Establishment of Syrian Hamster Fibroblast Culture in Albumin Fortified Medium (35166)

YUTAKA MATSUYA AND ISAO YAMANE

Microbiology Division, The Research Institute for Tuberculosis, Leprosy and Cancer, Tohoku University, Sendai, Miyagi, Japan

Mammalian cells can be carried in tissue culture for relatively short periods of time (anywhere from 5 to 100 cell generations) but a method for routine, long-term cultivation of cells has not yet been established. Hayflick and Moorhead (1, 2), Chang (3), Rothfels *et al.* (4), and Todaro and Green (5) have postulated that mammalian cells have a limited *in vitro* lifespan and cannot be carried as permanent cell lines without becoming heteroploid. Using enriched medium, Todaro and Green (6) have successfully grown both human and hamster cells for over 100 cell generations. We have attempted to duplicate this work using the lung cells of newborn hamsters to establish a serial culture method for these cells using the albumin supplemented medium described by Todaro and Green (6). Bovine plasma albumin fraction V was used to supplement the medium and was found to be as successful as crystalline serum albumin. Using this supplemented medium we were able to obtain a number of permanent cell lines from primary cultures of hamster cells (7).

Materials and Methods. Preparation of Syrian hamster cells. Cell samples were taken from 17- to 20-day-old hamster embryos, from 2- to 3-day-old hamsters and from random-bred adult hamsters. Primary cultures were prepared by the cell dispersion method. 0.25% trypsin (Nutritional Biochemical Corp., 1:300) or 0.1% pronase-P (Kaken Chemicals Co., Tokyo, Japan) in Hanks' solution was used to disperse the cells. The cells were collected after 15 min, centrifuged, and resuspended in the medium. Viable cells were counted and the known number was plated onto a series of petri dishes. The cultures were incubated in 5% CO_2 in air at 37° for 12 days without changing the medium. The cells were plated at 5×10^5 cells onto 60-mm petri dishes containing 5 ml of medium and transferred according to a constant transfer schedule. "A" cells were cultivated in square bottles and transferred every 3 to 4 days. The cultures were always reinoculated at the same cell density. In order to subculture the cells, 0.02% pronase plus 0.02% EDTA in Hanks' solution was used.

Media. The culture media supplemented with 10 to 20% bovine serum were prepared and designated as follows:

ST medium: Eagle's MEM plus 0.1% Bacto-peptone (Difco Labs, Detroit, Mich.).

BSA medium: ST medium plus 0.75% bovine albumin powder (Fraction V from bovine plasma, Armour Pharmaceutical Co., Kankakee, Ill.). The bulk medium was sterilized by passing through a pre-washed Seitz filter and adding 80 mg of kanamycin/liter.

Results. Use of pronase in dispersing cells. Pronase is an effective cell dispersing agent (8, 9). We have found it acts more rapidly than trypsin and is effective at lower concentrations. An experiment comparing the action of pronase and trypsin on cells in culture was performed as follows:

Cells released from the specimen at constant time intervals were harvested by centrifugation and the viable cells were counted in BSS containing 0.125% trypan blue. Parallel cultures were employed using both enzymes. After 2 hr, the released cells were counted. Four times as many cells were released with pronase than with trypsin. The released cells were immediately inoculated at 5×10^4 cells/plate and the growth rates of the cultures were compared. There was little difference in growth rate between the cells dispersed with pronase and those dispersed with trypsin (Fig. 1). With the use of BSA medium there

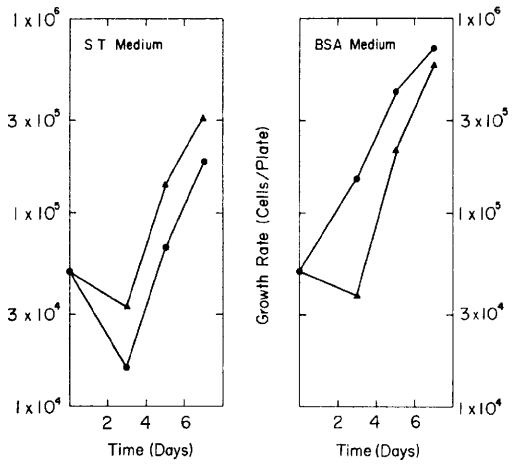

FIG. 1. Growth curves of lung cells of baby hamsters after digestion with pronase or trypsin: The specimen was digested with either 0.25% trypsin solution or 0.1% pronase solution for 0.5 hr and 5×10^4 cells were inoculated in ST medium and BSA medium, respectively. (●) pronase digested cells; (▲) trypsin digested cells.

was an initial lag phase in the growth of the pronase-treated cells which was not seen in the trypsinized cells. In spite of this, pronase was used in all further experiments to disperse and subculture the cells.

Use of albumin medium in primary cultures. Cells were obtained from lung, kidney, and liver of newborn hamsters, inoculated in either ST medium or BSA medium and incubated for 12 days in a CO_2 incubator. Although there was some difference in the number of cells that attached to the glass, depending on the tissue of origin, in every case the growth rate in BSA medium was superior to that in ST medium.

Cells cultivated in ST medium for 4 days and then transferred to BSA medium showed a considerable increase in growth rate after the medium was changed to BSA medium (Fig. 2). Therefore, the presence of the albumin does not appear to influence attachment of the cells to the glass. In addition, growth in BSA medium was enhanced regardless of whether pronase or trypsin were used as the cell dispersing agent (see Fig. 1).

Effect of the density of the inoculum in secondary cultures. Cells taken from hamster embryos or neonates, known to grow better in culture than those taken from adults, were used in the following experiments. Their growth rates in BSA medium were compared at various densities of the inoculum. Inocula of low density grew less well than those at the higher density, probably due to lack of cell–cell feeding. The relationship between density of the inoculum and the growth of cells from different organs is shown in Fig. 3. Lung cells grew better than cells of other organs at the low density of the inoculum, and embryo cells grew better than those from neonates. However, the growth potential in these sparse cultures could not be maintained and fell rapidly. The growth potential in dense cultures, in contrast, was maintained.

Serial transfer of cells of differing ages and tissues of origin. The long-term cultivation of lung cells from hamster embryos, neonates, and adults; liver cells from hamster neonates; and whole embryo cells was attempted. The density of the inoculum was kept at between 10^5 and 10^6 cells/plate. Parallel cultures were carried in both ST medi-

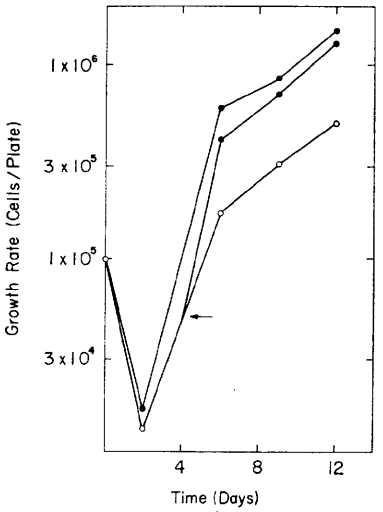

FIG. 2. Growth curves of lung cells of baby hamsters grown in ST medium and BSA medium: One hundred thousand cells digested for the primary culture, were inoculated in ST medium and BSA medium. Medium was changed every 4 days. The arrow indicates when ST medium was changed to BSA medium. After the cells had been carried in ST medium for 4 days, the cells were cultured in BSA medium. (○) ST medium; (●) BSA medium.

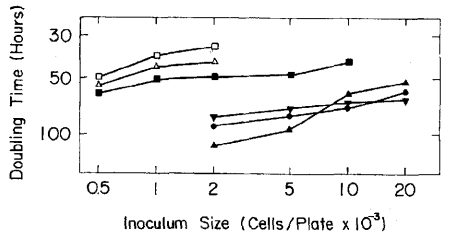

FIG. 3. Relation between the size of the inoculum and growth rate of the cells from different organs of the embryo and the neonate, in secondary culture: (△) whole embryo cells; (□) lung cells of embryo; (■) lung cells of neonate; (▲) kidney cells of neonate; (●) skin cells of neonate; (▼) liver cells of neonate.

um and BSA medium and transferred every 3 to 4 days without changing the medium during that interval. In addition, a culture of the liver cells was carried in the albumin medium supplemented with 0.01% egg albumin. Figure 4 illustrates the growth curves obtained.

Hayflick and Moorhead (1), Rothfels *et al.* (4), Todaro and Green (5), and Todaro *et al.* (10, 11) have previously shown that the lifespan of human, Syrian hamster, and mouse cells is limited when the usual culture conditions are employed. The growth rate of hamster cells begins to decline at the first transfer and is very low within 10 cell generations(10). We found this to be true with our hamster embryonic lung cell cultures on ST medium, however, when BSA medium was used, cells grew well at the 35th cell generation, at which time the experiment was terminated. The growth curve of a mixed hamster embryonic cell culture shows a linear increase in cell number during the initial 13 cell generations, a decline from the 13th to the 16th cell generation and another linear rise (Fig. 4). Cells from whole embryos were still growing well at the 30th generation, when the experiment was discontinued and the stock was frozen. In comparison, the cells grown on ST medium survived for only 5 cell generations.

Neonatal hamster lung cells designated "A," "B," "C," "D," and "E" were serially transferred on BSA medium. "D" cells were also grown in egg albumin-supplemented medium. The results obtained from a long-term study of these cultures shows that the cultures serially propagated for from 3 to 13 months and from 50 to 150 cell generations and that the cells were still growing well when the experiments were terminated after varied periods. Culture histories are summarized in Table I. Figure 5 illustrates the difference between growth of "A-1" cells on ST and BSA medium. The effective range of albumin concentration in the medium was found to be between 0.5 and 1.0%. Albumin at a concentration of 2% or greater markedly inhibits cell growth and at 0.2% does not enhance cell growth at all. The serum albumin allowed maintenance of hamster cells in a long-term culture, as shown in Fig. 6.

The same BSA medium could not maintain growth potential of adult hamster lung cells.

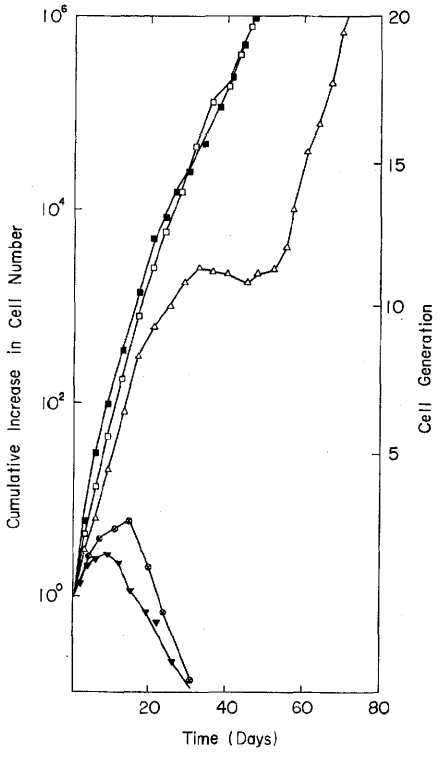

FIG. 4. Growth of hamster cells from animals of different ages and from different BSA medium was used. Beginning with the secondary culture: Each point represents one transfer: (△) whole embryo cells; (□) lung cells of embryo; (■) lung cells of neonate ("E" cells); (▼) liver cells of neonate; (⊗) lung cells of adult.

TABLE I. Culture History of Syrian Hamster Cells in Albumin-Fortified Medium.

Cultures	Culture vessel	Inoculum size ($\times 10^4$ cells/plate)	Time interval (days)	Cone of serum in medium (%)	Medium	Total no. of		Culture period (months)
						Cell generations	Transfers	
Lung cells of baby hamsters								
A	Bottle	50	3–4	20	BSA	150	110	13
B	Petri dish	50	3–4	20	BSA	76	70	9
C	Petri dish	50	3–4	20	BSA	60	50	6
D	Petri dish	20	3–4	5–10	BSA	100	40	5
					EA	78	40	5
E	Petri dish	3–20	3–4 7–8	3–10	BSA	50	25	3
Lung cells of hamster embryos	Petri dish	20	3–4	10	BSA	35	22	3
Whole embryo cells	Petri dish	20	3–4	10	BSA	30	26	3

After 6 transfers, the growth rate of the adult cells declined rapidly. Degenerative changes became apparent and it was impossible to continue serial tranfer of the cells (see Fig. 4). However, the lung cells obtained from the neonate could be maintained on BSA medium long enough to develop eventually into established lines. Liver cells of the neonate grown under the same conditions could not be maintained and growth stopped after several transfers. Although the addition of 0.01% egg albumin to the medium prolonged their growth somewhat they could not be developed into cell lines.

Discussion. With the development of effective cell culture techniques, many attempts have been made to develop established lines from diploid mammalian cells. However, although the cells grow initially, they could not be maintained. Occasionally a cell would undergo a chromosomal change from diploid to heteroploid and then an established line would be formed, with heteroploid characteristics but differing markedly from the cell of origin and from normal mammalian cells. Hayflick and Moorhead (1, 2) suggest that the finite lifetime of diploid mammalian cells *in vitro* may reflect aging or senescence at the cellular level. They suggest that this is an intrinsic property and is not related to the culture conditions employed. On the other hand, Todaro and Green (6) showed that diploid hamster and human fibroblasts could be serially cultivated for a prolonged time (at least for 100 cell generations) when grown on

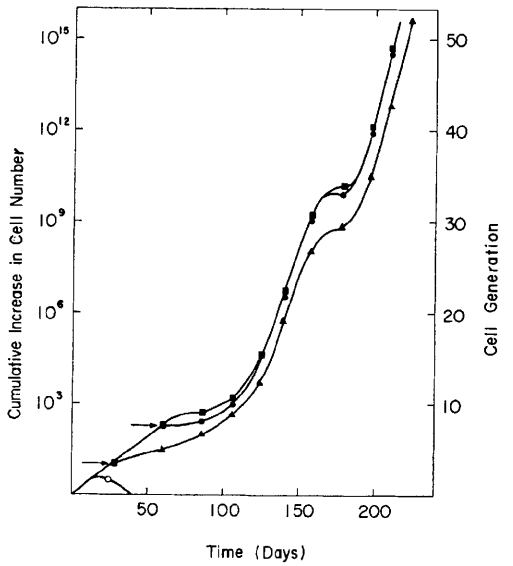

FIG. 5. Growth of "A" cells grown on medium containing different concentration of albumin: The left arrow indicates the beginning of the subculture from 0.75% BSA medium to 0.5% BSA medium and the right arrow shows the beginning of the subculture from 0.75% BSA medium to 1% BSA medium. Each point represents every 5th transfer: (○) ST medium; (●) 1% BSA supplemented ST medium; (■) 0.75% BSA supplemented ST medium; (▲) 0.5% BSA supplemented ST medium.

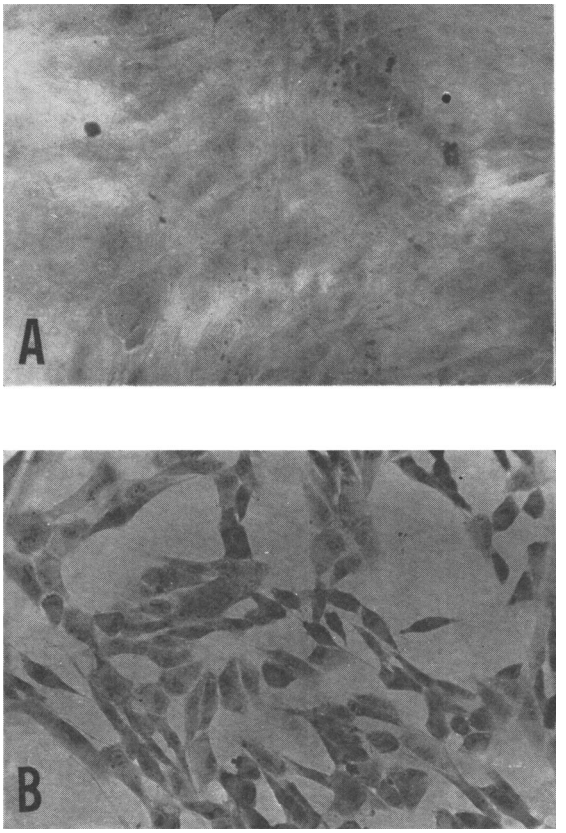

FIG. 6. Neonatal hamster lung cells from the primary cultures, maintained on either ST medium (A) or BSA medium (B) for a month without transfer; ×250.

an albumin supplemented medium. These cells continued to maintain a diploid karyotype.

It would be highly desirable to improve cell culture techniques to allow diploid cell strains to be maintained *in vitro* without alterations in their properties for prolonged periods of time. Previous studies with hamster embryo cells in this laboratory (12, 13) have shown that it is possible to produce hamster lines with a certain low probability (2 out of 10 cultures initiated). These result after about 3 months of declining growth rate.

The methods used here are similar to those previously used by Todaro and Green (6), however, the addition of crystalline albumin to the medium has been replaced with albumin fraction V at a concentration of 7.5 mg/ml. It has been shown that the immediate growth-promoting effect of albumin fraction V is followed by a substantial effect on the long-term cultivation of these cells. The experiments presented here show that nearly all cultures of lung cells of newborn hamsters eventually develop into established lines. These lines do not die out but begin a series of evolutionary changes expressed as various new properties, developing of chromosomal alterations, ability to grow at low inoculation density, ability to propagate continuously on ST medium and ability to produce tumors in animals. A long-term observation of these cultures reveals that diploid hamster cells can be propagated continuously on BSA medium for about 30 cell generations but the change in ploidy occurred between the 34th and the 45th generation at each of three independent culture series. These results have been published (7) and will be published (16).

While it is possible to produce established lines from mouse embryo cells (4, 5) and

Chinese hamster cells (14, 15) with a high degree of probability, Syrian hamster embryo cells (9, 11, 17) generally fail to develop into established lines. Under similar conditions the Syrian hamster cells can not be usually cultivated past 10 cell generations *in vitro*. With the addition of serum albumin fraction V, the hamster cells give rise to established cell lines with a high probability. It is apparent that the eventual fate of the hamster cells profoundly depends on the conditions of cell culture and especially the medium composition used. Some of the results are in agreement with Todaro and Green (6), although these authors could not produce established lines from the hamster embryo even after 100 cell generations. The growth activities of albumin may depend on the quantity of impurities that are present in the preparations. However, it is still not possible to decide on the albumin function whether it serves as an essential nutrient itself or whether it provides a dissociable element or an impurity which is responsible for the growth-promoting effect.

Summary. A successful routine method of developing cell lines from diploid hamster cells has been established. Bovine serum albumin fraction V was essential for serial long-term culture. Stimulation of growth was optimal at albumin concentrations between 0.5 and 1%. In the presence of albumin, hamster embryonic and neonatal lung cells and whole hamster embryonic cells developed into established lines.

The authors wish to thank Drs. G. J. Todaro (Viral Carcinogenesis Branch, National Cancer Institute, NIH, Bethesda, Maryland) and R. Pollack (Department of Pathology, New York University, School of Medicine, New York, N. Y.), for advice and for correcting the manuscript.

1. Hayflick, L., and Moorhead, P. S., Exp. Cell Res. **25**, 585 (1961).

2. Hayflick, L., Exp. Cell Res. **37**, 614 (1965).

3. Chang, R. S., J. Exp. Med. **113**, 405 (1961).

4. Rothfels, K. H., Kupelwieser, E. B., and Parker, R. C., *in* "Canadian Cancer Conference" (R. W. Begg, ed.), Vol. 5, p. 191. Academic Press, New York (1963).

5. Todaro, G. J., and Green, H., J. Cell Biol. **17**, 299 (1963).

6. Todaro, G. J., and Green, H., Proc. Soc. Exp. Biol. Med. **116**, 688 (1964).

7. Matsuya, Y., and Yamane, I., Exp. Cell Res. **50**, 652 (1968).

8. Wilson, B. W., and Lau, T. L., Proc. Soc. Exp. Biol. Med. **114**, 649 (1963).

9. Gwatkin, R. B. L., and Thomson, J. L., Nature (London) **201**, 1242 (1964).

10. Todardo, G. J., Nilausen, K., and Green, H., Cancer Res. **23**, 825 (1963).

11. Todaro, G. J., Wolman, S. R., and Green, H., J. Cell. Comp. Physiol. **62**, 257 (1963).

12. Tsuda, T., Tohoku J. Exp. Med. **86**, 380 (1965).

13. Yamane, I., and Tsuda, T., Tohoku J. Exp. Med. **88**, 171 (1966).

14. Yerganian, G., and Leonard, M. J., Science **133**, 1600 (1961).

15. Hsu, T. C., and Zenzes, M. T., J. Nat. Cancer Inst. **32**, 857 (1964).

16. Matsuya, Y., and Yamane, I., Tohoku J. Exp. Med. **101**, 35, 161, 251 (1970); **102**, 205 (1970).

17. Vogt, M., and Dulbecco, R., Proc. Nat. Acad. Sci. U.S.A. **49**, 171 (1963).

Received Apr. 7, 1970. P.S.E.B.M., 1970, Vol. 135.