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Cloning of myelomas and hybridomas in fibrin clots

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Myelomas and hybridomas were observed to proliferate normally when trapped in a fibrin clot. The fibrin clot was obtained by including fibrinogen in the cell culture medium and thrombin in the plastic dish. A clot formed within seconds and cloning efficiency was around 100%. This technique has all the advantages of semi-solid medium cloning but avoids toxicity to the cells and the exposure to high temperature associated with the soft-agar cloning technique.

Key words: Cloning; Semi-solid medium; Fibrin clot; Myeloma; Hybridoma

Introduction

Existing techniques for cloning non-adherent cell lines, such as myelomas and hybridomas (Köhler and Milstein, 1975), involve the use of limiting dilution in liquid media (Lernhardt et al., 1978) or growth at low cell concentrations in soft agar (Sharon et al., 1980). Limiting dilution in liquid media is the method preferred in many laboratories but because of cell clumping it is not definitive and involves a large number of plates, feeding, and assays. Despite the appealing simplicity of the soft-agar cloning techniques, the agar can sometimes be found to be toxic or may have been mixed with the cells at too high a temperature (Campbell, 1984). The cloning efficiency in soft agar varies between 0.01–50% for different agar batches, myelomas or hybridomas (Schreier et al., 1980).

The fibrin-clot cloning technique described here simplifies the recovery of monoclonal antibody-producing strains from large hybridoma populations. By preserving the advantages of semi-solid techniques, the fibrin-clot cloning technique adds a reproducible and highly efficient cloning alternative. The media have been optimized for F_0 myelomas and hybridomas derived from it, reaching 100% plating efficiency without the use of feeder cells.

Materials and methods

Chemicals and cells

RPMI 1640, DMEM, and fetal calf serum were obtained from Flow Laboratories (Ayrshire, Scotland). Lyophilized human fibrinogen was obtained from Kabi (Stockholm, Sweden), it was reconstituted with distilled water, dialyzed extensively against distilled water, lyophilized and kept at −20 °C in aliquots. Gentamicin was from Schering (Kenilworth, NJ), it was included in the medium at 50 μg/ml. Thrombin was from Miles Laboratories (Elkhart, IN).

Four non-producer mouse myeloma cell lines (SP2/0, NSO/U, X63/Ag 8.653, and F_0) and several anti-human C-reactive protein or anti-trout immunoglobulin (gift of Dr. Dominguez) anti-

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body-secreting hybridomas, derived from them by fusion with immunized mouse spleen cells, were obtained as described. These cells were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, at 37 °C with 5% CO_2/95% air (Iturralde and Coll, 1984; Coll, 1987 and Martinez and Coll, 1988).

Cloning in fibrin clots

The cell concentration was determined with a hemocytometer. Cell suspensions were prepared in RPMI 1640 supplemented with 4 mM L-glutamine, 0.5 mM pyruvate, 0.2 mM β-mercaptoethanol, 26% fetal calf serum, 50 μg/ml gentamicin, 10 mM Hepes pH 7 and 0.2 mg/ml of fibrinogen. Thrombin was added to the petri dish or the plastic bottles to give a final concentration of 2–4 NIH U/ml.

To study the cell culture media for optimal cloning conditions, cells were prepared at different cell concentrations in the above mentioned media in the absence or in the presence of additives. A piece of plastic containing 24 wells (model MRC-96 Linbro Chemical Co.) was put into each 100 × 20 mm petri dish containing 2–3 ml of sterile water. Thrombin was added to each well at 0.2–0.4 NIH U/well in a volume of 2 μl and, finally, 100 μl of the cell suspension were pipetted into each well. The dishes were incubated in a water-jacketed incubator at 37 °C in water-saturated air containing 5% CO_2.

Fixing and staining of the colonies

After incubation, the clots were removed from the wells of the microtiter plates onto a frosted end glass slide with the small end of a spatula. The clots were partially dehydrated by placing a rectangular piece of Whatman number 1 filter paper on their surfaces. A second piece of paper was applied on top of the first and allowed to remain long enough for the paper to become moist. Then the top paper was removed and five drops of 1.7% glutaraldehyde in 0.01 M sodium phosphate, 0.15 M sodium chloride, pH 7.4 were added to the paper and allowed to stand for 10 min. The excess of glutaraldehyde was absorbed with filter paper. The paper was removed by gentle pressure and the fixed clots were washed in water and air dried. The following staining procedures were used: either 10 min saturated toluidine blue in 25% acetic acid and 2 min in water (to score the number of colonies), or 20 min in basic Fuchsin of Ziehl (Merck, Schuchardt, F.R.G.) ten fold diluted in 0.5% acetic acid (to photograph the colonies). The stained clots were washed with water and allowed to dry and were covered with a cover slip using Permount (Fisher Sci. Co.) as mounting medium. Colonies containing more than 8 cells were scored at a magnification of 100 ×.

Cloning efficiency was calculated by the number of colonies/initial number of cells × 100. The initial number of cells plated was either counted in the initial suspension of cells, and adjusted for dilutions and volume dispensed into each well or scored in clots fixed and stained before culture. The scored initial number of cells was corrected by the percentage of trypan blue positive cells estimated before initiating the culture.

Results

Clots were formed by including in the cell culture medium, fibrinogen (0.2 mg/ml) and thrombin (2–4 NIH U/ml). Fibrinogen was added to the medium and thrombin was pipetted into the plastic container. The concentration of these components was not critical within these ranges, but thrombin had to be kept below 8 NIH U/ml and fibrinogen had to be kept below 1 mg/ml to avoid toxic effects. Fibrinogen-containing medium was poured into the plastic container and the mixture agitated gently. A clot formed in about 30 s. A few minutes after clotting an equal amount of fibrinogen-free medium was poured over the clot to prevent evaporation. Under the conditions described, the myelomas and hybridomas tested did not require a feeder layer to form macroscopic colonies from single cells. After 5–8 days of incubation, the overlayed medium was exchanged once. Colonies were picked up with pasteur pipettes after 8–10 days and grown in liquid media.

The non-productive myeloma cell lines cloned by this technique included SP2/0, NSO/U, X63/Ag 8653 and F_0. The hybridoma clones by this method included several anti-human C-reactive protein producers which have been described

elsewhere (Iturralde and Coll, 1984; Coll, 1987; Martinez and Coll, 1988), derived from X63/Ag 8653 myeloma and one anti-trout immunoglobulin producer derived from F_0 myeloma (1A6).

The number of F_0 colonies increased linearly with cell concentrations from 200 to 2400 cells/ml. Colony sizes increased from 17 to 24 cells per colony when the initial cell concentration was increased from 200 to 2400 cells/ml. Cloning efficiencies varied from 30 to 50% in medium containing 10% fetal calf serum, to 85% in medium containing 20% fetal calf serum.

To increase colony size at the lower cell concentrations, the F_0 cells were cultured in media containing 20% of fetal calf serum and different additives, and the number of cells per colony was scored after 3 days in culture. Table I shows that the inclusion of β-mercaptoethanol, mouse transferrin (Cappel, Cochranville, PA), or pyruvate increased the cloning efficiency up to 100%. The increase in fetal calf serum increased the number of cells per colony, corresponding to a cell cycle time of 14.7 h compared to 17 h for control cultures. No feeder layers were required to obtain 100% cloning efficiencies. Under these optimized conditions F_0 and the derived hybridoma 1A6 (producing anti-trout immunoglobulin antibodies) exhibited 100% cloning efficiency after 3 days and 100% and 87% cloning efficiency respectively after 8 days.

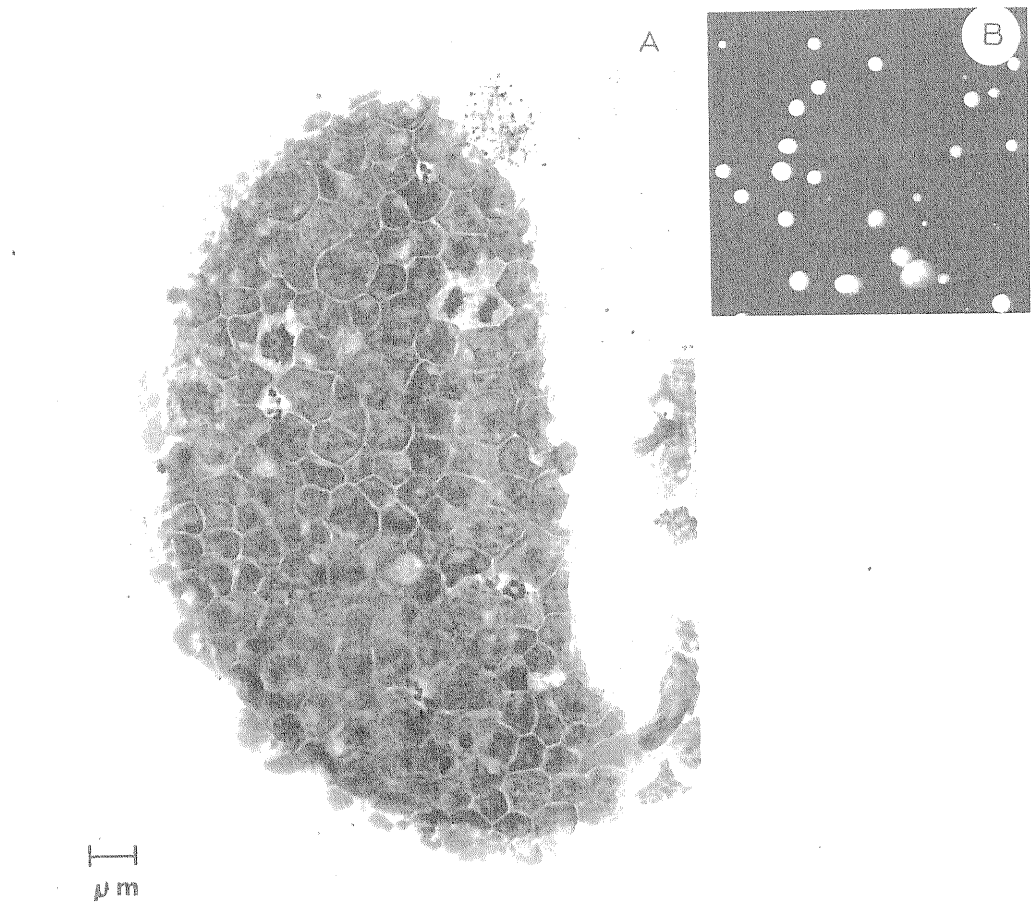

Fig. 1. Morphology of fixed anti-trout immunoglobulin hybridoma 1A6 colonies grown in fibrin clots. Anti-trout immunoglobulin hybridoma 1A6 cells were cultured at 100 cells/ml for 5 days in fibrin-clot cultures as described in the materials and methods section. *A*: After fixing and staining as indicated in the materials and methods section, ×500. *B*: After fixing and observed in dark field, ×10.

TABLE I

EFFECT OF ADDITIVES ON THE CLONING EFFICIENCY AND MEAN COLONY SIZE OF CULTURED F_0 MYELOMA CELLS

Additives	Final concentration	Cloning efficiency (%)	Cells per colony
Control	–	73	30
β-mercaptoethanol	0.2 mM	100	29
Mouse transferrin	1 mg/ml	100	30
Pyruvate	0.5 mM	100	39
Fetal calf serum	26%	85	51

Myeloma F_0 cells were cultured at 900 cells/ml in RPMI 1640 containing 20% fetal calf serum supplemented with 4 mM glutamine, 10 mM Hepes pH 7, and 50 μg/ml gentamicin, for 84 h. Further processing was as in the materials and methods section. At least 40 colonies were used to estimate colony size. Other additives that were tested but did not increase either cloning efficiency or colony size were: medium conditioned by the growth of F_0 (0.5–8%), insulin (200 μl/ml), Hepes (20 mM), human transferrin saturated with iron (0.4 mg/ml), mouse spleen cells (800000 nucleated cells/ml) and sodium bicarbonate (10 mM). Cloning efficiency was calculated by counting the cells in the fibrin clots prior to culture. Cloning efficiency was about three-fold lower when calculated from the number of cells in the initial suspension.

Fig. 1 shows the morphology of fixed anti-trout immunoglobulin hybridoma 1A6 colonies after 5 days in culture. Colonies of more than 100 cells were observed. Some colonies were perfectly spherical and others were more dispersed.

Discussion

This report describes a new technique for cloning myelomas and hybridomas in semi-solid medium formed by fibrinogen and thrombin. Medium requirements have been optimized to obtain F_0 cell cloning efficiencies close to 100% without the use of feeder cells. The cloning efficiency under limiting dilution conditions in liquid media, using one cell/ml with feeder cells in Costar trays (or one cell per 0.2 ml in microtiter plates) is close to 100% (Lernhardt et al., 1978). Although higher cell concentrations can be used in the soft-agar technique, its cloning efficiency varies greatly for different hybrid or myeloma cell lines (between 0.01–50%) (Sharon et al., 1980; Campbell, 1984). Soft-agar cloning is now less frequently used than limiting dilution cloning. In theory, it is a more certain method of assuring that the final clone is truly monoclonal, because the cells cannot be disturbed. Limiting dilution always carries the risk that two cells rather than one will give rise to a single clone after both have been put into a single well. Soft-agar presents technical difficulties in that it is necessary to use molten agar and the cells are vulnerable to damage at temperatures above 37 °C. In addition, batches of agar are highly variable and some may be toxic (Campbell, 1984). The method described here has all the above mentioned advantages of cloning in semi-solid medium with the addition of higher cloning efficiencies, due probably, to the use of more physiological substances (fibrinogen and thrombin). No feeder layers were necessary to obtain cloning efficiencies of 100 percent as calculated by counting single cells in the clot prior to culture. When the initial number of cells was calculated on the basis of the cell concentration in the suspension and adjusted for dilution and volume dispensed into each well, the maximum cloning efficiencies were 35%. The simultaneous detection of specific antibodies in association with the fibrinogen-thrombin cloning technique should also prove feasible, since both hemolysis and immunodiffusion can be performed in the soft-agar technique (Köhler and Milstein, 1975).

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