THE GROWTH OF CARTILAGE CELLS IN SOFT AGAR AND LIQUID SUSPENSION

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A considerable body of literature indicates that on monolayer culture, embryonic chick cartilage cells are obtained which morphologically and biochemically behave as chondrocytes (1). Earlier studies suggested that repeated monolayer culture results in "dedifferentiation" of chondrocytes to give fibroblast-like cells. Coon (2) has demonstrated that with appropriate media, in low density cultures, it is possible to obtain stable clones of polygonal cells which form metachromatic matrix. More recently, Bryan (3) has shown that the development of fibroblast-like

cells in cartilage cultures is probably due to overgrowth of fibroblasts from the original culture.

Since the enzymic steps involved in chondromucoprotein synthesis are now defined (4), we have attempted to develop methods of culture adequate for obtaining pure strains of cells for biochemical study.

Macpherson and Montagnier (5) have shown that in contrast to normal fibroblasts, polyoma-transformed BHK 21/3 cells which do not show contact inhibition grow in soft agar. Ichikawa, Pluznick, and Sachs (6) have demonstrated that cells taken from spleens of male SWR mice or from livers of embryos of an inbred strain of Swiss mice form macrophage and granulocyte colonies when grown in soft agar with feeder layers or conditioned medium from various types of cells. Because chondrocytes in monolayer cultures form multilayered nodules, an attempt was made to grow cartilage in soft agar.

METHODS

Epiphyses of tibias and femurs of 13-day-old chick embryos were dissected under sterile conditions and incubated for 1 hr in 1% trypsin in calcium-free phosphate-buffered saline. The mixture was first centrifuged at 50 *g* for 60 sec to remove large particles. The cells in the supernatant fluid were then sedimented at 100 *g* for 10 min and washed three times in medium with fetal calf serum. After resuspension, clumps were removed by centrifugation for 30 sec at 100 *g*. Under these conditions uniform suspensions of single cells were obtained. In order to obtain clones, dilutions of cells (10^3 cells per dish) were cultured on 60 mm Falcon tissue culture dishes (Falcon Plastics, Los Angeles, Calif.) in modified Eagle's medium containing a fourfold final concentration of vitamins and amino acids and 10% fetal calf serum. Cultures were not disturbed for 12 days. For growth in soft agar the technique of Macpherson and Montagnier (5) was used as described by Ichikawa, Pluznick, and Sachs (6). Difco bacto-agar (Difco Laboratories, Detroit, Mich.) and Difco tryptose phosphate was used with no purification. Fetal calf serum and F-12 powdered medium were obtained from Grand Island Biological Company, Grand Island, N.Y. Eagle's basic minimal medium with four times concentrated amino acids was obtained from Hyland Laboratories, Los Angeles, Calif., or prepared from constituents. Growth in agar occurred, using a number of different lots of fetal calf serum. Agar plates were fixed with 40% formalin for 3 min at room temperature for staining. After rinsing with water, 0.5% toluidine blue in 25% acetone was added for 1 min and the plates were rinsed several times with distilled water.

RESULTS AND DISCUSSION

Cartilage cells grown in plastic culture dishes show epitheloid cells and piled up nodules as demonstrated in Fig. 1. When cells obtained from epiphyses were placed in soft agar, within a few days cell division occurred. Additionally, matrix formation around individual cells was observed by phase microscopy and by metachromatic staining with toluidine blue. On further incubation, colonies increased in size until, after 3 wk more than 1000 cells were visible. Fig. 2 illustrates a chondrocyte colony in agar. Colonies of chondrocytes were also obtained when agarose was substituted for agar. No detailed studies of growth in agarose were undertaken.

In order to determine the specificity of the method, the growths in agar of several types of cells were compared. Two morphological types of cells were isolated from monolayer cultures of embryonic cartilage. One type of cell was polygonal with marked piling up and production of metachromatic matrix, while the other type was typically fibroblastic as previously reported (2, 3). Additionally, fibroblast cultures obtained from chick embryo skin, human skin and polyoma-transformed cells were studied. Representative clones which demonstrated polygonal cells showed a high level of $^{35}SO_4$ uptake as measured by chondroitin sulfate isolation after digestion with papain and precipitation with cetyl pyridinium chloride (7).

Table I summarizes the results of these experiments. Clones designated *Ag* in Table I were isolated from agar, grown as monolayers, and were then grown again in agar. Of the cells studied, only those isolated directly from cartilage or clones showing polygonal cells grew in agar and produced metachromatic matrix. Polyoma-transformed hamster cells (not shown in Table I) formed colonies in agar but did not produce metachromatic matrix.

In some experiments, F-12 medium with 10% fetal calf serum was utilized. The results in Table II show a much higher cloning efficiency with F-12 medium both on monolayers and in agar. Additionally in F-12 medium, clones grew faster and attained a larger size than those developing in Eagle's medium. Superior growth of embryonic chick cartilage cells in F-12 medium has been reported (8). Clones isolated from agar appear to grow slowly when placed in monolayer.

When liquid medium was placed on the surface

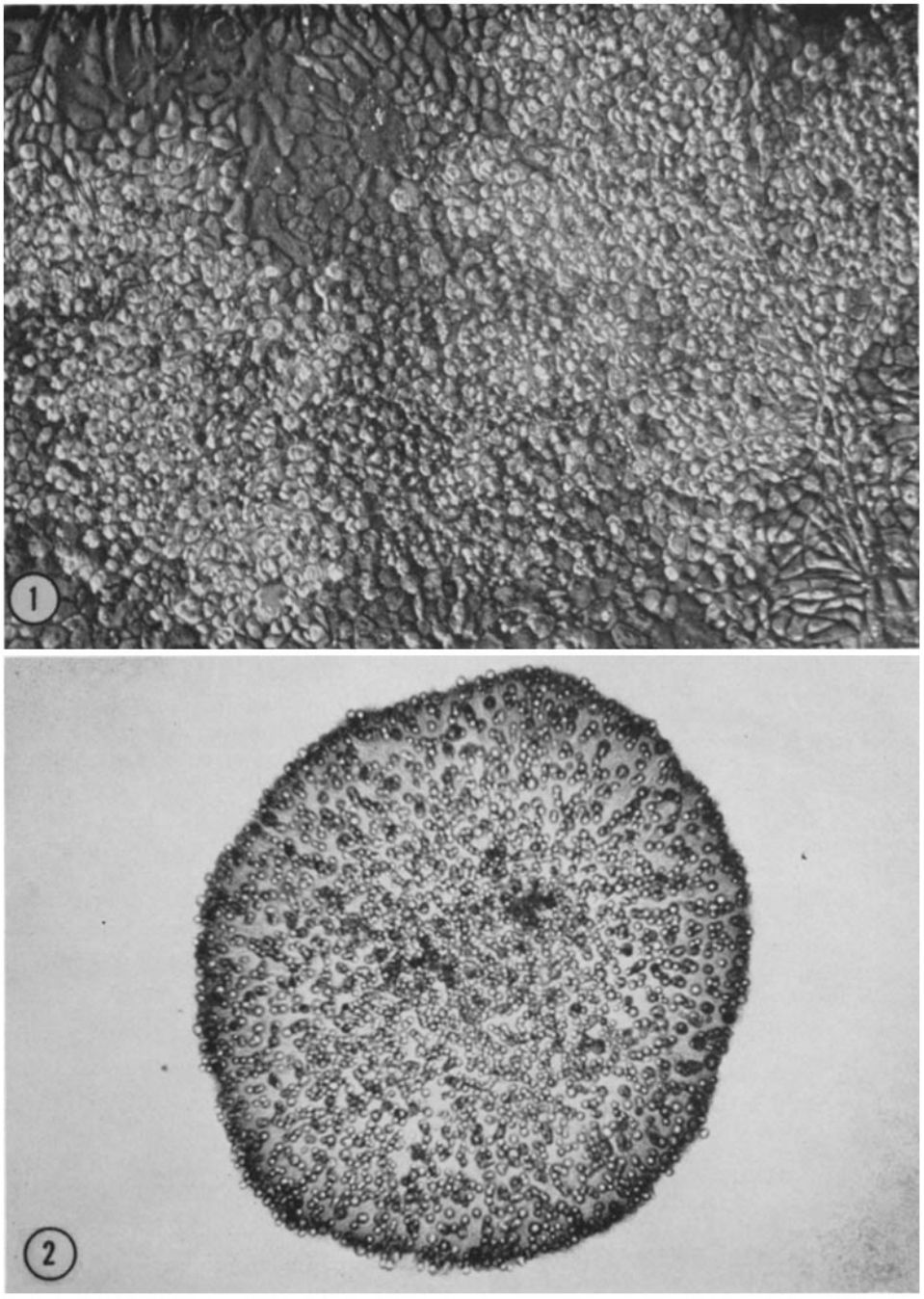

FIGURE 1 Phase contrast micrograph of cartilage cells grown on plastic culture dishes in modified Eagle's medium with 10% fetal calf serum. × 240.

FIGURE 2 Clone of cartilage cells grown in soft agar in F-12 medium with 10% fetal calf serum. Stained with toluidine blue. × 120.

TABLE I

Growth of Cartilage Cells in Agar in Modified Eagle's Medium

Results on primary culture represent three different primary isolations. Clones designated *C* were epitheloid polygonal type while those designated *F* were "fibroblast-like." The number of passages of each clone is indicated. Clones isolated directly from agar are designated *Ag*.

Origin of culture	Culture number	Cloning efficiency
		%
Primary embryonic chick cartilage	1	12.5
	2	7.7
	3	2.1
Clones obtained from monolayer cultures	C-1 4°	3.1
	C-1 5°	7.3
	C-2 3°	1.6
	C-2 4°	6.7
	C-3 3°	4.5
	C-5 2°	3.1
	C-7 3°	0.9
Clones obtained from agar	Ag-1 2°	6.0
	Ag-4 2°	5.6
	Ag-6 4°	2.3
	Ag-7 4°	1.3
Cells from suspension culture	I	6.1
	II	3.2
Fibroblast-like clones from monolayer	F-2 3°	0.21
	F-6 2°	0.04
Chick skin fibroblasts	1	0
	2	0
Human skin fibroblasts	1	0
	2	0

of agar plates, cartilage-like cells were observed to multiply in the liquid above the agar. Such cells when transferred to a spinner flask continued to multiply in suspension. In Eagle's medium containing 10% fetal calf serum, a maximum level of 5×10^5 cells/ml was achieved, while in F-12 medium containing 10% fetal calf serum a maximum level of 1×10^6 cells/ml was observed. This difference has not been studied in detail. The generation time in suspension culture in F-12 medium was approximately 48 hr. As shown in Tables I and II, such cultures maintained

TABLE II

Comparison of Modified Eagle's and F-12 Media

Type of cells	Method culture	Cloning efficiency	
		Eagle's medium	F-12
		%	
Primary embryonic chick cartilage	Agar	—	27
	Monolayer	—	25
	Monolayer	1.6	10
	Agar	2.1	8.5
Cells from suspension culture	Agar	3.2	21

their ability to grow in agar. After 2 days' growth in F-12 medium, approximately 0.1 mg of chondroitin sulfate/ml of medium was isolated from cultures containing 2×10^5 cells/ml. An increased number of cells in suspension has been noted by Holtzer and Abbott when chondrocytes were cultured on Falcon nontissue culture dishes (1).

The growth of chondrocytes in soft agar affords a new method for differentiating this cell type from other connective tissue cells and serves as a selective medium for cartilage cells. Experiments in monolayer culture with chick chondrocyte clones could not be carried out beyond the fifth transfer because of the appearance in cultures of large flat cells which become predominant at about the fourth transfer. When this occurs, the culture fails to multiply further. This observation is in accord with that of Bryan (3) and probably represents senescence observed with other diploid cells as described by Hayflick and Moorhead (9). With chick chondrocytes this phenomenon appears at about 30–35 doublings.

Cartilage cells share with tumor cells growth in soft agar and growth in liquid suspension. The capsule of chondromucoprotein which surrounds chondrocyte cells may prevent cell surface contact, thus permitting the formation of multilayered nodules. How this relates to growth in agar is not clear.

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REFERENCES

1. HOLTZER, H., and J. ABBOTT. 1968. *In* Stability of the Differentiated State. H. Ursprung, editor. Springer-Verlag, New York Inc., New York. 1.
2. COON, H. G. 1966. *Proc. Nat. Acad. Sci. U.S.A.* **55:** 66.
3. BRYAN, J. 1968. *Exp. Cell Res.* **52:**319.
4. STOOLMILLER, A. C., and A. DORFMAN. 1969. *In* Comprehensive Biochemistry. M. Florkin and E. H. STOTZ, editors. Elsevier, Amsterdam. **17:** 241.
5. MACPHERSON, I., and L. MONTAGNIER. 1964. *Virology.* **23:**291.
6. ICHIKAWA, Y., D. H. PLUZNICK, and L. SACHS. 1966. *Proc. Nat. Acad. Sci. U.S.A.* **56:**488.
7. HORWITZ, A. L., and A. DORFMAN. 1968. *J. Cell Biol.* **38:**358.
8. CAHN, R. D., H. G. COON, and M. B. CAHN. 1967. *In* Methods in Developmental Biology. F. H. Wilt and N. K. Wessells, editors. Thomas Y. Crowell Company, New York. 493.
9. HAYFLICK, L., and P. S. MOORHEAD. 1961. *Exp. Cell Res.* **25:**585.