*MAMMALIAN CELL GROWTH PROTEINS, I. GROWTH STIMULATION BY FETUIN**

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In earlier papers we described the presence and properties of a factor in the fetuin fraction of fetal calf serum which promotes growth of mammalian cells *in vitro* and enhances their stretching on glass and other surfaces.[1] Fetuin is an alpha-globulin, obtained by fractional precipitation with ammonium sulfate, whose major component is a glycoprotein of molecular weight 45,000.[1] A synthetic medium was described which when supplemented with both albumin and fetuin produces virtually 100 per cent efficiency of colony growth of single HeLa cells plated *in vitro*.[1] Another synthetic medium, F12,[2] was later developed in this laboratory which eliminated the need for albumin, greatly reduced the amount of fetuin required for maximal cell growth, and supported slow growth of some but not all cell strains without any protein supplement. Other laboratories have found or confirmed the presence of a growth-stimulating, alpha-globulin serum component,[3] but questions have been raised as to whether the active fraction of fetal serum is indeed fetuin, and whether growth-promoting proteins from adult sera are similar to or different from those found in fetal sera.[3h, 4] The divergences of interpretation which have arisen are due to a variety of causes, including the presence of several components[5] in the original fetuin described by Pedersen;[6] the ease with which active fetuin preparations can lose their biological activity;[1g] the lack of reliable, quantitative tests for titration of the biological activity of this growth-promoting factor; and the fact that various investigators have used different cells, media, and methodologies for assessment of the biological activities of their fractions. The present paper attempts to clarify further the relationship between the various materials that may be present in the fetuin fraction of fetal calf serum and the biological activity observed for the fraction as a whole.

Methods.—Cells: Three types of cells were used in these experiments: a standard strain of bovine ovary tissue culture cells isolated in this laboratory in 1966, the Chinese hamster ovary cell,[8] and the mouse L cell. Cells were farmed in F12 supplemented with 10% fetal calf serum by methods described previously.[7] Single cell platings were made in plastic Petri dishes (Falcon) as previously described.[7] Plating efficiencies, i.e., the fraction of single cells plated which develop into countable colonies, usually reached 75–100% in the presence of 0.50% or more of fetal calf serum which was always present in a set of control plates in each experiment. Plating efficiencies in test plates were routinely expressed as a fraction of the values obtained on the control plates.

Fetuin: Standard fetuin was prepared by $(NH_4)_2SO_4$ fractionation following the procedure of Fisher,[9] which was adapted from the Pedersen method,[6] but gives a higher yield of material with no sacrifice of specific biologic activity. It contains a small amount of albumin and of a high-molecular-weight component which has been regarded as a polymer of the major component.[5] In the experiments on more highly purified fetuin, the standard fetuin (which had already received four successive precipitations in 50% saturated $(NH_4)_2SO_4$ alternating with solubilization in saline, followed by water dialysis and filtration to eliminate euglobulins) was made the starting point for further purification. Three

more cycles of $(NH_4)_2SO_4$ fractional precipitation were administered, with the repeated elimination of all material precipitating at concentrations of $(NH_4)_2SO_4$ below 40% and above 45% of saturation.

Albumins: Crystalline bovine serum albumin prepared by alcohol precipitation (crystalline BSA-alcohol) was purchased from Armour or Pentex companies. Since the possibility exists that the alcohol might have changed the secondary structure of this protein, another crystalline albumin was also sought for comparison purposes. No other method completely free from objection was found for the preparation of crystalline bovine albumin, but a satisfactory crystalline horse albumin (crystalline HSA) was prepared by $(NH_4)_2SO_4$ precipitation from horse serum as described by Adair and Robinson.[10]

Antibodies: Antisera were prepared from sheep and rabbits by intramuscular injection of appropriate antigens plus Freund adjuvant (Difco). The antisera were fractionated and the gamma-globulin isolated by means of $(NH_4)_2SO_4$ precipitation.[11] The antifetuin gamma-globulin (AFGG) produced copious precipitate when treated with fetuin, no precipitate when treated with BSA-alcohol, and a trace of precipitate when treated with crystalline HSA. The anti-(BSA-alcohol) gamma-globulin (A-BSA-GG) produced a copious precipitate with BSA-alcohol, and with whole fetal calf serum, and a trace of precipitate with the standard fetuin preparation.

Experimental Results.—(1) *Effect of fetuin on single cell plating in F12:* Single cell plating would appear to be the most definitive method for quantitating cell reproduction.[12a] Single cells of the bovine ovary strain can grow in the basal medium alone exhibiting a variable plating efficiency response between limits of 5 and 90 per cent, depending on the particular batch of F12 used and on other adventitious conditions. However, the colony size obtained in the synthetic medium alone is always small and even after six days of growth, almost never exceeds 300 cells. By the addition of sufficient fetuin, the plating efficiency can be raised so as to approach 100 per cent and the growth rate approaches the best obtainable in the presence of the macromolecular fraction of fetal calf serum, i.e., a colony size of 3000–5000 cells in six days (Figs. 1*A–C*). The maximum growth rate is obtained in fetuin concentrations in the neighborhood of 50–100 μg/ml.[12b] While this procedure can be used as a crude determination of biological activity, the relatively high and variable plating efficiency of the cells in the basal medium and the anomalous action of low fetuin concentrations[12b] are disadvantageous, as compared with the methods described below.

(2) *Effect of albumin on single cell plating in F12:* Since albumin is the component present in largest amount as a contaminant of the standard fetuin preparations, albumin preparations were added to the F12 to see if these would affect the cell growth in a way resembling that of fetuin. Crystalline BSA-alcohol had no effect on the plating efficiency of the bovine ovary cells in concentrations below 50 μg/ml. In concentrations of 150 μg or higher, it reduced the plating efficiency uniformly to zero (Fig. 1*D*). However, addition of crystalline HSA to the basal F12 medium slightly stimulated the growth rate of the bovine ovary cells, but the maximum growth obtainable was clearly less than that achieved by fetuin (Fig. 1*E, F*). The existence of this albumin stimulation makes it necessary to examine carefully the possible contribution by the albumin fraction present in the original fetuin preparations to the biological activity observed.

(3) *The effect of antifetuin gamma-globulin (AFGG):* Growth of cells at a reduced rate in the basal medium alone might reflect their ability to synthesize suboptimal amounts of the macromolecular component needed for growth. In

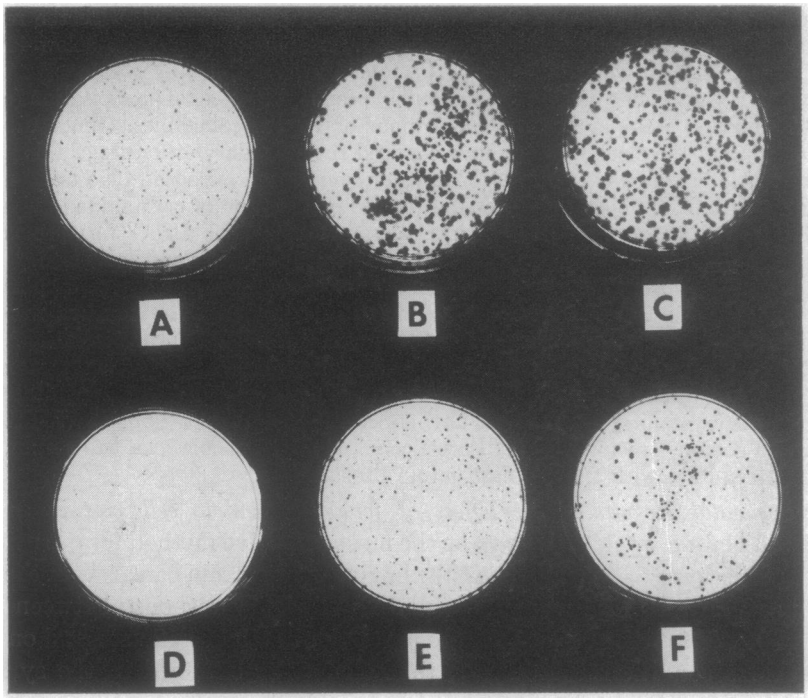

FIG. 1.—Typical plates demonstrating the effect of single cell growth in F12 with and without protein supplements. Each plate was seeded with 500 bovine ovary cells in the medium indicated and incubated for 6 days. (*A*) No supplement; (*B*) fetuin, 200 μg/ml; (*C*) fetal calf serum protein, 200 μg/ml; (*D*) crystalline BSA-alcohol, 200 μg/ml; (*E*) crystalline horse serum albumin, 200 μg/ml; (*F*) crystalline horse serum albumin, 500 μg/ml.

that case, addition of AFGG might suppress growth completely in the basal medium. Results shown in Figure 2 demonstrate that AFGG in concentrations of 25 μg/ml or higher completely inhibits the growth of bovine ovary cells in the basal medium. The gamma-globulin from the sheep inoculated with bovine albumin does not produce a precipitate with bovine serum albumin and had no demonstrable effect on cells. Anti-albumin serum produced from the rabbits produces copious precipitate with bovine serum albumin. Nevertheless, this albumin antibody also did not significantly inhibit cell growth, even when used in concentrations of 500 μg/ml or more (Fig. 2).

The specificity of the AFGG reaction was also tested by determining whether its cell inhibition could be neutralized by fetuin and by albumin. Addition of fetuin to AFGG completely neutralized its inhibitory effect on cell growth. However, the addition of neither crystalline BSA-alcohol nor crystalline HSA in any amount up to 200 μg/ml were capable of reversing the growth inhibition of any concentration of AFGG tested (Table 1). The conclusion appears justified that a component of fetuin different from albumin acts as the principal growth factor for these cells.

The specific neutralization of AFGG as demonstrated in Table 1 affords the basis for a quantitative titration of fetuin activity. By the use of a concentra-

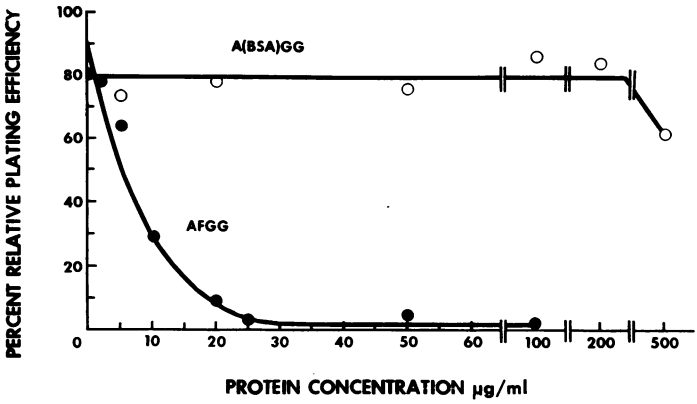

FIG. 2.—Demonstration that fetuin antibody (AFGG) strongly inhibits growth of bovine ovary cells while bovine serum albumin antibody (A-BSA-GG) has little or no effect. (*White circles*, albumin antibodies; *dark circles*, fetuin antibodies.)

tion of AFGG high enough to suppress growth in F12, it becomes unnecessary to subtract a blank or to count the cells per colony. Thus, with AFGG a simple colony count suffices to characterize the activity of any fetuin preparation.[12c]

The inhibitory effect of AFGG and its neutralization by fetuin is also exhibited on the cell-stretching reaction which has been previously described.[1h] However, cell stretching is more difficult to score than is colony counting, particularly in the neighborhood of the neutralization point, and so is less serviceable as a quantitative measure of biological activity.

(4) *Precise titration of fetuin activity in F12 plus BSA-alcohol:* The inhibitory activity of BSA-alcohol was found to make possible an alternative titration procedure based on the fact that addition of 150–200 μg/ml of BSA-alcohol lowers the plating efficiency of F12 to zero. Addition of fetuin in very small amounts was found to raise both the plating efficiency and the growth rate to maximal values, so providing a convenient and precise assay system. A typical titration curve is shown in Figure 3. The titration is not sensitive to the actual concentration of BSA-alcohol employed, 200–500 μg/ml yielding virtually identical results. Unlike the situation in the F12 medium alone, low concentrations of added fetuin did not cause an anomalous depression of plating efficiency.

TABLE 1. *Specific neutralization of growth-inhibiting power of AFGG by fetuin, but not by albumin.*

Concentration of anti-fetuin gamma-globulin (AFGG) (μg/ml)	Concentration of other proteins added (μg/ml)		Plating efficiency (%)
0		0	80.
20		0	9.0
20	Fetuin:	20	26.
20	Fetuin:	200	94.
100		0	0.30
100	Fetuin:	20	5.0
100	Fetuin:	200	70.
20	HSA:	200	0
10–200	BSA alcohol:	200	0–4

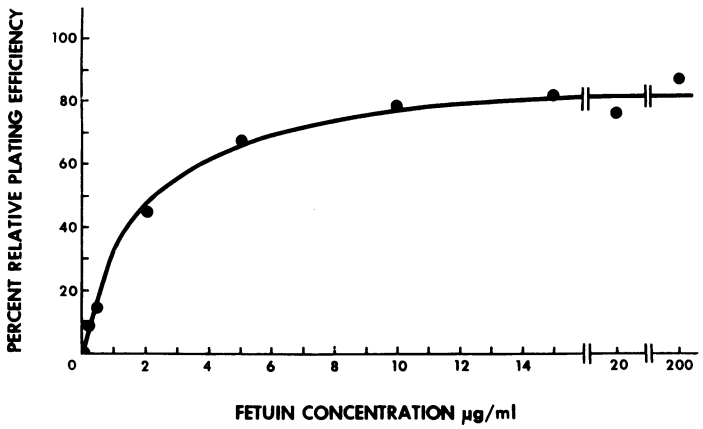

FIG. 3.—Titration curve showing action of fetuin in promoting single cell growth of the bovine ovary culture in the presence of F12 + BSA-alcohol, 200 μg/ml. The points presented represent the averaged values from six different sets of determinations. The average standard deviation of the means for all points taken at fetuin concentrations of 0.20 μg/ml or more was ±22%.

The BSA-alcohol titration has been the most sensitive and reliable method in our experience for titrating fetuin activity and is capable of routinely demonstrating the action of as little fetuin as 0.2 μg/ml. It therefore appears well suited for analysis of biological activity after further fractionation of fetuin preparations. It is of interest that, while BSA-alcohol inhibits the plating efficiency in F12 alone, the amount of fetuin required for maximum plating efficiency in BSA is equal to or less than that required for maximum cellular growth rate in F12 alone.

(5) *Further fractionation of fetuin activity:* Further fractionation of fetuin was undertaken in an effort to overcome the effects of the heterogeneity of this material in obscuring the identity of its active components. The standard fetuin preparation displays seven or eight distinct bands when 50 μg or more are subjected to disc gel[13] electrophoretic analysis (Fig. 4, *top*). However, the overwhelming majority of the mass of the sample lies in a single dense band which is in the alpha-globulin position and which yields a strongly positive Schiff test for glycoprotein. The principal problem is: does the biological activity of fetuin lie mainly in the principal band (with perhaps some small fraction of activity in the minor bands), or is it concentrated in one or more of the minor bands?

A series of protein fractionation procedures was attempted using *O*-(diethylaminoethyl) cellulose (DEAE chromatography)[1g] preparative disc gel electrophoresis, and fractional precipitation with $ZnSO_4$ and alcohol by the method of Spiro.[4b, 14c] In every case, the great majority of the biological activity was lost and could not be recovered in any fraction or combinations thereof.

Further purification (*Methods* section) was therefore undertaken with $(NH_4)_2SO_4$ since this material apparently causes minimal loss of biological activity. Titration of the specific biological activity of this new preparation by means of both AFGG neutralization and the BSA-alcohol test revealed it to be indistinguishable in specific activity from that of the standard fetuin.

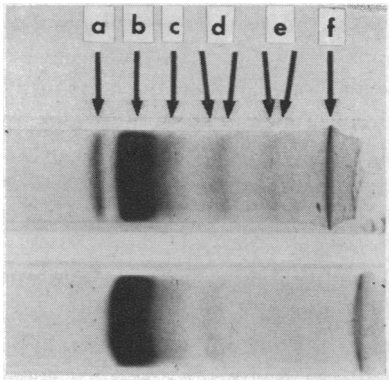

Fig. 4.—Comparison of the disc electrophoretograms of the standard fetuin (*top*) and the results of its further fractionation by means of $(NH_4)_2SO_4$ precipitation (*bottom*). The original material has one major band (*b*) and seven other bands, of which that in the (*a*) position corresponds to albumin, and that in the (*f*) position is the high-molecular-weight material which cannot enter the low pore-size gel. The refractionated material has retained all of the biological activity of the original; has lost the albumin band completely, and the (*e*) bands virtually completely; and retains decreased amounts of the (*c*), (*d*), and (*f*) materials.

Comparison of the gel electrophoretic pattern of this material with the standard fetuin reveals that considerable purification has been effected (Fig. 4). The principal band is unchanged in the purified preparation, but all the other bands have been either eliminated or markedly reduced. The only distinct minor bands which remain are the high-molecular-weight material which has passed through the initial gel but has been unable to penetrate into the main gel, and considerably reduced bands in the (*d*) and (*e*) positions.

The bands which have disappeared completely are obviously ruled out as representing carriers of the principal biological activity of fetuin. Since albumin is included in this group, the conclusion of the preceding section that the principal activity of fetuin is not that due to its contaminating albumin is confirmed. Moreover, since the specific biologic activity of the two samples in Figure 4 is identical, it is unlikely that this activity is carried exclusively on the minor bands (*c*), (*d*), (*e*), and (*f*) because these have undergone considerable diminution in the purified sample.[15d] We conclude that while some of the minor bands of the standard fetuin preparation might well possess some biological activity, the major activity resides in the (*b*) band which contains the majority of the mass of the preparation.

(6) *Applicability of these reactions to different mammalian cells and sera:* AFGG is capable of suppressing growth of a variety of other mammalian cells, including tissue culture cells of S3 HeLa (human carcinoma of the cervix), the Chinese hamster, and the mouse. AFGG, added to adult or to fetal bovine serum neutralizes completely their ability to support growth of standard tissue culture cell strains. Both human adult and fetal sera are similarly affected. Therefore, the reactions studied here appear to be fairly general for many mammalian systems. The responsible macromolecules are at least immunologically similar in fetal and adult sera, and since antibodies can be produced, they must be similar, though not identical, in different species.

Discussion.—These data indicate that the macromolecular serum fraction here investigated plays a fundamental role in mammalian cell reproduction, and may indeed represent a cell growth hormone. The quantitative methods described offer means for titration of the levels of this factor in various mammalian sera. They also permit investigating the suggestive relationships previously noted

between the rise in an alpha-globulin serum component and the onset of conditions involving great increase in cell division as in pregnancy, cancer, and recovery from injury by ionizing radiation[15] and other agents. The fact that both phytohemagglutinin, which initiates a cell division cycle in mammalian lymphocytes,[16] and erythropoetin, which presumably promotes cell division in the erythropoetic cell lineages,[17] are also glycoproteins, suggests the possibility that these molecules may share certain common cellular biochemical pathways with fetuin. The universality of sialic acid-containing substances in mammalian and bacterial membranes suggests their profound role in the cellular economy.

The effect of HSA on cells in F12 indicates that albumin may play some role in cell division, but one which appears of lesser importance than fetuin. This conclusion follows because the growth stimulation of HSA is less than that of fetuin, antibodies to albumin are not inhibitory as are those to fetuin, and addition of albumin cannot neutralize the inhibition of fetuin antibodies. Preliminary experiments have indicated that cells in tissue culture can synthesize both albuminlike and fetuinlike molecules. This intrinsic synthesis probably has contributed to the difficulties in unraveling the action of these agents.

Summary.—Addition of fetuin produces maximum reproduction of single bovine ovary cells in synthetic F12 medium. Crystalline albumin prepared by $(NH_4)_2SO_4$ stimulates growth slightly but crystalline alcohol-precipitated albumin has no effect in low concentrations and inhibits growth in high concentrations.

Fetuin antibody can be prepared in the sheep. It, but not albumin antibody, precipitates specifically with fetuin and destroys all growth of bovine ovary cells. This growth inhibition is reversed by fetuin addition, but not by albumin. Therefore, the action of fetuin on cell division is specific and different from that of albumin.

Fetuin activity can be accurately titrated by its neutralization of the growth-inhibitory action of antifetuin gamma-globulin, or more sensitively by measurement of its growth-promoting action on cells in the presence of alcohol-precipitated albumin.

In gel electrophoresis the standard fetuin preparation employed exhibits one major component and six or seven minor bands. While several purification methods completely destroyed the biological activity of the preparation, additional fractionation with $(NH_4)_2SO_4$ produced material with maximal specific biological activity, containing an unchanged major band, and only two minor components. Evidence is presented permitting the provisional conclusion that the principal component of the fetuin fraction contains the bulk of its biological activity.

A number of different mammalian cells respond to bovine fetuin, and several different adult and fetal mammalian sera are similarly deprived of their cell growth stimulatory power, by treatment with antifetuin gamma-globulin. Therefore, the factor studied here appears to be of general importance in mammalian cell growth.

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[12] (*a*) Puck, T., P. Marcus, and S. Cicciura, *J. Exptl. Med.*, **103**, 273 (1956). (*b*) It is noteworthy and as yet unexplained that in such titrations, when either fetuin or the macromolecular fraction of fetal calf serum are added in varying concentrations to test plates, the plates which receive 1–5 μg/ml of such supplement often exhibit a plating efficiency which is slightly below that obtained with either higher or lower concentrations. (*c*) In the presence of AFGG concentrations of about 10 μg/ml, the addition of 2 μg/ml of fetuin first drops the colony count markedly after which higher concentrations raise it, in an exaggeration of the anomalous effect noted in F12 alone. However, the titration method described in *Experimental Results*, section 4, is free from this interesting complication at low fetuin concentrations.

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