Improved Chemically Defined Basal Medium (CMRL-1969) for Primary Monkey Kidney and Human Diploid Cells

G. M. HEALY, S. TELEKI, A. V. SEEZFRIED, M. J. WALTON, AND H. G. MACMORINE
Connaught Medical Research Laboratories, University of Toronto, Willowdale, Ontario, Canada

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An improved tissue culture basal medium, CMRL-1969, supplemented with serum, has been evaluated by measuring the growth responses of primary cultures of trypsin-dispersed monkey kidney cells (PMKC) and of an established culture of a human diploid cell strain (HDCS). Medium H597, an early modification of medium 199 which has been used successfully in the preparation of poliomyelitis vaccine for 15 years, was used for comparison. In addition, parallel testing was done with Basal Medium Eagle (BME) widely used for the growth of HDCS. The improvements in basal medium CMRL-1969 are attributed to changes in amino acid concentrations, in vitamin composition, and, in particular, to enhanced buffering capacity. The latter has been achieved by the use of free-base amino acids and by increasing the dibasic sodium phosphate. The new medium has already been used to advantage for the production of polioviruses in PMKC where equivalent titers were obtained from cultures initiated with 70% of the number of cells required with earlier media. The population-doubling time was reduced in this system. Also, with small inocula of HDCS, the time required to obtain maximum cell yield was shorter with CMRL-1969 than with BME. Both media were supplemented with 10% calf serum. Maximum cell yields after repeated subcultivation in the new basal medium were greatly increased and the stability of the strain, as shown by chromosomal analysis, was not affected. Basal medium CMRL-1969 can be prepared easily in liquid or powdered form.

The first tissue culture medium reported from these laboratories, no. 199, was developed for the growth of freshly explanted chick embryo cells (12), which were kept alive for about 33 days. Medium 199 was used in the development of poliomyelitis vaccine (6, 14), but it soon became evident that certain modifications of medium 199, mostly in the nature of deletions, would greatly improve the medium for virus production. Accordingly, a modified medium 199 was developed, medium H597, which was used for poliovirus production in these laboratories for many years. The modifications of medium 199 consisted of the omission of the purines (adenine, guanine, xanthine, and hypoxanthine), pyrimidines (thymine and uracil), adenosine triphosphate, adenyl acid, ribose, and deoxyribose; Hanks balanced salt solution (BSS; 7) replaced that of Earle (5). These changes were introduced when continuing studies demonstrated that certain purines and pyrimidines were toxic to cells (9) and that the pentose sugars were dispensable. The higher concentrations of calcium chloride and sodium bicarbonate in Earles balanced salt solution led to serious complications during the processing of poliovirus in medium 199. These difficulties were avoided when Hanks balanced salt solution was used.

Recently, medium CMRL-1415 was developed for the growth of primary cultures of mouse embryonic tissue (10). An important change in this medium was the introduction of a stronger buffering system attributed, in part, to the use of certain free-base amino acids and to a fourfold increase in the concentration of dibasic sodium phosphate. A serious drawback to CMRL-1415 for large-scale procedures is the inclusion of expensive coenzymes. But other advantages in its formulation stimulated a re-examination of all the ingredients of medium H597. The result was the development of a new basal medium CMRL-1969; observations on the growth of primary cultures of trypsin-dispersed monkey kidney cells

1 CMRL-1969 is a registered trademark pending.
TABLE 1. Composition of chemically defined media CMRL-1969\(^a\) and H597

| Component                  | CMRL-1969 | H597 |
|----------------------------|-----------|------|
| L-Alanine                  | 25        | 25   |
| L-Arginine (FB)\(^b\)      | 58        | 58   |
| L-Aspartic acid            | 30        | 30   |
| L-Cysteine-HCl             | 0.10      | 0.10 |
| L-Cystine                  | 20        | 20   |
| L-Glutamic acid·H\(_2\)O   | 67        | 67   |
| L-Glutamine                | 200       | 100  |
| Glycine                    | 50        |      |
| L-Histidine (FB)\(^b\)     | 16.2      | 16.2 |
| L-Hydroxyproline           | 10        |      |
| L-Isoleucine               | 20        | 20   |
| L-Leucine                  | 60        | 60   |
| L-Lysine                   | 70        | 70   |
| L-Methionine               | 15        | 15   |
| L-Phenylalanine            | 25        | 25   |
| L-Proline                  | 40        | 40   |
| L-Serine                   | 25        | 25   |
| L-Threonine                | 30        | 30   |
| L-Tryptophan               | 10        | 10   |
| L-Tyrosine                 | 40        | 40   |
| L-Valine                   | 25        | 25   |
| p-Aminobenzoic acid        | 0.05      | 0.05 |
| Ascorbic acid              | 0.05      | 0.05 |
| d-Biotin                   | 1.00      | 0.01 |
| Calcium pantothenate       | 1.00      | 0.01 |
| Cholesterol                | 0.20      |      |
| Choline chloride\(^c\)     | 1.00      | 0.50 |
| Ethanol                    | 21.0      |      |
| Folic acid                 | 1.00      | 0.01 |
| Glutathione                | 0.05      | 0.05 |
| l-Inositol                 | 2.00      | 0.05 |
| Menadione                  | 1.00      | 0.01 |
| Nicotinamide               | 1.00      | 0.025|
| Nicotinic acid             | 1.00      | 0.025|
| Pyridoxal·HCl              | 1.00      | 0.025|
| Pyridoxine·HCl             | 1.00      |      |
| Riboflavin                 |          |      |
| Riboflavin-5-phosphate     | 0.10      |      |
| Sodium acetate·3H\(_2\)O   | 81.5      |      |
| Thiamine·HCl               | 1.00      | 0.01 |
| Tween 80                   | 5.0       |      |
| Vitamin A acetate          | 1.00      | 0.10 |
| Vitamin D (calciferol)     | 0.10      |      |
| Vitamin E (α-tocopherone)  | 0.01      |      |
| d-Glucose                  | 1,000     | 1,000|
| Phenol red                 | 20        | 20   |
| NaCl                       | 8,000     | 8,000|
| KCl                        | 400       | 400  |
| CaCl\(_2\)                 | 140       | 140  |
| MgSO\(_4\)·7H\(_2\)O       | 200       | 200  |
| Na\(_2\)HPO\(_4\)          | 180       | 180  |
| Na\(_2\)SO\(_4\)·H\(_2\)O  | 70        |      |
| KH\(_2\)PO\(_4\)           | 60        |      |
| NaHCO\(_3\)\(^d\)         | 560       | 980  |
| Fe(NO\(_3\))\(_3\)·9H\(_2\)O| 0.1       |      |

\(^a\) Registered trademark pending.
\(^b\) FB = free base.

TABLE 2. Concentration of inorganic buffering salts in media used

| Medium          | NaH\(_2\)PO\(_4\) | Na\(_2\)HPO\(_4\) | NaHCO\(_3\) | pH before incubation |
|----------------|-------------------|-------------------|-------------|----------------------|
| H597           | 48                | 980               | 750         | 6.7                  |
| BME (Earle's salts) | 14                | 0                 | 750         | 7.1                  |
| CMRL-1969      | 70                | 180               | 560         | 7.2                  |

\(^a\) Amount: 60 mg/l KH\(_2\)PO\(_4\).
\(^b\) For outgrowth of HDCS, NaHCO\(_3\) was increased twofold.

and of an established human diploid cell strain, WI-38 (8), in this medium have been most favorable.

MATERIALS AND METHODS

Cultures: PMKC. Primary monkey kidney cells (PMKC) were obtained as follows. The kidneys from healthy cynomolgus monkeys were decapsulated and weighed. After washing with phosphate-buffered saline (PBS), the tissue was minced and digested by conventional procedures with 0.25% trypsin (1:300) dissolved in PBS. A 5% suspension of cells (based on the weight of tissue) was prepared from sedimented cells in tissue culture medium containing 5% unheated bovine serum.

Cultures: HDCS. Human diploid cell strain (HDCS) was prepared as follows. Strain WI-38, derived from human fetal lung, was received from L. Hayflick at the 11th passage. It was serially transferred by using a 2:1 split ratio in Basal Medium Eagle (BME) in Earles balanced salt solution (2) containing 10% unheated calf serum and was frozen at the 16th passage. The thawed culture was again subcultured at 2:1 or 4:1 ratios to passage number 25 and tested as indicated below.

Media. CMRL-1969 and H597 were prepared from concentrated stock solutions as described previously (10) or by reconstituting a homogenous, dry, powdered mixture of the ingredients (15) in distilled water. The complete formulations of these media are shown in Table 1. BME was purchased in the powdered form from Grand Island Biological Co. The media were supplemented with 2% bovine serum (Connaught Medical Research Laboratories) for growth of the PMKC or with 10% calf serum (Hyland Laboratories) for growth of the HDCS. As the superior buffering capacity of tissue culture medium CMRL-1969 is attributed largely to an increase in the concentration of dibasic sodium phosphate over that in Hanks BSS, the concentrations of the inorganic buffering salts in the three outgrowth media under discussion are shown in Table 2.

Preparation of cultures. With the PMKC, replicate cultures were prepared in 250-ml flasks (Falcon Plat-
tics, Division of B-D Laboratories, Inc., Los Angeles) containing 35 ml of test medium. To each flask, 2 ml of a 5% cell suspension were added and the flasks were incubated at 37 C. Cell counts on the inoculum were made in a hemocytometer by means of dye-exclusion techniques. Complete fluid changes were made twice weekly.

With the HDCS, subcultivations were made in 40-
ml Falcon flasks (25 cm²) containing 10 ml of medium and incubated at 37 C. The number of cells inoculated is shown below. In all cases the determination of cell yield was based on the average count of at least three flasks.

Growth determinations. The population-doubling time was obtained by counting portions of the entire population of trypsin-dispersed cells from individual replicate cultures. Such counts were made with a hemocytometer or Coulter cell counter at intervals during the logarithmic phase of growth. The actual population-doubling time was calculated from the formula \( N = N_0 e^{kt} \) where \( N \) = cell population at time \( t \), \( N_0 \) = cell population at time \( t_0 \), \( 1/k \) = population-
doubling time (13).

RESULTS

The growth rates of PMKC were compared in the three test media, H597, CMRL-1969, and BME, each supplemented with 2% bovine serum. The results of a typical experiment (Fig. 1) indicate that the cells multiplied more rapidly and reached a higher population density in CMRL-1969 than in the other two media. The population-doubling times were 19, 21, and 22 hr for CMRL-1969, H597, and BME, respectively. CMRL-1969 has been used successfully in these laboratories for large-scale preparation of poliovirus fluids for the past 10 months. Cultures have been initiated with approximately 70% of the cells previously used with H597 without altering the titers of infectious virus, complement-fixing titers, or the antigenic potency of vaccines made from the virus fluids. There is evidence that the first two of these parameters have been increased slightly. A change in the third parameter would not be detected by the test method used.

A comparison of the growth properties of HDCS in BME and CMRL-1969 was made by inoculating flasks containing BME with \( 4 \times 10^6 \) cells, incubating for 24 hr, and replacing the culture fluids with either BME or CMRL-1969 supplemented with 10% calf serum. Both media were prewarmed to 37 C before use. In our laboratories, this inoculum of cells is considered to be approximately equivalent to a split-ratio of 12:1. Incubation of the cultures was continued and the cell yields were determined (Table 3). It can be seen that maximum cell yields were reached 1 day earlier with CMRL-1969 than with BME. Visual examination of the cultures indicated that the cells entered logarithmic phase earlier with the CMRL-1969 and that mature cells were somewhat larger. A further appraisal of the suitability of CMRL-1969 for the cultivation of WI-38 was made by determining the cell yields after repeated subcultivation in the new basal medium and in BME. At each transfer the flasks were inoculated with \( 1.6 \times 10^6 \) cells and incubated for 3 or 4 days until approximately the 35th passage was reached. Table 4 shows that the average cell yield per flask was higher at every passage in the CMRL-1969 than in the BME. The difference in cumulative totals indicates that the number of bottles avail-

![Fig. 1. Growth of primary monkey kidney cells in CMRL-1969, H597, and BME, all supplemented with 2% bovine serum.](image)

| Incubation at 37 C (days) | No. of cells per flask a |
|-------------------------|----------------------------|
|                         | Basal Medium Eagle | CMRL-1969 |
| 0                       | 0.4                  | 0.4       |
| 1                       | 0.4                  | 0.4       |
| 3                       | 1.0                  | 1.1       |
| 6                       | 3.6                  | 5.6       |
| 7                       | 5.6                  | 5.6       |

a Values to be multiplied by \( 10^4 \).
TABLE 4. Cell yields per flask and total number of cells produced by repeated subcultivations (3- to 4-day cycles) of WI-38 cells in Basal Medium Eagle and CMRL-1969, supplemented with 10% calf serum

| Incubation at 37°C (days)* | No. of cellsb |          |          |
|---------------------------|--------------|----------|----------|
|                           | Basal Medium Eagle | CMRL-1969 |          |
|                           | Flask avg. | Cumulative total | Flask avg. | Cumulative total |
| 0                         | 1.6       | 1.6       | 1.6      | 1.6       |
| 3                         | 4.8       | 5.4       | 5.4      | 5.4       |
| 4                         | 10.5      | 17.6      |          |          |
| 3                         | 24.2      | 41.8      |          |          |
| 4                         | 60.9      | 130.6     |          |          |
| 3                         | 137.0     | 310.2     |          |          |
| 4                         | 248.0     | 697.9     |          |          |
| 3                         | 355.0     | 1,124.1   |          |          |
| 4                         | 355.0     | 1,826.8   |          |          |

* Total: 28 days.

† Values to be multiplied by 10⁶.

‡ Cell doublings = 7.8.

§ Cell doublings = 10.1.

able at each subcultivation can be markedly increased by the use of CMRL-1969. It would appear, moreover, that the life span of WI-38 is prolonged in CMRL-1969, for good cell yields were still occurring in this medium at the termination of the experiment; in BME, however, the cells had stopped multiplying.

Chromosomal analyses were carried out on approximately 200 metaphase cells at passage 30 from each set of cultures. The results (Table 5) are shown along with an estimate of the acceptable upper limits of chromosomal abnormalities in samples of 200 cells as recommended for "normal" human diploid material (11). It can be seen that the observed abnormalities in cells serially transferred in both media were not different from each other and were below the acceptable upper limits for this cell strain. Although only two typical experiments are cited, the superior growth-promoting properties of basal medium CMRL-1969 for HDCS have been observed repeatedly.

Control cultures of HDCS in CMRL-1969, without the addition of serum, when subcultivated at a 2:1 split ratio gave confluent growth and remained in excellent condition for as long as 3 weeks. In the same period, in serum-supplemented medium, cultures retracted and deteriorated. Routine testing for Mycoplasma failed to give evidence that either the culture or the serum was contaminated. These preliminary experiments on the maintenance of HDCS in serum-free medium are the subject of continuing studies.

DISCUSSION

In the development of basal medium CMRL-1969, sodium bicarbonate was retained as part of the buffer system for tissues cultivated in closed vessels. In the presence of 0.07 g of NaH₂PO₄ and 0.18 g of Na₂HPO₄ per liter, a suitable pH for the growth of PMKC was achieved by the addition of 0.56 g of NaHCO₃ per liter. For the HDCS in which a much higher concentration of serum was used, twice this amount of NaHCO₃ was necessary for optimal results. In addition, substitution of the hydrochlorides of L-arginine and L-histidine with their free bases augmented the buffering capacity of the medium.

The 19 natural (L-form) amino acids present in the free form in mammalian plasma and in hydrolysates of nutritionally adequate proteins, included in earlier media from this laboratory, were retained. The levels for the essential amino acids were based on values previously established in this laboratory and upon those of Eagle et al. (4). The concentration of L-glutamine was increased to 200 mg/liter, double that present in H597.

In view of the interchangeability of vitamins and coenzymes (2), CMRL-1969 was formulated with B-group vitamins instead of the more expensive coenzymes present in CMRL-1415. Their concentrations were increased from 2 to 100 times the levels in medium H597. The lipid soluble vitamins A, D, and E; menadione; the two lipid sources, cholesterol and Tween 80; and ethanol, present in medium H597, were omitted as were ferric nitrate and sodium acetate.

Regardless of the method used for counting

TABLE 5. Karyological analyses of 200 cells of HDCS serially propagated in either serum-supplemented BME or CMRL-1969

| Chromosomal abnormality | Acceptable upper limits per 200 cellsb | Cultivated from passage 25 to passage 30 in |
|------------------------|---------------------------------------|----------------------------------------|
|                        |                                       | BME | CMRL-1969 |
| Breaks                 | 11/200                                | 3/200 | 4/205   |
| Unstable structural abnormals | 3/200 | 0/200 | 0/205   |
| Stable structural abnormals | 3/200 | 0/200 | 0/205   |
| Hyperploidy            | 3/200                                | 0/200 | 0/205   |
| Polyploidy             | 9/200                                | 0/200 | 4/205   |

* Cells were taken from the 30th passage.

† Interpolated from the data in reference 11.
viable cells in a trypsin-dispersed suspension of freshly excised tissue, the number bears little relation to the number of cells found attached to and growing on a glass surface after 24 to 48 hr of incubation. With PMKC, the first figure is commonly 10 times the latter. In the experiments cited, when either BME or H597 was the outgrowth medium, the cells recovered from the flask surfaces were 15 to 16% of the number inoculated. The recovery rate was raised to 20% with basal medium CMRL-1969, and this increase in population density was maintained throughout the entire incubation period. There is, in addition, evidence that the rate of growth in the new medium was also somewhat greater, but the main difference appears to be that resulting from an increase in the number of cells able to attach to the glass and multiply.

Our experience with the WI-38 strain of human diploid cells indicates that greater cell yields can be obtained with basal medium CMRL-1969 than with BME. It has already been shown (1) that replacement of glucose with galactose in Basal Medium Eagle results in extensive growth in the postconfluent period. This result was attributed, in part, to a reduction in the formation of lactic acid. A similar effect has been achieved in the presence of glucose by the increased buffering capacity of CMRL-1969. Greatly increased yields of cells have been achieved.

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