# The Use of Ultrafiltration for the Preparation of a Cell Growth Factor[1] (36464)

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(Introduced by V. P. Hollander)
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Methods have been developed in this laboratory for clonal growth of chondrocytes taken directly from adult rabbits (1). Serum was found to be an absolute requirement for growth of these cells in current media (2). Preliminary results have indicated that the addition of fetuin or fetuin-like growth factors to the medium was not sufficient for promoting growth of these cartilage cells in culture. The fractionation techniques commonly used to purify serum growth factors were found inadequate for obtaining a growth-promoting factor for these primary cells.

A series of Diaflo membranes of calibrated pore sizes was used to fractionate fetal bovine serum and to characterize its growth-promoting activity.

*Materials and methods.* Diaflo ultrafiltration membranes (Amicon Corp.) with molecular cutoff values from 500 to 100,000 daltons were used with a Model 50 ultrafiltration cell (Amicon).

Ultrafiltrations were done in a cold room at 4° and all the membranes were rinsed with 50 ml of cold $10^{-3}$ *M* sodium acetate at pH 7.5 before being used for the experiment.

Fetal bovine serum (Flow Laboratories) was used for all experiments. For ultrafiltration, 10 ml of serum was diluted with 4 vol of cold acetate buffer and ultrafiltered until the original serum volume remained in the cell. The ultrafiltrate was lyophilized and the residue was taken up in 10 ml of distilled water. This solution and the concentrate that remained in the cell were sterilized by filtration through 0.22 $\mu$ Millipore filters prerinsed with distilled water (3).

The protein concentration in serum and in each serum fraction was determined by a modified Lowry Method (4).

*Biological activity.* Rabbit ear cartilage cells were prepared and grown in culture as described before (2) except that the incubation period was reduced to 7 days and the colonies stained with 0.1% crystal violet. The percentage of the inoculated cells which gave rise to colonies with a diameter equal or larger than 0.5 mm was measured. This was defined as the plating efficiency (2). To facilitate comparison between experiments all data are presented as relative plating efficiency. This was accomplished by designating the plating efficiency obtained with 10% fetal bovine serum as 100% and comparing all other plating efficiencies to that value. Actual plating efficiencies obtained with 10% fetal bovine serum were always over 50%. The average diameter of the colonies obtained after 7 days of incubation in these conditions was also used as a growth index.

*Results. Relationship of plating efficiency to protein concentration.* For serum concentrations between 2 and 10% (v/v) in medium F12, a linear relationship was obtained between the plating efficiency and the protein content of the medium.

*Growth-promoting activity.* The growth-promoting activity of the fractions obtained by ultrafiltration is shown in Table I. When the plating efficiency and the colony size were used to compare the growth-promoting activity of the serum fractions, the activity was

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TABLE I. Biological Activity of Fractions.

| Fraction | Membrane | Protein Conc ($\mu$g/ml)[a] | Relative plating efficiency[b] Observed[c] | Expected[d] | Observed / Expected × 100 | Average colony size (mm) |
|---|---|---|---|---|---|---|
| None | — | 0 | 0 | 0 | — | — |
| Ultrafiltrate | UM-05 | 12 | 0 | 0 | — | 0.2< |
| | UM-2 | 14 | 0 | 0 | — | 0.2< |
| | PM-10 | 20 | 0 | 0 | — | 0.2< |
| | PM-30 | 25 | 0 | 0 | — | 0.2< |
| | XM-50 | 25 | 0 | 0 | — | 0.2< |
| | XM-100 | 1230 | 32.2 ± 7.2 | 40.0 | 80 | 1.5 |
| Concentrate | UM-05 | 2050 | 61.4 ± 4.4 | 59.5 | 103 | 0.5 |
| | UM-2 | 1960 | 57.9 ± 10.3 | 57.5 | 101 | 2.0 |
| | PM-10 | 2140 | 53.5 ± 3.1 | 61.8 | 87 | 1.5 |
| | PM-30 | 2070 | 45.3 ± 9.5 | 60.0 | 76 | 2.0 |
| | XM-50 | 1910 | 52.9 ± 11.8 | 56.0 | 94 | 2.0 |
| | XM-100 | 1140 | 28.0 ± 5.4 | 37.8 | 74 | 1.0 |
| 1:1 Mixture | UM-05 | 1030 | 25.1 ± 16.0 | 35.2 | 71 | 0.5 |
| | UM-2 | 990 | 28.4 ± 3.1 | 34.2 | 83 | 1.0 |
| | PM-10 | 1070 | 36.2 ± 7.1 | 36.0 | 101 | 1.0 |
| | PM-30 | 1200 | 26.6 ± 6.6 | 39.3 | 68 | 1.0 |
| | XM-50 | 980 | 23.8 ± 3.9 | 34.0 | 70 | 1.0 |
| | XM-100 | 1170 | 27.5 ± 3.8 | 38.5 | 71 | 1.5 |
| Whole fetal | | 810 | 30.0 ± 5.3 | 30.2 | 99 | 1.0 |
| bovine serum | | 1970 | 59.0 ± 5.7 | 58.1 | 101 | 2.0 |
| | | 3760 | 100.0 ± 8.1 | 100.8 | 99 | 2.5 |

[a] Protein concentration in the final medium before addition of the cell inoculum. (5.0 ml F12 plus 0.5 ml of fraction).

[b] All plating efficiencies are expressed as percentages of the plating efficiency obtained in control dishes containing medium F12 with 10% added fetal bovine serum (5.0 ml F12 plus 0.5 ml serum).

[c] Observed data are the means of two experiments each involving five Petri dishes per fraction. Standard deviations are calculated according to procedures described by Snedecor (26).

[d] Expected plating efficiencies are based on the concentration of protein added.

found only in the concentrate for all the membranes used except XM-100 where the activity was found both in the concentrate and in the ultrafiltrate.

*Absence of toxicity in small molecular fractions.* To eliminate the possibility that the ultrafiltrates might be toxic, they were mixed in equal amounts with concentrates from the same membrane fractionation and tested for biological activity. No toxicity of the ultrafiltrate was observed under these conditions.

*Extensive washing of the macromolecular fractions.* When fetal bovine serum was serially diluted and concentrated five times by ultrafiltration, the resulting concentrate was essentially inactivated. The biological activity could not be restored by the addition of the freeze-dried ultrafiltrate.

*Discussion.* There have been many reports of macromolecular growth factors from serum which partially or completely eliminate the need for whole serum in the culture media for a variety of cell lines (5–15). Jainchill and Todaro have reported that gamma globulin-free serum supports survival of the 3T3 and Balb/3T3 mouse cell lines for at least 4 weeks (26). The cells did not divide in these conditions. This absolute protein requirement for growth was lost however when the cells were transformed by tumor viruses SV 40 and MSV (27).

Although these reports contain much con-

flicting data, there seems to be fairly general agreement that globulins are probably involved. Growth-promoting activity was never found in the ultrafiltrate from the XM-50 membrane or any other membrane of smaller pore size. These data demonstrate that a macromolecular component of serum is essential for growth of the primary cultures. When mixed with macromolecular fractions, the ultrafiltrates appear to contain no growth-promoting or growth-enhancing activities.

The behavior of the growth-promoting activity on XM-50 and XM-100 membranes suggests a major component with a molecular weight of less than 100,000 daltons and indicates that essentially none of the active material occurs in serum with a molecular weight substantially below 50,000. The retention of the active material on these membranes is very similar to that of fetuin. This protein, which has a molecular weight of about 45,000 (16–19), was completely retained by the XM-50 membrane and partially retained by the XM-100 membrane (Michel Pagé, unpublished data). One of us (M.P.) has reported lately that fetuin can associate and dissociate easily (20). The fact that some growth-promoting activity was found both in the concentrate and in the ultrafiltrate from the XM-100 membrane could be explained by a similar phenomenon.

Despite similarities in behavior during ultrafiltration, fetuin and the growth-promoting component which is required by primary rabbit ear cartilage cells are not identical. We have assayed fetuin preparations (6, 15, 21) which had good growth-promoting activity for CHL, cow ovary cells, mouse L-cells and CHO cells (15, 21) and found that they are unable to promote growth in the primary cartilage cells. These preparations could not be reactivated by the XM-50 ultrafiltrate. Current data show that the serum activity which promotes growth of established cell lines does not necessarily apply to primary cells. The growth activity of fetal bovine serum which is required for the cartilage cells used for this experiment is much more labile than the activities which were described previously for established cell lines. Commonly used techniques such as gel filtration and ammonium sulfate or ethanol precipitation largely destroy the activity for rabbit cartilage cells while yielding fractions which are very active for growth of established cell lines (6, 15, 21, 27).

Although growth-promoting activity has been reported in this paper in terms of protein concentration which it tends to parallel, the active substance could easily be another macromolecule or small molecule bound to a protein. Several investigators working with established cell lines have stressed the importance of low molecular weight growth factors (22–25). It is very improbable that the inactivity of most of the ultrafiltrates could be due to a difference in their protein concentrations and the one in their respective concentrates. Irrespective of the protein concentration no activity was found in all the ultrafiltrates except the XM-100 ultrafiltrate.

The current study has shown that the growth-promoting activity for rabbit cartilage cells does not exist free in fetal bovine serum with a molecular weight significantly below 50,000 daltons.

If the growth activity is due to a small molecule bound to the macromolecular fraction, a dissociation of the former by extensive washings must cause an irreversible deactivation since the activity was never restored by the addition of freeze-dried ultrafiltrates to extensively washed concentrates. The loss of activity which accompanies extensive washing or standing in dilute solutions is more likely due to an irreversible denaturation. This preliminary characterization is expected to lead to development of conditions which stabilize the growth-promoting activity of fetal bovine serum for primary cultures.

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