DOUBLE-LAYER AGAR CELL CULTURES AS A METHOD OF STUDYING CELL GROWTH FACTORS

E. A. Cherepantseva, V. Ya. Shevlyagin, and A. D. Al'tshtein

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Transformed cells secrete substances which can induce proliferation of the same or other cells in semisolid agar [4]. These substances are known as tumor growth factors (GF) [2]. Various techniques can be used to detect GF, among which the most important is the study of conditioned culture fluid (CCF) [2, 7-9] or of acid-ethanol cell extracts [10] for the presence of these factors. The detection of GF, under these circumstances, will depend on their concentration, their physicochemical properties, and the proper choice of test cells.

It was shown previously that cells irradiated with ionizing radiation or treated with mitomycin C increase the cloning efficiency of transformed cells in semisolid agar [6]. Cells mounted in semisolid agar to increase the cloning efficiency of other cells, are known as feeder cells [6] or as filler cells [5]. In the light of modern data on the production of diffusing GF by cells [3], this phenomenon can be used to study the ability of the cells to form these factors while growing in semiliquid medium.

The aim of this investigation was to develop a method of double-layer agar cell cultures (DAC) which can be conveniently used to study the ability of some cells (donors) to produce GF for other cells (recipients) or test cells. The donor and recipient cells are placed in different layers of agar, separated by an intermediate layer. Under the microscope the two layers of cells can readily be distinguished and assessed in relation to colony formation.

EXPERIMENTAL METHOD

Transplantable lines of spontaneously transformed Syrian hamster fibroblast-like cells STHE and STHE-LM^8, obtained and generously supplied by G. I. Deichman (All-Union Oncologic Research Center, Academy of Medical Sciences of the USSR) [1], were used. The cells were cultured in MEM medium with 10% tryptose-phosphate broth (TPB), 5% embryonic calf serum (ECS), and antibiotics. The cells were cultured in semisolid agar [6] by the following method. First 1.2% Difco Noble agar was made up in triple distilled water; the agar, cooled to 43°C, was then mixed with an equal volume of a twofold concentrate of MEM medium (Flow Laboratories, England), containing 20% TPB and 5% ECS. The cell suspension was treated with 0.6% nutrient agar (1:1), to produce the required cell concentration in 0.3% agar. Six-well plates (Leningrad Medical Polymers Factory), 35 mm in diameter, were used. The scheme of the method is shown in Fig.1. The plates were incubated at 37°C in a moist atmosphere with 5% CO_2 for 7-12 days. The number of colonies (more than 8 cells) was counted under low power (50 ×) of an inverted microscope.

To obtain CCF of STHE cells, the growth medium of the cultures, once a monolayer was formed, was replaced by serum-free medium for 48 h. The CCF was filtered through millipore filters (0.22 μ) and kept at −20°C until required for testing.

EXPERIMENTAL RESULTS

Table 1 gives data on the effect of STHE cells, placed in the donor layer, on formation of colonies of STHE-LM^8 test cells. As these data show, STHE cells distinctly enhanced colony formation by STHE-LM^8 cells, and this activity of them depends on the concentration of donor cells.

N. F. Gamaleya Institute of Epidemiology and Microbiology, Academy of Medical Sciences of the USSR. Laboratory of Genetic Engineering, Institute of General Genetics Academy of Sciences of the USSR, Moscow. (Presented by Academician of the Academy of Medical Sciences of the USSR P. A. Vershilova.) Translated from Byulleten' Éksperimental'noi Biologii i Meditsiny, Vol. 102, No. 7, pp. 116-119, July, 1986. Original article submitted April 26, 1985.

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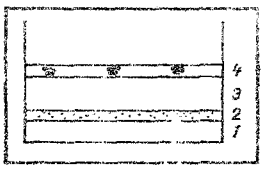

Fig. 1. Scheme of method of double-layer agar cell cultures: 1) Support (1 ml of 0.6% nutrient agar); 2) donor layer (donor cells in 0.25 ml of 0.3% nutrient agar) applied 30-120 min after support; 3) intermediate layer (1.5 ml of 0.3% nutrient agar) applied 4-16 h after donor layer, CCF, ECS, dialysis membrane, and so on, may be incorporated into it; 4) recipient layer (test cells in 0.25 ml of 0.3% nutrient agar) — applied to intermediate layer after 4-6 h.

TABLE 1. Effect of Dose of STHE Donor Cells on Colony Formation by STHE-LM[8] Test Cells

Number of test cells (number of cells per well)	STHE donors (number of cells per well)				
	0	10^4	$3 \cdot 10^4$	10^5	$3 \cdot 10^5$
10^4	256 (2,6 %)	262 (2,6 %)	600 (6 %)	960 (9,6 %)	1000 (10 %)
$3 \cdot 10^3$	0 (0 %)	4 (0,13 %)	220 (7,3 %)	307 (10 %)	340 (14 %)
10^3	0 (0 %)	0 (0 %)	51 (5,1 %)	138 (13,8 %)	145 (14,5 %)

Legend. A value signifying the number of colonies per well and the efficiency of colony formation (in parentheses) is used in Tables 1-5: $\frac{\text{number of colonies}}{\text{seeding dose}} \times 100$.

TABLE 2. Effect of STHE Donor Cells and CCF of STHE Cells on Colony Formation by STHE-LM[8] Test Cells

Donor layer	STHE-LM[8] test cells (number of cells per well)			
	$2 \cdot 10^4$	10^4	$5 \cdot 10^3$	$2{,}5 \cdot 10^3$
MEM medium	320 (1,6 %)	0 (0 %)	0 (0 %)	0 (0 %)
CCF of cells	510 (2,55 %)	130 (1,3 %)	0 (0 %)	0 (0 %)
STHE cells (10^5)	1000 (5 %)	480 (4,8 %)	270 (5,4 %)	140 (5,6 %)

Legend. CCF of STHE cells added to intermediate layer and accounts for one-quarter of the total volume of agar.

TABLE 3. Comparison of Donor Properties of STHE Cells Growing in Monolayer and Semisolid Agar

STHE-LM[8] test cells (number of cells per well)	MEM medium	STHE donor cells growing in monolayer in 0,3% agar	
$2 \cdot 10^4$	100 (0,5 %)	300 (1,5 %)	1000 (5 %)
10^4	9 (0,09 %)	150 (1,5 %)	460 (4,6 %)
$5 \cdot 10^3$	0 (0 %)	30 (0,6 %)	195 (3,9 %)
$2{,}5 \cdot 10^3$	0 (0 %)	0 (0 %)	125 (5 %)
$1{,}25 \cdot 10^3$	0 (0 %)	0 (0 %)	62 (5 %)
$6 \cdot 10^2$	0 (0 %)	0 (0 %)	34 (5,6 %)

Legend. Volume of agar above cell monolayer is equal to volume of layers in the DAC method.

TABLE 4. Determination of Dialysis Properties of GF by Use of the DAC Method

STHE donor cells (10^5)	Dialysis membrane	STHE-LM^8 test cells (number of cells per well)	
		10^4	10^3
—	—	670 (6,7%)	0 (0%)
—	+	720 (7,2%)	0 (0%)
+	—	2000 (20%)	350 (35%)
+	+	1000 (10%)	40 (4%)

Legend. The dialysis membrane was cut out of a dialysis tube (Serva, West Germany) along the diameter of the well and placed in the intermediate layer.

TABLE 5. Absence of Correlation between GF Production and Proliferation of Donor Cells

STHE-LM^8 donor cells (number of cells per well)	Agar concentration in donor layer (percent)	Proliferation of donor cells	STHE-LM^8 test cells (number of cells per well)				
			10^4	$3 \cdot 10^3$	10^3	$3 \cdot 10^2$	10^2
$5 \cdot 10^4$	0,3	+	520 (5,2%)	165 (5,5%)	45 (4,5%)	12 (4%)	4 (4%)
$5 \cdot 10^4$	0,6	—	560 (5,6%)	215 (7,1%)	80 (8%)	20 (6,6%)	6 (4%)
0	0,3	—	18 (1,8%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
10^5	0,3	+		620 (20%)		66 (22%)	
10^5 Irradiated	0,3	—		630 (21%)		60 (20%)	
0	0,3	—		0 (0%)		0 (0%)	

Legend. Monolayer of cells grown in a glass flask irradiated with γ-rays (8000 R).

Activity of CCF of STHE cells and of living STHE cells was compared by the DAC method according to their ability to stimulate colony formation (Table 2). It will be clear from Table 2 that the efficiency of colony formation by STHE-LM^8 cells is strongly dependent on the seeding dose of the cells. The most probable explanation of this phenomenon is the need for their own GF, formed by STHE-LM^8 cells. If CCF of STHE cells was included in the agar, colony formation was intensified, but it also remained dependent on the seeding dose of STHE-LM^8 cells. This suggests that although CCF of STHE cells contain CF, the set of these factors is insufficient for colony formation in the presence of low seeding concentrations of STHE-LM^8 cells. When the DAC method was used, STHE donor cells stimulated colony formation by STHE-LM^8 cells irrespective of the seeding dose of the latter, evidently by providing the complete set of GF required for colony formation by STHE-LM^8 cells. Incidentally, the use of STHE cells growing in a monolayer and not in agar as the donors stimulated colony formation by STHE-LM^8 cells only if the latter were present in high enough concentrations, i.e., their action was similar to that of isolated CCF (Table 3). By the DA method it is possible to use cells capable of proliferating in semisolid medium as donors. This enabled the role of proliferation in manifestation of the donor properties of the cells to be assessed. For these experiments STHE-LM^8 cells were used both as donors and as recipients. The donor properties of cells seeded in 0.3% agar (10-20% of cells formed colonies) or in 0.6% agar (proliferation absent), and also of cells irradiated with γ-rays and which had lost their ability to divide, was compared. One typical result is given in Table 5. Proliferation had no effect in principle on GF production.

Our experience of the use of the DAC method for different systems of donor and recipient cells, together with STHE and STHE-LM^8 cells suggests that this is a relatively simple and highly sensitive approach to the detection of GF produced by cells. In conjunction with the study of CCF, this method can shed light on the multiplicity of cellular GF which participate in stimulation of colony growth in semisolid medium, and it adds substantially to the results of investigation of CCF. The method has no limitations as regards either donor or recipient cells. The only important factor is that conditions must be created under which the recipient cells form colonies with low efficiency or not at all. This stipulation is usually easily met by the choice of seeding concentration of cells and the concentration of serum in the medium.

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FLUOROMETRIC DETERMINATION OF ORNITHINE DECARBOXYLASE ACTIVITY FROM ANIMAL TISSUES

T. T. Berezov and S. P. Syatkin

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Ornithine decarboxylase (ODC) limits the rate of synthesis of polyamines, whose level in animal tissues determines the rate of cell proliferation [13], and of hyperplasia and hypertrophy [7]. Of all known enzymes it has the shortest half-life (10-20 min), and has high lability in response to the action of hormones, drugs, and physicochemical factors [12]. These unique regulatory properties of ODC are responsible for the widespread use of this enzyme as a research tool in biology [7] and medicine, and in particular, in cardiology [8], pharmacology [12], neurology, and psychiatry [11], and in the study of fine mechanisms of regulation of metabolism. However, these investiations are hampered by the low level of ODC activity in normal tissues of intact animals and by the absence of a sensitive, yet technically convenient method of determination of the activity of this enzyme.

The aim of this investigation was to develop a highly sensitive and relatively easy method of determining ODC activity.

EXPERIMENTAL METHOD

Experiments were carried out on noninbred male albino rats weighing 120-180 g and on male C3HA mice, not exceeding 18-20 g in weight. The experimental tumors used included transplantable hepatoma 48, induced primarily in C3HA mice with orthoaminoazotoluene [2], and hepatoma G-27, induced primarily in noninbred albino rats with nitrosodiethylamine (NDEA) [6]. The liver affected with malignant disease, and the regenerating and normal liver also were investigated. Hepatocarcinogenesis was induced by NDEA. The carcinogen was injected intraperitoneally once a week for 2 months in a dose of 100 mg/kg body weight [1]. The total dose of NDEA received by each animal was 154 mg. Partial hepatectomy was performed by the method in [9].

The animals were decapitated and the liver quickly removed, perfused on ice with ice-cold distilled water, dried with filter paper, thoroughly freed from connective tissue, and weighed. Tumor tissue was taken in the logarithmic phase of growth, mainly from peripheral zones, and the possibility of necrotic tissue being present in the sample was excluded.

ODC does not withstand freezing, and for that reason the supernatant of a 33% freshly prepared homogenate (weight: volume = 1:2), obtained by centrifugation at 15,000 rpm for 20 min, was used as the source of the enzyme. The tissue was homogenized in 0.05 mM phosphate

Department of Biochemistry, Medical Faculty, Patrice Lumumba Peoples' Friendship University, Moscow. Translated from Byulleten' Eksperimental'noi Biologii i Meditsiny, Vol. 102, No. 7, pp. 119-122, July, 1986. Original article submitted June 26, 1985.

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