

## Monoclonal Antibody (5G6.4) against Ovarian Carcinoma Shows Inhibition of *in Vitro* Colony Formation<sup>1</sup>

LORNA RODRIGUEZ-RODRIGUEZ, M.D.,\* MONICA LIEBERT, PH.D.,†  
RONALD NATALE, M.D.,‡ AND RICHARD WAHL, M.D.†

\*Department of Obstetrics and Gynecology, Division of Gynecological Oncology, University of Michigan; †Division of Nuclear Medicine, University of Michigan; and ‡Department of Medical Oncology, University of Michigan, Ann Arbor, Michigan 48109-0028

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Monoclonal antibodies (MAbs) have the potential for diagnosis and therapy of cancer. 5G6.4 is a MAb of the IgG2a class which was produced by immunization of BALB/c mice with human ovarian carcinoma (Ov Ca) cells. To further characterize 5G6.4, its effect on cell growth was tested using a human Ov Ca cell line established in our laboratory. A clonogenic assay was set up in 48-well plates in a double agar system. The cells were plated and 5G6.4 was added at different concentrations. Control plates consisted of cells with media without MAb. Negative control plates were also prepared using the same concentrations of an isotype-matched antimelanoma MAb, 225.28s. Colony formation (CF) was reduced to 50% or less of control with increasing amounts of 5G6.4 up to 50  $\mu\text{g}/\text{ml}$ . Although CF was still depressed at concentrations above 50  $\mu\text{g}/\text{ml}$ , the inhibition did not follow a directly proportional line; instead, it followed a bell-shaped curve. Plates with the control MAb, 225.28s, did not show this response. Similar results were obtained with cells from malignant Ov Ca ascites in the same clonogenic assay. Our study suggests that in the evaluation of the *in vitro* effect of MAb on growth, the concentration of MAb is crucial and may not show a linear response and that 5G6.4 may have a direct therapeutic effect by blocking the growth of Ov Ca cells. 5G6.4 is presently under study for therapy in an animal model. © 1987 Academic Press, Inc.

### INTRODUCTION

Monoclonal antibodies (MAbs) have the potential for use in therapy of cancers [1,2] and have been used in some clinical trials in humans [3-8]. The results have been varied, and in some cases interaction of the host's immune system has appeared to be important in positive responses [3,9]. Studies have concentrated on complement activation or cell cooperation, such as antibody-dependent cell-mediated cytotoxicity (ADCC; [10-12]). Yet another group of monoclonal antibodies is capable of directly inhibiting the growth of cells [13,14].

We have begun evaluation of a monoclonal antibody, 5G6.4, reactive with human ovarian carcinoma (Ov Ca) cells. Unlike some MAbs to leukemia which

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cause modulation or loss of antigen expression [15], the 5G6.4-defined antigen does not appear to modulate (Wahl and Liebert, unpublished). Radioiodinated 5G6.4 localizes well in subcutaneous human ovarian tumors in nude mice, with tumor:blood ratios of 5:1 to 10:1 [16,17]. Although 5G6.4 does bind to some normal human tissues (skin and kidney) [16], its high expression on Ov Ca cells and good localization in human tumor-bearing nude mice suggest promise as an immunolocating or immunotherapeutic agent. In this study, we demonstrate antigen expression on Ov Ca cells by flow cytometry and radiolabeled antibody binding.

To further evaluate the usefulness of 5G6.4 for immunotherapy, we tested its ability to block growth of these cells using an *in vitro* colony formation assay.

### MATERIALS AND METHODS

*Cell lines.* The methods for initiation and maintenance of the cell line used in this study have been previously described [18]. Briefly, the cell line (MI OV) was maintained in RPMI 1640 media supplemented with 10% heat-inactivated fetal bovine serum, 1% nonessential amino acids, 100 IU penicillin/ml, 100  $\mu$ g streptomycin/ml, 2 mM glutamine. The cells were grown in T-25 tissue culture flasks and kept in a 37°C incubator at a 5% CO<sub>2</sub> atmosphere.

*Collection of cells.* Malignant effusion from a patient of documented recurrent carcinoma was used to retest the assay with a fresh tumor. Three liters of ascitic fluid was centrifuged (1000 rpm for ten min). The cells (TA OV) were then resuspended in RPMI and an aliquot was counted in a hemocytometer with trypan blue to identify the viable cells. The cells were resuspended into an adequate volume of RPMI in order to get the predetermined cell density needed for the clonogenic assay.

*Monoclonal antibodies.* 5G6.4 is an IgG2a murine monoclonal antibody prepared by immunizing BALB/c mice with HTB77, a human ovarian carcinoma cell line. It is directed against an epithelial tumor-associated antigen, found on the basal layer of various epithelia, proximal tubules of the kidney, ovarian tumors and several other types of tumors [16]. 225.28s is a monoclonal antibody reactive with a high-molecular-weight chondroitin sulfate antigen found in melanoma cells [19]. It is of the IgG2a subclass. UPC-10 is a murine myeloma IgG2a protein. Purified UPC-10 was purchased from Litton Bionetics (Rockville, MD). Antibodies were purified by affinity chromatography on staphylococcal protein A columns [20]. Purified antibodies were sterilized by filtration through a 0.22  $\mu$ m filter (Millipore, Bedford, MA) and were dialyzed against RPMI without serum overnight at 4°C under sterile conditions. The protein concentration was checked using the Bradford protein assay [21] immediately before use.

*Flow cytometry.* Washed tissue-cultured cells were incubated with 5G6.4 at 20  $\mu$ g/ml in 2% bovine serum albumin in phosphate-buffered saline (0.01 M phosphate, pH 7.2, 0.14 M NaCl; BSA/PBS). Cells incubated in BSA/PBS without antibody were used as a control. The cells were incubated for 1 hr on ice and washed three times with BSA/PBS. Then the cells were resuspended in 1 ml of fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Cappel, Cochranville, PA) diluted 1/100 in BSA/PBS and the mixture was incubated for 1 hr on ice. The cells were washed three times with BSA/PBS and resuspended

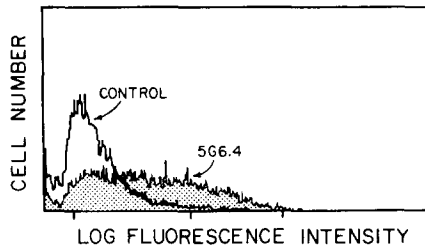


FIG. 1. Flow cytometry of MI Ov Ca cells without (no shading) or with (shaded) 5G6.4.

in 1 ml BSA/PBS. The fluorescence of cells was observed by fluorescence microscopy and analyzed using an Epics C flow cytometer (Coulter Diagnostics, Hialeah, FL).

*Iodination.* Antibodies were radiolabeled with  $^{125}\text{I}$  using "Iodogen" as previously described [22]. Cell-binding assays were performed as previously described [23].

*Soft agar clonogenic assay.* An agar underlayer of 0.5% molten Bacto-agar (Difco Laboratories, Detroit, MI) was poured over the bottom of 48-well plates. After the agar solidified, the tumor cell-plating layer was poured over the top. Tumor cell-plating density was  $2 \times 10^3$  cells/well, suspended in 0.3% molten Bacto-agar. The culture media added to both agar layers was RPMI on the bottom layer and the same media in which the cell lines grow on the top layer. After solidification of the agar the plates were incubated at  $37^\circ\text{C}$  with 5%  $\text{CO}_2$  overnight. Twenty-four hours later, different concentrations of MAb diluted in RPMI were added to the top layer. The same volume of RPMI, without any antibody, was used as control. The cells were incubated again, and after 7–14 days, colonies were visible. Colonies were defined as aggregates of greater than 30 cells. Colony formation in control plates were compared to the number of colonies growing in the cultures with the different concentrations of MAb overlays. Results of colony formation are given as percentage of control. All experiments were done in triplicate and the person reading the plates for colonies did not know which plates had antibody overlay nor their concentrations. MI OV cell line experiments were repeated three times and the fresh tumor (TA OV) experiment was performed once.

## RESULTS

The reaction of 5G6.4 with MI Ca Ov cells is shown in Fig. 1 by flow cytometry. Cells incubated with an equal concentration of an isotype-matched control antibody (UPC-10) showed fluorescence intensity similar to cells incubated without antibody (data not shown). In the beginning of these experiments, 225.28s was used as a control antibody because the high-molecular-weight chondroitin sulfate antigen was thought not to be expressed by Ov Ca cells. In the course of these experiments, the 225.28s was shown to bind to Ov Ca cells by immunofluorescence microscopy. In a binding assay using radioiodinated antibodies, Ov Ca cells bound 3.5 times more 5G6.4 than 225.28s (data not shown). 225.28s has been reported to have an *in vitro* effect on melanoma cell growth due to ADCC [12]. In our hands, its

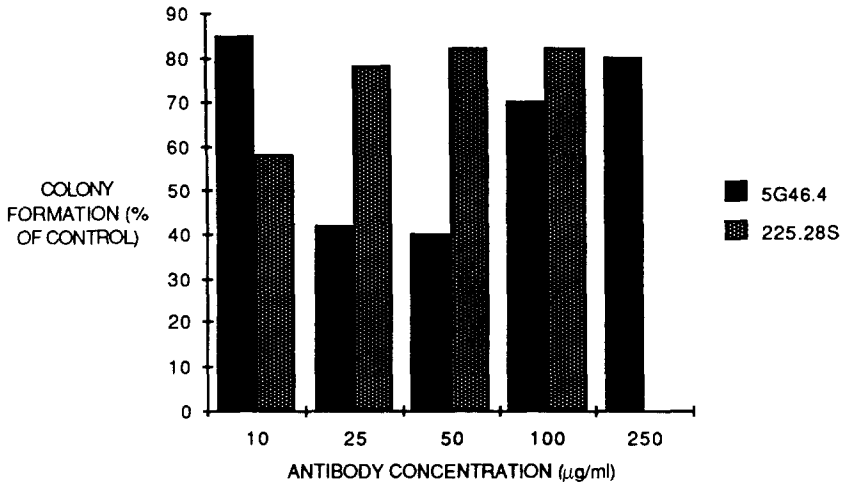


FIG. 2. Colony formation inhibition of MI Ov Ca cells in the presence of 5G6.4 (solid) or 225.28s (shaded). 225.28s was not tested at 250  $\mu\text{g}/\text{ml}$ .

*in vitro* effect on Ov Ca colony formation was inconsistent possibly due to a low level of antigen expression.

Figures 2 and 3 show the percentage of colony formation of the two different tumor cells used in relation to the different concentrations of 5G6.4 and 225.28s. The curves for both cell populations show a bell-shaped distribution for 5G6.4. The optimum concentration for best inhibition of tumor colony formation by 5G6.4 was 50  $\mu\text{g}/\text{ml}$  for both cell populations. For MI OV colony formation was inhibited to 40–55% of control and for the malignant effusion it was 11% of control ( $P < 0.05$  compared to control using Student's *t* test).

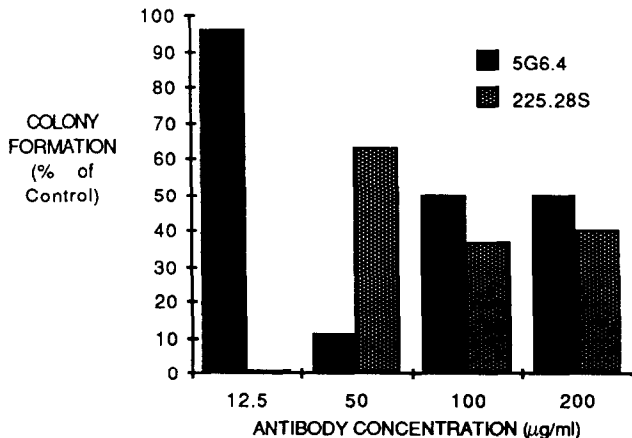


FIG. 3. Colony formation inhibition of TA Ov Ca cells in the presence of MAb 5G6.4 (solid) or 225.28s (shaded). 225.28s was not tested at 12.5  $\mu\text{g}/\text{ml}$ .

## DISCUSSION

We report here the ability of a MAb, 5G6.4, to block the growth of human Ov Ca cells in a tumor colony-forming assay in a nondose-related fashion. These results suggest that 5G6.4 may have potential immunotherapeutic usefulness but also indicate that the dose of antibody used for immunotherapy may be important. These results may be especially significant because of the nature of the colony-formation assay used. Growth of colonies measured in this assay is reportedly due to cells responsible for continued tumor growth and metastasis ("stem cells") [24].

Our results differ from those reported for several other MAbs capable of *in vitro* growth suppression. A MAb binding to transferrin receptor was previously shown to block cell growth *in vitro*, but in a dose-dependent fashion [13,25]. Prolonged exposure of melanoma cells to MAb R-24, an anti-GD3 ganglioside antibody, was shown to arrest cell growth, again in a dose-dependent fashion [14]. While the first assay looks at the growth inhibition of the whole malignant cell population, the second assay (used in our study) focuses on the growth inhibition of stem cells, which hypothetically are responsible for totipotent and continuous tumor growth.

The mechanism of growth inhibition by 5G6.4 remains to be determined. The unusual response to 5G6.4 (i.e., inhibition at 25 and 50  $\mu\text{g/ml}$ , lost at lower or higher concentrations of antibody) suggests some biological feedback. R-24, the anti-ganglioside GD3 MAb, appears to act through changes in membrane attachment sites [14]. Antitransferrin receptor may block uptake of essential nutrients [13,25]. Ov Ca cells have been reported to have growth factor receptors and to produce growth factors [26], both of which could act as targets for antibodies capable of growth inhibition. However, the 5G6.4 antigen does not resemble growth factors or receptors described previously [13,25,26]. Further, the antigen reactive with 5G6.4 appears to be a glycoprotein rather than a ganglioside or glycolipid, so the activity does not appear to be similar to R-24 (Wahl *et al.*, unpublished).

These results suggest that MAb 5G6.4 may have a direct immunotherapeutic effect by blocking the growth of Ov Ca "stem cells." We are continuing the investigation of this activity and testing this MAb *in vivo* in a nude mouse model.

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