ABSTRACT
A cultured renal carcinoma cell line, ACHN, continued to proliferate in the absence of exogenous growth factors supplied by fetal calf serum. ACHN cells had been previously used as the immunogen for the production of monoclonal antibody 5F4. Subsequent immunohistochemical studies showed that 5F4 strongly and selectively reacted with renal carcinoma cells compared with normal renal epithelium, suggesting that expression of the substance to which it was bound was amplified as a result of malignant transformation. Because renal carcinoma cells are relatively undifferentiated with regard to function except for elements to ensure sustained growth, the immunoreactivity patterns led to the hypothesis that 5F4 reacted with some component of a growth-stimulating pathway. The hypothesis was supported in the present work by showing that 5F4 specifically inhibited the growth of ACHN cells; ACHN cells prominently secreted a protein with a molecular mass of 178 kilodaltons that was immunoreactive with 5F4; the protein was partially purified by simple lyophilization of serum-free conditioned medium; both ACHN conditioned medium and the lyophilizate stimulated the growth of quiescent human fibroblasts and BALB/3T3 cells as well as serum-deprived human renal carcinoma cell lines ACHN, A498, Caki-1, and Caki-2. Specific immunoabsorption of the protein by affinity chromatography with 5F4 removed the growth-stimulating activity. The results demonstrated a novel growth-promoting substance secreted by ACHN cells with autologous activity as well as activity for human and murine fibroblastic cell lines and other renal carcinoma cell lines. The growth-promoting activities were specifically blocked by monoclonal antibody 5F4.

INTRODUCTION
It has been proposed that malignant neoplastic cells secrete substances which support their growth in the absence of exogenous growth factors (1). This mechanism explains the aggressive growth pattern of neoplastic cells both locally and as metastases to otherwise foreign parts of the body where specific stimulatory factors may not be present. In addition, these growth factors could sometimes function to stimulate the growth of normal cells surrounding the tumor to explain at least two classical pathological features of malignant neoplasia, neovascularization and desmoplasia (2, 3). It can be deduced that autologous production of these growth factors by the neoplastic cells has evolved to promote their independent survival.

It can be hypothesized that direct interference with autologous growth-promoting mechanisms would specifically inhibit growth of neoplastic cells. There have been a few experiments performed to support this hypothesis. For example, Masui et al. showed that monoclonal antibodies which bind to the epidermal growth factor receptor inhibited in vitro tumor cell growth apparently by altering physiological functions of the receptor (4). Refinement of these methods of tumor cell growth inhibition could lead to practical methods of cancer control through the use of specific substances that block autologous growth-stimulating pathways.

For the present work, it was hypothesized that anti-renal cell carcinoma monoclonal antibody 5F4 would inhibit the growth of cultured renal carcinoma cells through binding and inactivation of some component of a growth-stimulating pathway. The hypothesis was formulated through recognizing and connecting certain fundamental properties of renal cell carcinoma and the immunoreactivity pattern of the antibody with the tumor cells. Renal carcinoma cells are relatively undifferentiated with regard to function except for elements to ensure relentless, aggressive growth. These elements should thus be prominent components of the cell. Monoclonal antibody 5F4 produced a prominent, coarse, granular, cytoplasmic staining of the various histological varieties of renal carcinoma cells by avidin-biotin immunoperoxidase staining of both frozen and formalin-fixed, paraffin-embedded tissue sections (5, 6). The antibody reacted only focally and weakly, if at all, with normal renal cortical epithelium in the same sections. The results of these immunohistochemical studies implied that 5F4 reacted with a substance that was amplified in expression as a result of malignant transformation and thus was essential for continued aggressive growth of the cells.

A series of experiments are presently reported that show the continued growth of a renal carcinoma cell line, ACHN, in the absence of exogenous growth factors supplied by fetal calf serum and specific inhibition of this autonomous growth through incubation with monoclonal antibody 5F4. A growth-promoting substance secreted by ACHN cells that was immunoreactive with monoclonal antibody 5F4 and that showed growth-stimulating activity for cultured human and murine fibroblasts as well as other human renal carcinoma cell lines was identified.

MATERIALS AND METHODS
Demonstration of Autonomous Growth of ACHN. Cells (5 × 10⁴) were seeded into 35-mm culture dishes in triplicate for each test or control and allowed to adhere overnight in DMEM with 10% FCS in a standard tissue culture environment of 37°C and 5% CO₂. The cells were then incubated in fresh DMEM without FCS for two additional periods of 8 and 16 h in order to remove traces of FCS (7). In order to establish a reference point for proliferation, the average number of cells in three of the dishes was determined by trypsinization and counting in a hemacytometer. Fresh DMEM was replaced into the experimental dishes, and fresh DMEM with 10% FCS was added to the control dishes.

After 23 h, 2 μCi of tritiated thymidine were added to some of the dishes in order to determine if the cells that had been incubated in the absence of FCS had become quiescent (2) or showed continued DNA synthesis that would be characteristic of autonomous growth stimulation. After an additional hour of incubation, the cells were washed with HBSS, precipitated and washed with cold 10% TCA, and extracted with 0.3 N NaOH-1% SDS (8). The extracts were added to scintillant and β counted.

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2 The abbreviations used are: DMEM, Dulbecco's modified Eagle's medium; EGF, epidermal growth factor; FCS, fetal calf serum; GPS, growth-promoting substance; HBSS, Hanks' balanced salt solution; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline, pH 7.4; PBST, phosphate-buffered saline:0.1% Tween 20; pH 7.4; SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid; TGF-α, transforming growth factor α.
In order to determine actual cell growth in the absence of FCS which would be characteristic of autonomous growth, the actual number of cells in the dishes after incubation for 96 h with a change of medium at 48 h was determined by trypsinization and counting in a hemacytometer. The cell lines used in the above experiments included ACHN, OS2.4, CCD-41Sk, and BALB/3T3 clone A31. ACHN was derived from malignant cells in the pleural effusion of a 22-yr-old man with widely metastatic renal cell carcinoma. It had been used as the immunogen for the production of monoclonal antibody 5F4. The antibody (5F4) reacts with a characteristic coarsely granular, perinuclear, cytoplasmic pattern with the cells by both immunofluorescent and avidin-biotin complex immunoperoxidase staining. OS2.4 was derived from the primary culture of a canine osteosarcoma. This cell line shows no detectable reactivity with 5F4 by immunostaining or immunoblotting detergent extracts and thus was used as a negative control for the growth modulation experiments described below that included 5F4 in the medium. CCD-41Sk was derived from cutaneous fibroblasts in a 21-yr-old man. It represented a normal cell line that does not proliferate in the absence of exogenous growth factors. BALB/3T3 cells are contact-inhibited fibroblasts that do not proliferate in the absence of exogenous growth factors and that have been shown to be stimulated by other growth factors extracted from renal carcinoma cells (2, 9, 10).

Blockage of Autonomous Growth of ACHN Specifically by 5F4. ACHN and OS2.4 cells showed autonomous growth in the above experiments and, thus, were used to investigate the hypothesis that 5F4 would inhibit cellular proliferation through binding to some substance in an autologous growth stimulation pathway. Cells (5 x 10^5) were seeded into 35-mm dishes, adhered overnight, then incubated overnight in fresh DMEM for two additional periods of 8 and 16 h in order to remove traces of FCS (7). Fresh DMEM was added to each dish along with Protein A affinity-purified anti-renal cell carcinoma monoclonal antibody 5F4 or an irrelevant monoclonal antibody, MOPC21, at concentrations of 0.025, 0.25, 2.5, and 25 µg/ml. MOPC21 was prepared by Protein A affinity chromatography of supernatant from a culture of P3X63Ag8 (ATCC TIB 9). It is an IgGl murine monoclonal antibody like 5F4 and does not react with either ACHN or OS2.4 by either immunofluorescence or immunoblotting cellular extracts. The antigen it recognizes is unknown. It represented a control for possible nonspecific inhibitors of cellular growth that might have been introduced during antibody purification. After 23 h of incubation, 2 pCi of tritiated thymidine were added to the cultures for 1 h, and the labeled cells were washed, precipitated, extracted, and β counted as above. Viability of the treated and untreated cells was determined by staining duplicate dishes with acridine orange and ethidium bromide.

Preparation of Conditioned Medium and Identification of a Secreted Protein Immunoreactive with 5F4. ACHN and other renal carcinoma cell lines A498, Caki-1, Caki-2, and A-704 were grown to confluence in 25-cm² tissue culture flasks in DMEM with 10% FCS, then washed, and incubated in fresh DMEM for two additional periods of 8 and 16 h as above in order to remove traces of FCS (7). Fresh DMEM was added to each dish, collected after 48 h, combined, and passed through a 0.22-µm pore size filter to finally produce conditioned medium (7). The protein concentration of the conditioned medium was determined by TCA precipitation and a copper complex assay (Pierce). The conditioned medium and appropriate molecular weight standards were electrophoresed in 7% SDS-polyacrylamide gels under reducing conditions. The gels were either fixed in methanol and acetic acid and silver stained for molecular weight determination of the separated proteins or were used for transfer of the separated proteins to nitrocellulose paper according to the method of Towbin et al. (11). Nitrocellulose paper containing the transferred proteins was washed with PBS, blocked for nonspecific protein binding by incubation in PBS with 0.1% Tween 20 and 1% bovine serum albumin, and then incubated overnight with either 5F4 supernatant or Protein A affinity-purified 5F4 (25 µg/ml). Supernatant from P3X63Ag8 or Protein A affinity-purified MOPC21 (25 µg/ml) was used as a negative control. The filters were washed twice for 15 min each in PBST, incubated for 30 min with alkaline phosphatase-labeled, affinity-purified, anti-mouse IgG, washed again twice in PBST, and then once again in 0.5 M Tris-0.5 M NaCl–0.2 M MgCl2, pH 9.5. Color was developed by incubation of the labeled filters with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate in the pH 9.5 Tris-magnesium buffer for 30 min.

In some experiments, immunoreactivity was blocked by overnight preincubation of Protein A affinity-purified 5F4 with various concentrations of an electrophoretically pure M, 178,000 protein, designated GPS, which was prepared by lyophilization of dialyzed conditioned medium as described below. 5F4 (50 µg/ml) was mixed with equal volumes of GPS in serial dilutions from 1000 to 1 µg/ml and incubated overnight. This solution, which had a final 5F4 concentration of 25 µg/ml, was then used as the primary reagent for immunoblotting electrophoretically pure GPS (10 µg) as described above.

Lyophilization. It was seen from the above electrophoretic gels that the ACHN cells secreted relatively large amounts of a M, 178,000 protein that was immunoreactive with 5F4. The conditioned medium showed no detectable background proteins, suggesting that lyophilization would produce a relatively pure concentrate of the antigen. Ten ml of the conditioned medium were dialyzed versus three changes of a 100x volume of distilled water at 4°C for a total period of 24 h. The dialyzed conditioned medium was frozen, and the water was removed by sublimation overnight in a vacuum. The lyophilized was reconstituted in sterile HBSS, and the protein concentration of the reconstituted specimen was determined by a copper complex assay (Pierce) and finally diluted with sterile HBSS to appropriate concentrations for the growth stimulation experiments below.

Stimulation of Quiescent Fibroblasts and Serum-deprived Renal Carcinoma Cells by the Conditioned Medium and Lyophilized. CCD-41Sk, BALB/3T3, ACHN, A498, Caki-1, Caki-2, and A-704 cells (5 x 10^5) were seeded into 35-mm² dishes and allowed to adhere overnight. The cells were washed and incubated in fresh DMEM for two additional periods of 8 and 16 h in order to remove traces of FCS (7). Quiescence of the fibroblasts and autonomous growth of the renal carcinoma cells were induced by incubating in fresh DMEM for an additional 24 h as described above. Conditioned medium from the ACHN cells and the reconstituted lyophilitated at final protein concentrations of 100, 10, and 1 µg/ml were added to the cultures for 23 h. In addition, the conditioned medium was adsorbed by a 10-fold excess of 5F4 by passage over an Affi-gel HZ column prepared by binding the antibody according to the manufacturer's directions (Biorad). This adsorbed preparation was also added to the quiescent fibroblast and serum-deprived renal carcinoma cultures to demonstrate that the growth-stimulating factor was specifically removed by adsorption with 5F4. Tritiated thymidine (2 µCi) was finally added to each culture for 1 h, followed by washing, precipitation, extraction, and β counting as described above.

RESULTS

Demonstration of Autonomous Growth of ACHN. Uptake of tritiated thymidine after incubation in DMEM without FCS for 24 h and expressed as a percentage of control dishes that had been incubated in DMEM with 10% FCS averaged 54% for ACHN, 39% for OS2.4, 7% for CCD Sk41, and 1% for BALB/3T3 (Fig. 1). The actual increase in cell number over a 96-h incubation period in DMEM without FCS averaged 5.1-fold for ACHN, 5.0-fold for OS2.4, 0.9-fold for CCD Sk41, and 0.8-fold for BALB/3T3 (Fig. 2). The results essentially show that ACHN and OS2.4 continue to proliferate, albeit at a somewhat reduced rate, in the absence of exogenous growth factors supplied by FCS. CCD-41Sk and BALB/3T3 become quiescent.

Blockage of Autonomous Growth of ACHN Specifically by 5F4. Monoclonal antibody 5F4, at a concentration of 25 µg/ml, strongly inhibited the incorporation of tritiated thymidine by ACHN after incubation for 24 h (Fig. 3). At this antibody concentration, the cells incorporated thymidine at a rate of 7% of that of matched cultures without added 5F4. The cells showed greater than 99% viability by staining with acridine orange and ethidium bromide. There was a slight inhibition.

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Fig. 1. Thymidine uptake of ACHN, OS2.4, CCD-41Sk, and BALB/3T3 cells cultured in DMEM in the absence of fetal calf serum for 24 h. The results are expressed as a percentage of thymidine incorporation by matched cultures grown in the presence of 10% fetal calf serum. It can be seen that ACHN and OS2.4 maintain substantial thymidine incorporation in the absence of exogenous growth factors, while CCD-41Sk and BALB/3T3 cells become quiescent. Columns, mean; bars, SD.

Fig. 2. Actual increase in cell number of ACHN, OS2.4, CCD-41Sk, and BALB/3T3 cells when grown in DMEM in the absence of fetal calf serum for 96 h. It can be seen that ACHN and OS2.4 cells continue to divide in the absence of exogenous growth factors, while CCD-41Sk and BALB/3T3 cells cease division and begin to degrade. Columns, mean; bars, SD.

Fig. 3. Specific inhibition of thymidine incorporation by ACHN cells through addition of monoclonal antibody 5F4 to the culture medium. The results are expressed as the ratio of thymidine incorporation by cultures in the presence of antibodies to incorporation by matched cultures with DMEM only. It can be seen that 5F4, at a concentration of 25 µg/ml, substantially inhibited thymidine uptake by ACHN cells. 5F4 had no effect on OS2.4 cells at this concentration, suggesting that the effect was related to antigen binding. MOPC21 had no effect on either cell line at any concentration, diminishing the possibility of nonspecific toxicity caused by the addition of purified antibodies to the culture medium. ■, ACHN + 5F4; □, ACHN + MOPC21; △, OS2.4 + 5F4; □, OS2.4 + MOPC21.

(87% of matched cultures without 5F4) of thymidine incorporation at a 5F4 concentration of 2.5 µg/ml. There was no detectable effect at lower concentrations. An identically processed irrelevant monoclonal antibody, MOPC21, had no effect on thymidine incorporation by the cells at the same concentrations. 5F4 had no effect on thymidine incorporation by OS2.4, a cell line which showed autonomous growth, but which also had no detectable reactivity with the antibodies by immunoblotting nonionic detergent extracts or by immunocytochemistry.

Preparation of Conditioned Medium and Identification of a Secreted Protein Immunoreactive with 5F4. The conditioned medium from ACHN cells had a TCA-precipitable protein concentration of 0.03 mg/ml. SDS-PAGE showed a single prominent M, 178,000 band with no densitometrically detectable background proteins (Fig. 4). The protein was not detectable in electrophoretic gels of conditioned media from the other renal carcinoma cell lines. Western immunoblotting of the conditioned medium from ACHN with 5F4 showed a positive reaction with the band (Fig. 5). Preincubation of Protein A affinity-purified 5F4 (25 µg/ml) with GPS at concentrations ranging from 500 to 62 µg/ml blocked detectable immunoreactivity with GPS (10 µg) bound to nitrocellulose paper (Fig. 6). A weak reaction was seen after blocking with GPS at 31 µg/ml. The reaction increased in intensity as the concentration of blocking GPS was further reduced.

Fig. 4. Electrophoresis and silver stain of conditioned medium (1.5 µg of protein) from ACHN cells in a 7% SDS-polyacrylamide gel under reducing conditions. There is a single prominent band at a migration corresponding to a molecular mass of 178 kilodaltons (Kdal).

Fig. 5. Immunoblotting of conditioned medium from ACHN cells separated by SDS-PAGE and transferred to nitrocellulose paper. Lane A shows the reaction of monoclonal antibody 5F4 with the 178-kilodalton (Kdal) band seen in Fig. 4. Lane B is a negative control and shows the lack of reaction of MOPC21, an irrelevant monoclonal antibody, with the band.
conditioned medium with 5F4 because the ACHN cells, unlike the renal cell line ACHN, can be blocked by adding protein A without GPS blocking, was the positive control. By preincubation with GPS at a concentration of 125 μg/ml, it was observed that the immunoreactivity of 5F4 was blocked overnight. This solution, which contained 5F4 at a final concentration of 25 μg/ml, without GPS blocking, was the positive control.

DISCUSSION

It has been shown that autonomous growth of a renal carcinoma cell line, ACHN, can be blocked by adding protein A affinity-purified monoclonal antibody 5F4 to the culture medium. It was demonstrated that ACHN cells secrete a M, 178,000 protein into serum-free culture medium that was immunoreactive with monoclonal antibody 5F4. The protein directly stimulated the uptake of tritiated thymidine by quiescent human and murine fibroblasts as well as by serum-deprived human renal carcinoma cell lines. These findings suggest that the protein is a novel renal cell carcinoma-associated, growth-promoting substance with autologous stimulatory activities as well as activities for both human and murine fibroblasts.

Most other growth-promoting substances associated with renal cell carcinoma have had different characteristics. Chodak et al. (9) described the partial purification of a M, 17,000 protein through heparin-Sepharose chromatography of extracts of human renal tumors. This protein stimulated bovine capillary endothelial cell proliferation and DNA synthesis in BALB/3T3 cells. It also reacted with polyclonal antiserum raised against a peptide fragment of basic fibroblast growth factor. Mydlo et al. (2) described the extraction of a similar substance from renal adenocarcinomas.

In another report, Mydlo et al. (12) described relatively increased levels of mRNA for TGF-α and for the epidermal growth factor receptor in extracts from five freshly resected Stage I renal cell carcinoma of the clear cell variety. Avidin-biotin immunoperoxidase staining of frozen sections from two of the same cases showed increased reactivity of neoplastic cells that had been incubated with antibodies against TGF-α and the EGF receptor. TGF-α has a mass of 5.6 kilodaltons and the EGF receptor has a mass of 170 kilodaltons. EGF secreted by the distal tubes of the murine kidney has a relatively high mass of 130 kilodaltons. Although it is generally believed that tumors do not secrete EGF, but rather the more primitive, yet strongly related TGF-α (13), Lau et al. (15) reported positive staining of formalin-fixed, paraffin-embedded sections of renal cell carcinoma with mouse polyclonal antiserum reactive with human EGF. Although these findings suggested that monoclonal antibody 5F4 might have reacted with renal EGF or the EGF receptor, there are many reasons why this was not so. Nakamoto et al. (10) were unable to detect either EGF or TGF-α by radioimmunoassay of conditioned supernatant from ACHN cells. The secreted protein reactive with 5F4 directly stimulated the growth of human fibroblasts, thus it was a factor and not a receptor.
The present work demonstrates a novel mechanism of monoclonal antibody-mediated growth inhibition of cultured tumor cells by direct neutralization of a growth-promoting substance secreted into the medium. Cultured cells have been previously inhibited in growth by monoclonal antibodies through blockage of the function of the EGF receptor as well as by modulating the classical immune mechanisms of complement-mediated and effector cell cytotoxicity (4,16). Monoclonal antibody 5F4 is an IgGl isotype and has not shown complement-mediated cytotoxicity or modulation of effector cell function in previous unpublished experiments. Continued work should elucidate the autologous growth stimulation pathways in malignant cells in order to gain insight into possible new methods of growth inhibition using specific reagents that interrupt those pathways.

The present work also establishes a function for the antigen defined by monoclonal antibody 5F4. Continued study of this antigen at a molecular level will give fundamental insight into the discrete cellular changes causing renal cell carcinoma, because it is apparent that the antigen's gene is amplified in expression as a result of malignant transformation of certain renal epithelial cells. It will be of interest to determine if the gene is activated as a result of loss of regulation secondary to inactivation of a recessive oncogene. This mechanism has been postulated for renal cell carcinoma because of various common cytogenetic changes and specific deletions on chromosome 3 (17–19).

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Blockage of Autonomous Growth of ACHN Cells by Anti-Renal Cell Carcinoma Monoclonal Antibody 5F4

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