STIMULATION OF CLONAL GROWTH OF NORMAL FIBROBLASTS WITH SUBSTRATA COATED WITH BASIC POLYMERS

WALLACE L. McKEEHAN and RICHARD G. HAM

From the Department of Molecular, Cellular and Developmental Biology, University of Colorado, Boulder, Colorado 80309

ABSTRACT

Improved media have reduced the amount of serum protein required for clonal growth of normal human and chicken fibroblast-like cells. In the presence of limiting amounts of serum protein, attachment of colonies to tissue culture plastic surfaces is weak. Treatment of the culture surface with polylysine or other basic polymers causes the cells to adhere much more tightly. Growth is also improved on the surfaces treated with basic polymers, and further reductions in the concentration of serum are possible. At sufficiently low protein concentrations, growth of some types of cells is totally dependent on the use of a treated surface. Several different types of normal human and chicken fibroblast-like cells show improved growth on polylysine-coated surfaces, but no improvement was obtained in growth of a line of SV-40 transformed WI-38 cells. Acidic and neutral polymers are generally inactive. Collagen and gelatin improve growth slightly, but the effect is much less than that obtained with basic polymers. Both natural and synthetic polymers with an excess of basic groups are active, including histone, polyarginine, polyhistidine, polylysine, polyornithine, and protamine. The only critical requirement appears to be a polymer that carries a positive charge at a physiological pH.

The amount of serum protein required for clonal growth of normal human and chicken cells has been reduced significantly by recent improvements in medium formulation (9, 16).[1] During experiments seeking to determine the role of the serum protein that was still required, we observed that colonies grown in the presence of limiting amounts of serum protein were poorly attached to the culture surface. Such colonies frequently came loose as sheets of cells during the mild mechanical disturbances involved in removing the medium and fixing and staining the cultures.

Polylysine-coated surfaces have been used to attach live myoblasts, amoeba, and sea urchin eggs firmly to surfaces used in microscope observation (15). This is believed to be accomplished through adsorption of polylysine molecules to the surfaces, followed by interaction between cationic sites on the attached molecules and anionic sites on the surface of the cells.

We have tested polylysine and a variety of other synthetic and natural polymers for their effect on the attachment of vertebrate cells grown in the presence of minimal amounts of serum protein to culture surfaces. The attachment is much more stable on culture surfaces that have been coated

[1] McKeehan, W. L., K. A. McKeehan, S. L. Hammond, and R. G. Ham. 1976. Improved medium for clonal growth of human diploid cells with limiting concentrations of serum protein. *In Vitro* (*Rockville*). In press.

with synthetic or natural polymers that carry positive charges. Moreover, clonal growth is markedly stimulated on such surfaces. For some types of cells at very low concentrations of serum protein, clonal growth is totally dependent on the use of a culture surface coated with a basic polymer. The results presented in this paper demonstrate a direct relationship between the nature of the culture substratum and the amount of serum protein required for clonal growth.

MATERIALS AND METHODS

Chemicals

Dextrans, alginic acid, histone, protamine, hyaluronic acid, polyvinylpyrrolidone, bovine serum albumin, and all synthetic polyamino acids were purchased from Sigma Chemical Co., St. Louis, Mo. Methyl cellulose was obtained from Fisher Scientific Co., Pittsburgh, Pa, gelatin from Difco Laboratories, Detroit, Mich., and collagen from Worthington Biochemical Corp., Freehold, N. J. Heparin and *N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid (HEPES) were obtained from Calbiochem, San Diego, Calif.

Amino acids, vitamins (except folinic acid) and other organic chemicals used in preparation of media were obtained from Sigma. Folinic acid was purchased from Grand Island Biological Co., Grand Island, N. Y. and major inorganic salts and $Na_2SiO_3 \cdot 9H_2O$ from Fisher Scientific. The remaining trace elements were SpecPure grade obtained from Johnson Matthey Chemicals Ltd., London, England.

Media, Serum, and Serum Proteins

Clonal growth experiments with human cells were done in medium MCDB 104 (Table I). Details of the development of MCDB 104, which is the most recent in a series of media developed in this laboratory specifically for clonal growth of human diploid cells (9, 16), are being published elsewhere.[1]

Clonal growth experiments with chicken embryo fibroblasts were done in medium MCDB 201 (Table I),

TABLE I*
Composition of Media

Components	MCDB 104	MCDB 201	Components	MCDB 104	MCDB 201
	moles/liter	*moles/liter*		*moles/liter*	*moles/liter*
Amino acids			Thiamine · HCl	1.0×10^{-6}	1.0×10^{-6}
L-Alanine	1.0×10^{-4}	1.0×10^{-4}	Vitamin B_{12}	1.0×10^{-7}	1.0×10^{-7}
L-Arginine · HCl	1.0×10^{-3}	3.0×10^{-4}	Other organic compounds		
L-Asparagine · H_2O	1.0×10^{-4}	1.0×10^{-3}	Adenine	1.0×10^{-5}	1.0×10^{-6}
L-Aspartic acid	1.0×10^{-4}	1.0×10^{-4}	Choline chloride	1.0×10^{-4}	1.0×10^{-4}
L-Cysteine · HCl · H_2O	5.0×10^{-5}	2.0×10^{-4}	D-Glucose	4.0×10^{-3}	8.0×10^{-3}
L-Glutamic acid	1.0×10^{-4}	1.0×10^{-4}	*i*-Inositol	1.0×10^{-4}	1.0×10^{-4}
L-Glutamine	2.5×10^{-3}	1.0×10^{-3}	Linoleic acid	1.0×10^{-8}	3.0×10^{-7}
Glycine	1.0×10^{-4}	1.0×10^{-4}	Putrescine · 2HCl	1.0×10^{-9}	1.0×10^{-9}
L-Histidine · HCl · H_2O	1.0×10^{-4}	1.0×10^{-4}	Sodium pyruvate	1.0×10^{-3}	5.0×10^{-4}
L-Isoleucine	3.0×10^{-5}	1.0×10^{-4}	Thymidine	3.0×10^{-7}	3.0×10^{-7}
L-Leucine	1.0×10^{-4}	3.0×10^{-4}	Major inorganic salts		
L-Lysine · HCl	2.0×10^{-4}	2.0×10^{-4}	$CaCl_2 \cdot 2H_2O$	1.0×10^{-3}	2.0×10^{-3}
L-Methionine	3.0×10^{-5}	3.0×10^{-5}	KCl	3.0×10^{-3}	3.0×10^{-3}
L-Phenylalanine	3.0×10^{-5}	3.0×10^{-5}	$MgSO_4 \cdot 7H_2O$	1.0×10^{-3}	1.5×10^{-3}
L-Proline	3.0×10^{-4}	5.0×10^{-5}	NaCl	1.0×10^{-1}	1.3×10^{-1}
L-Serine	1.0×10^{-4}	3.0×10^{-4}	$Na_2HPO_4 \cdot 7H_2O$	3.0×10^{-3}	5.0×10^{-4}
L-Threonine	1.0×10^{-4}	3.0×10^{-4}	Trace elements		
L-Tryptophane	1.0×10^{-5}	3.0×10^{-5}	$CuSO_4 \cdot 5H_2O$	1.0×10^{-8}	1.0×10^{-8}
L-Tyrosine	3.0×10^{-5}	5.0×10^{-5}	$FeSO_4 \cdot 7H_2O$	5.0×10^{-6}	6.0×10^{-6}
L-Valine	1.0×10^{-4}	3.0×10^{-4}	H_2SeO_3	3.0×10^{-8}	5.0×10^{-8}
Vitamins			$MnSO_4 \cdot 5H_2O$	1.0×10^{-8}	5.0×10^{-10}
d-Biotin	3.0×10^{-8}	3.0×10^{-8}	$Na_2SiO_3 \cdot 9H_2O$	5.0×10^{-7}	5.0×10^{-7}
Folinic acid (calcium leucovorin)	1.0×10^{-9}	1.0×10^{-8}	$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	1.0×10^{-9}	5.0×10^{-10}
DL-α-Lipoic acid (DL-6,8-Thioctic acid)	1.0×10^{-8}	1.0×10^{-8}	NH_4VO_3	5.0×10^{-9}	5.0×10^{-11}
Niacinamide	5.0×10^{-5}	5.0×10^{-5}	$NiCl_2 \cdot 6H_2O$	5.0×10^{-10}	5.0×10^{-12}
D-Pantothenic acid (Hemi-calcium salt)‡	1.0×10^{-6}	1.0×10^{-6}	$SnCl_2 \cdot 2H_2O$	5.0×10^{-10}	–
Pyridoxine · HCl	3.0×10^{-7}	3.0×10^{-7}	$ZnSO_4 \cdot 7H_2O$	5.0×10^{-7}	1.0×10^{-7}
Riboflavin	3.0×10^{-7}	3.0×10^{-7}	Buffers and indicators		
			HEPES	5.0×10^{-2}	3.0×10^{-2}
			NaOH§	2.6×10^{-2}	1.6×10^{-2}
			Phenol red	3.3×10^{-6}	3.3×10^{-6}
			Final pH of medium‖	7.6	7.6

* The alphabetical listings in this table do not reflect the grouping of components or the sequence of their addition used during preparation of media. For detailed instructions for preparation of MCDB 104, see footnote one. Except for altered quantities of nutrients, the preparation of MCDB 201 is very similar. Both media must be equilibrated with 5% carbon dioxide in air to yield the results described in this paper.

‡ Molar concentration refers to pantothenate ion.

§ Enough NaOH is added to bring the pH of each batch of medium precisely to 7.6. The concentrations indicated in the table are approximate.

‖ The pH is measured with the medium equilibrated with air at room temperature (25°C). When equilibrated with 5% carbon dioxide and 95% air at 37°C, the pH of media MCDB 104 and MCDB 201 is 7.20 and 7.05, respectively.

which was developed in this laboratory specifically for that purpose. Fetal bovine serum (FBS) was obtained from Flow Laboratories, Inglewood, Cal. Bulk fetal bovine serum protein (FBSP) was prepared by extensive dialysis of EDTA-treated FBS as described previously (16).

Cells and Cultures

Diploid fibroblasts (strain WI-38) from human fetal lung tissue (10) were obtained from Dr. L. Hayflick (Stanford University School of Medicine, Stanford, Calif.). Stock monolayer cultures were maintained in MCDB 104 supplemented with 1.0 mg per ml FBSP, and subcultured as described elsewhere (16).[1]

Human diploid cells derived from fetal lung (passage 7), newborn foreskin (passage 7), and amniotic fluid (passage 4) were obtained from Dr. Charles Waldren (University of Colorado Medical Center, Denver, Colo.). These cultures were initiated and maintained in medium F12 (8) supplemented with 7.5% (vol/vol) FBS plus 7.5% (vol/vol) human cord serum. Upon receipt in this laboratory, the cells were transferred and maintained as stock cultures in medium MCDB 104 supplemented with 1.0 mg per ml FBSP.

WI-38 cells transformed by SV40 virus (WI-38 VA-13 Subline 2RA) were obtained from Dr. Keith Porter (University of Colorado, Boulder, Colo.) who originally obtained them from Dr. A. J. Girardi (Wistar Institute, Philadelphia, Pa.). Stock cultures were maintained in MCDB 104 supplemented with 2.5 mg per ml FBSP.

Primary cultures of chicken embryo fibroblasts were initiated from 10-day embryos in medium MCDB 201 supplemented with 2.5 mg per ml FBSP. They were subcultured in MCDB 201 with 1.0 mg per ml FBSP, and were generally used between the second and fifth passages.

Measurement of Clonal Growth

Cell suspensions prepared from monolayer stock cultures (16)[1] were diluted with protein-free medium to a concentration of 1,000 cells per ml for human cells or 1,500 cells per milliliter for chicken cells. Corning no. 25010 plastic tissue culture dishes (60 × 15 mm) (Corning Glass Works, Science Products Div., Corning, N.Y.), treated as indicated in the text and containing 5.0 ml of medium, were inoculated with 0.10 ml of the diluted cell suspension (100 human cells or 150 chicken cells). The cultures were incubated in a humidified 5% CO_2 atmosphere at 37°C for 14 days for human cells and 10 days for chicken cells. At the end of the incubation period, the medium was removed, and the colonies were fixed for 5 min with a solution containing 2% (vol/vol) glutaraldehyde and 0.05 M sodium cacodylate buffer (pH 7.0). The colonies were then rinsed with H_2O and stained for 5 min with 0.1% (wt/vol) crystal violet solution. Excess stain was removed by washing with H_2O.

Cell growth was quantitated by measuring the area and density of colonies photometrically. The measuring device consisted of an International Rectifier B2M photocell (International Scientific Instruments, Inc., Mountain View, Calif.) masked with a 5.0-mm diam round opening, and a variable light source. The illumination was adjusted until an output of 50 μA was obtained with a portion of the petri dish that contained no colonies over the opening. The colony to be measured was then placed over the opening, and the reduction in photocell output was recorded.

The "colony size" measurements obtained in this manner are proportional both to the area and to the density of the colony and accurately reflect the number of cells per colony. The colony size measurements reported throughout this paper are averages of the values obtained from at least five separate colonies per petri dish. The colony size measurement responds to small differences in clonal growth much more precisely than plating efficiency, which tends to remain near constant except under drastic conditions leading to almost complete growth failure. Therefore, the effects on "clonal growth" discussed in this paper refer primarily to changes in the number of cells formed from a single cell during the incubation period, rather than to the fraction of cells forming colonies or to colony morphology.

Substrate Coating Procedure

The procedure used for coating culture surfaces is very similar to that described by Mazia et al. (15). All of the following steps were performed aseptically and at ambient room temperature. All components used for coating the culture substrate were dissolved in H_2O at a concentration of 0.10 mg per ml and sterilized by passage through Millipore type GS (0.22 μm) filter membranes. 0.5 ml of the solution was pipetted into each 60 × 15-mm plastic tissue culture dish. Care was taken to be sure that the solution was spread over the entire dish surface. After 5 min, the solution was removed with a Pasteur pipette, and the surface was washed with 1.5 ml of sterile H_2O. The H_2O was subsequently removed with a Pasteur pipette. Care must be exercised to remove all solutions completely from the culture surface since basic polymers seriously inhibit growth when added directly to the culture medium. In some experiments, the culture surface was coated and washed with nonsterile solutions, and the coated surface was sterilized by UV-irradiation.

RESULTS

The study of the effects of polymer-coated surfaces on clonal growth of normal cells was facilitated by the development of improved growth media that support formation of visible colonies from single cells with much smaller amounts of dialyzed FBSP than previously required (16).[1] Medium MCDB 104 (see footnote one and Table I) supported detectable clonal growth of WI-38 cells with a supplement of dialyzed FBSP of less than 100 μg per ml (protein equivalent to 0.20%

(vol/vol) whole FBS). Under this condition, the colonies that formed were more loosely attached to the culture dish surface than those grown with higher amounts of FBSP supplement. Colonies were often washed from the surface by the fixing and staining procedure used to quantitate cell growth. Although the improved fixing procedure described in Materials and Methods reduced the detachment of colonies from the surface, it was apparent that the degree of adhesion of cells and colonies to the dish surface before fixation was affected by the amount of FBSP in the medium.

Fig. 1 shows the effect of coating the culture surface with polylysine on colony formation by a variety of cell types. Media supplemented with 500 μg per ml FBSP (protein equivalent to 1.0% (vol/vol) whole FBS) were used for this series of experiments. Clonal growth of human diploid strain WI-38 (passage 20) is markedly stimulated by the polylysine-coated surface (Fig. 1*a*). Colonies grown on the coated surface are generally less dense, and the cells migrate out from the center of the colonies over the culture surface rather than stacking up on top of each other. Clonal growth of human fibroblasts at lower passage numbers derived from fetal lung (passage 9), newborn foreskin (passage 9), and amniotic fluid (passage 6) is almost dependent on the polymer-coated surfaces in medium MCDB 104 supplemented with 500 μg per ml FBSP (Fig. 1*b*, *c*, and *d*). Clonal growth of chicken embryo fibroblasts (passage 3) in medium MCDB 201 containing 500 μg per ml FBSP supplement is also markedly increased by the polylysine-coated surface (Fig. 1*e*). In contrast to the results with normal cells, clonal growth of WI-38 cells transformed by infection with SV 40 virus is

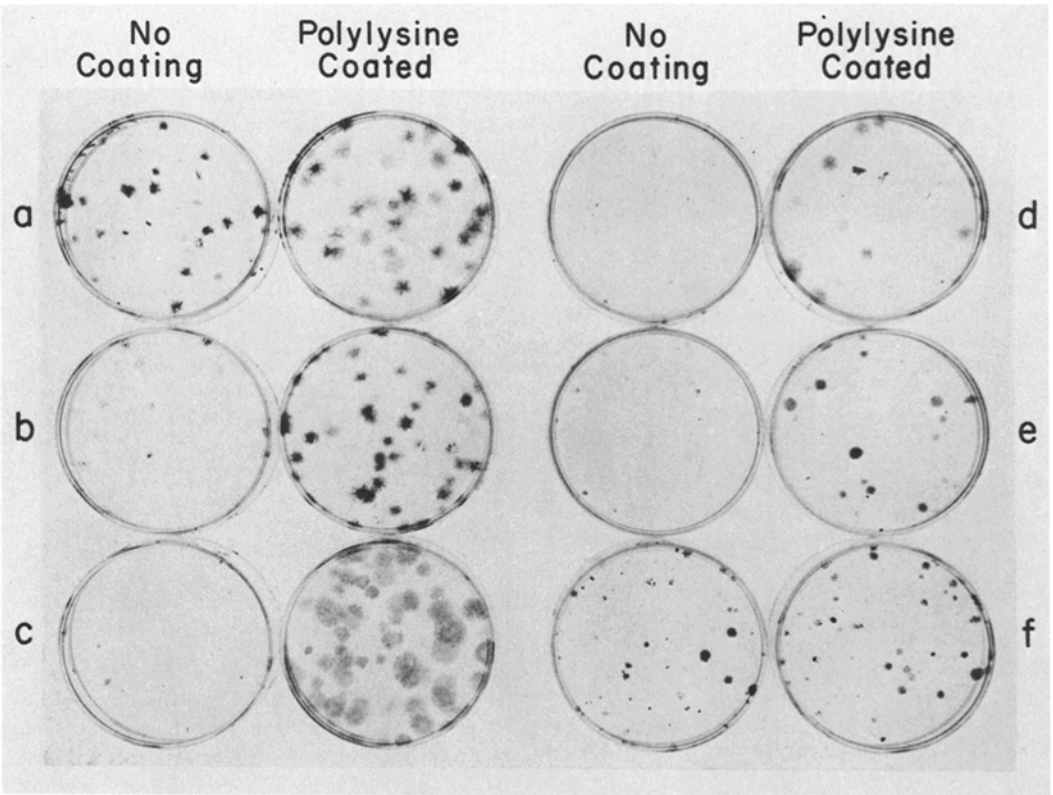

FIGURE 1 The effect of the polylysine-coated culture surface on clonal growth of human and chicken fibroblasts. Culture dishes were coated with polylysine where indicated, and the cells were grown in media containing 500 μg per ml FBSP. The colonies were fixed and stained as described in Materials and Methods. (*a*) WI-38 cells (pasage 20), (*b*) human embryonic lung cells (passage 9), (*c*) human newborn foreskin cells (passage 9), (*d*) human amniotic fluid cells (passage 6), (*e*) chicken embryo cells (passage 3), and (*f*) WI-38 VA-13 Subline 2RA cells.

much less affected by the basic polymer-coated surface (Fig. 1*f*).

The degree of stimulation of clonal growth of the normal cells that is observed with polylysine-coated surfaces is related to the amount of FBSP in the medium. Fig. 2 illustrates the quantitative requirements for FBSP for clonal growth of newborn foreskin fibroblasts on untreated and on polylysine-coated surfaces. Significantly larger amounts of FBSP are needed for clonal growth without polylysine than with it. At concentrations of FBSP around 100 μg per ml, clonal growth is completely dependent on the coated surface.

To determine the specificity of the type of surface coating that is beneficial to clonal growth, a variety of synthetic and natural substances were used to coat dish surfaces, and the coated dishes were tested for effect on growth of WI-38 cells. In the presence of 500 μg per ml FBSP (Table II), both synthetic and natural polymers with basic properties were found to stimulate growth markedly. This is in contrast to an assortment of acidic and neutral polymers which have no effect or inhibit growth. Coating the dishes with collagen or gelatin gives a detectable stimulation of growth, but it is only a fraction of that obtained by coating with the basic polymers. Some apparent benefit is obtained by coating the dishes wth FBSP if the subsequent clonal growth test is performed at ex-

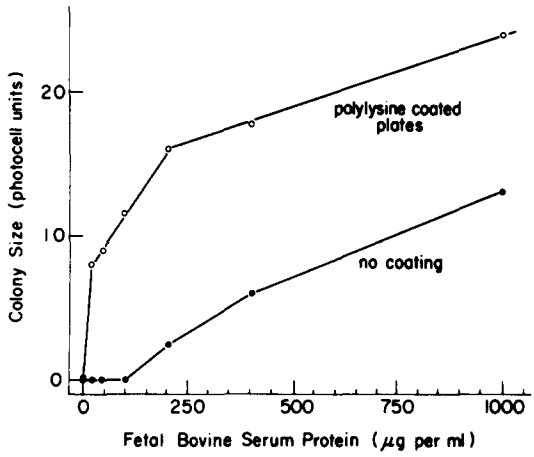

FIGURE 2 The effect of the polylysine-coated surface on growth response of human newborn foreskin fibroblasts to FBSP. Culture dishes were coated with polylysine where indicated. The cells were grown in medium MCDB 104 with the indicated amounts of FBSP. The resulting colonies were fixed and stained, and their size and density were measured photometrically as described in Materials and Methods. The average reading in photocell units for the colonies of foreskin fibroblasts shown in Fig. 1*c* is 16.0. This represents a single cell multiplication factor in excess of 12 doublings.

TABLE II

Effect of Different Culture Surface Coating Agents on Clonal Growth of WI-38 Cells

Coating agent	*Percent of clonal growth on polylysine-coated plates
None	54
DEAE-dextran	72
Histone‡	118
L-lysine	55
L-lysyl-L-lysine	55
Poly-L-arginine·HCl (mol wt 15,000–50,000)	95
Poly-L-histidine (mol wt 5,000–15,000)	94
Poly-D-lysine·HBr (mol wt 30,000–70,000)	100
Poly-DL-ornithine·HBr (mol wt 3,000–15,000)	111
Protamine§	140
Sodium alginate	30
Dextran sulfate (mol wt 500,000)	33
Heparin (Na salt)	44
Hyaluronic acid (Na salt)‖	46
Poly-D-glutamic acid (Na salt) (mol wt 50,000–100,000)	26
Dextran (mol wt 500,000)	47
Methylcellulose (15 centipoises)	28
Poly-L-asparagine (mol wt 5,000–10,000)	46
Poly-L-proline (mol wt 10,000–30,000)	55
Polyvinylpyrrolidone (mol wt 360,000)	36
Bovine serum albumin	50
Collagen	75
FBSP	55
Gelatin	62

* Cell culture plastic petri dishes were treated with the indicated polymer as described in Materials and Methods. The amount of polymer and the length of treatment were saturating for effect on clonal growth for all polymers tested. WI-38 cells were grown into colonies in medium MCDB 104 containing 500 μg per ml FBSP in the treated dishes. Colonies were fixed and stained, and colony size was measured photometrically as described in Materials and Methods. Untreated dishes and dishes treated with poly-D-lysine were included as controls in all experiments. For direct comparison of responses from different experiments, all colony size measurements are reported as percentages of the values obtained for the poly-D-lysine controls in each experiment. The value for no treatment is an average from all of the experiments.

‡ Sigma Type II-A from calf thymus.

§ Sigma grade IV from salmon.

‖ Sigma grade IIIS from human umbilical cord.

tremely low concentrations of serum protein. However, this may be primarily a nutritional effect. No benefit is seen from coating with FBSP under the test conditions in Table II (500 μg per ml of FBSP in the culture medium).

DISCUSSION

Improvements in the defined portion of the culture medium have eliminated at least 90% of the serum protein requirement for clonal growth of fibroblast-like cells, both from chicken embryos and from human amniotic fluid, fetal lung, and newborn foreskin. In addition, the subculturing procedures (9, 16)[1] that are currently used appear to have eliminated cellular dependence on the antitryptic action of serum proteins. The cells will attach and flatten in the total absence of added serum proteins, and in dense cultures will undergo several rounds of division before exhausting nutrient reserves.

With minimal amounts of serum protein added to the improved media, the cells in the developing colonies tend to be firmly attached to one another, but only very loosely attached to the culture surface. Entire colonies are easily dislodged from the culture surface as coherent sheets of cells, even when the cultures are handled very gently. Treatment of the culture surface with polylysine efficiently overcomes the problem of detachment of colonies and permits the serum concentration to be reduced to the point where it becomes nutritionally limiting. Under such conditions, the attachment of the cells to the culture surface appears to be stronger than their attachment to one another. The colonies that are formed are flat and diffuse, with the cells tightly flattened on the culture surface and scarcely in contact with one another at all.

The literature contains several reports of enhanced cellular attachment and spreading in the presence of basic proteins and polyamino acids (12, 13, 15, 26). There are also reports that histones and basic polyamino acids stimulate uptake of calcium and of macromolecules (22, 23, 31). Access of nutrients to the cells may be one of the factors that limit cellular population density (4, 25). If such is the case, polylysine and other cationic polymers might stimulate growth either by increasing cellular surface area as a result of greater flattening or by direct enhancement of nutrient uptake. It is interesting to note that the amount of serum needed to initiate DNA synthesis in HeLa or BHK21 cells that are attached and flattened is much less than the amount needed for rounded cells in suspension culture (1, 18).

Growth of "normal" cells is generally considered to be "anchorage dependent" (24). We have not yet evaluated fully the implications of obtaining reasonable clonal growth under conditions of marginal attachment. However, the improvement in growth that accompanies improved attachment on surfaces coated with basic polymers is consistent with the anchorage dependence concept.

It is difficult to compare previous literature reports on cellular attachment, flattening, and growth either with one another or with the present work because of the diversity of cell types, culture media, culture surfaces, and experimental techniques that have been used. Two operationally distinct forms of cellular attachment and flattening seem to be involved. One occurs rapidly in the absence of serum or other proteins, and is delayed significantly by the presence of serum proteins (5, 7, 17, 26, 32). The other is slower, and in many cases has been reported to be dependent on specific serum proteins (6, 12, 29). Inhibition of trypsin may be an important part of the action of serum (28), but specific attachment and growth-promoting effects also appear to be involved (6, 11).

The exact nature of cellular attachment to culture surfaces is not understood. The electron microscope reveals a region 450 Å thick that is filled with electron-dense material between the plasma membrane and the actual culture surface. Components from serum bind tightly to the culture surface (20, 26). In addition, material of cellular origin is present between the cells and the culture surface (3, 20, 21, 33). When cells are removed with EGTA, a layer of substrate-attached material containing both serum and cellular components is left on the culture surface (3). This residual "microexudate" is rich in high molecular weight acidic proteoglycans (27). Surfaces coated with microexudate from rapidly proliferating cultures support enhanced growth of cells at low population density, although there is no effect with larger inocula (30, 33).

Culp (2) has proposed a model in which the negatively charged proteoglycans of the microexudate from the cells bind to the culture surface, which is normally coated with serum proteins. The proteoglycans on the culture surface then cross link through Ca^{++}-bridges to negatively charged

components of the cell surface. If this model is valid, the basic polymers in our experiments would form a surface that is electrostatically favorable to accommodate a carpet of acidic cell-derived microexudate, and might possibly also stimulate deposition of the microexudate. The fact that cells can be harvested from polylysine-coated surfaces by normal trypsinization procedures without loss of viability suggests that attachment is of the same general nature as that on untreated surfaces (our unpublished results).

In the past, it has generally been assumed that a negatively charged surface is desirable for cell growth, and that cationic bridges are involved in the attachment and flattening process (14, 19). Polylysine significantly reduces the net negative charge of Corning tissue culture petri dishes as measured by crystal violet binding (19). However, we are not certain whether the coating generates a net positive charge or merely reduces the net negative charge. Data presented by Macieira-Coelho and Avrameas (13) suggest that cultured cells will attach to positively-charged surfaces.

Unmodified "bacterial" polystyrene petri dishes have hydrophobic surfaces and will not support attachment or clonal growth of cells in our current media. After treatment with polylysine, they do support both cellular attachment and the formation of visible colonies, although these results are not equal to those obtained on "tissue culture" dishes coated with polylysine. Glass petri dishes coated with polylysine are equivalent in growth-supporting ability to treated tissue culture plastic dishes. A net surface charge appears to be needed to obtain sufficient attachment of polylysine to promote optimum growth.

Although surfaces coated with the natural basic polymers, histone and protamine, stimulate clonal growth somewhat better than surfaces coated with polylysine (Table II), we prefer the synthetic, chemically defined polylysine as the coating agent of choice to avoid the possible introduction of biologically derived trace contaminants into the culture system. The fact that coating the culture surfaces with any of a variety of natural and synthetic polymers that are positively charged at physiological pH will improve clonal growth suggests that the critical factor is the positive charge rather than the chemical nature of the polymer. If this is the case, it should be possible to introduce a chemical modification during the manufacturing process that will result in culture vessels with positively charged surfaces that are far better for cellular growth than those that are currently available.

We are grateful to Dr. George Veomett for bringing to our attention the use of polylysine-coated surfaces in microscopy. We thank Kerstin McKeehan for excellent technical assistance and Karen Brown for assistance in preparation of the manuscript. This work was supported by contract no. 223-74-1156 from the Bureau of Biologics, United States Food and Drug Administration and grant no. AG00310-02 from the National Institute on Aging.

Received for publication 26 April 1976, and in revised form 21 July 1976.

REFERENCES

1. CLARK, G. D., M. G. P. STOKER, A. LUDLOW, and M. THORNTON. Requirement of serum for DNA synthesis in BHK21 cells: effects of density, suspension and virus transformation. *Nature (Lond.).* **227:**798–801.
2. CULP, L. A. 1974. Substrate-attached glycoproteins mediating adhesion of normal and virus-transformed mouse fibroblasts. *J. Cell Biol.* **63:**71–83.
3. CULP, L. A., and J. F. BUNIEL. 1976. Substrate-attached serum and cell proteins in adhesion of mouse fibroblasts. *J. Cell Physiol.* **88:**89–106.
4. DULBECCO, R., and J. ELKINGTON. 1973. Conditions limiting multiplication of fibroblastic and epithelial cells in dense cultures. *Nature (Lond.).* **246:**197–199.
5. EASTY, G. C., D. M. EASTY, E. J. AMBROSE. 1960. Studies of cellular adhesiveness. *Exp. Cell Res.* **19:**539–548.
6. FISHER, H. W., T. T. PUCK, and G. SATO. 1958. Molecular growth requirements of single mammalian cells: the action of fetuin in promoting cell attachment to glass. *Proc. Natl. Acad. Sci. U. S. A.* **44:**4–10.
7. GRINNELL, F. 1974. Studies on the mechanism of cell attachment to a substratum. Evidence for three biochemically distinct processes. *Arch. Biochem. Biophys.* **160:**304–310.
8. HAM, R. G. 1965. Clonal growth of mammalian cells in a chemically defined, synthetic medium. *Proc. Natl. Acad. Sci. U. S. A.* **53:**288–293.
9. HAM, R. G., S. L. HAMMOND, and L. L. MILLER. 1976. Critical adjustment of cysteine and glutamine concentrations for improved clonal growth of WI-38 cells. *In Vitro (Rockville).* In press.
10. HAYFLICK, L., and P. S. MOOREHEAD. 1961. The serial cultivation of human diploid strains. *Exp. Cell Res.* **25:**585–621.
11. KLEBE, R. J. 1974. Isolation of a collagen-dependent cell attachment factor. *Nature (Lond.).* **250:**248–251.

12. LIEBERMAN, I., and P. OVE. 1958. A protein growth factor for mammalian cells in culture. *J. Biol. Chem.* **233:**637–642.
13. MACIEIRA-COELHO, A., and S. AVRAMEAS. 1972. Modulation of cell behavior in vitro by the substratum in fibroblastic and leukemic mouse cell lines. *Proc. Natl. Acad. Sci. U. S. A.* **69:**2469–2473.
14. MARTIN, G. R., and H. RUBIN. 1974. Effects of cell adhesion to the substratum on the growth of chick embryo fibroblasts. *Exp. Cell Res.* **85:**319–333.
15. MAZIA, D., G. SCHATTEN, and W. SALE. 1975. Adhesion of cells to surfaces coated with polylysine. Applications to electron microscopy. *J. Cell Biol.* **66:**198–200.
16. MCKEEHAN, W. L., W. G. HAMILTON, and R. G. HAM. 1976. Selenium is an essential nutrient for growth of WI-38 human diploid fibroblasts in culture. *Proc. Natl. Acad. Sci. U. S. A.* **73:**2023–2027.
17. NORDLING, S. 1967. Adhesiveness, growth behavior and charge density of cultured cells. *Acta Pathol. Microbiol. Scand. Suppl.* **192:**1.
18. PAUL, D., M. HENAHAN, and S. WALTER. 1975. Changes in growth control and growth requirements associated with neoplastic transformation in vitro. *J. Natl. Cancer Inst.* **53:**1499–1503.
19. RAPPAPORT, C., J. P. POOLE, and H. P. RAPPAPORT. 1960. Studies on properties of surfaces required for growth of mammalian cells in synthetic medium. *Exp. Cell Res.* **20:**465–510.
20. REVEL, J. P. and K. WOLKEN. 1973. Electron microscope investigations of the underside of cells in culture. *Exp. Cell Res.* **78:**1–14.
21. ROSENBERG, M. D. 1960. Microexudates from cells grown in tissue culture. *Biophys. J.* **1:**137–159.
22. RYSER, H. J. P., and R. HANCOCK. 1965. Histones and basic polyamino acids stimulate the uptake of albumin by tumor cells in culture. *Science (Wash. D. C.).* **150:**501–503.
23. SCHELL, P. L. 1976. Uptake of polynucleotides by mammalian cells. XIV. Stimulation of the uptake of polynucleotides by poly(L-lysine). *Biochim. Biophys. Acta.* **340:**323–333.
24. STOKER, M., C. O'NEILL, S. BERRYMAN, and V. WAXMAN. 1968. Anchorage and growth regulation in normal and virus-transformed cells. *Int. J. Cancer.* **3:**683–693.
25. STOKER, M. G. P. 1973. Role of diffusion boundary layer in contact inhibition of growth. *Nature (Lond.).* **246:**200–203.
26. TAYLOR, A. C. 1961. Attachment and spreading of cells in culture. *Exp. Cell Res. Suppl.* **8:**154–173.
27. TERRY, A. H., and L. A. CULP. 1974. Substrate-attached glycoproteins from normal and virus-transformed cells. *Biochemistry.* **13:**414–425.
28. WALLIS, C., B. VER, and J. L. MELNICK. 1969. The role of serum and fetuin in the growth of monkey kidney cells in culture. *Exp. Cell Res.* **58:**271–282.
29. WEISS, L. 1959. Studies on cellular adhesion in tissue culture. I. The effect of serum. *Exp. Cell Res.* **17:**499–507.
30. WEISS, L., G. POSTE, A. MACKEARNIN, and K. WILLETT. 1975. Growth of mammalian cells on substrates coated with cellular exudates. I. Effect on cell growth at low population densities. *J. Cell Biol.* **64:**135–145.
31. WHITFIELD, J. F., A. D. PERRIS, and T. YOUDALE. 1968. The role of calcium in the mitotic stimulation of thymocytes by detergents, agmatine and poly-L-lysine. *Exp. Cell Res.* **53:**155–165.
32. WITKOWSKI, J. A., and W. D. BRIGHTON. 1972. Influence of serum on attachment of tissue cells to glass surfaces. *Exp. Cell Res.* **70:**41–48.
33. YAOI, Y., and T. KANASEKI, T. 1972. Role of microexudate carpet in cell division. *Nature (Lond.).* **237:**283–285.