Evaluation of Methods for Reestablishment of L-Cell Suspension Cultures Directly from Liquid Nitrogen Stored Stocks

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Methods were developed and evaluated for the preservation of tissue cells grown in suspension culture and the reestablishment of suspension cultures directly from inoculum stored at -175°C. The factors investigated were processing pH, temperature of processing, freezing medium, and method of inoculation of the starter suspension cultures from the frozen stock (-175°C). Three parameters, cell viability, cell size, and growth potential in suspension culture after freezing, were used to evaluate the various factors. The results indicate that cells processed at 4°C, frozen at 1°C per min to -50°C in a medium containing 5% dimethyl sulfoxide plus 10% bovine serum at concentrations of 2 x 10⁷ to 4 x 10⁷ cells/ml, and stored at -175°C will reestablish suspension cultures directly from frozen seed. A 1-ml amount of frozen stock inoculated into 99 ml of medium routinely produced 2 x 10⁸ to 3 x 10⁹ viable cells/ml (2 x 10⁸ to 3 x 10⁹ total cells) in suspension culture in 4 to 5 days. Inoculum preserved by this procedure grew equally well in either serum-free or serum-containing growth medium.

Previous methods for the preservation of mammalian cells in liquid nitrogen required one or several monolayer or centrifugation steps, or both types of steps, to build up a suspension culture from inoculum stored in the frozen state (1, 5, 7, 9, 11; A. E. Greene et al., Cryobiology 3: 383-384). At the 1966 Annual Meeting of the Tissue Culture Association, Merchant, Walker, and Parker reported that, after repeated transfers through 1 to 2 years of growth, suspension cultures of the L-M cell line were genetically different from the monolayer culture.

To maintain genetic homogeneity of the cultures and follow stock culture procedures as outlined by Walker et al. (12), it was necessary to develop a method which would allow one to go directly from suspension culture to liquid nitrogen preservation and back to suspension culture without an intermediate monolayer step. A method for preservation of inoculum which fits the above specifications was developed and evaluated on the basis of cell count, viability, and growth potential in suspension. The method allows suspension cultures of mammalian cells to be started with inoculum directly from liquid nitrogen storage.

MATERIALS AND METHODS

Cell lines. Two variants of the mouse connective tissue L cell line (Earle; 3) were used; one, the L-M, was derived from NCTC clone 929 of Hsu and Merchant (4) and adapted to grow in suspension culture in serum-free 199 peptone medium, and the second, L-DR, was obtained from W. F. Daniels at Fort Detrick. The L-DR variant, used to test procedures developed with the L-M cell line, required bovine serum in the growth medium.

Media. The L-M cell line was propagated in medium 199 with Hanks salts and L-glutamine (Grand Island Biological Co., Grand Island, N.Y.). It also contained 0.5% peptone (Difco) and 0.12% Methocel (15 centipoise, USP grade, Dow Chemical Co., Midland, Mich.). This medium is referred to as 199P. The L-DR cell line was grown in Eagle's minimal essential medium (MEM) as modified by Daniels et al. (2). It contained MEM plus 10% bovine serum and 0.12% Methocel (15 centipoise); it was fortified with 0.25% lactalbumin in hydrolysate, 260 μg of cysteine per ml, and 50 μg of ascorbic acid per ml; and is referred to as modified MEM. The pH of each medium was adjusted to 7.2 with 6% sodium bicarbonate. Medium 199P was sterilized by autoclaving, and modified MEM was sterilized by filtration through a Seitz filter.
Experimental design. A simple factorial design was employed. The factors and levels of interest were the effect of (i) processing temperature, 25 °C versus 4 °C; (ii) pH, 7.0 versus 7.4; (iii) bovine serum, 0% versus 10% addition; and (iv) inoculation, direct versus step-wise. See Fig. 2 for the experimental design and results.

Preparation of L-M cell culture suspension. Cell growth was maintained in Merchant-type spinner flasks on 50/50 splits in 199P medium without antibiotics. A working volume of 3,600 ml of culture was produced by three serial builds that ended in 1-liter Merchant-type spinner flasks containing 600 ml of culture medium. Six such cultures were pooled to yield 3,600 ml of cell culture with an average count of 1.75 × 10⁴ cells/ml and 97% viability at pH 6.9.

The pooled suspension of cells was subdivided into 16 200-ml samples. Half of the samples were concentrated at room temperature (25 °C), and the other half were concentrated at 0 to 4 °C. Filter-sterilized dimethyl sulfoxide (DMSO), to a final concentration of 5%, was added to the supernatant medium. This was used to resuspend a portion of the 25 °C concentrated cells, and to another portion of the supernatant medium DMSO, to a final concentration of 5%, and bovine serum, to a final concentration of 10%, were added. The same treatments were performed with the 4 °C supernatant medium. Further subdivisions were made, and pH levels were adjusted in appropriate samples to either 7.0 or 7.4. Sedimented cells were resuspended with the aid of sterile glass beads to 10% of the original volume. Each sample (preparation) was distributed in 1-ml portions in 2-ml Wheaton glass ampoules, flame-sealed, control-rate frozen at 1 °C per min to −50 °C, and stored immediately in a liquid nitrogen refrigerator, modified with a dry well for vapor phase storage at −175 °C. Total processing time was 8 hr.

Cell enumeration. Cell counts were obtained directly in a hemacytometer by using the erythrocin B dye exclusion method (9) and with an electronic cell counter (Coulter Electronics, Hialeah, Fla.). Cell size distribution determinations were done with the Coulter cell-size analyzer and automatic plotter.

Inoculation of growth medium. Two methods of inoculation, direct and step-wise, were employed. In the direct method, 1 ml of frozen cells was rapidly thawed in a water bath at 37 °C with constant agitation and added to 99 ml of medium. In the step-wise method, the contents of one ampoule, after thawing, were added to 9 ml of medium, allowed to stand at room temperature for 10 min, and then added to 90 ml of medium in a 250-ml Erlenmeyer shaker flask. Cell and viability counts were made 1, 3, and 4 hr after inoculation and daily thereafter.

Growth potential in suspension culture. The shaker culture system described previously (13) was utilized to test the growth potential of L-M cells after the various processing procedures as outlined above. A growth medium of 199P containing 50 μg of penicillin and 50 μg of streptomycin per ml, adjusted to pH 7.0 with 6% sodium bicarbonate, was used. Each 250-ml Erlenmeyer flask contained 100 ml of medium and was incubated at 36 °C on a reciprocating shaker.

RESULTS

Cell viability. Losses of viable cells during prefreeze processing and after freezing plus 24 hr of storage at −175 °C are shown in Table 1. The viability of the pooled suspension culture before freezing was 97%, and the theoretical cell count was about 3 × 10⁷ cells/ml. Prefreeze processing includes all processing steps from unconcentrated cell culture to concentrated culture in flame-sealed glass ampoules immediately before controlled-rate freezing. Cells processed at 25 °C suffered marked losses in viability during processing. Slight protection during concentration was noted in preparations adjusted to pH 7.4; however, cell preparations processed at pH 7.4 suffered a marked loss in viable cell count during freezing in contrast to processing at pH 7.0, at which no further losses occurred. This loss was observed regardless of the presence or absence of 10% bovine serum. In contrast, cells processed at 4 °C suffered only about a 4% loss in viability during processing. Of the three main variables, temperature, serum, and pH, only temperature was statistically significant at the 99% level. Serum and temperature interacted at the 95% level, with serum giving added protection at 25 °C but not at 4 °C.

Cell size distribution. Comparisons of cell size distribution of the same preparations before and after freezing are shown in Fig. 1. A shift to smaller sizes occurred when cells were processed at 25 °C. There was an indication that serum maintained cell size; however, pH had no effect. With material processed at 4 °C, serum-free cell preparations showed a shift to larger sizes at both pH levels. Little or no change could be seen in the cell size distribution of cell preparations containing 10% bovine serum at either pH level. Cell size appeared to be a good measure of cell viability in this case, and a shift to smaller sizes indicated poor cell viability.

Growth potential in serum-free medium. To evaluate the growth potential more fully, two methods of inoculating the suspension cultures were tested: namely, direct (1 ml into 99 ml of medium) and step-wise (1 ml into 10 ml for 10 min and then 10 ml into 90 ml of medium). Inspection of the data plotted in Fig. 2 shows that only cells processed at 4 °C grew, whereas those processed at 25 °C either did not grow or grew very slightly through 3 days of incubation.

Statistical analysis of cell growth in media without serum showed a main effect caused by the temperature of processing (p =< 0.05), with growth being initiated at the 4 °C but not at the 25 °C processing temperatures, and an interaction between serum and the inoculation method signi-
Table 1. Effect of processing conditions on the viable cell counts and viability of L-M cells

| Serum | Process step | 25 C | 4 C |
|-------|--------------|------|-----|
|       | pH 7.0       | pH 7.4 | pH 7.0 | pH 7.4 |
|       | VCC/μl | Viability | VCC/μl | Viability | VCC/μl | Viability | VCC/μl | Viability |
| None  | Prefreeze  | 1.50 × 10^7 | 46.5 | 2.03 × 10^7 | 66.0 | 2.84 × 10^7 | 93.2 | 2.75 × 10^7 | 87.7 |
|       | Postfreeze | 1.48 × 10^7 | 44.6 | 1.14 × 10^7 | 35.2 | 2.59 × 10^7 | 77.2 | 2.87 × 10^7 | 80.7 |
| 10%   | Prefreeze  | 1.68 × 10^7 | 47.9 | 2.42 × 10^7 | 75.1 | 3.00 × 10^7 | 90.6 | 3.07 × 10^7 | 89.2 |
|       | Postfreeze | 1.47 × 10^7 | 45.0 | 1.49 × 10^7 | 42.0 | 2.85 × 10^7 | 78.6 | 2.89 × 10^7 | 81.2 |

* Viable cell count and viability of pooled suspension culture before processing were 1.63 × 10^6/μl and 94.1%, respectively. Theoretical total population was 3.3 × 10^7 VCC/μl.

b VCC, viable cell count. Each value is an average of eight values; two replicates, two technicians, and two assay methods (hemocytometer and Coulter counter).

c Viability was determined by the dye exclusion test (erythrocyn B). Each value is an average of four values (two replicates and two technicians).

Fig. 1. Cell size distribution of L-M cell preparation prepared for freezing and after freezing and thawing. Symbols: ○, prefreeze; ●, postfreeze.

Significant at the 95% level. Direct inoculation was better than step-wise inoculation when cell preparations contained no serum, but, when 10% bovine serum was present, the inoculation method did not differ significantly. All other primary effects and interactions were not significant.

Confirmation of the significant variables of the factional design. Three separate lots of L-DR cell
Suspensions (modified MEM) were processed by using the following conditions: freezing medium; modified MEM containing 10% serum and 5% DMSO (pH 7.0); and 4 C processing temperature. They were control-rate frozen at 1 C per min and stored at −175 C. The cell concentrations of the frozen cells were $1.9 \times 10^7$, $3.3 \times 10^7$, and $6.7 \times 10^7$ viable cells per ml per ampoule with 78, 89, and 83% viability, respectively. Suspension cultures were initiated from 1 ml of frozen, concentrated cell suspensions in 99 ml of modified MEM with 0.12% Methocel in a 250-ml Erlenmeyer flask (Fig. 3). When cell populations reached approximately $2 \times 10^6$ viable cells/ml (3 to 6 days, depending on the cell density at inoculation), the entire contents of the flask were transferred to 300 ml of medium (modified MEM) in a 1-liter Erlenmeyer flask. A cell population of $2 \times 10^6$ viable cells/ml in 400 ml of medium was attained 3 days after transfer. As shown in Fig. 3, all three lots reached the same final concentration, $10^9$ viable cells in total, but there was a 3-day difference in the time required to reach this concentration which depended on the initial inoculum level. In as little as 6 days without centrifugation, medium changes, or the use of antibiotics, we obtained 400 ml of cell suspension, at ca. $2 \times 10^6$ cells/ml with 95% viability, inoculated directly from liquid nitrogen storage (1 ml into 100 of medium transferred to 300 ml) without the usual intermediate monolayer step.

**Fig. 2.** Growth potential in suspension cultures of stored frozen L-M cell preparations. Symbols: ○, per cent viability; ●, viable cells per milliliter.
DISCUSSION

All three parameters (cell viability, cell size, and growth potential), used to test fractionally four variables at two levels, demonstrated that temperature of processing was the only significant primary variable; however, the presence of serum (10%) in the medium used for freezing and storing the cells and the method or inoculation of the growth medium (in a single rather than a two-step series) both interacted with temperature. The fourth variable tested, pH, did not cause a significant variance. Thus, temperature of processing was the most important variable to be controlled (10). Three possible reasons are (i) slowing of cellular metabolism such that toxic products of metabolism do not accumulate rapidly, (ii) the nutrients in the freezing medium are depleted rapidly by the high cell concentrations, or (iii) the toxic effects of DMSO may be less at 4°C than at 25°C (4), or all three. This latter possibility was supported both by data obtained before freezing and storage and by the one-step dilution method of inoculation (1 ml per 100 ml of medium), which was superior to the two-step dilution series. The reason could be that the faster DMSO is diluted after thawing the lower its toxicity. We feel that the results obtained are due to a combination of (i) and (iii).

It was of interest that cell size correlated very closely, in this case, with cell viability and growth potential. It appears that, as the cells get smaller than the norm during processing and freezing (for the cell line under study), the lower the cell viability and growth potential after freezing and storage. Optimal conditions appear to increase cell size slightly above the norm during processing before freezing.

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