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Effects of a Serum Spreading Factor on Growth and Morphology of Cells in Serum-Free Medium

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A heat-sensitive, trypsin-sensitive factor that promoted growth and spreading of cells in serum-free, hormone-supplemented medium was partially purified from human serum. The major portion of the proteins in these preparations migrated upon SDS-polyacrylamide gel electrophoresis with a mobility consistent with molecular weights between 60,000 and 90,000. The spreading activity, which we have termed serum spreading factor, stimulated growth and spreading of a wide variety of cell types. The serum spreading factor was similar to fibronectin in that it showed an affinity for the plastic cell culture substrate but was shown to be distinct from fibronectin by several criteria. This factor may prove useful in studies of cell attachment and spreading and in studies of the relationship of cell shape and cell proliferation.

Key words: **serum spreading factor, cell proliferation, cell morphology, cell substratum, serum-free medium**

Historically, supplementation of culture medium with serum has been required for maintenance and growth of animal cells in culture. Our laboratory and others have shown recently that for many cell types it is possible to replace the usual serum supplement in culture medium with specific combinations of nutrients, hormones, and purified serum proteins [1–3]. Among the serum proteins stimulatory for growth of some cell types in serum-free medium are factors that mediate the proper attachment and spreading of cells on the plastic or glass culture substrate. In particular, cold-insoluble globulin (CIg), the plasma form of fibronectin, has been useful in this regard [4–6]. In the process of developing serum-free media for various cell lines, we have become involved in the biochemical and biological characterization of another factor present in human serum that appears to be distinct from CIg by several criteria. We have termed this activity serum spreading factor.

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This factor was first reported to exist in human serum by Holmes in 1967 [7]. Two of us (Barnes and Sato) have previously reported that the serum spreading factor is active on the MCF-7 line of human breast cancer cells [8]. Subsequently, we found that this factor influenced growth and spreading of a wide variety of cell types in serum-free medium. These include cell lines derived from rat glioma (C6), mouse neuroblastoma (N18TG-2), mouse embryonal carcinoma (F9, 1003), rat ovary (RF-1), and mouse embryo (3T3, SV40-transformed 3T3), as well as a mouse preadipocyte line (1246) and human fetal lung fibroblasts (WI38) [9, 10]. In this paper, we report the preliminary biochemical characterization of the partially purified serum spreading factor and describe some of the biological effects of these preparations on the C6, 1246, and SV40-transformed 3T3 cell lines in serum-free medium.

MATERIALS AND METHODS

Materials

Bovine insulin, human transferrin, 4-(2-hydroxyethyl)-1-piperazineethene-sulfonic acid (Hepes), crude pancreatic trypsin, soybean trypsin inhibitor, ovalbumin, bovine serum albumin (BSA), linoleic acid, sodium chloride, calcium chloride, potassium and sodium carbonate, and sodium bicarbonate were obtained from Sigma Chemical Company. Fatty-acid-free BSA was obtained from Miles. Antibiotics and powdered formulations of Ham's F12 and Dulbecco-modified Eagle's medium (DME) were obtained from Grand Island Biological Company. Bovine fibroblast growth factor (FGF), multiplication stimulating activity (MSA), human CIg, and rabbit antiserum to human CIg were obtained from Collaborative Research, Inc. Plastic cell culture labware was obtained from Falcon. Glass beads (Number 1014, Class IV-A) were obtained from Ferro, Cataphote Division, Jackson, Miss. Materials for SDS-polyacrylamide gel electrophoresis (SDS-PAGE) were obtained from Biorad. Plates for double immunodiffusion were obtained from Hyland. Fetal calf serum was obtained from Reheis. Freunds complete adjuvant was obtained from Difco.

Preparation and Characterization of Serum Spreading Factor

Partially purified serum spreading factor was prepared by a modification of the procedure of Holmes [7]. Outdated human plasma was dialysed overnight against 0.8% sodium chloride, and clotted by the addition of 1 mg/ml calcium chloride, and the clot removed by low speed centrifugation (700g, 30 min). The resulting serum was adjusted to pH 8.0 with 1 N sodium hydroxide, and 80 ml was put on a 50 cm by 2.5 cm column previously packed with acid-washed glass beads and equilibrated with 0.6 M sodium bicarbonate (pH 8.0). Bed volume of the column was approximately 250 cm^3. The flow rate of the column was approximately 2 ml/min. The chromatography was carried out at room temperature. The column was eluted sequentially with 250 ml of 0.6 M sodium bicarbonate (pH 8.0), 125 ml 0.6 M sodium bicarbonate-0.2 M sodium carbonate (pH 9.5), 125 ml 0.15 M potassium bicarbonate-0.05 M potassium carbonate (pH 9.5), and 300 ml of 0.6 M potassium bicarbonate-0.2 M potassium carbonate (pH 9.7). Ten milliliter fractions were collected. Spreading activity in fractions eluted from the column was routinely identified by microscopic examination 60–90 min after the addition of 20–50 μl of the fractions to C6 cells seeded in 35 mm diameter plates in 2 ml of serum-free medium containing 2.5 mg/ml BSA. A quantitative spreading assay is described below.

Peak fractions eluted with 0.6 M potassium bicarbonate-0.2 M potassium carbonate were pooled and concentrated with Amicon CF 25 Centriflo membrane cones. The potassium carbonate buffer was exchanged for 10 mM potassium hydroxide by repeated concentration and dilution utilizing the Centriflo membrane cones, and the preparation was sterilized by filtration. All tubes used for collection and storage of the serum spreading factor preparations were polypropylene. Protein assay procedure was the method of Bradford [11]. SDS-PAGE was carried out as described by Weber and Osborn [12]. Polyacrylamide concentration was 10%.

Antiserum to the serum spreading factor preparations was raised in rabbits by injections of the antigen in Freunds complete adjuvant on three occasions at 2 week intervals. At each injection, 400 μg of the serum spreading factor preparation was injected subcutaneously at multiple sites. Antiserum was collected 2 weeks after the third injection. Double immunodiffusion plates were developed in 48 h at room temperature in a humidified atmosphere.

Experiments designed to determine if the serum spreading factor preparations were inactivated by incubation with trypsin were carried out using trypsin covalently bound to polyacrylamide beads (Sigma T8386). Approximately 100 μg of the spreading factor preparation was incubated overnight at room temperature in 1 ml of phosphate buffered saline (PBS) containing 20 units of insoluble enzyme. The spreading activity in aliquots of this incubation mixture was assayed as described, after removal of the beads by centrifugation. Aliquots of controls, in which the trypsin-bead complex was incubated in PBS without the spreading factor and then removed by centrifugation, had no morphological effect on cell spreading induced by active spreading factor preparations. Incubation of spreading factor with a trypsin-bead complex, in which the trypsin had been previously inactivated, had no effect on the spreading activity.

Experiments designed to determine which of the components appearing on stained SDS-polyacrylamide gels was the active protein were carried out by cutting gels of unreduced, unboiled samples of the serum spreading-factor preparations into 25 slices, homogenizing each slice in a glass dounce with 2 ml of serum-free medium, and pretreating plates with this medium as described under Cell Culture Experiments below. Under these conditions, the active factor attached to the plastic culture dish (see Results), while most of the SDS was removed before cells were plated for the standard spreading assay.

Cell Culture Experiments

The C6 rat glioma cell line (C6BU-1) was obtained from Dr. M. Nirenberg, National Institutes of Health. Other C6 sublines from different sources responded in a manner identical to the response of these cells with regard to the properties reported in this paper. Stock cultures of these cells were maintained in DME containing 1.2 g/l sodium bicarbonate, 25 mM Hepes (pH 7.3), 190 IU/ml penicillin, 0.2 mg/ml streptomycin, 25 μg/ml ampicillin, and 10% fetal calf serum. The synthetic nutrient medium used for experiments with C6 was a mixture of 3 parts DME to 1 part Ham's F-12, containing 1.2 g/l sodium bicarbonate, 25 mM Hepes (pH 7.3), 190 IU/ml penicillin, 0.2 mg/ml streptomycin, 25 μg/ml ampicillin, and a mixture of trace elements as previously described [2]. For experiments studying growth and spreading of C6, exponentially growing cells from stock cultures were removed from plates with a solution of 0.1% (W/V) trypsin in a Ca-Mg-free PBS containing 0.9 mM EDTA. The detached cells were suspended in the serum-free, synthetic nutrient medium described above containing 0.2 mg/ml soybean trypsin inhibitor,

and centrifuged; the pellet was suspended in fresh serum-free medium without trypsin inhibitor. Cells from the suspension were inoculated into 35 mm culture dishes (2.5×10^4 cells/plate) containing 2 ml of serum-free medium. Supplements as indicated in the figures and tables (eg, insulin, transferrin, FGF) were added to the plates as small volumes of concentrated sterile stocks.

For the C6 growth assay, the number of cells attached to the culture dish was determined 5 days after plating by detaching the cells with the trypsin-EDTA solution and counting the resultant cell suspensions with a Coulter counter, model Z_f. Greater than 99% of the total number of cells in the dish were found to be attached to the dish and counted by this procedure. For the C6 spreading assay, 100 to 250 cells in 5–8 random fields at each concentration of spreading factor were examined and the number of completely rounded cells determined and expressed as a percentage of the total number of cells counted. In experiments in which plates were treated with serum spreading factor preparations, dishes were incubated for 24 h at 37°C with 1 or 2 ml of medium supplemented with the spreading factor at the indicated concentrations and then thoroughly washed with medium before adding the cells. Plates were incubated with the washes for 2 h at 37°C.

The 1246 line is an adipogenic clone derived from a phenotypically unstable myogenic line, T984 [13], isolated from a mouse teratocarcinoma, and was obtained from Dr. M. Darmon, UCSD. Experiments with the 1246 cells were carried out essentially as described for C6 except that the basic nutrient medium for 1246 was a 1:1 mixture of DME and Ham's F12, and cells were detached from flasks or plates by incubating in Ca-Mg-free PBS containing 0.9 mM EDTA, without the use of trypsin or trypsin inhibitor. SV40-transformed Balb/c-3T3 (SV-3T3) cells were obtained from Dr. R. Holley, Salk Institute. Experiments with SV-3T3 cells were carried out essentially as described for C6.

RESULTS

Serum Spreading Factor

Partially purified serum spreading factor was prepared by passing human serum at pH 8.0 over glass beads and eluting with sodium and potassium carbonate buffers (see Materials and Methods). Fractions were collected in 10 ml aliquots. An elution profile of the glass-bead chromatography of human serum (80 ml) under these conditions is shown in Figure 1. The relative sizes of the OD_{280} peaks eluted with the different buffers varied somewhat with different serum batches. This was particularly true with the material eluted with water (fractions 46–50) and the material eluted with 0.15 M potassium bicarbonate-0.05 M potassium carbonate (fractions 59–60). Most of the serum protein passed through the column unretarded (fractions 1–15). Cell spreading activity was found in fractions 1 through 40 and fractions 68 through 73. The latter fractions represent material eluted with 0.6 M potassium bicarbonate-0.2 M potassium carbonate. Fractions 69–71 were pooled, concentrated, desalted, and sterilized. Approximately 3 mg of protein was recovered in fractions 69–71 from approximately 5 g of protein in the starting material. The spreading promoting activity in the material was lost upon incubation at 100°C for 5 min or upon incubation with trypsin.

The SDS-PAGE pattern of the material in fractions 69–71 is shown in Figure 2. Most of the protein in reduced samples of the spreading-factor preparations migrated on SDS-PAGE in a manner consistent with molecular weights between 60,000 and 90,000.

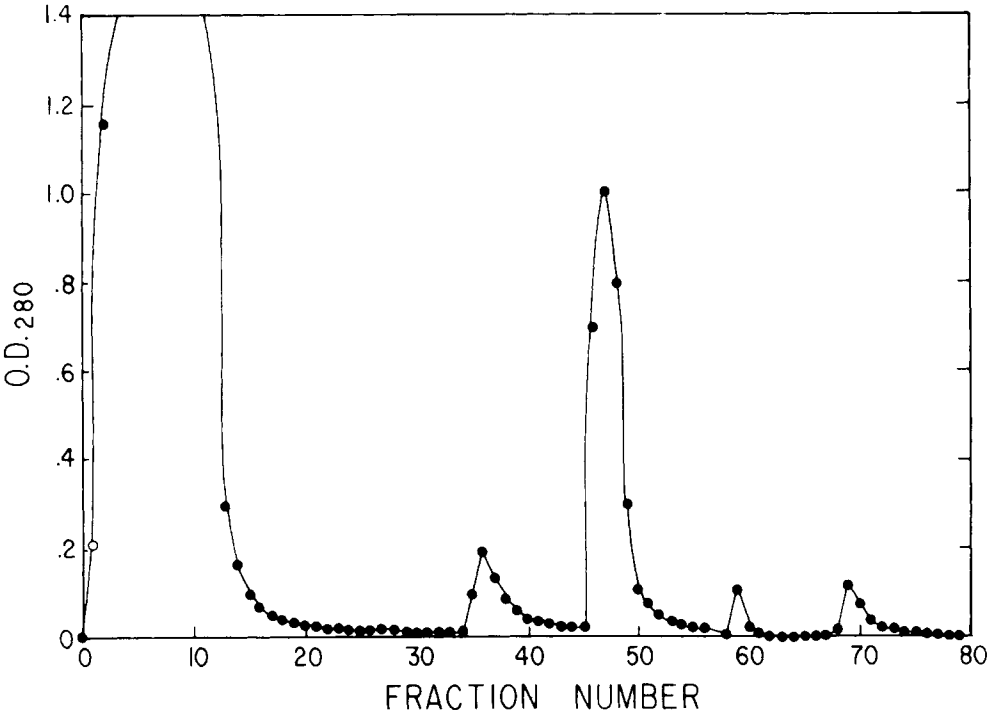

Fig. 1. Elution pattern: glass-bead chromatography of human serum at alkaline pH.

Proteins of higher mobility (15,000–60,000 daltons) were also present. The major band in this area appeared to have a molecular weight of approximately 70,000–80,000, running between BSA (molecular weight 67,000) and human transferrin (molecular weight 86,000). We estimate that this band represents about one third of the total protein in the preparations. No protein migrating in a manner consistent with a molecular weight of 100,000 or higher was detected on the gels. SDS-PAGE of unreduced samples of the serum spreading factor preparations resulted in a pattern similar to that of Figure 2, although the bands were less well resolved.

Fibronectin or CIg is reported to stimulate adhesion or spreading of some types of cells in culture [5, 6, 14–16]. The serum spreading factor described in this paper appeared to be distinct from CIg by several criteria. No 220,000 dalton protein could be detected on SDS-PAGE of spreading factor preparations reduced with mercaptoethanol (Fig. 2). Also, no precipitin band was detected in double immunodiffusion plates in which antiserum to human CIg was tested against the spreading factor preparations (Fig. 3). Likewise, no precipitin band was detected when antiserum raised against the spreading factor preparations was tested against human CIg. Each of these antisera produced precipitin bands against their respective antigens. Finally, active preparations of the spreading factor have been made in our laboratory using as a starting material plasma which had been passed through a gelatin-conjugated sepharose column, which removes CIg [17]. Comparison of theoretical

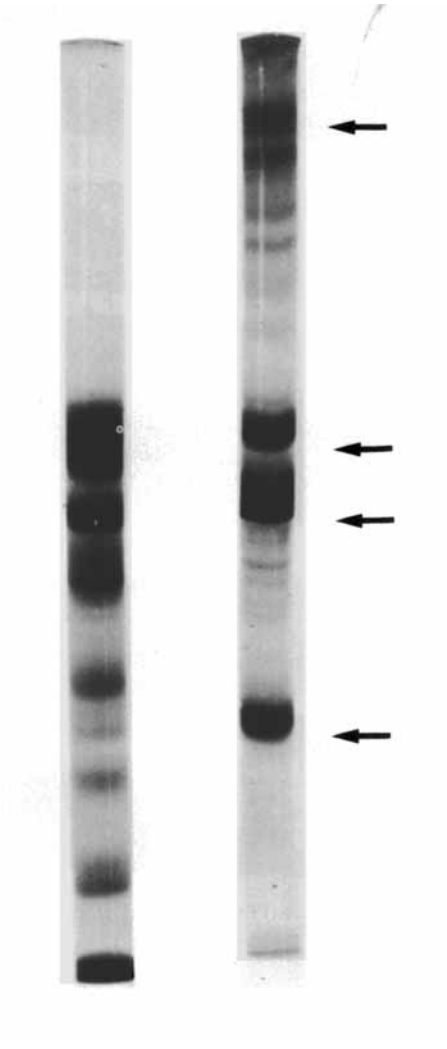

Fig. 2. SDS-polyacrylamide gel electrophoresis of serum spreading factor. Left: 15 μg serum spreading factor, reduced; right: 5 μg molecular weight standards, reduced. Standards, top to bottom (arrows): human CIg; human transferrin; BSA; ovalbumin.

vs actual yield of CIg subsequently eluted from such a column with 8 M urea indicated that all of the CIg had been removed from the plasma which passed through the column under the conditions of the experiment.

Effects of Serum Spreading Factor on C6 Rat Glioma

Figure 4 shows micrographs of C6 cells after 3 days in the presence of serum-free medium containing insulin (25 μg/ml), transferrin (25 μg/ml), FGF (50 ng/ml), and varying concentrations of the partially purified serum spreading factor. Insulin, transferrin, and

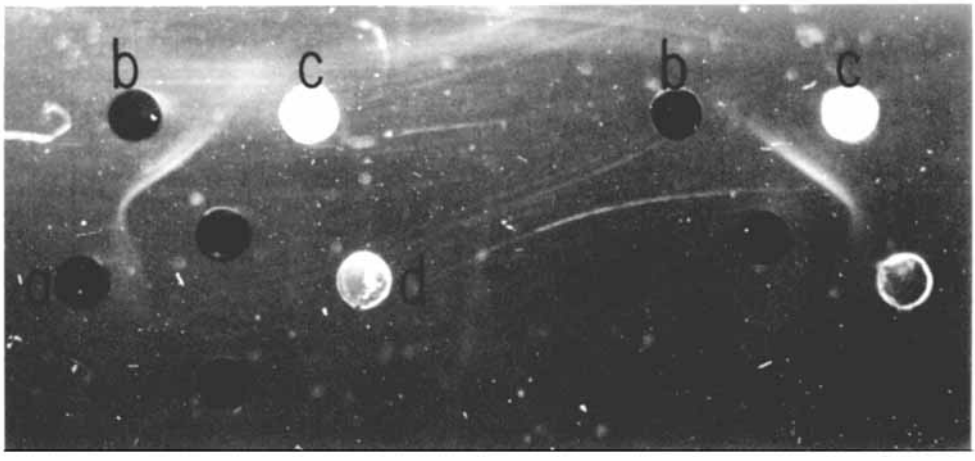

Fig. 3. Double immunodiffusion of serum spreading factor, CIg, and antiserum to each. a: 1 μg of serum spreading factor; b: 2 μg of serum spreading factor; c: 2 μg of CIg; d: 1 μg CIg. Center well, left, 5 μl antiserum to serum spreading factor; center well, right, 5 μl antiserum to CIg.

FGF have been reported previously to be stimulatory for growth of C6 in serum-free medium [18]. Although some effect of the spreading factor on cell morphology could be detected at doses as low as 60 ng/ml, maximum effect was seen at about 4 μg/ml. At this dose, the morphology of the C6 cells was similar to that of cells in medium containing 10% serum. Under the conditions of Figure 4, the spreading factor preparations caused a dose-dependent increase in cell number in addition to influencing cell spreading. Addition of the spreading factor in the absence of insulin, transferrin, and FGF did not cause an increase in cell number.

It is possible to quantitate both the growth-stimulatory effect and the cell-spreading effect. Figure 5 shows the dose-response relationship of spreading-factor preparations for both cell spreading and cell number. Cell spreading was assayed by counting the number of rounded, completely unspread cells in several random fields at each concentration of spreading factor and expressing the number of rounded cells as a percentage of the total number of cells counted. As the concentration of spreading factor was increased from 0 to 5 μg/ml, the number of unspread cells decreased from near 100% to about 5%, and the final cell number increased 3.5-fold. At high concentrations of the spreading factor (10–25 μg/ml), the stimulatory effects were not seen. The data of Figure 5 suggest that there is a reasonably good correlation between the concentration dependency for the spreading activity in the spreading factor preparations and the concentration dependency for the growth promoting activity in these preparations. CIg had no effect on cell number for C6 under these conditions. However, effects of CIg on cell number in the presence of insulin, transferrin, and FGF may be seen if the culture dishes are first coated with polylysine [19].

Serum at concentrations as high as 500 μg of protein/ml added to C6 cultures containing insulin, transferrin, and FGF did not substitute for the spreading factor in regard to cell spreading. This would suggest that the factor responsible for the spreading activity in

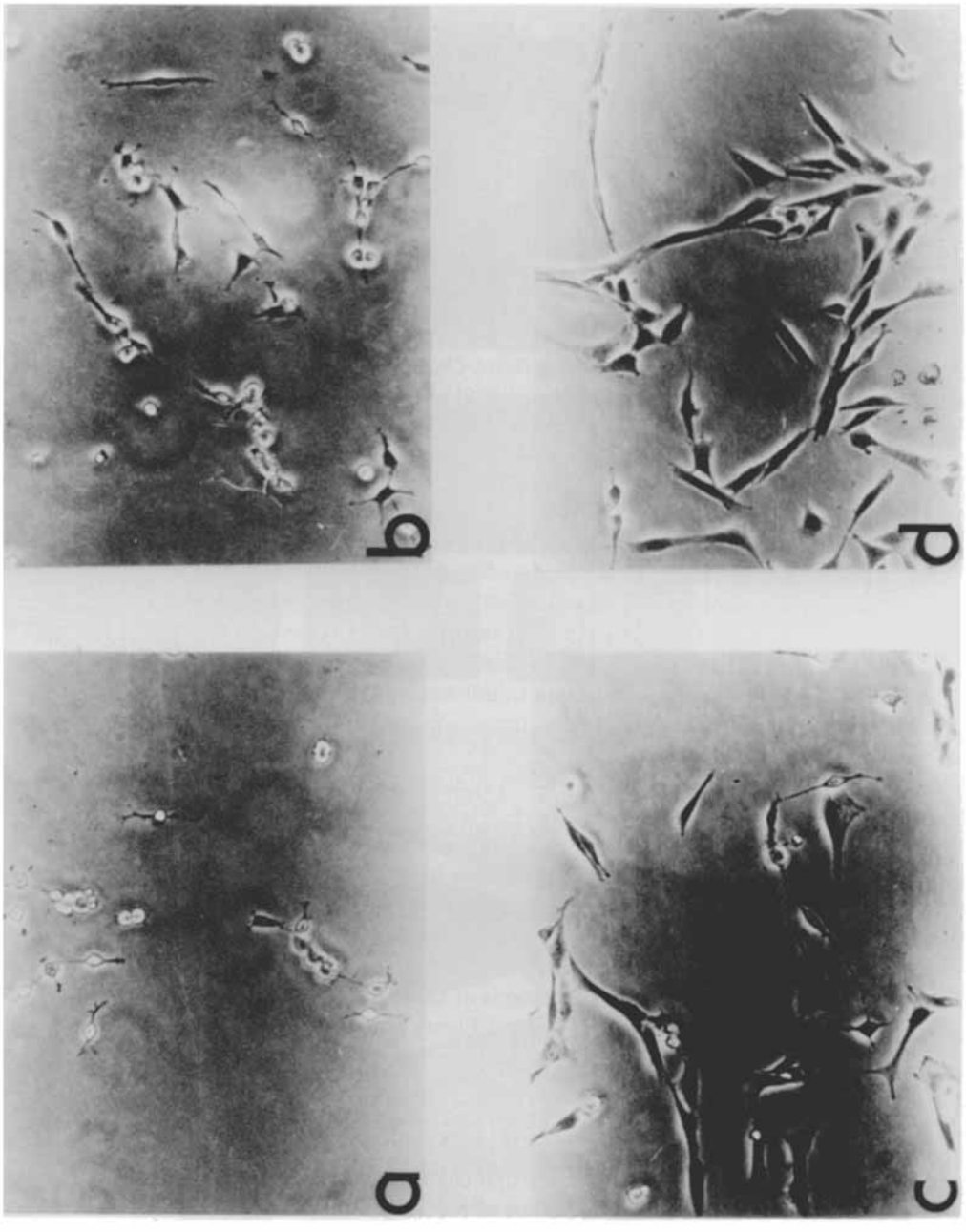

Fig. 4. Effect of serum spreading factor on the morphology of C6 rat glioma. Cells were plated as described in Materials and Methods in serum-free medium containing insulin (25 μg/ml), transferrin (25 μg/ml), and FGF (50 ng/ml) plus no spreading factor (a), or spreading factor at 60 ng/ml (b), 1.3 μg/ml (c), and 3.3 μg/ml (d). Pictures were taken 3 days after plating.

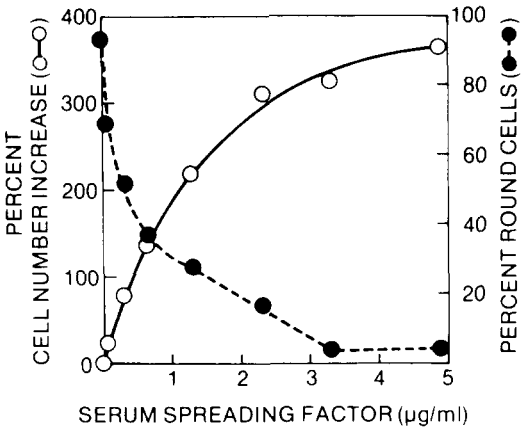

Fig. 5. Effect of serum spreading factor on growth and spreading of C6. Cells were plated as described in Materials and Methods in serum-free medium containing insulin (25 μg/ml), transferrin (25 μg/ml), FGF (50 ng/ml), and the indicated concentrations of the serum spreading factor. Growth and spreading assays were carried out as described in Materials and Methods. Zero percent cell number increase represents cell number in the presence of insulin, transferrin, and FGF in the absence of serum spreading factor. Cell number in this condition was 1.5×10^5 cells/35 mm plate. Plating efficiency under the conditions of this experiment for C6 cells was near 100%.

the preparation was purified 100-fold or more from serum. Such a statement may be misleading, however, since inhibitors of spreading, such as albumin, exist in serum and are separated from the spreading activity by the isolation procedure.

In the experiment described by Figure 5, the spreading factor was added directly to plates containing the serum-free medium in which the cells were incubated. We have found that the factor apparently has an affinity for plastic, and it was possible to pretreat the cell culture dishes with the spreading factor before the cells were added and produce effects identical to those produced if the spreading factor was added directly to the medium. In Figure 6 the increase in cell number due to the spreading factor showed the same concentration dependence whether the spreading factor was added directly to the medium or the dishes were preincubated with medium containing varying concentrations of the spreading factor. The concentration-dependent effect of the spreading factor on cell morphology (as shown in Fig. 4 and Fig. 5) was also maintained on dishes pretreated with the spreading factor. Since some of the inactive proteins in the serum spreading factor preparations probably do not stick to the culture dish, the precoating procedure may be effecting a further purification of the spreading and growth promoting activity.

Effects of Serum Spreading Factor on 1246 Mouse Teratocarcinoma Cells

As with C6, the serum spreading factor caused an increase in cell number for the 1246 cell line in serum-free medium in the presence of insulin, transferrin, and FGF (Table I), and addition of the spreading factor alone did not cause an increase in cell number. Also as with C6, the spreading factor was active on 1246 cells if dishes were pretreated with it rather than adding it to the medium. Unlike C6, the 1246 line showed a similar response to CIg under these conditions, although the response to CIg was somewhat smaller

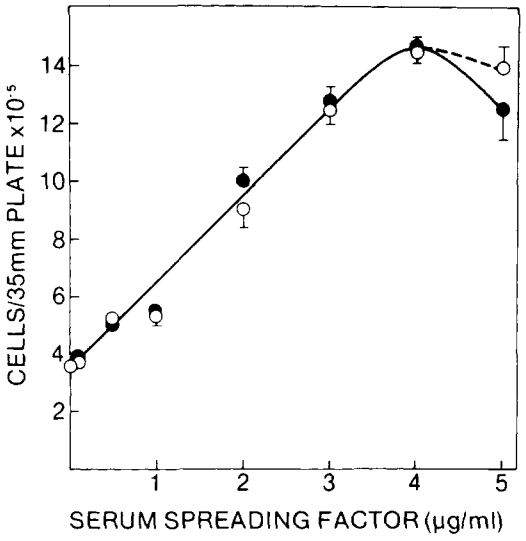

Fig. 6. Effect of serum spreading factor pretreated dishes on growth of C6. Cells were plated, dishes pretreated, and growth assayed as described in Materials and Methods. (•): serum spreading factor added to the medium; (○): serum spreading factor treated dishes.

than the response to the spreading factor. CIg had no effect if added to medium containing insulin, transferrin, FGF, and the spreading factor. The serum spreading factor can also replace the CIg requirement for some other cell lines (eg, RF-1, SV40-transformed 3T3) in serum-free, hormone-supplemented medium [9].

The 1246 cells were capable of attaching and spreading on the culture dish to a greater extent than C6 in the presence of insulin, transferrin, and FGF and the absence of either CIg or the spreading factor. However, addition of the spreading factor did result in a morphological change for these cells from a flattened, epitheloid appearance to a fibroblastic and spindle-shaped morphology, which was more similar to the morphology of these cells in serum-containing medium (Fig. 7). This difference in morphology was maintained in cultures at both low and high cell densities. Under conditions in which cell spreading was inhibited, such as if cells were plated in the presence of 1 mg/ml BSA, spreading of 1246 cells was found to be completely dependent on the presence of the serum spreading factor.

Effects of Serum Spreading Factor on SV40-Transformed 3T3 Cells

A line of SV40-transformed Balb/c-3T3 cells can be grown in serum-free monolayer culture in a medium consisting of a 3:1 mixture of DME and Ham's F12 supplemented with insulin (250 μg/ml) or MSA (100 ng/ml), transferrin (500 ng/ml), fatty-acid-free BSA (1 mg/ml), and linoleic acid (5 μg/ml) [6]. A properly conditioned substratum is also required for the growth of these cells in the absence of serum. This requirement could be met by pretreating plastic tissue culture surfaces with either serum spreading factor or CIg (Fig. 8). As shown, cells plated into either serum spreading factor or CIg-pretreated dishes divided logarithmically with a mean generation time of 20 h and, in each case, reached a final cell density of 2×10^5 cells/cm^2; both of these growth parameters are similar to those

TABLE I. Effect of CIg and Serum Spreading Factor on the Growth of 1246 Cells

Medium supplements[a]		Cells/35 mm plate × 10^{-5}
Insulin + transferrin		0.60
Insulin + transferrin + FGF		1.90
Insulin + transferrin + FGF + CIg,	0.3 μg/ml	2.20
	1.0 μg/ml	2.53
	3.0 μg/ml	3.00
Insulin + transferrin + FGF		
+ serum spreading factor,	0.3 μg/ml	3.10
	1.0 μg/ml	3.70
	3.0 μg/ml	3.80

[a]Concentrations of insulin, transferrin, and FGF were 10 μg/ml, 5 μg/ml, and 100 ng/ml, respectively. Cell number was determined 3 days after plating.

for cells cultured in serum-supplemented medium. There was no significant increase in the number of cells per dish when these cells were seeded directly onto bare plastic surfaces in the absence of either of these proteins.

While the serum spreading factor and CIg acted similarly in that they both were capable of conditioning the tissue culture substratum, making it more amenable for cellular growth, the growth response of SV40 Balb/c-3T3 cells as a function of the concentration used to precoat dishes was quite different for each of these factors (Fig. 9). Under the experimental conditions employed, CIg was maximally active at 25 μg/dish; a half-maximal effect occurred at 3 μg/dish. The highest level of CIg tested (100 μg/dish) was as effective as lower but optimal doses. In contrast, the serum spreading factor was maximally active at 1 μg/dish, while a half-maximal response was seen at 0.25 μg/dish. Increasing the amount of spreading factor in the preincubation medium above 5 μg/dish resulted in a gradual decline in the number of attached cells per dish. At a concentration of 50 μg/dish, the spreading factor preparation was ineffective in supporting cell attachment and growth. Both the spreading factor and CIg, at optimal levels, promoted the attachment and growth of SV-3T3 cells to a similar extent, and a mixture of an optimal amount of each of these factors was no better than when each were added individually.

DISCUSSION

We have described experiments in which a partially purified factor from human serum influenced growth and spreading of several cell types in culture in serum-free medium. Our laboratory has found that the serum spreading factor is also active on a number of other widely divergent cell types in culture [8–10]. Preliminary experiments indicate that the active factor is a protein of molecular weight between 70,000 and 80,000, which composed approximately one third of the total protein in the preparations used in the experiments described in this paper.

It should be emphasized that the glass-bead column procedure described for the preparation of the spreading factor did not remove all of the spreading activity from serum, and we do not wish to suggest that this factor is the only protein in serum capable of influencing cell spreading in vitro. Indeed, CIg, a high molecular weight serum component,

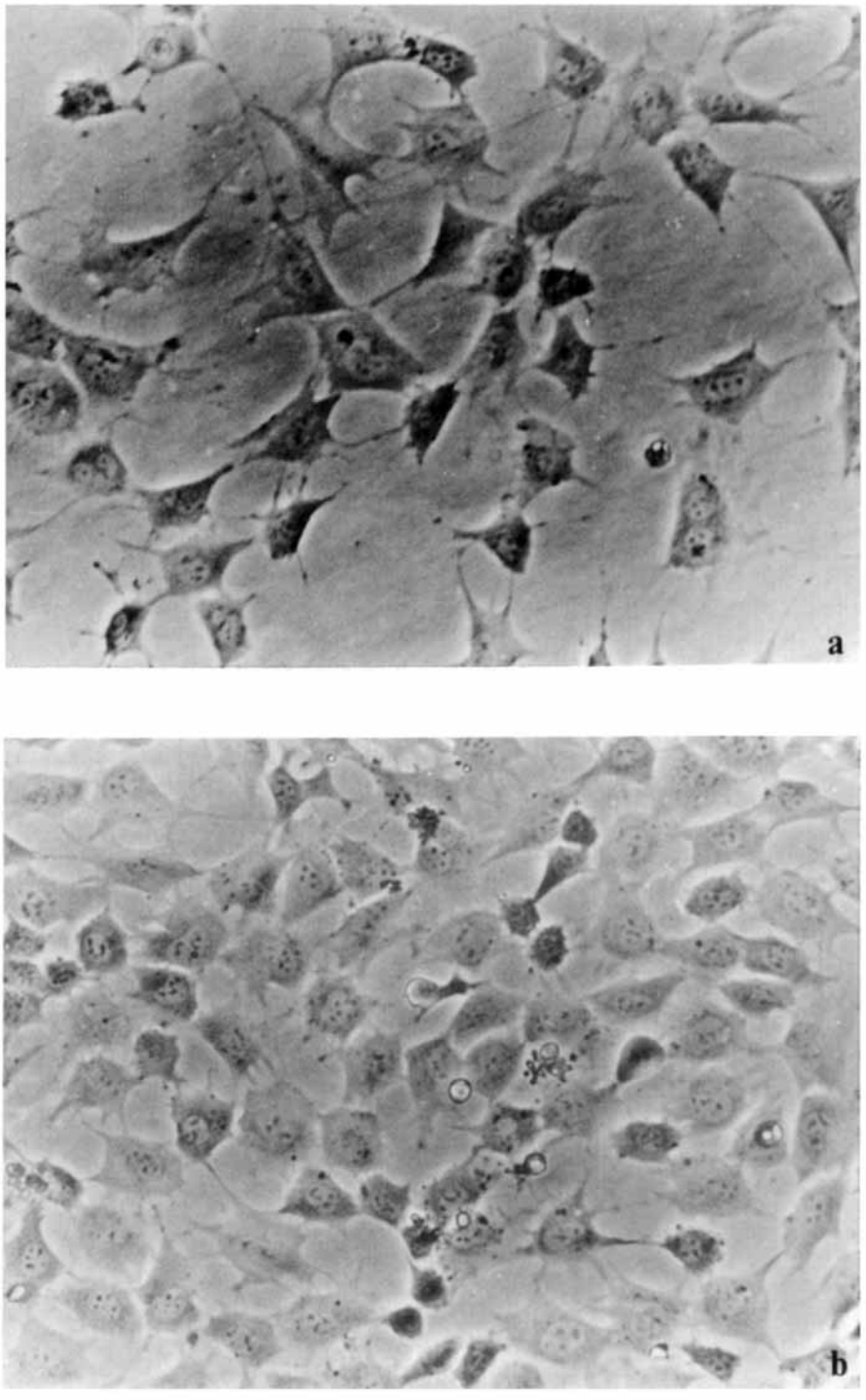

Fig. 7. Effect of serum spreading factor on morphology of 1246 cells. Medium contained as supplements: insulin, transferrin, and FGF (a); insulin, transferrin, FGF, and CIg (b); insulin, transferrin, FGF, and serum spreading factor (c); fetal calf serum (d). Concentrations of the supplements were: insulin, 10 μg/ml; transferrin, 5 μg/ml; FGF, 100 ng/ml; CIg, 3 μg/ml; serum spreading factor, 3 μg/ml; fetal calf serum, 10%.

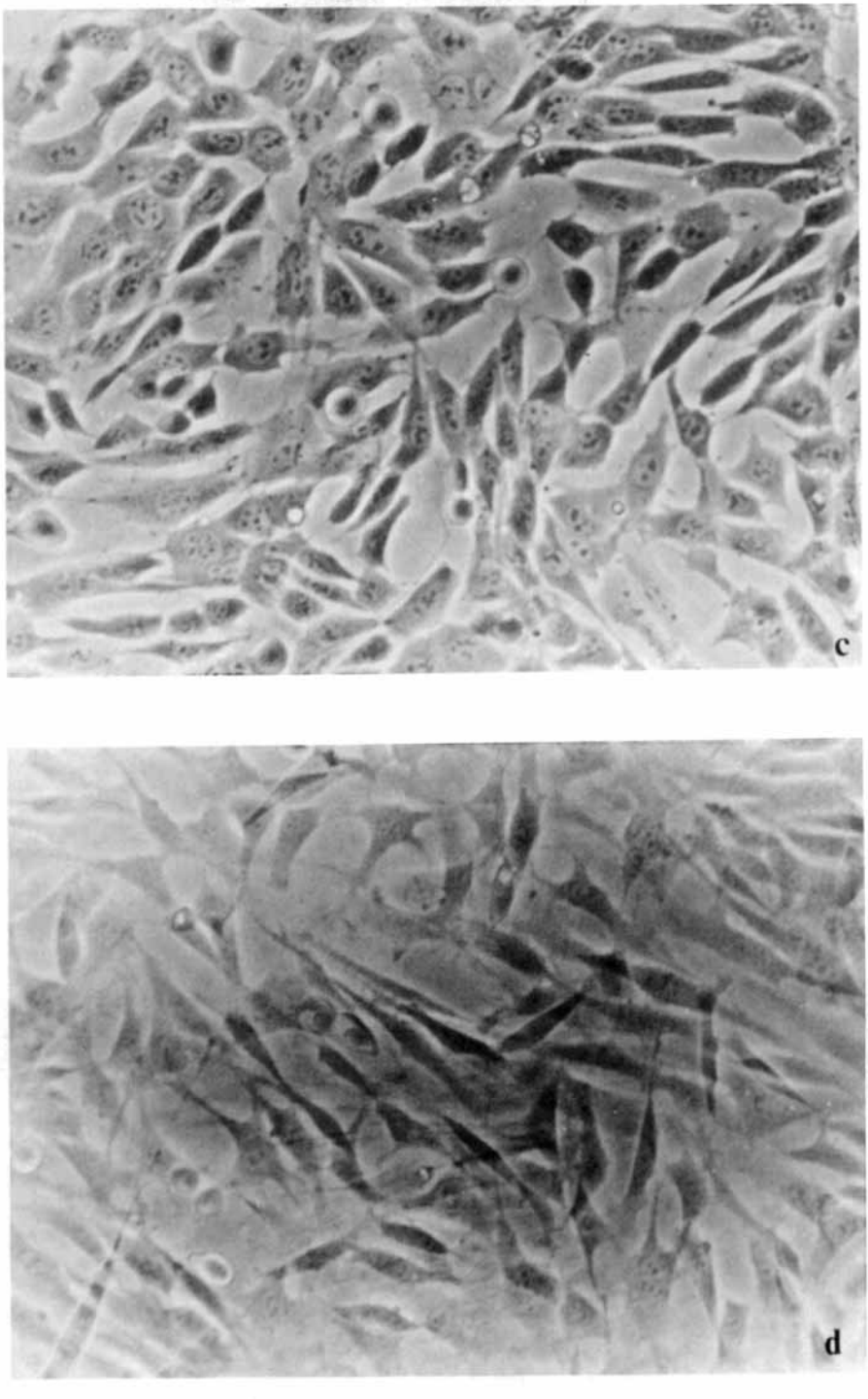

Fig. 7. (continued)

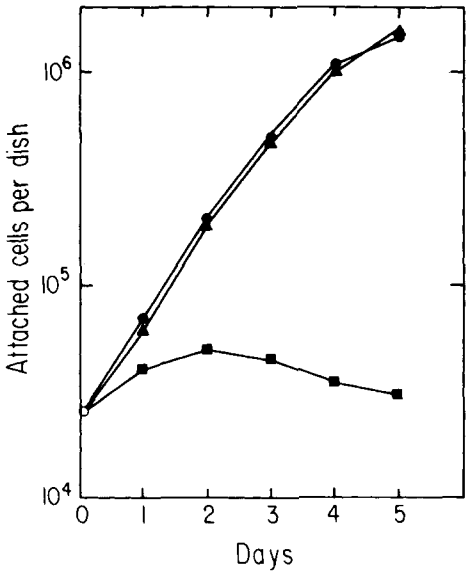

Fig. 8. Effect of serum spreading factor and CIg on growth of SV-3T3 in serum-free medium. Cell culture dishes (35 mm) were pretreated as described in Materials and Methods with 1 ml/plate of serum-free medium (■), serum-free medium supplemented with 5 μg/ml serum spreading factor (●), or 5 μg/ml CIg (▲). SV-3T3 cells (2.5×10^4) were seeded in these plates in serum-free medium and, after the cells attached (3 h), the medium was supplemented with MSA (50 ng/ml), transferrin (5 μg/ml), fatty-acid-free BSA (1 mg/ml), and linoleic acid (5 μg/ml).

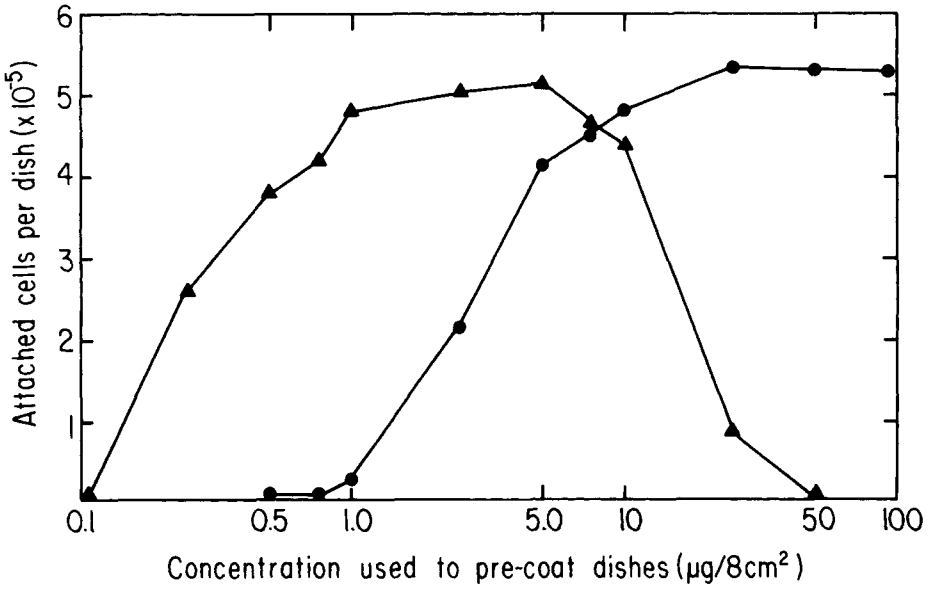

Fig. 9. SV-3T3 growth response as a function of the concentration of serum spreading factor or CIg used to pretreat cell culture dishes. Cell culture dishes were precoated with the indicated concentrations of serum spreading factor (▲) or CIg (●) in 1 ml of serum-free medium as described in Materials and Methods. Two milliliters (2.5×10^4 cells) of a trypsinized cell suspension in medium containing insulin (250 ng/ml), transferrin (5 μg/ml), fatty-acid-free BSA (1 mg/ml) and linoleic acid (5 μg/ml) were plated into each dish. Four days later the number of attached cells in duplicate dishes was determined. Data shown are the average of the duplicate plate cell counts.

is capable of stimulating growth and spreading of several cell types at concentrations comparable to the concentrations that we found to be effective for the spreading factor [4–6, 14–16]. It is likely that several serum factors, some of them as yet unidentified, may play a role in cell adhesion in vitro.

The serum spreading factor shared several properties with CIg. Both exhibit an affinity for plastic and glass surfaces, both are capable of stimulating growth and spreading some cell lines, and both mediate cytokinesis of RF-1 rat ovary cells in serum-free, hormone-supplemented medium [4–6, 14–16]. However, the serum spreading factor seems to be distinct from CIg, since no CIg monomer was detected upon SDS-PAGE of reduced serum spreading factor samples, and no cross reactivity could be demonstrated between antiserum to human CIg and the serum spreading factor preparations or antiserum to the serum spreading factor and human CIg. Knox et al [20] have recently reported that the major portion of the spreading activity in fetal calf serum is separable from CIg by gel filtration and appears in the fractions of serum proteins with molecular weights in the range of 60,000 to 90,000. This factor and the serum spreading factor described in this paper also show some similarity to one of the components of a partially purified serum fraction that promoted cell spreading and has been described by Grinnell [21]. The relationship of the serum spreading factor to these and other serum fractions reported to affect cell spreading [22–24] remains to be determined.

The serum spreading factor preparations used in this study were clearly heterogeneous by examination upon SDS-PAGE, and the possibility exists that the spreading promoting activity and the growth promoting activity reside on different molecules. However, two lines of evidence suggest that the activity may be in the same molecule. The first is the data of Figure 5, in which the concentration dependence of the spreading-factor preparations for both cell spreading and cell number increase were similar. The second is the observation that both the growth-promoting and spreading-promoting activities were absorbed to the cell culture dish if the dishes were incubated with medium containing the spreading factor and these activities could not be removed by several subsequent washes of serum-free medium.

One might speculate that the spreading factor caused an increased cell number for the C6 or 1246 lines 3–5 days after plating simply by improving the initial plating efficiency in cultures to which it had been added. Experiments with both of these lines in which the number of cells attached to the dish 20 h after plating were measured showed that this was not the case and that the same number of cells attached to the dish in the presence or absence of the spreading factor, although gross morphology of the cells under the two conditions was quite different. It is possible that the effect of the spreading factor on cell number was due to a decreased cell generation time in cultures in which it was present. Alternatively, the presence of the spreading factor may allow a higher percentage of the cells plated at the beginning of the experiment to go through the initial rounds of cell division, as is the case with either the spreading factor or CIg for the RF-1 cell line [4, 9]. We have not carried out experiments designed to differentiate between these two possibilities.

Effects of the serum spreading factor and CIg on SV-3T3 cells are shown under two different plating conditions in Figure 8 and Figure 9. In the conditions of Figure 8, cells were first seeded onto pretreated plates in serum-free medium without insulin, transferrin, linoleic acid, or BSA. After the cells had attached to the dish, the above supplements were added. Under these conditions cells attached well, but cell growth was seen only on plates pretreated with CIg or the spreading factor. In the conditions of Figure 9, cells were plated onto pretreated dishes directly in medium containing insulin, transferrin, linoleic acid, and

BSA. The presence of BSA at the time of seeding in this experiment inhibited cell attachment, and this inhibition was reversed by pretreatment of dishes with CIg or the spreading factor. Thus, in Figure 9, part of the increase in cell number due to the spreading factor or CIg was the result of the ability of these factors to promote cell adhesion. However, Figure 8 shows that pretreatment of plates with CIg or the spreading factor caused an increase in cell number even under conditions in which cell adhesion was not a consideration. It may be that these cells, like the RF-1 rat ovary line, require the spreading factor or CIg to carry out cytokinesis.

The striking loss of stimulation at the high concentrations of the spreading factor preparations in Figure 9 may be due to an inhibitory contaminant in these preparations. If this is the case, however, such a contaminant must show an affinity for plastic cell culture surfaces, as do the spreading and growth promoting activities. Alternatively, high concentrations of the spreading factor may act in some way to reverse the stimulatory effect of lower concentrations. Such effects in serum-free medium are well documented in the case of some hormones [29–32]. We have preliminary data indicating that, if cells were pretreated in suspension with concentrations of the spreading-factor preparations that were effective for pretreating dishes and then plated on untreated dishes, cell spreading was extremely poor compared to that seen if untreated cells were plated on pretreated dishes. Cells pretreated in suspension in such a manner and seeded onto pretreated plates spread as well as cells that were not pretreated with the serum spreading factor preparations before plating onto pretreated dishes.

The existence of a purified factor capable of stimulating adhesion and spreading of cells in culture would allow many kinds of interesting experiments, and we are engaged in the further purification of the active 70,000–80,000 dalton protein from the spreading factor preparations used in the experiments described in this paper. Intriguing questions may be asked regarding the role of this protein in vivo. For instance, it is possible that this protein, like fibronectin, is a component of basement membrane [25–28]. The purified factor also should be useful in vitro in studies of the interaction of normal and metastatic and nonmetastatic neoplastic cells with the substratum, studies examining the relationship of cell shape and cell growth, and studies of the signals and processes that control cell shape.

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