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Preparation of a Monoclonal Antibody to a Melanoma Growth-Stimulatory Activity Released Into Serum-Free Culture Medium by Hs0294 Malignant Melanoma Cells

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Autostimulatory growth factors may contribute to the ability of malignant cells to escape normal growth controls. We have previously shown that Hs0294 human malignant melanoma cells release into culture medium an acid-soluble, heat-stable, trypsin-sensitive, autostimulatory monolayer mitogen which can be purified from acetic acid extracts of conditioned medium by gel filtration, reverse-phase high-performance liquid chromatography, and preparative electrophoresis. The majority of this melanoma growth-stimulatory activity (MGSA) resides in a 16-Kd moiety, though bioactivity is also associated with 24–26 and < 14-Kd forms of MGSA (Richmond and Thomas: J Cell Physiol 129:375, 1986). In order to further characterize this growth factor, monoclonal antibodies were prepared against a partially purified preparation of the autostimulatory melanoma mitogen. Monoclonal antibody clones were selected based on supernate inhibition of ^{3}H-thymidine incorporation in serum-free Hs0294 melanoma cultures. One of these, termed FB2AH7, slows, but does not completely block, the growth of Hs0294 cells in serum-free medium in a dose-dependent manner. This antibody does not slow the growth of normal rat kidney fibroblasts, which neither produce nor require this mitogen, in either serum-free medium or medium containing 0.8% calf serum. This monoclonal antibody also blocks the mitogenic effects of partially purified preparations of this melanoma growth stimulatory activity (MGSA) on both Hs0294 cells and normal rat kidney fibroblasts. The FB2AH7 antibody has been demonstrated to bind MGSA by Western blot and by immunoprecipitation procedures. Western blot analysis of reverse-phase high-performance liquid chromatography purified growth factor demonstrated that FB2AH7 antibody binds to the 16-Kd and ~13–14-Kd forms of MGSA. FB2AH7 antibody can be used in immunoprecipitation experiments to bind the ~13–16-Kd forms of MGSA. The specificity of the binding of FB2AH7 antibody for MGSA but not other growth

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factors has been demonstrated in a modified dot blot assay. These data thus support the hypothesis that MGSA is an autostimulatory melanoma mitogen distinct from other growth factors.

Key words: monoclonal antibody, melanoma growth stimulatory activity, serum free culture medium, Hs0294 malignant melanoma cells

Malignant cells may escape normal growth controls by producing autostimulatory growth factors [3]. Transformation may be associated with production of and/or response to several main classes of growth factors: (1) transforming growth factor-alpha (TGFα) [4,5]; (2) transforming growth factor-beta (TGFβ) [6–8]; (3) platelet-derived growth factor (PDGF)-like polypetides [9,10]; (4) bombesin [11]; (5) insulin-like growth factor-II-like peptides [12,13]; and (6) interleukin-3 (multicolony stimulating factor) [14]. Work with a variety of cell lines [15–24] supports a role for these and other tumor-derived growth factors in the control of proliferation of malignant cells.

Monoclonal antibodies to growth factors or their receptors can in some instances neutralize the effect of these mitogens. This provides a useful approach to defining the roles of specific factors in the control of proliferation of both malignant and nonmalignant cells and their roles in the expression of other features of the transformed phenotype. In the case of TGFα, antibodies to the EGF receptor can block the biological effects of this peptide, including its ability to stimulate anchorage-independent growth of nontransformed cells [25]. In a study examining a possible autostimulatory role of TGFα in colony formation by A2058 melanoma cells, however, a monoclonal antibody to the EGF receptor was unable to block soft agar colony formation by these cells although they release TGFα. Intact EGF receptor could not be detected in these cells. These data suggest that TGFα may not play an autocrine role in the anchorage-independent growth of this cell line. Alternatively, it may act through a receptor other than the EGF receptor or function intracellularly, bypassing the need for secretion followed by binding to a plasma membrane receptor [26].

In studies with PDGF, antiserum to this peptide can inhibit [^{3}H]-thymidine incorporation in simian sarcoma virus–transformed NIH 3T3 cells and simian sarcoma virus–transformed normal rat kidney (NRK) fibroblasts which release a PDGF-like moiety into culture medium, suggesting an autostimulatory role for this factor in these cells [27]. In a clonal human osteosarcoma cell line, however, monoclonal antibodies to PDGF had no detectable effect on growth rate, although both release of a PDGF-like moiety into culture medium and expression of PDGF receptors on the cell surface were demonstrated. These data suggest that if the PDGF-like factor plays a role in the growth of these particular cells, it does so in a way such that extracellularly added antibodies do not affect it [28]. Alternatively, these antibodies may not recognize the active epitope of these PDGF-like factors. Antibodies to PDGF can also block certain of the morphologic and functional consequences of transformation of some cell lines by simian sarcoma virus, the oncogene product of which is closely related to PDGF [29,30]. These findings suggest that transformation by this virus is at least partially mediated by interaction of the v-sis gene product with the PDGF receptor.

Antibodies to somatomedin-C (Sm-C) block the ability of this peptide to stimulate DNA synthesis in Balb/C 3T3 cells [31,32]. Human fibroblasts and porcine smooth muscle cells have been shown to release Sm-C-like peptides when stimulated

by PDGF. An autocrine role for these Sm-C-like peptides has been suggested by the finding that [^{3}H]-thymidine incorporation by these cells in response to PDGF is partially blocked by antibodies to Sm-C [32].

Finally, monoclonal antibodies to the tetradecapeptide bombesin, which is secreted by human small cell lung cancer cell lines, can block the attachment of this peptide to its cell-surface receptors. These antibodies inhibit the growth of small-cell lung cancer cells in vitro and significantly slow the growth of small-cell lung cancer xenografts in nude mice [11]. These data suggest that bombesin can function as an autostimulatory growth factor in these cells.

We reported data suggesting that cells from the human malignant melanoma cell line Hs0294 are able to grow in monolayer cultures in the absence of serum or exogenous mitogens because they release an autostimulatory growth factor [21–23]. This mitogen has recently been purified from melanoma-conditioned medium by a series of gel filtration, reverse-phase high-performance liquid chromatography and preparative electrophoresis procedures [23a]. Though melanoma growth stimulatory activity was associated with proteins over a molecular weight range of 26–< 14 Kd, the majority of the bioactivity was associated with a 16-Kd polypeptide which elutes from a μBondapak C-18 column with 35% acetonitrile/0.05% trifluoroacetic acid. The mechanism of action proposed for this mitogen involves extracellular release and binding to a cell-surface receptor. This would provide an opportunity for antibodies to inhibit this autocrine activity. In order to further investigate this hypothesis and to further characterize the responsible mitogen(s), monoclonal antibodies were prepared against a partially purified preparation of this melanoma growth-stimulatory activity (MGSA). Hybridoma clones were selected on the basis of ability of supernatant fluids to inhibit [^{3}H]-thymidine incorporation in the Hs0294 cells which release this mitogen. Using this screening procedure a monoclonal antibody has been identified which slows the growth of Hs0294 cells and partially blocks the effect of exogenous MGSA on these cells. Western blot analysis and a modified dot blot assay demonstrate that the FB2AH7 antibody binds to the 16-Kd and ~ 14-Kd polypeptide in the reverse-phase high-performance liquid chromatography (RP-HPLC) preparation of MGSA.

These data thus provide further support for the hypothesis that the melanoma mitogen (MGSA) produced by Hs0294 cells which has been recently purified by Richmond and Thomas [23a] is autostimulatory and that it is distinct from previously described growth factors.

MATERIALS AND METHODS

Cells

Hs0294 cells were obtained from Naval Bioscience Laboratories (Oakland, CA). NRK cells were from the Viral Carcinogenesis Laboratory, National Cancer Institute (Frederick, MD). Cells were maintained as previously described [21].

Initial Preparation of Monoclonal Antibodies

The monoclonal antibody FB2AH7 was prepared against an acetic acid extract of culture medium conditioned by Hs0294 cells. Serum-free medium was collected from these cells, ultracentrifuged (100,000g for 30 min), lyophilized, dissolved in 1N acetic acid, dialyzed (Spectrapor-3, 3,500d exclusion, Spectrum Medical Industries Inc., Los Angeles, CA), lyophilized, extracted with 1 N acetic acid, and applied to a

Bio-Gel P-30 column (Bio-Rad, Richmond, CA), all as previously described [21]. The eluant from the Bio-Gel column was pooled into two fractions based on apparent molecular weight: (1) >22 Kd and (2) 6–22 Kd. The 6–22-Kd material contains the MGSA. These two fractions were then lyophilized. Five-hundred-micrograms dry weight from these two fractions was used to prepare hybridomas according to the method of Kohler and Milstein [33], with minor modifications. Supernates from cloned hybridomas were screened for ability to inhibit ^{3}H-thymidine incorporation in serum-depleted Hs0294 cells. Five wells of hybrids were selected on the basis of this assay; cells from these wells were cloned by the limiting dilution method and expansion cultures of single clones were reassayed. Eleven clones, including FB2AH7, were positive in the melanoma growth inhibition assay; these clones were expanded and supernates were collected for further characterization.

Isolation and Characterization of Monoclonal Antibodies

Monoclonal antibodies for the studies described below were prepared according to the following procedure [34]: The antibody-producing cells were maintained in Corning 150-cm^2 tissue culture flasks (American Scientific Products, McGaw Park, IL) in medium composed of 1,000 ml deionized water, 14.04 g reinforced minimal essential media (Eagle; Grand Island Biological Company, Grand Island, NY), 350 mg glutamine (Gibco), 1 ml insulin/transferrin/selenium (5 mg/5 mg/ 5 μg) (Collaborative Research, Inc., Waltham, MA), 40 mg gentamycin, 2.5 mg amphotericin B, 2.2 g sodium bicarbonate (Fisher Scientific Company, Fair Lawn, NJ), 100 ml heat inactivated fetal bovine serum (Gibco), 12 ml 1M HEPES (N′-2-hydroxyethylpiperazine-N-ethanesulfonic acid) (Gibco), and 12 ml of a 0.1 M 2-mercaptoethanol solution diluted 1:20 in deionized water. The pH of the medium was adjusted to 7.3.

For isolation of the antibody, conditioned medium was clarified by centrifugation (1,500g for 5 min) and precipitated by dropwise addition of an equal volume of 4 M ammonium sulfate. The antibody was collected by centrifugation (5,600g for 30 min) and the supernate was reprecipitated. The combined pellets were then dialyzed against deionized water (pH 7.4–7.8) (Spectrapor 6, 50,000d exclusion, Spectrum), lyophilized, and then chromatographed on a 90 × 2.5 cm AcA 34 Ultrogel (LKB) column, eluting with 0.05 M sodium phosphate buffer, pH 7.2. The antibodies eluted in the excluded fractions of the Ultrogel column. The antibody-containing fractions were pooled, dialyzed against deionized water, lyophilized, and stored at −20°C. Presence of antibody was verified on double immunodiffusion plates (Hyland Diagnostics, Malvern, PA) using rabbit antiserum to mouse immunoglobulins (Calbiochem-Behring Corp., La Jolla, CA). For some experiments monoclonal antibodies produced in the peritoneal cavity of pristane-primed Balb/c mice were treated in a similar fashion.

The monoclonal antibodies were characterized as to heavy-chain class and light-chain type by an immunodot assay as previously described [35].

[^{3}H]-Thymidine Incorporation Assay

Both NRK and Hs0294 cells were assayed according to a modification of the procedures of Iio and Sirbasku [36] essentially as described [22,23]. Both cell lines were serum depleted at the time of assay. Number of cells plated and additions of antibody preparations and partially purified preparations of MGSA were as described in Results; ^{3}H-thymidine (specific activity 21.5 Ci/mmol) was obtained from New

England Nuclear (Boston, MA). Wheaton scintillation vials (225288) were from VWR Scientific (Atlanta, GA) and Scintiverse-II was from Fisher Scientific.

Cell Number Assay

Cell number assays were performed as previously described [21,22] under conditions as described in Results. Monoclonal antibody was added to treated cultures every other day at the time of change of culture media. Hs0294 cells were studied under serum-free conditions in all experiments.

Immunoprecipitation and Western Immunoblot Procedure

Hs0294 cells (1×10^6) were injected intrascapularly into athymic nude (nu/nu) mice (Charles River) and allowed to grow until large tumors developed, at which time the mice were killed by cervical dislocation. MGSA was purified from acid ethanol extracts of these Hs0294 tumors by sequential molecular sieve chromatography and reverse-phase high-performance liquid chromatography (RP-HPLC) as previously described [21,23]. RP-HPLC was performed on equipment from Waters Associates (Milford, MA) using HPLC grade solvents from Fisher Scientific. A μBondapak C-18 Radial-Pak cartridge (Waters) was used. Elution was accomplished at room temperature with a gradient of acetonitrile: water: trifluoroacetic acid 6:94:0.05 to 60:40:0.05 over 1 hr at a flow rate of 1 ml/min. Fractions eluting at $35 \pm 3\%$ acetonitrile were lyophilized and reconstituted with 4 mM HCl. For immunoprecipitation, the samples were precipitated with FB2AH7 or a mouse nonspecific monoclonal MF2 using the sandwich technique with antimouse Ig(GAM) antibodies. Some precipitations were performed in the presence of 0.1% Triton X-100. The immunoprecipitates were then resolved on a gradient polyacrylamide gel (4% stacking gel, 12–18% resolving gel) according to the procedure of Laemmli [37], using double-strength sample buffer. Electrophoresis was continued until the bromophenol blue tracking dye was within 1 cm of the gel bottom, using a current of 20 mA for the stacking gel and 35 mA for the resolving gel. Gels were stained with silver and scanned using an LKB laser densitometer (model 2212). For Western blots, the RP-HPLC MGSA samples were loaded in duplicate so that one-half could be probed with FB2AH7 antibody and the other half with human myeloma IgM. The electrophoretic transfer and immunoblot were accomplished with a modification of the procedures of Towbin [47]. To increase the visualization of the immunoreaction, the Vectastain ABC kit was utilized (Vector Laboratories).

Enzyme-Linked Immunoadsorbent Assay (ELISA) for MGSA

Immunoreactivity of purified preparations of MGSA or other growth factors for the monoclonal antibody FB2AH7 is determined by an ELISA that is a modification of the procedures of Hawkes [48] for dot-immunobinding assays for monoclonal antibodies. Purified growth factors were obtained from the following sources: epidermal growth factor (EGF), platelet-derived growth factor (PDGF), nerve growth factor (NGF), and interleukin-1 (1L-1) were from Collaborative Research; fibroblast growth factor (FGF) was from Sigma; insulinlike growth factor-1 (IGF-I) from AmGen; recombinant interleukin-2 (1L-2) was the kind gift of Dr. Sam Newcom, Emory University; transferrin was from Cal BioChem; purified transforming growth factor-beta (TGF_β) was the kind gift of Dr. Harold Moses. In the dot-blot assay, the antigen is bound to the nitrocellulose paper by spotting antigen (100–1 ng) on small circles of

nitrocellulose paper placed in wells of 96-well microtiter plate; then the dot blot procedure is followed according to modification of Hawkes procedures as previously described.

Statistical Analysis

Experiments having more than one set of data points were analyzed by analysis of variance techniques followed by the Student-Newman-Keuls procedure to determine specific differences between groups. In addition, paired comparisons were done using t-tests when interactions were present. All tests were conducted at the .05 level of significance.

Classification of Monoclonal Antibodies

Monoclonal antibody FB2AH7, the subject of this report, is of the IgM Kappa class (IgM_K). Another of the hybridoma clones prepared and characterized in this study, termed FB2AG6, is also of the IgM_K class but had no relevant specificity and was used as a control in some experiments. For Western blots and immunoprecipitation experiments, a human myeloma IgM and mouse monoclonal IgA antibodies with no relevant specificity were used. For the modified dot blot assay, the control antibodies included an IgM_K monoclonal antibody to α-lipoteichoic acid and a mouse monoclonal IgA.

RESULTS

Effect of Monoclonal Antibody FB2AH7 on the Growth of Hs0294 and NRK Cells

In order to determine whether monoclonal antibody FB2AH7 could block the effects of endogenously produced MGSA, 10 μg of Ultrogel-purified FB2AH7 was added to actively growing Hs0294 cells, which release MGSA, and NRK cells, which do not. The [^{3}H]-thymidine incorporation was then measured during an 18-hr period of continuous labeling. As a control, 10 μg of Ultrogel-purified FB2AG6 monoclonal antibody was also added to Hs0294 cells. As shown in Figure 1, FB2AH7 antibody inhibited [^{3}H]-thymidine incorporation into Hs0294 cells 39% ($P < .05$), whereas [^{3}H]-thymidine incorporation into NRK cells was not significantly affected ($P > .05$). Control antibody FB2AG6 did not significantly inhibit [^{3}H]-thymidine incorporation into Hs0294 cells ($P > .05$) (Fig. 1).

Cell number experiments were used to further define the effect of monoclonal antibody FB2AH7 on the growth of Hs0294 and NRK cells. The effect on Hs0294 cell growth was dependent upon amount of antibody added (Fig. 2). Addition of 0.01 μg of antibody at 48-hr intervals had little effect, whereas a maximum effect (treated cultures 59% of control) was observed with addition of 1 μg of FB2AH7. Addition of 10 or 100 μg of antibody (10 μg/10 μl) did not significantly enhance this effect. There was no detectable direct cytotoxic effect of the antibody or effect on cell attachment. In these experiments treated cultures slightly more than doubled in cell number relative to number of cells plated over the 7–9-day period of the assay.

The time course of the growth inhibition caused by this antibody was also characterized. At relatively low cell density and high antibody concentration there was no net increase in cell number over 9 days in treated cultures while control cultures increased fourfold over this time period. Little net growth was seen in control

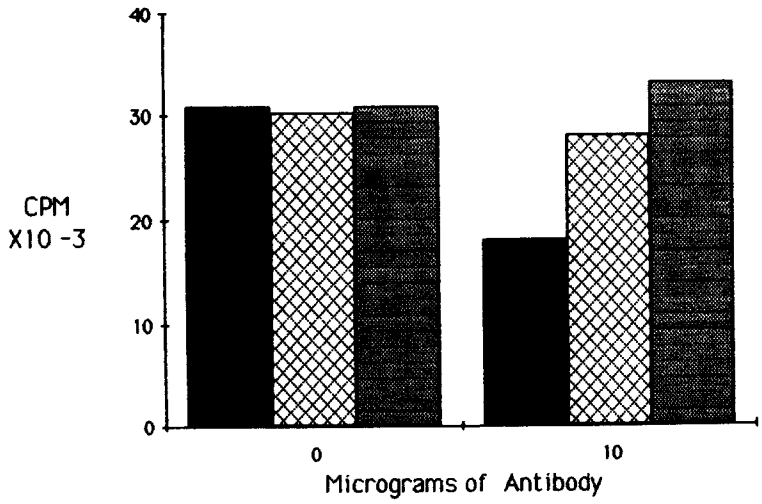

Fig. 1. Effect of monoclonal antibodies on [^{3}H]-thymidine incorporation in Hs0294 and NRK cells. Ten thousand Hs0294 cells were plated in 1 ml of Ham's F-10 medium plus 10% fetal bovine serum in Wheaton scintillation vials. NRK cells were plated at a density of 6,000 cells per vial in Dulbecco's modified Eagle's medium plus 10% calf serum. Cells were incubated at 37°C in a 95% air/5% CO_2 environment. Twenty-four hours later cells were washed twice with phosphate-buffered saline (PBS) and then fed with serum-free medium. The next day treated cultures received 10 μg of Ultrogel-purified FB2AH7 or FB2AG6 monoclonal antibody as indicated. Six hours later, 5 μCi of [^{3}H]-thymidine was added per vial and cultures were then incubated as described above for 18 hr. The medium was decanted and cells were fixed and counted as previously described [22]. N=8 for Hs0294 controls and 5 for each treatment group; n=11 for NRK controls and 4 for FB2AH7-treated cells. Actual cpm ± SD: Hs0294 control, 30,102±6,218; Hs0294 plus FB2AH7, 17,999 ± 2,800 ($P<.05$); Hs0294 plus FB2AG6, 33,141 ± 3002; NRK control, 30,223± 8,581; NRK plus FB2AH7, 28,099 ± 5,677. Black bars, the effect of FB2AH7 on Hs0294 cells; crosshatched bars, the effect of FB2AH7 on NRK cells; densely crosshatched bars, the effect of FB2AG6 on Hs0294 cells.

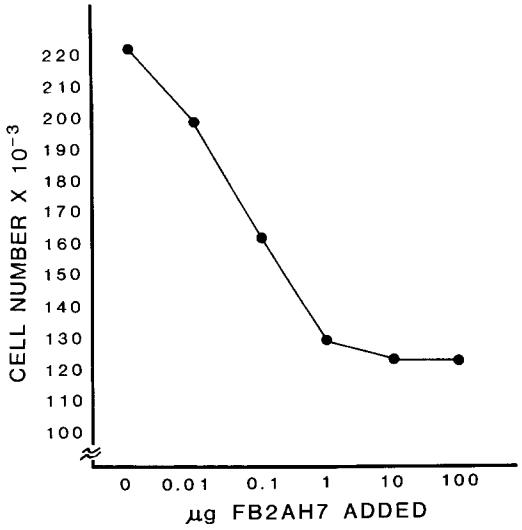

Fig. 2. Effect of addition of different amounts of antibody FB2AH7 on growth of Hs0294 cells. This graph represents pooled data from two experiments. Hs0294 cells were plated at 50,000 or 60,000 cells per ml in Ham's F-10 medium plus 10% fetal bovine serum. After attachment the cells were washed twice with PBS and given fresh serum-free F-10 medium. Medium was changed and antibody added in the amounts shown every other day. Cells were counted on a hemocytometer on the seventh to ninth days in serum-free culture medium. N=6 for control and the 0.01, 1, and 10 μg antibody additions; N=4 for the other points. Growth inhibition became significant ($P<.05$) with addition of 1 μg of antibody. The SEM averaged 12.5% of the mean.

cultures over the first 5 days of the experiment, but untreated cells entered a rapid growth phase at about that time. This growth phase was inhibited by the antibody ($P=.003$ control versus treated; Fig. 3). Under conditions of twice the initial cell number and one-half the antibody there was a modest increase in cell number in both treated and control cultures by the fifth day in culture. Control cultures grew significantly between day 5 and day 9 ($P=.0001$), but there was no significant increase in cell number in treated cultures during this period ($P=.116$). Thus, by the ninth day treated cultures had only 58% the number of cells found in untreated cultures ($P=.002$) (Fig. 3). The inhibition of growth was not seen when monoclonal antibody of similar titer from ascites fluid was used, possibly because of residual mitogens

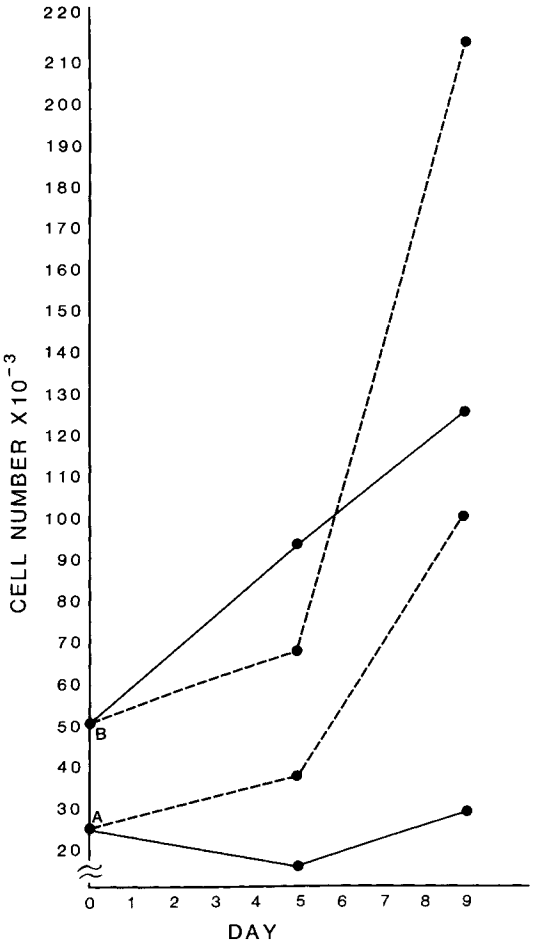

Fig. 3. Effect of monoclonal antibody on growth of Hs0294 cells. In experiment A, Hs0294 cells were plated in Falcon 35-mm^2 dishes at a density of 25,000 cells per dish and then changed to serum-free F-10 as described in the legend to Figure 2; 20 μg of FB2AH7 was added to treated cultures every other day along with media changes, and cells were counted on the fifth and ninth days in serum-free medium. N=3 for treated (——) and 5 for control (---) cultures. Experiment B was identical except that the initial plating density was 50,000 cells per well, cells were plated in 24-well plates, and only 10 μg of FB2AH7 were added every other day. N=4 for both treated (——) and control (---) cultures. SEM averaged 8.8% of the mean.

from the ascites fluid. Similarly, there was variation between antibody preparations in the ability of antibody prepared from supernatant fluid to produce this growth inhibition, possibly because the antibody is of the IgM class and partially purified preparations of antibody and antigen were used.

The effect of monoclonal antibody FB2AH7 on the growth of NRK fibroblasts was also evaluated both in the presence and absence of serum. When NRK cells were plated at an initial density of 25,000 cells per ml and then maintained on serum-free DME for 9 days with and without addition of antibody FB2AH7, there was no growth in either control or treated cultures (day 9 cell counts 18,000 and 38,000, respectively). In the presence of DME medium containing 0.8% calf serum, by the sixth day of the experiment control dishes averaged 187,000 cells per dish compared with 162,000 for treated cultures. By the ninth day of culture controls had further increased to 380,000 cells per dish, compared to 338,000 cells per dish in the treated group ($P = .237$). These experiments demonstrate that monoclonal antibody FB2AH7 has no significant effect on the growth of NRK cells, which neither produce nor depend on MGSA, compared to the effect seen with Hs0294 cells.

Effect of Monoclonal Antibody FB2AH7 on the Ability of Partially Purified Preparations of MGSA to Stimulate [^{3}H]-Thymidine Incorporation in Hs0294 and NRK Cells

In order to determine whether the growth inhibitory effect of monoclonal antibody FB2AH7 resulted from an interaction with a factor in the preparations of MGSA, the ability of this antibody to inhibit the stimulation of [^{3}H]-thymidine incorporation caused by the addition of acetic acid extracts of Hs0294-conditioned medium to Hs0294 and NRK cells was tested. In the case of the Hs0294 cells, concentrations of acetic acid extracts of Hs0294-conditioned medium of 0.1, 1, 10, 20, and 50 μg/ml of culture medium were added with or without 20 μg of Ultrogel-purified FB2AH7. The dose-response curve for NRK cells is different; accordingly, concentrations of 0.1, 1, 10 and 20 μg/ml of culture medium were added with and without 10 μg FB2AH7. As shown in Figure 4a, there was significant ($P < .05$) stimulation of [^{3}H]-thymidine incorporation in Hs0294 cells by addition of 10-μg and 20-μg amounts of conditioned media protein that was blocked by addition of 20 μg of FB2AH7 antibody. This block was not overcome by addition of up to 50 μg of conditioned media protein. With NRK cells, 1 μg of conditioned media protein stimulated a significant ($P < .05$) increase in [^{3}H]-thymidine incorporation compared to control cultures that was not seen in the presence of 10 μg of FB2AH7 (Fig. 4b).

Western Immunoblot Procedure

As previously described, after RP-HPLC purification the majority of the MGSA elutes at 35±3% acetonitrile and is associated with a 16-Kd moiety [23,38]. When an RP-HPLC-purified preparation of MGSA was subjected to gel electrophoresis under nonreducing conditions and then electrophoretically transferred to nitrocellulose paper, the major polypeptide recognized by the FB2AH7 antibody migrated at 16 Kd and ~13–14 Kd (Fig. 5). Western immunoblots performed with control human myeloma IgM antibodies revealed some non-specific interaction of human IgM with the 16-Kd but not the 13–14-Kd forms of MGSA in the transblotted protein. However, the peroxidase positivity for the myeloma IgM was weaker than with FB2AH7. In some experiments, the FB2AH7 antibody and control antibodies exhibited some

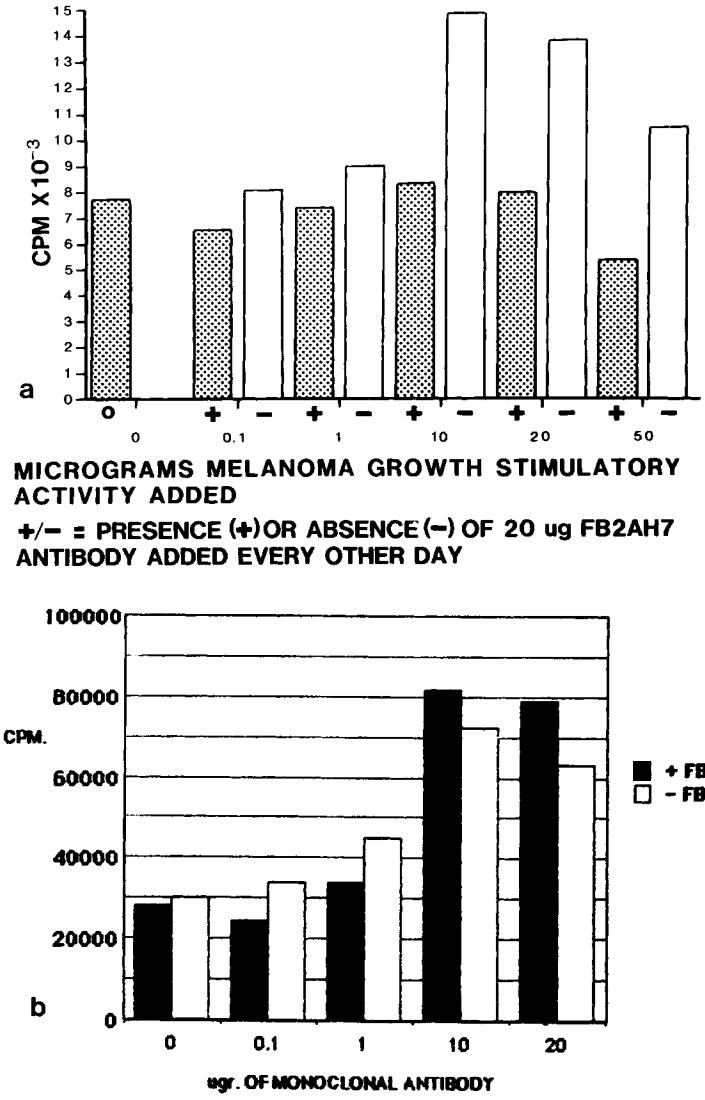

Fig. 4. **a**: Effect of monoclonal antibody FB2AH7 on stimulation of ^{3}H-thymidine incorporation in Hs0294 cells by an acetic acid extract of Hs0294-conditioned medium (CM). Six thousand Hs0294 cells were plated in 2 ml of Ham's F-10 medium plus 10% fetal bovine serum in Wheaton scintillation vials and incubated at 37°C in a 95% air/5% CO_2 environment. The next day cells were washed twice with phosphate-buffered saline prior to addition of serum-free Ham's F-10. After a 24-hr incubation the culture medium was changed again and each vial received μg quantities of acetic acid extracts of Hs0294 CM with or without 20 μg of FB2AH7 as indicated in the chart. Incubation was continued for another 8 hr; then each vial received [^{3}H]-thymidine (5 μCi per vial). Cultures were subsequently incubated as described above for 16 hr; then the medium was decanted and cells were fixed and counted as previously described [22]. Control counts per min ± standard error of the mean, 7,715 ± 716. Average standard error as a percent of the mean was 8%. Significant stimulation ($P < .05$) was seen with 10 μg, 20 μg, and 50 μg of CM, but in no groups which received monoclonal antibody FB2AH7. The difference between treated groups with and without monoclonal antibody FB2AH7 was statistically significant ($P < .05$) at the 10-, 20-, and 50-μg amounts. N = 3 for each treatment group and 6 for controls. **b**: Effect of monoclonal antibody FB2AH7 on stimulation of [^{3}H]-thymidine incorporation by Hs0294 CM on NRK-49F cells. Six thousand NRK-49F cells were plated in 2 ml of DME medium plus 10% calf serum as described in the legend to Figure 4a. The assay was conducted as described for Figure 4a, with the amounts of CM and FB2AH7 indicated.

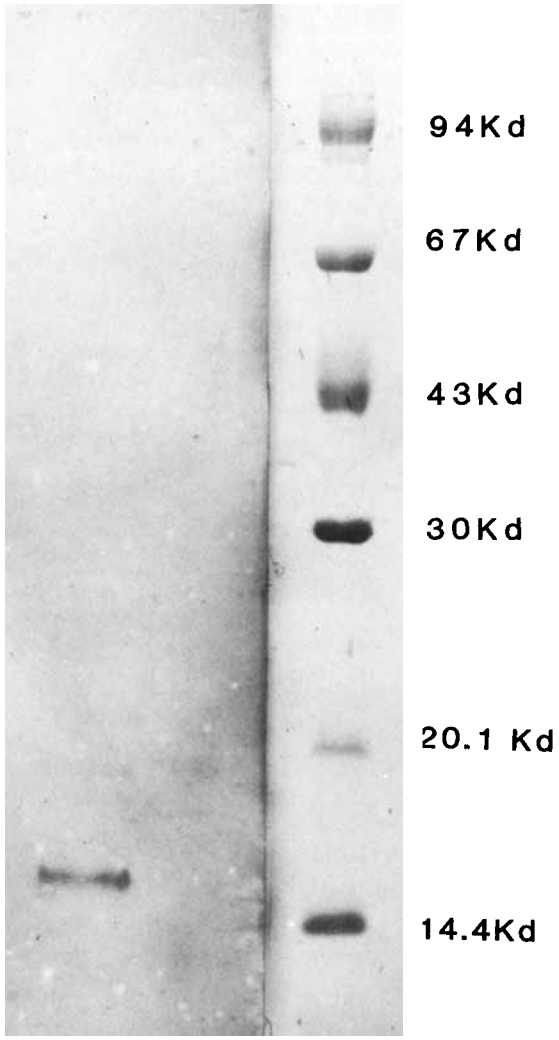

Fig. 5. Western immunoblot of RP-HPLC-purified MGSA activity with antibody FB2AH7. MGSA was purified through RP-HPLC and subjected to SDS polyacrylamide gel electrophoresis as described in the text. This photograph illustrates the binding of monoclonal antibody FB2AH7 to a 16-Kd moiety in this preparation (**left lane**). Standards (**right lane**) (Pharmacia) were phosphorylase b, Mr 94,000; bovine serum albumin, Mr 67,000, ovalbumin, Mr 43,000; carbonic anhydrase, Mr 30,000, soybean trypsin inhibitor, Mr 21,000, and α-lactalbumin, Mr 14,400.

apparent nonspecific binding to the carbonic anhydrase and phosphorylase B-molecular weight markers. At the present time, we cannot explain the nonspecific interactions of IgM antibodies but this problem appears to occur with other IgM antibodies as well and may be the result of proteins with the sugar groups on the IgM.

Immunoprecipitation Experiments

Immunoprecipitation experiments with FB2AH7 and control human myeloma IgM also revealed that there is some nonspecific interaction of IgM with proteins in

the same size range as MGSA (Fig. 6a). When the control antibody is allowed to prebind these proteins prior to precipitating with FB2AH7, the 16–17-Kd moiety emerges as the dominant protein which is specifically precipitated by FB2AH7, and the contribution of the 14- and <14-Kd moieties to the total immunoprecipitate is diminished (Fig. 6b). Growth factors as well as immunoglobulins of the IgM class are known to be sticky and exhibit problems with non-specific interaction with proteins. We found that addition of 0.1% Triton X-100 to the immunoprecipitation buffer reduced but did not completely eliminate this problem.

Modified Dot Blot ELISA

The specificity of FB2AH7 for MGSA has been demonstrated by utilizing a modified dot blot enzyme-linked immunoassay (ELISA). Purified preparations of PDGF, EGF, FGF, NGF, IGF-I, 1L-1, 1L-2, transferrin, and insulin did not bind FB2AH7, while RP-HPLC-purified preparations of MGSA did specifically bind FB2AH7. IGF-1 preparations did weakly bind FB2AH7, but the binding was marginal and not concentration dependent. TGFβ did bind FB2AH7 at the 100- and 50-ng

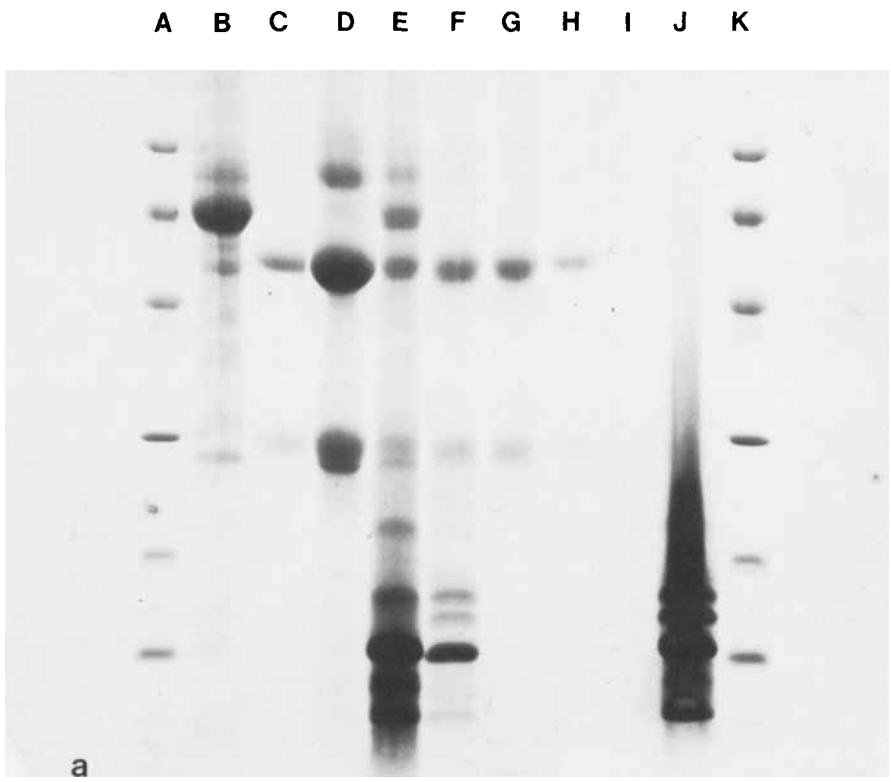

Fig. 6. **a**: Bio-Gel Pool B preparations of MGSA (0.5 ml) were cleared by centrifugation, combined with 0.5 ml of a 1 mg/ml solution of FB2AH7 or control nonspecific MF2 antibody, and placed at 4°C overnight. The next day 0.5 ml of goat antimouse Ig(GAM) antibodies (1mg/ml) were added; after a 2-hr incubation at 25°C, the precipitate was collected by centrifugation, washed three times with PBS, and electrophoresed under reducing conditions according to the procedures of Laemmli [37]. From left to right, lanes **A–K** are standards, FB2AH7 antibody, antimouse IgM_K, FB2AH7 plus antimouse Ig precipitate, MGSA precipitated with FB2AH7 and antimouse IgM_K, control MF2 and mouse Ig precipitate from MGSA preparation, MF2 plus antimouse Ig precipitate, MF2, blank, Bio-Gel Pool B MGSA, and standards (top to bottom: 94; 67; 43; 30; 20.0; and 14.4 Kd).

dilutions but only minimally at 20 ng. Control monoclonal antibodies (IgA and IgM_K) did not bind to MGSA preparations in this assay.

DISCUSSION

These data are compatible with the hypothesis that monoclonal antibody FB2AH7 recognizes a 16-Kd autostimulatory mitogen produced by Hs0294 malignant mela-

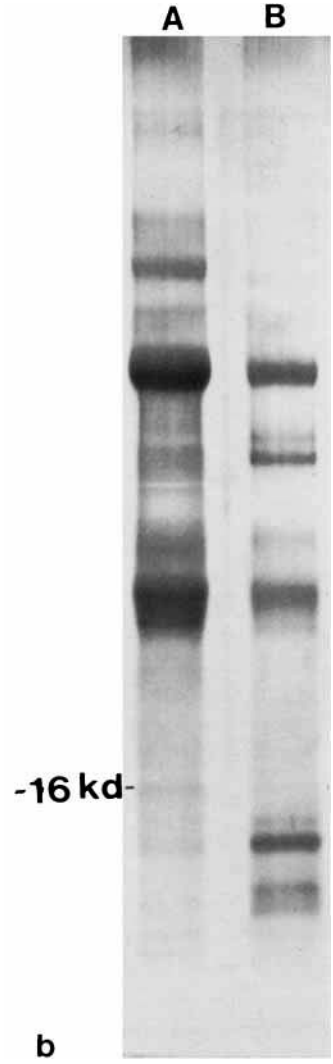

Fig. 6. **b**: MGSA preparations from Bio-Gel Pool B were precipitated with the nonspecific MF2 antibody as described above except that 0.1% Triton X-100 was included in the sample buffer. The precipitate was collected by centrifugation, washed three times with PBS containing 0.1% Triton X-100, and frozen at −80°C. The supernatant was incubated with FB2AH7 antibody overnight at 4°C; then antimouse IgM_K antibodies were added to precipitate the FB2AH7 antigen-antibody complexes. After collecting the precipitate by centrifugation and washing three times with PBS containing 0.1% Triton X-100, the precipitates were solubilized and electrophoresed under nonreducing conditions according to procedures of Laemmli. Lane **A** visualizes the FB2AH7 precipitate and lane **B** the non-specific MF2 precipitate. Note the 16-Kd band lane A which is only faintly visible in lane B.

noma cells. Though there are some problems regarding nonspecific binding with MGSA and the FB2AH7 IgM in immunoprecipitation experiments, these nonspecific interations are greatly reduced when the antigen is fixed on nitrocellulose paper or in cells [49]. Further supporting evidence comes from the recent demonstration that antibody FB2AH7 can be bound to Sepharose and utilized to purify MGSA from acetic acid extracts of Hs0294-conditioned medium [50]. In these experiments, both high and low molecular weight forms of MGSA were bound to and eluted from FB2AH7. Preparative electrophoresis of the eluant followed by MGSA bioassay revealed that the majority of the bioactivity resides in the ~16-Kd band. MGSA has also been recently purified from Hs0294 conditioned medium by Richmond and Thomas [23a]. Though MGSA bioactivity was found to reside in a family of polypeptides (~9–26 Kd) the major bioactive form of MGSA was demonstrated to migrate with the 16-Kd band. The growth of Hs0294 cells is not completely blocked by the addition of FB2AH7 antibody to MGSA. This may indicate that the cells are not totally dependent on this factor for proliferation, although growth is enhanced when it is available. This could be due in part to production of other autocrines. Alternatively, the growth factor may have a higher affinity for its receptor than for the antibody, such that receptor binding cannot be completely blocked. The receptor binding epitope may be distinct from the antibody reactive epitope. Finally, some of the factor may exert a major portion of its bioactivity through an intracellular mechanism, as has been suggested for certain cells which both release and respond to PDGF [28].

Some of these considerations may also account for the delayed effect of the antibody in cell number experiments. Alternatively, it is possible that the contribution of the antigen recognized by monoclonal antibody FB2AH7 to the growth of these cells is most apparent at higher cell densities (resulting from longer periods in culture) because larger numbers of cells can more effectively "condition" the culture medium. The primary effect of MGSA may be to enhance the synthesis or release of another mitogen, as has been shown for the effect of PDGF on fibroblasts and smooth muscle cells [32]. The delayed effect of the antibody in cell number experiments could also be explained if the primary effect of the FB2AH7 antigen is to render cells competent to respond to other growth factors [39]. Brief exposure to the antigen before antibody additions were begun would render some cells competent for one or two cycles of cell division, which for these cells in serum-free medium would take 3–6 days. Thus, the effect of the antibody would not become apparent until cells began to enter a second or third round of division. These issues and the issue of the specificity of the response of these cells to the antibody will be best answered when pure preparations of antibody and antigen are available; it may also be necessary to develop other antibodies to this antigen, preferably of the IgG class. Though FB2AH7 did not bind PDGF, IL-1, IL-2, NGF, FGF, EGF, transferrin, or insulin, the interation with IGF-1 and TGFβ further indicates problems with nonspecific binding. This is probably due to the inherent stickiness of these growth factors, combined with the problems of nonspecific interactions with IgM antibodies. Neither TGFβ nor IGF-1 stimulates ^{3}H-thymidine incorportion in Hs0294 cells as MGSA does [23,23a]. Therefore, it is unlikely that the growth-inhibitory effects of FB2AH7 are associated with the binding of TGFβ or IGF-I. Rather, the growth-inhibitory effects of FB2AH7 appear to be the result of the specific binding of MGSA to FB2AH7 antibody. However, at this time we cannot rule out the possibility that these three growth factors share a common

epimer, although when the FB2AH7 antibody is used in immunohistochemical studies, tissues which are known to contain TGFβ and IGF-I are not positive for the FB2AH7 antigen [49].

The mechanism by which FB2AH7 antibody inhibits growth of Hs0294 cells is apparently different from that of other melanoma-associated antibodies with growth-inhibitory properties. The melanoma-assoicated antigen p97, a 97-Kd glycoprotein, is structurally and functionally related to transferrin [40]. Monoclonal antibodies to p97 did not kill melanoma cells in the absence of an attached toxin [41]. A monoclonal antibody against the transferrin receptor, however, was able to inhibit the growth of M21 human melanoma cells in nude mice without an attached toxin [42]. A monoclonal antibody directed against the disialoganglioside GD_3 caused rounding up and growth inhibition in vitro of melanoma cells expressing high levels of this antigen but had no effect on the morphology or growth of melanoma and nonmelanoma cells with low levels of GD_3 [43]. A human monoclonal antibody to the disialoganglioside GD_2 has been reported to delay the growth of a human melanoma cell line in nude mice [44]. Monoclonal antibodies to the core glycoprotein for chondroitin sulfate proteoglycan had little effect on the growth of melanoma cells in liquid culture [45,46], but they did inhibit the growth of these cells in soft agar cultures [45] and in nude mice [46]. The mechanism by which these monoclonal antibodies inhibit growth presumably involves binding to the cell-surface components. The mechanism proposed for monoclonal antibody FB2AH7 is that it binds to a mitogen released by the cells and thereby prevents binding of the growth factor to its receptor. The 16-Kd form of this autostimulatory melanoma mitogen is apparently different from other known growth factors.

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