	.3	1002
	5	5.0	1.0	13.8	.9	1007
6 "	2	10.0	1.0	15.4	4.6	680
	3	1.0	0	2.0	.3	1001
	4	14.0	5.0	21.1	5.8	171
15 "	1			4.1	.4	1426
	2			3.6	0	220
	3			3.7	0	1637

* Percentages calculated from Table I.
† Tetraploids including endoreduplicated.
‡ Endoreduplicated.

were frozen directly and after several days at —80°C were found to be capable of growing and to have retained a normal complement of chromosomes(7), but no counts were presented nor was storage extended, so we are unable to make comparisons with the present study.

Endoreduplication is a phenomenon which has been observed with increasing frequency in recent years (*cf.* ref. 8 for a summary of earlier work). Originally most such cases were from cells of individuals with chromosomal aberrations, leukemia, cancer, or from individuals or cells exposed to X-irradiation or chemotherapeutic agents. However, it has also been observed in cells from normal individuals. In the study by Schwarzacher and Schnedl(8), a fascia biopsy from a normal healthy individual was used to initiate duplicate cultures, which were then grown under identical conditions. One culture had a distribution of tetraploid and endoreduplicated chromosomes comparable to other normal cultures, while the other had unusually high frequencies of both. Unlike the tissue used in the present study, neither the tissue nor the cells were frozen at any time. It is conceivable that more endoreduplications have been observed in cells from pathological tissues because they have been scrutinized more closely than normal controls. It seemed worthwhile to scan under reduced magnification some slides which we had of chromosome preparations from skin cultures of a patient with Down's syndrome (trisomy 21) and a normal control. While no endoredupli-

cations were observed, the frequency of tetraploid cells was 10 times higher in the Down's syndrome (30% *vs.* 3%). The reason for this is not clear. It was believed that the increased tetraploidy and endoreduplication observed with cultures from the frozen tissue fragments might be attributable to exposure to dimethylsulfoxide and/or the low temperatures, but the observations of Schwarzacher and Schnedl(8), and our results with the Down's syndrome, where there was no such exposure, have made these possibilities seem less tenable. Notwithstanding, if normal ploidy is a consideration, the use of frozen tissue fragments for tissue culture is precluded.

Summary. Tissue fragments frozen and stored at —90°C for 15 months were capable of initiating growth in culture. Chromosomal analysis revealed that such cells had a high incidence of tetraploidy and endoreduplication.

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Received July 11, 1966. P.S.E.B.M., 1966, v123.

Boracéia Virus. A New Virus Related to Anopheles B Virus.* (31526)

OSCAR DE SOUZA LOPES, OSWALDO P. FORATTINI, ILEANA E. M. FONSECA, JOSÉ P. G. LACERDA, LIA A. SACCHETTA, AND E. X. RABELLO

Virus Section, Instituto Adolpho Lutz, São Paulo, Department of Parasitology, School of Hygiene and Public Health, University of São Paulo, and Department of Zoology, Department of Agriculture, São Paulo, Brazil

During the studies of the epidemiology of arbovirus made in Casa Grande region of the State of São Paulo, a virus was isolated from mosquitoes. This virus has been named Boracéia after a mountain located near the site of isolation.

Methods. The methods used for identification have been described(1). Tissue culture studies were done with the BHK-21 cell line. The growth medium was a modified Eagle's with 20% calf serum, and the maintenance medium included 2% calf serum. Neutralization testing in BHK-21 cells was done by a constant serum—varying virus technique. Serum virus mixtures were incubated for one hour at 37°C prior inoculation.

Isolation and characterization. The virus, SP Ar 395, was isolated from a pool of 95 *Anopheles (Kerteszia) cruzii* collected in the Boracéia field station on March 30, 1962. Of 12 baby mice inoculated with the mosquito suspension, one sickened with evidence of central nervous system disturbance on the seventh day, when a suspension of brain was passed intracerebrally (i.c.) to another group of 2-day-old mice. The remaining 11 mice were dead by the ninth day. By the second passage, the average survival time was 5.0 days after i.c. inoculation and 9.0 days after intraperitoneal (i.p.) inoculation. Nearly half of the adults inoculated i.c. became paralyzed and died; the rest recovered. Adults inoculated i.p. showed no signs of disease.

In BHK-21 cells SP Ar 395 showed a

* These studies were partially supported by USPHS research grant RF-1, by a grant from The Rockefeller Foundation and by the Fundo de Pasquisas do Instituto Adolfo Lutz.