Characterization of human serum spreading factor with monoclonal antibody

(cell adhesion/WI-38 fibroblasts/MCF-7 breast carcinoma/platelet/plasma)

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ABSTRACT Serum spreading factor is a glycoprotein isolated from human serum that promotes spreading of a variety of cell types on culture dishes. We developed mouse hybridoma lines secreting monoclonal antibody to serum spreading factor that markedly inhibited the rate of serum spreading factor-promoted spreading of both fibroblastic and epithelial cells in culture. Fibronectin-promoted cell spreading was unaffected by monoclonal antibody to serum spreading factor, and the factor appeared to be distinct by several criteria from fibronectin or laminin. Human serum-promoted cell spreading was partially inhibited by monoclonal antibody to serum spreading factor. The antibody recognized primarily two forms of serum spreading factor that migrated in NaDodSO₄/polyacrylamide gel electrophoresis in a manner consistent with molecular weights of 65,000–70,000 and 75,000–78,000. In addition to being found in plasma, serum spreading factor was also found associated with washed human platelets.

Among the functions that serum serves for cells in culture is the provision of factors that allow proper attachment and spreading of cells on the plastic or glass surface of the culture vessel (1). One such factor is fibronectin; forms of this cell spreading-promoting protein are found in plasma and basal lamina and on cell surfaces (2). Barnes and co-workers have described another cell spreading-promoting glycoprotein, which has been termed serum spreading factor (3–10). This activity was first reported by Holmes to exist in a preparation isolated from human serum by glass bead column chromatography (11). In addition to acting in serum-free cell culture in a manner similar to fibronectin on a variety of cell types, preparations of human serum spreading factor are also capable of mediating effects that cannot be duplicated by fibronectin on the growth, morphology, and differentiative capacity of some cell types (3–6). To better define the nature of the activity in the serum spreading factor preparations, we derived mouse hybridomas secreting monoclonal antibody to human serum spreading factor. Here, we report the isolation of the hybridoma lines and describe studies using monoclonal antibody for the characterization of serum spreading factor.

MATERIALS AND METHODS

Cell Culture. Stock cultures of WI-38 and MCF-7 cells were maintained in a 1:1 mixture of Ham's F12 and Dulbecco's modified Eagle's (DME) media supplemented with sodium bicarbonate at 1.2 g/liter, 10 mM Hepes (pH 7.4), and antibiotics (F12:DME medium)/10% fetal calf serum. P3-X63-AG8 mouse plasmacytoma cells were maintained in DME medium supplemented with bicarbonate and antibiotics, 1 mM sodium pyruvate, 0.1 mM 8-azaguanine, and 10% horse serum. Hybridomas were derived as described (12). For the experiments in Figs. 1, 2, and 3, cell culture dishes (35-mm diameter) were exposed

prior to plating cells to the following series of 1-hr room temperature incubations (1 ml per plate), washing twice with phosphate-buffered saline (P_i/NaCl) after each: (i) serum spreading factor preparation or fibronectin (Bethesda Research Laboratories) at 4 µg/ml in P_i/NaCl, (ii) bovine serum albumin at 1 mg/ml in P_i/NaCl, (iii) monoclonal antibody to serum spreading factor or nonspecific mouse IgG at 15 µg/ml in P_i/NaCl. For the experiment of Fig. 8, the pretreatment of plates with human serum (1% in F12:DME medium) was carried out for 20 hr at 37°C. Cell spreading was scored 1.5 hr after plating cells onto pretreated dishes in F12:DME medium. Incubation with bovine serum albumin after the initial incubation of the dishes with spreading factor was necessary to prevent nonspecific adsorption of mouse IgG to the plastic culture dish and subsequent inhibition of cell spreading unrelated to a specific interaction of serum spreading factor with monoclonal antibody. The bovine serum albumin treatment was not, however, essential to show that serum spreading factor promoted cell spreading; obvious spreading-promoting activity was seen within 15 min after plating cells onto dishes pretreated with serum spreading factor only, while the rate of spreading of cells plated onto dishes receiving no pretreatment of any kind was considerably slower.

Preparation of Serum Spreading Factor, Monoclonal Antibody, and Blood Cell Extracts. Serum spreading factor was partially purified from human serum by glass bead column chromatography (10) and concanavalin A-Sepharose affinity chromatography. Monoclonal antibody was isolated from serum-free conditioned medium from hybridoma cultures by protein A-Sepharose affinity chromatography. For the preparation of platelet extracts, platelets were centrifuged from outdated platelet-rich plasma and the platelet-poor plasma supernatant was removed and assayed for serum spreading factor in the experiment of Fig. 9. The pellet of platelets was washed twice with P_i/NaCl/1 mM EDTA, suspended in a small volume of the same buffer, and lysed by freeze-thaw, and the lysate was centrifuged at 100,000 × g for 1 hr. The supernatant from this centrifugation was assayed for serum spreading factor in the experiment of Fig. 9. Erythrocyte extract was prepared in an identical manner. Extracts of fresh platelets contained specific anti-serum spreading factor binding activity comparable with that found in the experiment of Fig. 9.

Enzyme-Linked Immunosorbent Assay (ELISA). The procedures are based on techniques described previously (13–15). Immunochemicals were obtained from Bethesda Research Laboratories and Cappel Laboratories. For the experiments of Figs. 5 and 6, 96-well microtiter dishes containing antigen prepara-

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Abbreviations: ELISA, enzyme-linked immunosorbent assay; DME medium, Dulbecco's modified Eagle's medium; F12:DME medium, 1:1 mixture of Ham's F12 and DME media supplemented with Hepes and antibiotics; Inh-Mc1, monoclonal antibody to human serum spreading factor secreted by the Inh-Hy1 hybridoma cell line; P_i/NaCl, phosphate-buffered saline.

tions in $P_i/NaCl$ (100 μ l per well) at the concentrations necessary to give the indicated amount of antigen preparation per well were incubated overnight. Monoclonal antibody was used at a final concentration of 10 μ g/ml in $P_i/NaCl$ containing bovine serum albumin at 0.5 mg/ml. Rabbit antisera were used at a final dilution of 1:200 in $P_i/NaCl$. Peroxidase-conjugated goat anti-rabbit IgG and anti-mouse IgG were used at a 1:1,000 dilution in $P_i/NaCl$. Antibody incubations were 1 hr at room temperature. 2,2'-Azino-di-(3-ethylbenzthiazolinesulfonic acid) was the peroxidase-dependent chromogen. The reaction was inhibited after 15 min, contents of the wells were diluted with water, and the extent of reaction was determined by measuring the absorbance at 415 nm. The experiment of Fig. 7 was carried out in a similar manner, except that 35-mm-diameter cell culture plates and appropriately larger volumes of solutions were used. For the experiment of Fig. 9, the appropriate samples were diluted with $P_i/NaCl$ containing bovine serum albumin at 1 mg/ml to give the indicated final concentrations of sample. These dilutions (100 μ l) were incubated for 20 hr in microtiter wells with 100 μ l of $P_i/NaCl$ containing bovine serum albumin at 1 mg/ml and monoclonal antibody at 0.5 μ g/ml. The contents of these wells were transferred to other wells previously saturated with serum spreading factor, and the mixtures were incubated for 1 hr at room temperature. The contents of the wells were removed and the amount of antibody bound to the serum spreading factor-coated wells was determined as described above. Immunoperoxidase localization of monoclonal antibody to serum spreading factor on nitrocellulose blots (immunoblots) of 7.5% polyacrylamide gels after NaDodSO₄/polyacrylamide gel electrophoresis was carried out by adaptation of the procedure of Towbin (13), using 3-amino-O-ethylcarbazole as the peroxidase-dependent chromogen.

RESULTS

Monoclonal Antibody to Human Serum Spreading Factor.

Mice were immunized initially by subcutaneous injection of a partially purified preparation of serum spreading factor; 15 wk later, a booster immunization of electrophoretically purified serum spreading factor was given intravenously. The fusion was carried out 4 days after the second injection and the cells were distributed evenly among the wells of two 24-well cell culture trays. Two weeks after plating, some cells from each well were suspended in F12:DME medium containing bovine serum albumin at 0.5 mg/ml and seeded into wells previously treated with a preparation of serum spreading factor. In cell populations taken from 4 of the 48 wells, 20% or more of the cells attached to the antigen-treated substratum; less than 1% of cells taken from the other 44 wells attached. This phenomenon was taken as a possible indication of cell surface expression of antibody to serum spreading factor. Conditioned media from the four wells suspected to contain cells expressing antibody to serum spreading factor were tested by an ELISA and found to contain antibody to serum spreading factor. Cloned lines were established from cells from each of these wells. Media from three of the four cloned hybridoma lines inhibited serum spreading factor-promoted spreading of several cell types in culture; these lines were recloned twice, and monoclonal antibodies to serum spreading factor produced by these lines were determined to be IgG. One of the lines (Inh-Hy1) was propagated continuously in serum-free medium (16), and antibody (Inh-Mc1) produced by this line was used in the experiments described below.

The effect of Inh-Mc1 on the morphology of WI-38 human embryonic lung fibroblasts plated on culture dishes previously treated with a preparation of serum spreading factor is shown in Fig. 1. It has been shown that previous treatment of dishes with serum spreading factor is as effective as adding the material

FIG. 1. Inhibition by Inh-Mc1 of serum spreading factor-promoted spreading of WI-38 fibroblasts. Cells were photographed 1.5 hr after plating onto dishes previously treated with the following: $P_i/NaCl$ (A), serum spreading factor at 4 μ g/ml (B), serum spreading factor (4 μ g/ml) followed by Inh-Mc1 at 15 μ g/ml (C), or serum spreading factor (4 μ g/ml) followed by nonspecific mouse IgG at 15 μ g/ml (D). ($\times 120$).

directly to the medium into which the cells are plated (5). Previous treatment of plates with serum spreading factor resulted in rapid spreading of WI-38 cells subsequently seeded on these plates; cell spreading was decreased on serum spreading factor-treated plates exposed to an additional pretreatment with Inh-Mc1 but not on serum spreading factor-treated plates exposed, instead, to an additional pretreatment with an equal amount of nonspecific mouse IgG (Figs. 1 and 2). Although the rate of cell spreading in both control and Inh-Mc1-treated plates was markedly reduced compared with the rate of spreading in serum spreading factor-treated plates, the cells spread eventually under all conditions. Spreading of human fibroblasts in the absence of exogenous spreading-promoting factors has been observed previously and may result from the spreading-promoting action of fibronectin synthesized by the cells (1). Inh-Mc1 did not inhibit fibronectin-promoted spreading of WI-38 cells (Fig. 2). As shown in Fig. 3, Inh-Mc1 also inhibited the spreading-promoting effect of serum spreading factor on epitheloid MCF-7 human mammary carcinoma cells. Fibronectin did not promote MCF-7 cell spreading under the conditions of Fig. 3.

The spreading-promoting activity in partially purified serum spreading factor preparations is associated with two protein

FIG. 2. Quantitation of inhibition by Inh-Mc1 of serum spreading factor-promoted spreading of WI-38 fibroblasts. Bars: a, control (no spreading-promoting protein); b, serum spreading factor at 4 μ g/ml; c, fibronectin at 4 μ g/ml; d, serum spreading factor followed by Inh-Mc1 at 15 μ g/ml; e, fibronectin followed by Inh-Mc1; f, serum spreading factor followed by nonspecific mouse IgG at 15 μ g/ml; g, fibronectin followed by nonspecific mouse IgG.

FIG. 3. Inhibition by Inh-Mc1 of serum spreading factor-promoted spreading of MCF-7 mammary carcinoma cells. Cells were photographed 1.5 hr after plating onto dishes previously treated with the following: $P_i/NaCl$ (A), serum spreading factor at 4 $\mu g/ml$ (B), serum spreading factor (4 $\mu g/ml$) followed by Inh-Mc1 at 15 $\mu g/ml$ (C), or serum spreading factor (4 $\mu g/ml$) followed by nonspecific mouse IgG at 15 $\mu g/ml$ (D). ($\times 120$.)

components with molecular weights of approximately 70,000 and 80,000 (7). A minor spreading-promoting component of molecular weight approximately 65,000 also is observed. As shown in Fig. 4, Inh-Mc1 recognized two areas of immunoblots of reduced electrophoretically separated serum spreading factor preparations, corresponding to the areas found previously to exhibit spreading-promoting activity. We estimate the molecular weights of these two components to be 65,000–70,000 and 75,000–78,000. Some preparations of serum spreading factor also exhibited a minor antibody-binding region in the molecular weight range of 60,000–64,000 (data not shown), corresponding to the minor spreading-promoting band previously reported.

Nonidentity of Serum Spreading Factor and Other Spreading-Promoting Proteins. Experiments using double immuno-

FIG. 4. Identification of proteins in serum spreading factor preparations recognized by Inh-Mc1. (A) Polyacrylamide gel after Na-DodSO₄/polyacrylamide gel electrophoresis. Samples in lanes a–c were reduced and boiled; those in lanes d–f were reduced but not boiled. Lanes: a and d, molecular weight standards (5 μg each); b and e, serum spreading factor preparation (15 μg); c and f, fibronectin (15 μg). Molecular weight standards (top to bottom) were myosin, 200,000; galactosidase, 116,000; phosphorylase, 92,000; bovine serum albumin, 67,000; ovalbumin, 43,000. (B) Immunoblot of the gel represented in A. Antibody is localized in two bands corresponding to two bands in lane e, the reduced unboiled serum spreading factor preparation.

diffusion have shown that rabbit antiserum to human serum spreading factor preparations does not recognize human fibronectin and that rabbit antiserum to human fibronectin does not recognize human serum spreading factor (5). Inh-Mc1 did not inhibit fibronectin-promoted cell spreading (Fig. 2), and neither monoclonal antibody nor polyvalent rabbit antiserum to human fibronectin inhibits serum spreading factor-promoted cell spreading (7). As shown in Fig. 5, Inh-Mc1 bound to serum spreading factor previously adsorbed to microtiter wells but did not bind to fibronectin-treated wells. Inh-Mc1 also did not bind to human chondronectin (17) in this assay (data not shown). Fig. 6 shows that fibronectin-treated wells contained immunologically active fibronectin that was recognized by rabbit antiserum to human fibronectin; this anti-fibronectin antiserum did not recognize serum spreading factor. Fig. 6 also shows that rabbit antiserum to mouse laminin (15) did not recognize human serum spreading factor; this antiserum crossreacts with human laminin. The relationships between human serum spreading factor and other cell spreading-promoting proteins (1, 18, 19) are not clear. However, rabbit antiserum to human epibolin (18) shows some crossreactivity with human serum spreading factor preparations (D.B. and K. Stenn, unpublished data).

Inhibition of Serum-Promoted Cell Spreading by Monoclonal Antibody to Serum Spreading Factor. We used our monoclonal antibody in experiments designed to determine whether serum spreading factor in human serum contributes significantly to the cell spreading-promoting capacity of that serum under cell culture conditions. Plastic cell culture plates were incubated overnight with medium containing human serum at a variety of concentrations and the amount of serum spreading factor that remained associated with the plates after several washes was determined, as well as the capacity of these plates to promote WI-38 cell spreading. When compared with plates incubated with 1% serum, plates incubated with higher serum concentrations showed decreases in both the amount of serum spreading factor present on the plate (Fig. 7) and cell spreading-

FIG. 5. ELISA of Inh-Mc1 binding to fibronectin and serum spreading factor. $\circ$, Inh-Mc1 vs. serum spreading factor preparation; $\bullet$, Inh-Mc1 vs. fibronectin.

FIG. 6. ELISA of antilaminin and antifibronectin antibody binding to laminin, fibronectin, and serum spreading factor preparations. $\circ$, Antilaminin vs. laminin; $\square$, antifibronectin vs. fibronectin; $\bullet$, antilaminin vs. serum spreading factor; $\blacksquare$, antifibronectin vs. serum spreading factor.

promoting ability. Similar observations have been made regarding decreased fibronectin binding to cell culture dishes at higher serum concentrations (20). The percentage of spread cells 1.5 hr after plating on dishes previously treated with 1% serum was equivalent to that of cells on dishes previously treated with serum spreading factor or fibronectin (Figs. 2 and 8); an additional preincubation of 1% serum-treated plates with Inh-Mc1 resulted in a reduction in this percentage (Fig. 8). The data of Fig. 8 lead to the conclusion that serum spreading factor contributed a significant portion of the spreading-promoting capacity of serum under the conditions of the experiment.

Human Platelet-Associated Serum Spreading Factor. The results of an assay of specific Inh-Mc1-binding activity in platelet-poor human plasma, soluble P_i /NaCl extracts of washed

FIG. 7. ELISA of Inh-Mc1 binding to human serum-treated and serum spreading factor-treated cell culture dishes. $\bullet$, Human serum-treated dishes; $\circ$, serum spreading factor-treated dishes.

FIG. 8. Inhibition of human serum-promoted spreading of WI-38 fibroblasts by Inh-Mc1. Percent spread cells was determined on plates previously treated with the indicated concentration of Inh-Mc1 ($\circ$) or with mouse IgG at 10 μ g/ml ($\square$). All plates were treated with 1% human serum prior to treatment with mouse IgG or Inh-Mc1.

platelets and erythrocytes, and a partially purified serum spreading factor preparation are shown in Fig. 9. This assay provides a measure of the relative amount of serum spreading factor per mg of protein in these preparations. The specific Inh-Mc1-binding activity in the soluble platelet extract was about 60% of that in platelet-poor plasma; serum spreading factor was not detected in erythrocyte extracts. Because serum spreading factor was found associated with washed human platelets, experiments similar to that of Fig. 7 were carried out with a range of concentrations of platelet-free plasma, serum prepared from platelet-free plasma, and serum prepared from plasma containing a normal concentration of platelets. The amount of serum spreading factor, and the spreading-promoting activity associated with the culture dishes, did not differ markedly among plates previously treated with the same concentration of three preparations. Although only a small portion of the total amount of serum spreading factor in blood is associated with platelets, it is possible that this factor, which mediates phenomena related to cell adhesion in culture, may be involved in platelet adhesion or aggregation. Fibronectin also has been reported to occur in platelet-associated form, but its importance in platelet function is unclear (21, 22).

DISCUSSION

Monoclonal antibody to serum spreading factor inhibited the rate of serum spreading factor-promoted spreading of cells in culture (Figs. 1, 2, and 3). As indicated in *Materials and Methods*, some inhibition of cell spreading was also observed under conditions in which nonspecific mouse IgG was adsorbed onto dishes; this nonspecific effect of IgG was prevented by incubation of the dishes with bovine serum albumin subsequent to the incubation with spreading factors. The following results indicate that inhibition by Inh-Mc1 of cell spreading on serum spreading factor-treated plates is due to specific inhibition of

FIG. 9. Relative amounts of serum spreading factor per mg of protein (specific Inh-Mc1-binding activity) in plasma (●), platelet extract (□), erythrocyte extract (■), and partially purified serum spreading factor preparations (○). Samples (100 μ l) at various protein concentrations were incubated for 20 hr with 100 μ l of Inh-Mc1 at 0.5 μ g/ml in P_i /NaCl containing bovine serum albumin at 1 mg/ml, and unbound antibody remaining free at the end of this incubation was determined by transfer of the samples to serum spreading factor-coated microtiter wells and subsequent incubations.

serum spreading factor activity by the antibody and not due simply to the nonspecific spreading-inhibiting effect of mouse IgG associated with the plate as an antibody-antigen complex. (i) We have isolated a cloned hybridoma line producing monoclonal antibody to serum spreading factor that binds to serum spreading factor-treated dishes but does not inhibit serum spreading factor-promoted cell spreading. (ii) In experiments in which plates previously treated with a mixture of fibronectin and serum spreading factor (both at concentrations optimal for cell spreading) were used, no inhibition of cell spreading by Inh-Mc1 was observed, although ELISA measurements indicated that the same amount of antibody was associated with the dish as in experiments in which serum spreading factor, but not fibronectin, was present and inhibition of cell spreading by Inh-Mc1 was observed.

Immunoblots of the proteins of electrophoretically separated serum spreading factor preparations indicate that Inh-Mc1 recognizes the two proteins shown previously to be associated with the cell spreading-promoting activity of these preparations; the form having a molecular weight of $\approx 70,000$ appears to be more abundant in human serum than the higher molecular weight form (7). Based on the data of Figs. 7 and 9, we estimate that human plasma contains serum spreading factor at ≈ 0.5 mg/ml. Although we have presented data in this paper regarding effects on human cells only, serum spreading factor is capable of influencing cell spreading of cultured cells of other species (5–7), and Inh-Mc1 is capable of inhibiting these effects. Proteins analogous to human serum spreading factor may exist in sera from other species; we have identified a factor in fetal calf serum with biochemical and spreading-promoting properties similar to human serum spreading factor (8). None of our monoclonal antibodies to human serum spreading factor showed any binding activity against sera from other animals, presumably representing inability of the antibodies to recognize analogous molecules of different species rather than an absence of the factor in other sera.

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- Grinnel, F. (1978) *Int. Rev. Cytol.* 53, 65–144.
- Yamada, K. M. & Olden, K. (1978) *Nature (London)* 275, 179–184.
- Barnes, D. & Sato, G. (1979) *Nature (London)* 281, 388–389.
- Barnes, D. & Sato, G. (1980) in *Cell Biology of Breast Cancer*, eds. McGrath, C. M., Brennan, M. J. & Rich, M. A. (Academic, New York), pp. 277–287.
- Barnes, D., Wolfe, R., Serrero, G., McClure, D. & Sato, G. (1980) *J. Supramol. Struct.* 14, 47–63.
- Barnes, D. W., Darmon, M. & Orly, J. (1981) in *Cold Spring Harbor Symposium on Animal Cell Proliferation* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY), Vol. 9, pp. 155–167.
- Hayman, E. G., Engvall, E., A'Hearn, E., Barnes, D., Pierschbacher, M. & Ruoslahti, E. (1982) *J. Cell Biol.* 95, 20–23.
- Hayman, E. G., Barnes, D., Pierschbacher, M., Engvall, E. & Ruoslahti, E. (1982) *J. Cell Biol.* 95, 121 (abstr.).
- Barnes, D., Silnutzer, J., See, C. & Shaffer, M. (1982) *J. Cell Biol.* 95, 119 (abstr.).
- Barnes, D., van der Bosch, J., Masui, H., Miyazaki, K. & Sato, G. (1981) *Methods Enzymol.* 79, 368–391.
- Holmes, R. (1967) *J. Cell Biol.* 32, 297–308.
- Oi, V. T. & Herzenberg, L. A. (1980) in *Selected Methods in Cellular Immunology*, eds. Mishell, B. & Shiigi, S. (Freeman, San Francisco), pp. 351–372.
- Towbin, H., Staehelin, T. & Gordon, J. (1979) *Proc. Natl. Acad. Sci. USA* 76, 4350–4354.
- Engvall, E. (1980) *Methods Enzymol.* 70, 419–439.
- Timpl, R., Rohde, H., Robey, P. G., Rennard, S. K., Foidart, J.-M. & Martin, G. R. (1979) *J. Biol. Chem.* 254, 9933–9937.
- Murakami, H., Masui, H., Sato, G. H., Sueoka, N., Chow, T. P. & Kano-Sueoka, T. (1982) *Proc. Natl. Acad. Sci. USA* 79, 1158–1162.
- Hewitt, A. T., Varner, H. H., Silver, M. H., Dessau, W., Wilkes, C. M. & Martin, G. R. (1982) *J. Biol. Chem.* 257, 2330–2334.
- Stenn, K. S. (1981) *Proc. Natl. Acad. Sci. USA* 78, 6907–6911.
- Whateley, J. G. & Knox, P. (1980) *Biochem. J.* 185, 349–354.
- Grinnell, F. & Feld, M. K. (1982) *J. Biol. Chem.* 257, 4888–4893.
- Bensusan, H. B., Koh, T. L., Henry, K. G., Murray, B. A. & Culp, L. A. (1978) *Proc. Natl. Acad. Sci. USA* 75, 5864–5868.
- Santoro, S. A. & Cunningham, L. W. (1979) *Proc. Natl. Acad. Sci. USA* 76, 2644–2648.