PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 163, 406–410 (1980)

Uniform Production and Bulk Storage of P388 Murine Lymphoma Cells for Antitumor Assay (40787)[1]

FREDERICK KLEIN AND ROBERT T. RICKETTS

Frederick Cancer Research Center P.O. Box B Frederick, Maryland 21701

Mammalian cell cultures are being used extensively for the screening of potentially useful therapeutic agents in cancer chemotherapy. Foley and Epstein (1) discussed in depth the different methodologies in the propagation of various cell lines and their usefulness in assay systems for evaluating potential antitumor activity. Experience (1, 2) has shown that an assay system, to be effective, must incorporate cells that can be grown both *in vitro* and *in vivo* for comparative purposes as well as to maintain tumor specificity. The mouse lymphoma P388 cell line was chosen to meet these criteria.

The purpose of this report is to present a plan for an old but effective methodology for large volume production of suspended cell culture, and to discuss in detail some growth requirements of P388 cells, and, in particular, their high sensitivity to light. In addition, we report on the storage stability of bulk-frozen P388 cells tested for cell density, viability, passage ability, and assay capability over a period of 166 days.

Materials and methods. The murine P388 lymphoma cells used in these studies were obtained from Mr. B. Haase, University of Wisconsin. This cell line was originally isolated by Dawe and Potter (3) and chosen by Haase for the NCI contract screening program. Working seed stocks were prepared using dimethyl sulfoxide (DMSO) and double-strength horse serum (20%) for storage in liquid nitrogen. New stocks were initiated every month to insure good growth and uniformity of production.

For seed and production cultures, Fisher's medium for leukemic cells of mice was supplemented with 10% horse serum, 2 m*M* L-glutamine and 50 μg/ml gentamicin. The pH of the medium was adjusted to 7.0 ± 0.1. Horse serum was chosen for this study to maintain consistency with the procedures of outside laboratories involved in the screening process.

Seed and production cultures were grown both in shake bottles and production plastic (Corning No. 25140) or glass (Bellco No. 7730-38260) roller bottles. Seed cultures were kept in the growth phase to initiate production levels in roller bottles. A 2-day transfer protocol was used during scale-up to each successive increase in culture volume. After starter cultures were initiated, the inoculum was split using a 1:4 liquid to culture bottle ratio during scale-up until achieving production roller bottle level. The scale-up to production volumes is detailed in Fig. 1.

Cells to be utilized in the assay were obtained from production roller bottles by concentration in a Beckman centrifuge at 4°C at 1820*g* for 10 min. The cell pellet was resuspended to a concentration of 1×10^7 viable cells/ml by gentle pipetting and washing to avoid clumping and loss of cell viability.

Similar cell concentrates were used for bulk storage experimentation. From each of several lots, 10 samples consisting of 44 ml/100 ml glass serum bottle each of concentrated cells ranging in cell density from 17 to 25×10^6 viable cells/ml were resuspended in Fisher's medium supplemented with 20% horse serum, 2 m*M* L-glutamine, and 10 or 7.5% glycerol or DMSO. The concentrated cell samples were cooled and frozen in stages at 4 and −20°C for 1 hr each followed by prolonged storage at −80°C (Revco freezer) until used. At intervals single samples were withdrawn from stor-

---

[1] The U.S. Government's right to retain a nonexclusive royalty-free licence in and to the copyright covering this paper, for governmental purposes, is acknowledged.

0037-9727/80/030406-05$01.00/0

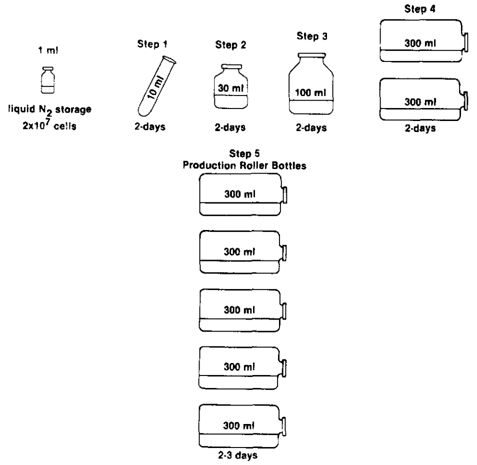

FIG. 1. Scale-up of P388 cells to production volume. Step 1, 1 ml of frozen inoculum was diluted in 9.0 ml of medium in roller tube (25 × 150 mm); Step 2, 10 ml quantities of inoculum from Step 1 were added to 20 ml of medium in a 100 ml serum bottle; Step 3, 30 ml of inoculum from Step 2 were added to 70 ml of medium in a 500 ml serum bottle; Step 4, transfer enough culture to give 4–8 × 10^5 viable cells/ml/roller bottle; Step 5 repeat as in Step 4, but increasing the number of rollers to production volume desired. All roller apparatus operated at 1 rpm, shakers at approximately 100 rpm. All cultures were maintained at 37°C.

age and fast thawed in a water bath at 37°C with agitation. Observations were made for (i) cell density; (ii) cell viability (determined by dye exclusion); (iii) passage potential through four serial passages in culture; and/or (iv) sensitivity to the antitumor agent tubercidin. Cytotoxicity assays were performed using the method of Perlman *et al.* (4) as modified by Garretson (to be published). The concentrations of tubercidin on 12.7-mm paper disks were 400, 200, 100, and 50 μg/ml. Zones of inhibition were measured using a millimeter rule.

Regression analysis (5) was used to examine the characteristics of cell growth after exposure to light and dark environments, and the relation between viable cell density and percentage cell viability and storage time. Appropriate statistical comparisons were based on Student's t tests using the $P < 0.05$ as the probability level for rejecting null hypotheses (6).

Results and discussion. Initially, readaptation of the cell line to suspension culture was required. Some difficulty was experienced in the propagation of the P388 cell line in regular spinner flasks and/or shaker type vessels. This difficulty was attributed to an imbalance between culture volume and vessel size resulting in improper dissolved oxygen partial pressure (pO_2). In our laboratory these growth difficulties were circumvented by the use of roller bottles which provide greater air space to culture volume ratios, thereby giving a suitable oxygen tension for the growth of these cells in suspension culture.

Developmental work on the roller bottle production system was accomplished in unlighted incubators. Production cultures were moved to a modern fluorescent lighted incubator where inconsistent growth patterns were observed. Light intensity of the surface of the roller bottles was observed to be approximately 50 fc. Numerous investigators have reported (7–10) that fluorescent light produces chromatid breaks in adult mouse cells, and that light-induced damage appears enhanced by increasing the concentration of oxygen in the gas phase (pO_2) of the culture. It appears that the chromatid damage induced by light is mediated through production of H_2O_2 in the culture medium or in the cells (7). Studies by Sanford *et al.* (11) on primary cultures of mouse cells indicate higher chromosome damage rates in horse serum-supplemented media than with fetal bovine serum supplements. If similar breaks are found in mouse malignant lines such as P-388, these could also be synergistic or even potentiating to light-induced damage. In an effort to test the effect of fluorescent light and varying pO_2, three experiments were conducted; (i) to determine the direct effect of light on cell growth, (ii) to test the effect of replenishing pO_2 by permitting enrichment daily when sampling of the roller bottles occurred, and (iii) to test the effect of pO_2 depletion by sampling roller bottles on test once, and then discarding.

In the first experiment cells were incubated in roller bottles in both dark and lighted environments. The results are shown in Fig. 2A. In the dark environment, cell growth was uninhibited, reaching den-

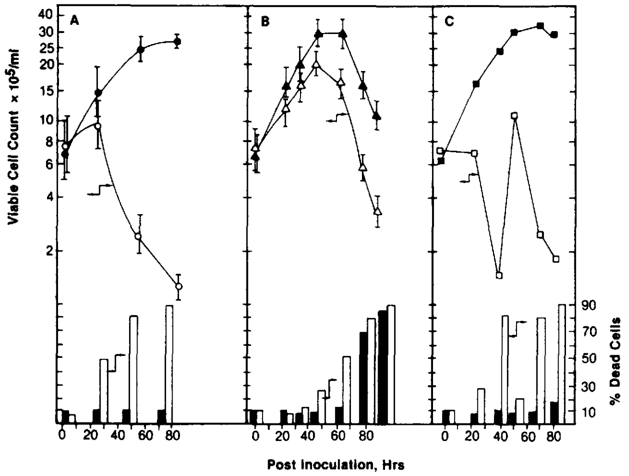

FIG. 2. Growth and viability of P388 mouse lymphoma cells relative to time post inoculum (three replications). (A) Cells incubated in darkness (●, ■) and in light (○, □). (B) Cells sampled twice daily incubated in darkness (▲, ▮) and in light (△, ▯). (C) Cells sampled once and terminated, incubated in darkness (■, ▮) and in light (□, ▯). I indicates ± 1 SE.

sity levels of 2.5 to 3.0 × 10^6 viable cells/ml with a doubling time of about 35 hr. Cell viability was maintained above the 90% level. In the lighted environment, the cells grew for 24 hr with a doubling time of about 80 hr and then entered log death phase. Cell viability deteriorated to less than 10% viable cells. This experiment clearly demonstrated that direct fluorescent light on the roller bottle surface affects cell growth.

In the second experiment (Fig. 2B), the pO_2 was altered in the growing cultures by opening the flasks to sample twice daily. In that manner, the pO_2 in the medium was replenished after each sampling in both the dark and lighted environments. In the dark environment, the cell growth continued through about 48 hr at the same relative cell density and viability levels as was observed in the first experiment. After 48 hr cell growth, the cultures appeared to enter the log death phase, and the viability dropped to less than 15%. In the lighted environment, the cell density and viability paralleled the growth pattern observed in the dark environment, however, the cell density in the lighted environment remained about one-half that of cells grown in the dark environment. Cell growth in light environment with direct pO_2 replenishment seemed to be enhanced up to 60 hr. After 60 hr, a negative effect on cell growth occurred that possibly was the result of a combination of pO_2 and light.

In the third experiment, a series of individual roller bottles was incubated in both dark and lighted environments. Samples were taken once from each bottle and that bottle discarded. The results are shown in Fig. 2C. Cell growth and viability in the dark environment were similar to that observed in the first experiment (Fig. 2A). Cell density levels were again at the level of 2.5 to 3.0 × 10^6 viable cells/ml. Cell cultures grown in the lighted environment were erratic with low density and viability levels.

Based on the above three experiments, it was evident that light on the surface of the roller bottle resulted in a marked deleterious effect on cell growth. After these test findings, P388 cell production was limited to the dark environment.

Typical cell production runs using this methodology of growing cells in suspension are shown in Fig. 3. The number of roller bottles per production batch, the total volume of cell culture produced, and the viable cell count per production batch are presented in Figs. 3A–C. An average of 2.5 × 10^6 viable cells/ml of cell culture was produced per production batch with greater than 90% viability. After each cell produc-

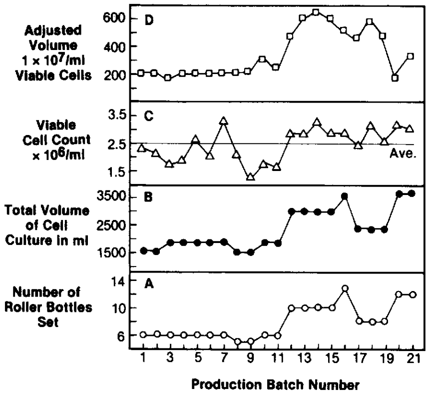

FIG. 3. Production parameters.

tion batch was obtained, the cell density was adjusted to a constant of 1.0 × 10^7 viable cells/ml as required for bioassay use. Production volume (Fig. 3D) varied with bioassay requirements, but subsequent to the first 21 production runs detailed in Fig. 3, output was stabilized at 3.5 liters of cell culture having densities up to 3.5 × 10^6 viable cells/ml providing at least 1 liter of 1 × 10^7 viable cells/ml cell concentrate made available twice weekly.

The stability of P388 cells stored at −80°C was tested and the results shown in Figs. 4, 5, and 6. Cells resuspended in 10% glycerol medium showed immediate toxic effects prior to freezing, and storage at −80°C caused rapid deterioration (data not shown). The results with cells resuspended in 7.5% DMSO and stored at −80°C for 166 days are shown in Fig. 4. No significant loss was observed in viable cell density or cell viability percentage during this time period as confirmed by regression analyses giving zero slopes (i.e., change in cell density or percentage viability relative to storage time).

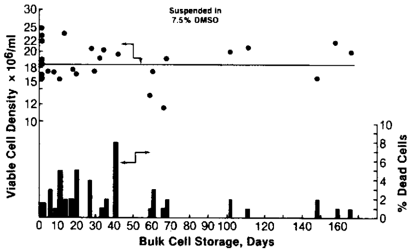

FIG. 4. Storage stability of P388 cells at −80°C.

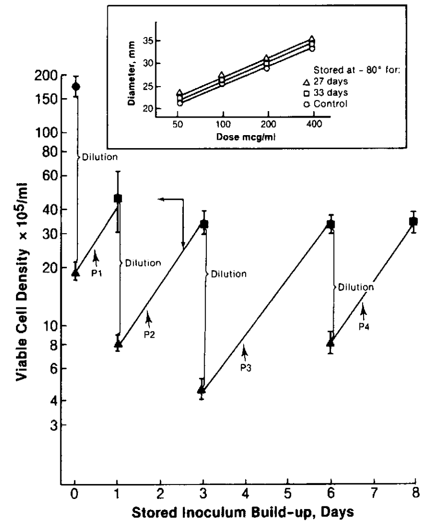

FIG. 5. (A) Postinoculum cell culture potential. (B) Assay testing of P_4 cells. I Range of cell densities of lots stored at −80°C for 4, 11, 27, and 33 days, respectively.

The ability of cells after bulk storage to replicate and pass through four serial passages—to facilitate rapid inoculum build-up—was tested, Fig. 5A. It was clearly indicated that P388 cells, resus-

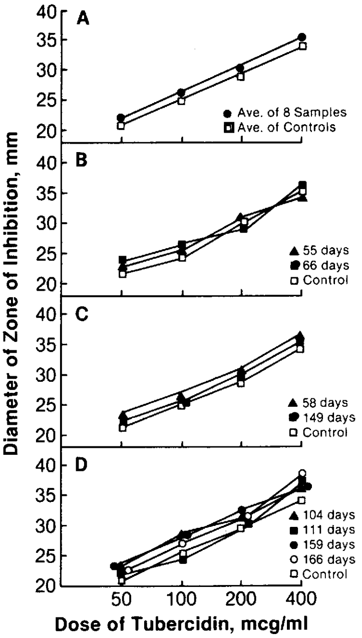

FIG. 6. Assay testing of stored cells after indicated days storage.

pended in 7.5% DMSO and stored at −80°C up to 30 days, could be used as seed stocks for rapid build up of cell cultures to production volumes. Assays conducted using the test agent tubercidin on cells on the fourth passage after storage for 27 or 33 days gave parallel results with cells from serial culture (control), Fig. 5B.

Another experiment demonstrated the feasibility of taking bulk stored cells from the −80°C freezer, thawing rapidly, and using for assay immediately after adjusting the cell concentrate to the proper density (Fig. 6). In Fig. 6A, a test of statistical significance indicated that cells stored up to 166 days were slightly more sensitive (i.e., the zone of inhibition was larger) than fresh cells. After proper dilution of the cells for assay, approximately 3.0% DMSO remained in the cell suspension, resulting in a final DMSO concentration of 1% in the agar. It is possible that this 1% DMSO was responsible for the increased sensitivity.

The ability to store bulk quantities of concentrated P388 cells represents a significant advantage in providing cells for immediate use in bioassay procedures without the unavoidable delays involved in the coordination of cell culture harvests with sample harvests of agents of potential interest.

Summary. P388 mouse lymphoma cells have been produced in quantity with little variability. Difficulties (i.e., O_2 deficiencies in spinner flasks and lighted environment for roller bottles) first encountered in the scale-up of a single vial to production volume quantities were overcome. Bulk lots of stored cells proved to be as effective as fresh cells for bioassay, and also provided a rapid method for obtaining production quantities of cells. This reproducible production capability and uniformity, coupled with the flexibility offered by bulk storage provides for a high degree of assay uniformity between and among groups involved in screening antitumor agents.

Research sponsored by National Cancer Institute (NCI) under contract NO1-CO-75380 with Litton Bionetics, Inc. We gratefully acknowledge I. A. De Armon for statistical analyses and support in experimental design, and L. M. Gilbert and P. M. Clark for technical assistance in the many laboratory tasks required. The helpful discussions with A. L. Garretson are also acknowledged.

---

1. Foley, G. E., and Epstein, S. S., Exp. Cell Res. **42**, 157 (1966).
2. Herzenberg, L. A., and Roosa, R. A., Exp. Cell Res. **21**, 430 (1960).
3. Dawe, C. J., and Potter, M., Amer. J. Pathol. **33**, 603 (1957).
4. Perlman, D., Lummis, W. L. and Geiersbach, H. J. J. Pharm. Sci. **58**, 633 (1969).
5. Dixon, W. J., and Brown, M. B., "BMDP-77, Biomedical Computer Programs," P-Series. Univ. of Calif. Press, Los Angeles (1977).
6. Snedecor, G. W., and Cochran, W. G., "Statistical Methods," 6th ed. Iowa State Univ. Press, Ames (1967).
7. Parshad, R., Sanford, K. K., Jones, G. M., Tarone, R. E., Proc. Nat. Acad. Sci. USA. **75**, 1830 (1978).
8. Parshad, R., Sanford, K. K., Jones, G. M., Price, F. M., Taylor, W. G., Exp. Cell Res. **104**, 199 (1977).
9. Bradley, M. O., and Sharkey, N. A. Nature (London) **266**, 724 (1977).
10. Wang, R. J., In Vitro **12**, 19 (1976).
11. Sanford, K. K., Parshad, R., Handleman, S. L., Price, F. M., Gantt, R. R., and Evans, V. J., In Vitro **15**, 488 (1979).

Received September 4, 1979. P.S.E.B.M. 1980, Vol. 163.