Chemical Mutagenesis at the Phosphoribosyltransferase Locus in Cultured Human Lymphoblasts

(ethylmethane sulfonate/6-thioguanine resistance/Lesch-Nyhan syndrome)

KOKI SATO*, RONALD S. SLESINSKI†, AND JOHN W. LITTLEFIELD‡

Genetics Unit, Children's Service, Massachusetts General Hospital and Department of Pediatrics, Harvard Medical School, Boston, Mass. 02114

Communicated by Paul C. Zamecnik, March 1, 1972

ABSTRACT The presence of selectable genetic markers in long-term human lymphoblast cultures would facilitate cell hybridization experiments on the biosynthesis of immunoglobulins, as well as other studies. This work reports the induction with ethylmethane sulfonate of 6-thioguanine-resistant, phosphoribosyltransferase-deficient mutants in a lymphoblast line from a patient with infectious mononucleosis. These cells were unusually sensitive, with a D_0 value of 28 μ g of ethylmethane sulfonate per ml; the sensitivity curve followed a biphasic pattern suggesting the presence of 3% resistant cells. Ethylmethane sulfonate increased the frequency of mutants resistant to 6-thioguanine over 100-fold, to about 2×10^{-4} ; nitrosoguanidine was less effective. Almost all the mutants contained considerably less than 1% of the hypoxanthine-guanine phosphoribosyltransferase (EC 2.4.2.8) activity of wild-type cells. The mutation did not appear to result from loss of an X chromosome.

Almost all of the genetic markers presently available in diploid human cells in culture have been derived from patients with inherited metabolic disorders (1). This is because it has been difficult to induce mutants with chemical or physical agents in human fibroblasts, in contrast to heteroploid animal cell cultures (2, 3). Recently Albertini and DeMars have reported the induction with x-rays of mutations in human fibroblasts causing resistance to 8-azaguanine and deficiency in hypoxanthine-guanine phosphoribosyltransferase (EC 2.4.2.8) (4). Still the finite life-span of human fibroblasts (5) restricts their use for experiments in which repeated cloning, perhaps followed by extensive growth, is desirable.

Long-term cultures of human lymphoblast or lymphocytoid cells have considerable potential for genetic, immunological, and biochemical studies. They are readily available from patients with lymphoproliferative disorders as well as from healthy individuals (6-9), and have an apparently indefinite life-span (10). They grow rapidly in suspension to high cell densities, retain a diploid or near-diploid karyotype in most of the cells of the culture (11-17), and maintain the differentiated ability to synthesize and secrete immunoglobulins and other immunologically active factors (18). Most, if not all, of these lines contain the Epstein-Barr virus (14, 19-21).

Abbreviations: EMS, ethylmethane sulfonate; NG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; TG, 6-thioguanine; HAT, hypoxanthine-amethopterin-thymidine medium.

* Present address: Research Institute for Microbial Diseases, Osaka University, Osaka, Japan.

† Present address: Department of Biology, Kansas State University, Manhattan, Kans. 66502.

‡ Send reprint requests to Dr. Littlefield at the Massachusetts General Hospital.

Further investigation of biosynthetic control mechanisms in these differentiated human cells would be facilitated if mutant lines with selectable genetic markers were available, e.g., for studies with cell hybrids. Recently, lymphoblast lines lacking hypoxanthine-guanine phosphoribosyltransferase have been derived from a patient with the Lesch-Nyhan syndrome (22). But it would be useful to introduce this marker into other lines, and to produce other selectable markers as well. The present study reports the chemical induction of 6-thioguanine-resistant, phosphoribosyltransferase-deficient diploid and near-diploid mutants in a lymphoblast line derived from a patient with infectious mononucleosis.

MATERIALS AND METHODS

Cells. A culture of an established human lymphoblast cell line, PGLC 33H, was initiated by Dr. P. R. Glade from the peripheral blood of a female patient with infectious mononucleosis, and has been in continuous culture for about 5 years. Chromosome analyses of this cell line in our laboratory have shown a bimodal distribution with about equal numbers of cells with 46,XX and 47,XX,C+ karyotypes. The PGLC 33H cell line was cloned twice in succession in soft-agarose medium by one of us (R.S.), and a subline designated L33-6-1 with rapid doubling time (18-22 hr) was selected for further studies. Early chromosome analyses on L33-6-1 showed apparently normal 46,XX karyotype, but repeated analyses during these studies demonstrated regeneration of a 46,XX/47,XX,C+ mosaic population similar to the uncloned PGLC 33H line.

Cell Culture Maintenance. Lymphoblast cells were grown in static cultures in Roswell Park Memorial Institute (R.P.M.I.) Medium 1640 supplemented with 20% (v/v) fetal calf serum (Grand Island Biological Co.). Previously, one of us (R.S.) had shown that 1640 medium produced a somewhat faster growth rate and higher concentration of cells in the stationary phase than several other media, including Eagle's Medium, Waymouth's Medium, F10 medium, F12 medium, and R.P.M.I. 1634. Concentrations of fetal calf serum between 15 and 20% in 1640 medium gave optimum growth rates, whereas concentrations above and below this range resulted in decreased growth. Cultures were passaged weekly into fresh medium in 30-ml T-flasks (Falcon Plastics) by dilution of the culture to a minimum cell density of 10^5 cells/ml. Initial cell densities of 10^5 cells/ml and above permitted early growth at the maximum rate and higher final cell densities. No antibiotics were used in the culture medium. Periodic examinations for *Mycoplasma* contamination were performed in the

laboratory of Dr. L. Dienes at the Massachusetts General Hospital.

Mutagen Treatment. Exponentially growing cells were treated with various concentrations of ethylmethane sulfonate (EMS) (Eastman Organic Chemicals) and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NG) (Aldrich Chemical Co.) at 37° in 1640 medium. Cells were exposed to EMS for 24 hr or to NG for either 2 to 24 hr. Then the cells were centrifuged, resuspended in fresh medium, and either immediately plated in agarose medium, e.g., in experiments for determination of cell survival, or allowed to grow for expression of the mutation before exposure to selective medium. Survivors of the mutagen treatment were quantitated by plating between 2×10^2 and 2×10^6 cells per dish in agarose medium for each mutagen concentration. EMS and NG were stored frozen (−20°) at 5 mg/ml in phosphate-buffered saline (pH 7.2) and at 1 mg/ml in acetate buffer (pH 5.0), respectively, and small aliquots were thawed just before use.

Cell Plating in Agarose-Medium. Lymphoblast cells were plated in soft agarose (Marine Colloids) containing 1640 medium by a method modified from that described by Coffino and Scharff (23, 24). A confluent monolayer of fibroblast cells in a 60-mm petri dish was used as a basal "feeder" layer, and it was overlaid with 2.5 ml of a 0.24% agarose-medium "separating" layer. Then the lymphoblast cells contained in 2.0 ml of 0.22% agarose-medium were added on top of the separating layer. Subsequently they were fed with 0.5–1.0 ml of liquid 1640 medium at 24 hr after plating and then once a week. Small but distinct colonies were usually visible by 2 weeks after plating, but not counted until 3–4 weeks. Colonies were isolated by suction with a Pasteur pipette and dispersed into 1.0 ml of medium in a 30-mm petri dish. For reproducible results and good plating efficiencies, it is important that both gels be firm enough. Thus the 0.24% agarose-medium was prepared by adding 1.0 ml of sterile 2.4% agarose in 0.9% sodium chloride solution at 39° to 9.0 ml of 1640 medium at 39° with care to rinse all the agarose from the pipette, and the 0.22% agarose-medium was prepared in a similar fashion.

The plating efficiency of the L33-6-1 subline averaged 30%, much as did the uncloned PGLC 33H line and others of its sublines. Considerably lower values (2–3%) occurred in the absence of a feeder layer. Plating efficiency was proportional to the number of cells inoculated up to about 100 resultant colonies per plate; more colonies were difficult to count accurately.

Selections for 6-Thioguanine-Resistant Mutants and for Revertants. The forward selection of mutants resistant to 6-thioguanine (TG) was based on the inability of cells deficient in phosphoribosyltransferase to use this toxic purine analog, so that they survived in its presence (25). The selection for reverse mutants that had regained the enzyme was based on the inability of enzyme-deficient cells to grow in the presence of the folate antagonist amethopterin (Methotrexate, Lederle Labs.) at a concentration of 10^{-5} M, supplemented with 10^{-4} M hypoxanthine and 4×10^{-5} M thymidine (HAT medium) (25). Because L33-6-1 cells were unusually sensitive to growth inhibition by thymidine, 10^{-6} M deoxycytidine was added routinely to the HAT medium in all these studies (26). Forward selection was performed by plating from 10^4 – 10^7 cells in 2.0 ml of agarose-medium containing 5–10 μ g of TG/ml. In this case the monolayers of feeder cells were fibro-

blasts resistant to TG from a patient with the Lesch-Nyhan syndrome. For the selection of revertant cells in HAT medium, feeder fibroblasts from normal individuals were used.

Determination of Enzyme Activities. Phosphoribosyltransferase activities in control and mutant cells were assayed by a modification of the method described previously (27) because lymphoblasts, like fibroblasts, contain an active nucleotidase. Cells growing exponentially at a density of 4 – 6×10^6 cells/ml were harvested by centrifugation, rinsed twice with 0.9% saline solution, resuspended in 0.01 M Tris·HCl buffer (pH 7.4) at a concentration of 10^6 cells/0.1 ml, and stored frozen at −20°. Extracts were prepared immediately prior to enzyme assay by sonication for 2 min in a Raytheon model DF101 sonicator and clarification by centrifugation at about $6000 \times g$ for 10 min at 4°. The supernatant fluid was used for protein analysis (28), and for enzyme assay as follows: 0.01 ml extract, 0.01 ml of 5 mM 5-phosphorylribose-1-pyrophosphate (Sigma), and 0.03 ml of a mixture containing 1 volume of [14 C]hypoxanthine (Calatomic, 8.55 mCi/mM), 1 volume of 0.1 M MgCl₂, 4 volumes of 0.05 M phosphate buffer (pH 7.4), 2 volumes of 0.1 M Tris·HCl (pH 7.4), and 2 volumes of H₂O were incubated at 37° for 60 min. Heat-inactivated extract was substituted in the blank reaction mixture. After cooling, 0.01 ml of reaction mixture was spotted on prewashed polyethyleneimine thin-layer chromatographic plates (Polygram Cel 300 PEI, Brinkman Instruments) on top of marker inosine and inosinic acid. Plates were developed for 2 hr with H₂O. The inosine and inosinic acid spots were located, cut out, eluted with 0.5 ml of 0.5 M MgCl₂, diluted with 10 ml of scintillation fluid [toluene–Triton X-100 (3:1, v/v)], and counted at 85% efficiency in a Nuclear-Chicago Unilux II counter.

RESULTS

Sensitivity of Lymphoblasts to EMS and NG. The survival of lymphoblast cells after treatment with various concentrations of the mutagens, EMS and NG, is shown in Fig. 1, *a* and *b*.

FIG. 1. Survival of L33-6-1 human lymphoblasts after treatment with various concentrations of EMS for 24 hr (*a*) or NG for 2 hr (*b*) [see *Methods*]. For each point the range of 2–5 replicate dishes is plotted. 10^5 cells exposed to 0.05 or 0.1 μ g of NG per ml were plated per dish; fewer cells were plated for all the other points.

TABLE 1. Spontaneous and induced frequency of human lymphoblast mutants resistant to 6-thioguanine

Treatment	Concentration ($\mu\text{g/ml}$)	Total number of cells plated* ($\times 10^{-5}$)	Total number of resistant colonies recovered	Mutation frequency ($\times 10^5$)†
None		180.4	29	0.161
Ethylmethane sulfonate	25	32.2	37	1.15
	50	33.4	63	1.89
	100	12.4	106	8.55
	150	7.0	45	6.43
	200	44.0	772	17.5
Nitrosoguanidine	0.005	28.0	3	0.107
	0.01	26.0	11	0.423
	0.025	28.0	28	1.00
	0.05	34.2	102	2.98
	0.1	82.5	32	0.388
	0.5	48.0	89	1.86

* The sum of several experiments with 10^6 or less cells plated per 60-mm petri dish to permit good recovery of mutants (see Table 2).

† These values have not been corrected for plating efficiency, which may have been particularly depressed after NG (see text).

Only three points were obtained for NG, but repetition of these experiments gave entirely similar results. Although only data for 2 hr of exposure to NG are shown, the same pattern was obtained with exposure for 24 hr; these results are consistent with those of others (29, 30) that indicate that NG is unstable in tissue culture medium with loss of mutant induction and cytotoxic properties in 2–6 hr.

A biphasic survival curve was obtained for EMS (and perhaps for NG) (Fig. 1, *a* and *b*), suggesting the presence of two populations of cells with different sensitivity. The D_0 value (37% survival dose) for the initial portion of the curve in Fig. 1*a*, representing the major cell population, was 28 μg of EMS per ml. This D_0 value is similar to that for an SV40-transformed human fibroblast line (Littlefield, J. W., and Radner, B., unpublished) but lower than that for Chinese hamster cells (110 μg of EMS/ml) (31) and for untransformed human fibroblasts (Littlefield, J. W., and Radner, B., unpublished). Also a lymphoblast line (MG 57) originated here from a patient with infectious mononucleosis was not as highly sensitive as L33-6-1, nor could this line be mutated with EMS, although it contained very rare spontaneous TG-resistant mutants.

Extrapolation of the linear portion of the curve for the resistant population in Fig. 1*a* indicates that about 3% of the cells have increased resistance to EMS. The D_0 value for this minor component of the L33-6-1 culture is intermediate between that for the majority of L33-6-1 cells and the less sensitive cell types mentioned above.

Induction and Selection of 6-Thioguanine-Resistant Mutants. The sensitivity of L33-6-1 lymphoblast cells to TG was determined by plating 10^2 – 10^4 cells in agarose-medium containing various concentrations of TG. The plating efficiency was 34% in the absence of TG, 4% at 0.5 μg of TG/ml, and

0.02% at 1.0 μg of TG/ml; no colonies appeared at higher concentrations. A concentration of 5–10 μg of TG/ml was chosen for the selection of resistant mutants, and TG at this level was incorporated into both agarose layers and the liquid medium added weekly.

The frequency of resistant mutants obtained with and without mutagen treatment is summarized in Table 1. Spontaneous mutants occurred, but mutagen treatment resulted in increased numbers of colonies, according to the agent and dose used. EMS appeared to be more effective than NG; the maximum mutant frequency values show that EMS produced a 109-fold increase over the spontaneous frequency, while NG produced only a 19-fold increase. However, there may be an extended depression of plating efficiency following treatment with NG, as noted in baby hamster kidney cells (29). This might relate to chromosomal damage known to result from NG, or to the unusual sensitivity of L33-6-1 lymphoblasts to NG (Fig. 1*b*). Furthermore, mutant lymphoblast clones induced by NG were difficult to isolate because of continued slow growth, and none were characterized in detail as were the clones from EMS (see below).

Density Dependence of Mutant Recovery. Initial experiments suggested that mutant frequency varied with cell density, as is well recognized with hamster and human cells (3, 4, 29). Reconstruction experiments were performed to quantitate the recovery of a few mutant cells in the presence of many wild-type cells, and vice versa. Thus, 200 cells from a TG-resistant subline (T5-1) were plated with 10^6 – 10^7 wild-type L33-6-1 cells in 2.0 ml of agarose-medium containing TG; the reciprocal experiment involved plating 200 wild-type L33-6-1 cells with 10^6 – 10^7 TG-resistant cells in agarose containing HAT medium. As shown in Table 2, the recovery of TG-resistant cells was only slightly decreased by the simultaneous presence of up to 10^6 sensitive cells. However, 10^7 sensitive cells decreased the recovery of the resistant cells by depleting the medium or perhaps by crossfeeding 6-thioguanine. In the reciprocal experiment with HAT medium the presence of 10^6 or 10^7 TG-resistant cells reduced the recovery of wild-type cells by one-half and two-thirds, respectively. Thus, while up to 10^6 cells per dish can be used efficiently in the forward selection for TG-resistance, only 10^5 cells per dish or less seem advisable for the selection of revertants.

TABLE 2. Recovery of rare mutant (T5-1) or wild-type (L33-6-1) lymphoblasts selected in the presence of many wild-type or mutant cells

Selection	Number of cells plated per dish		Number of colonies recovered per dish*	Plating efficiency (%)
	T5-1	L33-6-1		
6-Thioguanine	200	0	68, 65	33.3
	200	10^6	68, 61	32.3
	200	10^6	51, 42	23.3
	200	10^7	6, 2	2.0
HAT medium	0	200	56, 61	29.3
	10^6	200	33, 28	15.3
	10^6	200	19, 17	9.0
	10^7	200	4, 5	2.3

* Values for duplicate dishes are given.

Characterization of Isolated Mutants. Mutants isolated in the above experiments with EMS were recloned in the presence of 5 μ g of TG/ml, and examined with respect to phosphoribosyltransferase activity, chromosome number, and frequency of revertants. As shown in Table 3, there was a marked reduction of phosphoribosyltransferase activity in these spontaneous or induced mutant clones. The residual activities were equal to or less than those in lymphoblasts (22) or fibroblasts (32) derived from boys with the Lesch-Nyhan syndrome; thus in the UM-10 line, which Dr. A. D. Bloom kindly sent us (22), the enzyme activity was 1.4% of that in wild-type lymphoblasts. Incidentally, possible contamination here of the L33-6-1 line with the UM-10 line was excluded since none of the TG-resistant mutants in Table 3 had the XY karyotype of UM-10. Both X chromosomes were retained by most of the cells in the clones listed in Table 3; only a small number of cells in a few of the clones lacked a C-group chromosome, which could be an X chromosome. Therefore, resistance to TG in lymphoblasts, as in fibroblasts (4), appears attributable usually to mutagenesis at the phosphoribosyltransferase locus rather than to selective loss of an X chromosome.

Preliminary cytogenetic studies indicated that six of the 16 mutants examined had predominantly a 46,XX karyotype. Five mutants were mixtures of cells with 46,XX and either 47,XX,C+ or 47,XX,F+ karyotypes, and two mutants had 47,XX,C+ karyotypes with various different extra C-group chromosomes. The other mutants karyotyped were mixtures of cells with additional C-, E-, or F-group chromosomes. Variation of chromosome number from diploidy to near-diploidy may be an inherent characteristic of this cell line, since the L33-6-1 clone was observed to change from 46,XX to a 46,XX/47,XX,C+ mosaic population like the PGLC 33H parental line. Similar chromosomal variations, mosaicism, and extra C-group chromosomes have been described in lymphoblast cells (11, 15, 16).

Amethopterin strongly inhibits dihydrofolate reductase (33), and cells lacking the phosphoribosyltransferase cannot use exogenous hypoxanthine to circumvent this block. Thus, in medium supplemented with hypoxanthine, thymidine, and deoxycytidine, T5-1 cells were markedly inhibited by an amethopterin concentration of 3×10^{-8} M (0.02% plating efficiency), and completely inhibited at higher concentrations, while the plating efficiency of wild-type cells was not affected at the high concentration of amethopterin (10^{-5} M) routinely used in HAT medium (Table 2).

Revertant cells that had regained the phosphoribosyltransferase were sought by selection of each of the TG-resistant lines listed in Table 3 with agarose containing HAT medium and deoxycytidine. No spontaneous revertants were found among at least 2.4×10^6 cells of each line, including a total of 1.5×10^7 cells for T5-1. Furthermore, no revertants were produced by treatment of T5-1 or T8-1 cultures with 100 μ g of EMS/ml (1.2×10^7 cells examined) or 0.02 μ g of NG/ml (6.6×10^6 cells examined).

DISCUSSION

Diploid and near-diploid human lymphoblast clones resistant to the purine analog, 6-thioguanine, and markedly deficient in hypoxanthine-guanine phosphoribosyltransferase have been isolated after mutagenesis with ethylmethane sulfonate. Deficiency of this enzyme also occurs in fibroblasts from boys

TABLE 3. Characteristics of 6-thioguanine-resistant lymphoblast mutants

Mutant	Mutagen	Concentration (μ g/ml)	6-Thio- guanine (μ g/ml)*	Enzyme activity (%)†	Chromosome number‡
T5-1	EMS	100	5	0.0	46
T8-1	EMS	100	5	1.2	46
T9-1	EMS	100	10	0.9	46
T10-1	EMS	150	10	0.2	46/47/48/49
T11-1	EMS	150	10	0.2	46/47
T13-1	EMS	150	10	0.5	46/47
T14-1	EMS	150	5	0.0	47
T21-1	EMS	150	10	0.0	48/49
T30-1	EMS	100	5	0.2	47
T37-1	EMS	100	10	0.5	46/47
T42-1	None	—	5	0.0	46
T01-1	None	—	5	0.1	46/47/48/49
T04-1	None	—	5	0.4	46/47
T05-1	None	—	5	0.1	46
T06-1	None	—	5	0.2	46
T07-1	None	—	5	0.4	46/47

* 6-Thioguanine concentration used for selection of resistant mutants.

† % of the hypoxanthine-guanine phosphoribosyltransferase activity of wild-type L33-6-1 cells, which averaged 232 nmol of substrate used per mg of protein per hr in three determinations.

‡ At least 20 cells were counted for chromosome number and seven cells were karyotyped for each mutant; where only one chromosome number is given, this occurred in the majority of cells examined.

with the Lesch-Nyhan syndrome (32) and in the 8-azaguanine-resistant clones that Albertini and DeMars have found in human fibroblast cultures exposed to x-rays (4). Although the phosphoribosyltransferase locus is on the X chromosome and the L33-6-1 lymphoblast line was derived from a female patient, mutants were obtained after a single exposure to the mutagen, presumably because only a single X chromosome is active in individual female cells (34). Loss of this active X chromosome was not found in these clones; and, by analogy to the Lesch-Nyhan syndrome, these mutations probably involved the structural gene for the phosphoribosyltransferase (35, 36).

It is interesting that TG-resistant mutants could not be produced with EMS in another lymphoblast line (MG-57), although rare spontaneous mutants were present; this line was also less sensitive to EMS than L33-6-1. About half its cells were 46,XY and half 45,X. Perhaps this sort of difference between lines somehow relates to differences in chromosome constitution. Alternatively, the relatively high frequency of spontaneous TG-resistant mutants in the L33-6-1 line, and the ease of mutagenesis, suggests some sort of instability in this culture of the phosphoribosyltransferase locus, the X chromosome, or all chromosomes, as in heteroploid lines of mouse and hamster cells.

The sensitivity of L33-6-1 to EMS (and perhaps that to NG) followed a biphasic cell survival pattern similar to that reported for Chinese hamster cells (30). In yeast cells biphasic sensitivity to x-ray treatment is due to the increased resistance of budding cells (37); perhaps lymphoblasts at a certain stage of the cell cycle are more resistant to mutagens. Alternatively, certain of the cells in the L33-6-1 population with unusual

karyotypes might be less sensitive to EMS, and repeated exposure to high concentrations of mutagen may provide a method to select these resistant cells for further study. Finally, the biphasic survival curve may relate to the state of the Epstein-Barr virus in individual cells.

It is currently uncertain how often long-term lymphoblast lines develop chromosome changes with near-diploidy and mosaicism (15); these may be more likely in lines derived from patients with Burkitt's lymphoma or infectious mononucleosis, and perhaps relate again to the presence of the Epstein-Barr virus. But despite uncertainties as to the role and effects of the virus, lymphoblasts have several advantages over fibroblasts for somatic cell genetic experiments, such as indefinite lifetime permitting repeated cloning and rapid growth in suspension providing large yields. They provide a new system with human cells for the investigation of mutagens and mutagenesis. The present studies on TG-resistant mutants suggest that it will not be difficult to introduce resistance to 6-thioguanine into at least some lymphoblast lines, and perhaps to introduce other drug-resistant or auxotrophic markers as well. Thus, during this work, Dr. R. Byrne of Johns Hopkins University told us of his similar studies on 8-azaguanine resistance in Burkitt's lymphoma cells exposed to ethylmethane sulfonate. Such marked lines will permit hybridization experiments designed to investigate, for example, the controls of immunoglobulin synthesis, especially including intriguing phenomena such as allelic exclusion (38) and marked deficiencies of IgA due to dominant inheritance (39) or associated with the 18q-syndrome (40).

We are grateful to Drs. P. R. Glade, A. D. Bloom, and G. E. Moore for providing us with lymphoblast lines, to Dr. R. Byrne for information about his experiments, to Mrs. A. Broekman for the karyotypes, and to Mrs. B. Radner for some of the enzyme analyses. This work was supported by research grants CA-04670 from the National Cancer Institute and P-526 from the American Cancer Society, plus a fellowship to R. S. from the Massachusetts Chapter of the Arthritis Foundation.

- Krooth, R. S. & Sell, E. K. (1970) *J. Cell. Physiol.* **76**, 311-330.
- Kao, F. T. & Puck, T. T. (1968) *Proc. Nat. Acad. Sci. USA* **60**, 1275-1281.
- Chu, E. H. Y. & Malling, H. V. (1968) *Proc. Nat. Acad. Sci. USA* **61**, 1306-1312.
- Albertini, R. & DeMars, R. (1970) *Science* **169**, 482-485.
- Hayflick, L. & Moorhead, P. S. (1961) *Exp. Cell Res.* **25**, 585-621.
- Moore, G. E., Gerner, R. E. & Franklin, H. A. (1967) *J. Amer. Med. Ass.* **199**, 519-524.
- Foley, G. E., Lazarus, H., Farber, S., Uzman, B. G., Boone, B. A. & McCarthy, R. E. (1965) *Cancer* **18**, 522-529.
- Glade, P. R., Kasel, J. A., Moses, H. L., Whang-Peng, J., Hoffman, P. F., Kammermeyer, J. K. & Chessin, L. N. (1968) *Nature* **217**, 564-565.
- Moore, G. E., Grace, J. T., Jr., Citron, P., Gerner, R. & Burns, A. (1966) *N.Y. State J. Med.* **66**, 2757-2764.
- Moore, G. E. & McLimans, W. F. (1968) *J. Theor. Biol.* **20**, 217-226.
- McCarthy, R. E., Junius, V., Farber, S., Lazarus, H. & Foley, G. E. (1965) *Expt. Cell Res.* **40**, 197-200.
- Moore, G. E., Ito, E., Ulrich, K. & Sandberg, A. A. (1966) *Cancer* **19**, 713-723.
- Huang, C. C. & Moore, G. E. (1969) *J. Nat. Cancer Inst.* **43**, 1119-1128.
- Zajac, B. A. & Kohn, G. (1970) *J. Nat. Cancer Inst.* **45**, 399-406.
- Macek, M., Seidel, E. H., Lewis, R. T., Brunschwig, J. P., Wimberly, I. & Benyesh-Melnick, M. (1971) *Cancer Res.* **31**, 308-321.
- Steel, C. M. (1971) *Nature*, **223**, 555-556.
- Huang, C. C., Minowada, J. & Horoszewicz, J. S. (1971) *Proc. Soc. Exp. Biol. Med.* **137**, 183-190.
- Glade, P. R. & Hirschhorn, K. (1970) *Amer. J. Pathol.* **60**, 483-493.
- Gerber, P., Whang-Peng, J. & Monroe, J. H. (1969) *Proc. Nat. Acad. Sci. USA* **63**, 740-747.
- Miller, M. H., Stitt, D. & Miller, G. (1970) *J. Virol.* **6**, 699-701.
- Nonoyama, M. & Pagano, J. S. (1971) *Nature New Biol.* **233**, 103-106.
- Choi, K. W. & Bloom, A. D. (1970) *Science* **179**, 89-90.
- Coffino, P., Laskov, R. & Scharff, M. D. (1970) *Science* **167**, 186-188.
- Pluznik, D. H. & Sachs, L. (1965) *J. Cell. Comp. Physiol.* **66**, 319-324.
- Littlefield, J. W. (1966) *Exp. Cell Res.* **41**, 190-196.
- Xeros, N. (1962) *Nature* **194**, 682-683.
- Littlefield, J. W. (1963) *Proc. Nat. Acad. Sci. USA* **50**, 568-576.
- Lowry, O. H., Rosenbrough, J. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265-275.
- Orkin, S. H. & Littlefield, J. W. (1971) *Exp. Cell Res.* **66**, 69-74.
- Barranco, S. C. & Humphrey, R. M. (1971) *Mutat. Res.* **11**, 421-429.
- Kao, F. T. & Puck, T. T. (1969) *J. Cell. Physiol.* **74**, 245-257.
- Fujimoto, W. Y. & Seegmiller, J. E. (1970) *Proc. Nat. Acad. Sci. USA* **65**, 577-584.
- Werkheiser, W. C. (1961) *J. Biol. Chem.* **236**, 888-893.
- Lyon, M. (1968) *Annu. Rev. Genet.* **2**, 31-52.
- Kelley, W. N. & Meade, J. C. (1971) *J. Biol. Chem.* **246**, 2953-2958.
- Rubin, C. S., Dancis, J., Yip, L. C., Nowinski, R. C. & Balis, M. E. (1971) *Proc. Nat. Acad. Sci. USA* **68**, 1461-1464.
- Beam, C. A., Mortimer, R. K., Wolfe, R. G. & Tobias, C. A. (1954) *Arch. Biochem. Biophys.* **49**, 110-122.
- Herzenberg, L. A., McDevitt, H. O. & Herzenberg, L. A. (1968) *Annu. Rev. Genet.* **2**, 209-244.
- Stocker, F., Ammann, P. & Rossi, E. (1968) *Arch. Dis. Childhood* **43**, 585-588.
- Feingold, M., Schwartz, R. S., Atkins, L., Anderson, R., Bartsocas, C. S., Page, D. L. & Littlefield, J. W. (1969) *Amer. J. Dis. Child.* **117**, 129-136.