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Immunology

Inhibition of tumor growth by a monoclonal antibody reactive with an oncogene-encoded tumor antigen

(immunotherapy/*neu* oncogene)

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ABSTRACT **The *neu* oncogene encodes a 185-kDa transmembrane glycoprotein tumor antigen, termed p185. We have recently described a monoclonal antibody reactive with a cell surface domain of the p185 molecule. *In vivo* treatment with this anti-p185 monoclonal antibody was able to significantly inhibit the tumorigenic growth of *neu*-transformed NIH 3T3 cells implanted into nude mice. Such treatment had no effect on the tumorigenic growth of Ha-*ras*-transformed NIH 3T3 cells. Furthermore, anti-p185 antibody treatment was able to inhibit the growth of the rat neuroblastoma cells from which the *neu* oncogene was initially isolated. These results demonstrate that a monoclonal antibody reactive with the extracellular domain of an oncogene-encoded protein can exert a significant antitumor effect; such antibodies may prove useful in the therapy of certain malignancies.**

The advent of monoclonal antibody technology (1) has allowed investigators to produce large amounts of homogeneous immunoglobulin specifically reactive with tumor cell membrane antigens (2, 3). Therapeutic trials of such monoclonal antibodies are now underway in both animal models and cancer patients, with some encouraging preliminary results (4–10). However, many of the antitumor antibodies identified thus far react with antigens whose expression bears no obvious relationship to the neoplastic state of the tumor cell. Continued expression of such antigens may be unnecessary for maintenance of the neoplastic state, permitting the appearance of nonantigenic variants in the tumor cell population. These nonantigenic variants may then be responsible for tumor relapse. More effective treatment might be achieved by the administration of monoclonal antibodies directed against antigens whose continued expression is required for neoplastic cell growth. For example, monoclonal antibodies reactive with certain growth factors or their receptors have been shown to mediate antitumor effects *in vitro* and *in vivo* (11–13).

Among the antigens necessary for neoplastic cell growth are those encoded by activated oncogenes. These genes and their products are thought to play a critical role in neoplastic transformation (14–16). Furthermore, some of these oncogenes appear to encode proteins expressed on the cell surface. The products of the *erbB*, *fms*, *neu*, and *ros* oncogenes are transmembrane glycoproteins possessing extracellular domains (17–21); the *sis* oncogene product may also be found in a membrane-associated form on the surface of tumor cells (22). The proteins encoded by activated oncogenes may represent an important class of tumor antigen for the targeting of monoclonal antibody-mediated tumor therapy.

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The *neu* oncogene, initially identified by transfection of DNA from several ethylnitrosourea-induced rat neuroblastomas, has been shown to encode a transmembrane glycoprotein of 185 kDa (p185) (17). Amplification and rearrangement of the *neu* oncogene have been detected in several human tumors, suggesting that this gene may also play a role in the development of these malignancies (refs. 23–25 and A. Schechter and R.A.W., unpublished data). We have described (18) a hybridoma that secretes an IgG2a monoclonal antibody reactive with a cell surface domain of the rat p185 molecule. This anti-p185 monoclonal antibody exerts a direct cytostatic effect on *neu*-transformed cells growing in soft agar and can also mediate *in vitro* cytotoxic effects on *neu*-transformed cells in the presence of complement or spleen cells (ref. 26 and J.A.D., V.C.L., and M.I.G., unpublished data). We therefore examined the effects of anti-p185 antibody administration on the *in vivo* growth of *neu*-transformed cells.

MATERIALS AND METHODS

Cell Lines. Cell lines used in these studies have been described (17, 18). B104 is a rat neuroblastoma that contains an activated *neu* oncogene capable of transforming NIH 3T3 cells in DNA transfection assays. B104-1-1 is a *neu*-transformed NIH 3T3 cell line generated by passaging B104-derived transforming DNA sequences through two cycles of DNA transfection. XHT-1-1a is a Ha-*ras*-transformed NIH 3T3 cell line generated by transfection of proviral DNA from Harvey sarcoma virus-infected cells. Cell lines were cultured in 100-mm tissue culture dishes (Costar, Cambridge, MA) containing 10 ml of Dulbecco's modified Eagle's medium (DMEM, KC Biological, Lenexa, KS) supplemented with 10% heat-inactivated fetal bovine serum, 1% penicillin/streptomycin/Fungizone mixture (M. A. Bioproducts, Walkersville, MD), and 100 μg of gentamicin sulfate (M. A. Bioproducts) per ml. Cell lines were passaged twice weekly at 1:20 dilution following release from the culture dish surface with trypsin/Versene (M. A. Bioproducts). Cell lines were maintained in a humidified 5% CO_2 incubator at 37°C and were replaced from frozen stocks every 2–3 months.

Experimental Animals. Inbred, congenitally athymic BALB/c nude (*nu/nu*) mice were obtained from the National Cancer Institute animal colony (Frederick, MD) and from the University of California Cancer Center animal colony (San Diego, CA). Male and female mice were used in different experiments with comparable results; within each experiment only animals of a single sex were used. Female BDIX rats were obtained from the National Cancer Institute animal colony (Frederick, MD).

Abbreviation: HBSS, Hanks' balanced salts solution.

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Monoclonal Antibodies. Monoclonal antibody 7.16.4, a mouse IgG2a antibody reactive with the rat *neu* oncogene-encoded p185 molecule, has been described (18). Hybridoma cells secreting antibody 7.16.4 were washed free of serum and injected intraperitoneally into pristane-primed, x-irradiated (400 rads; 1 rad = 0.01 Gy) C3D2 F_1 mice to induce ascites. After the development of ascites (in 7–14 days), ascites fluid was tapped with a 19-gauge needle, cellular elements and debris in the fluid were removed by centrifugation at 1000 × *g* for 10 min, and the fluid was stored at −70°C. Immunoglobulin was purified from ascites fluid by protein A-Sepharose affinity chromatography as described (26). Control monoclonal antibody 9BG5, a mouse IgG2a antibody reactive with the hemagglutinin of reovirus type 3, was generously provided by G. Gaulton (University of Pennsylvania).

Tumor Cell Implantation and Measurement of Tumor Growth. Tumor cells were released from tissue culture dishes with trypsin/Versene (EDTA) and were washed three times in Hanks' balanced salts solution (HBSS). Viable tumor cells (10^6) were then injected subcutaneously in the mid-dorsum of the experimental animals. Growing tumors were measured with vernier calipers on the days indicated, and tumor area was calculated as the product of tumor length and width, a method that has been shown to correlate with tumor weight (27). Data are presented as mean ± SEM. Significance was determined by comparing the means of different groups at the days shown, using Student's *t* test.

Immunofluorescent Staining. Cells were removed from tissue culture dishes with buffered EDTA (Versene, M. A. Bioproducts) and washed twice in FACS medium [HBSS (GIBCO) supplemented with 2% fetal bovine serum, 0.2% sodium azide, and 10 mM Hepes]. Cells (10^6) in 0.1 ml of FACS medium were incubated with antibody or control supernatant, in an additional 0.1 ml, for 1 hr at 4°C. Cells were diluted in 2.5 ml of FACS medium, pelleted by centrifugation at 1000 × *g*, and washed twice more with 2.5 ml of FACS medium per wash. After the final wash, the cell pellet was gently resuspended and cells were incubated with 0.1 ml of fluorescein isothiocyanate-conjugated rabbit anti-mouse IgG (reactive with heavy and light chains, Miles) diluted 1:50 in FACS medium, for 1 hr at 4°C. Cells were then diluted and washed as after the first incubation. The cell pellet was resuspended, and the cells were fixed in 0.5 ml of phosphate-buffered saline containing 2% paraformaldehyde. Samples were run on a Coulter Epics V flow cytometer (85 channels per logarithmic unit of fluorescence). Each analysis was conducted on 10,000 cells.

RESULTS

Treatment with an Anti-p185 Monoclonal Antibody Inhibits the Tumorigenic Growth of *neu*-Transformed NIH 3T3 Cells. NIH 3T3 cells transformed by the *neu* oncogene (cell line B104-1-1) are highly tumorigenic. As shown in Fig. 1 (triangles), injection of 10^6 B104-1-1 cells into nude mice produced rapidly growing fibrosarcomas that killed their hosts after 15–20 days. When nude mice injected subcutaneously with 10^6 B104-1-1 cells were injected intraperitoneally with ascites fluid containing the anti-p185 antibody 7.16.4 on days 0 and 1 following subcutaneous tumor implantation, the tumorigenic growth of the B104-1-1 cells was inhibited significantly (Fig. 1, filled circles, $P < 0.05$ at all days measured). In contrast, injection of a control ascites fluid containing an IgG2a monoclonal antibody of an irrelevant specificity (anti-reovirus) had no effect on the tumorigenic growth of B104-1-1 cells (Fig. 1, open circles). Not only was tumor growth significantly inhibited in animals treated with antibody 7.16.4, but the median survival of tumor-bearing mice was almost doubled, from 18 days to 34 days, by treatment with the anti-p185 antibody; treatment with the control ascites fluid did not extend the survival of treated animals.

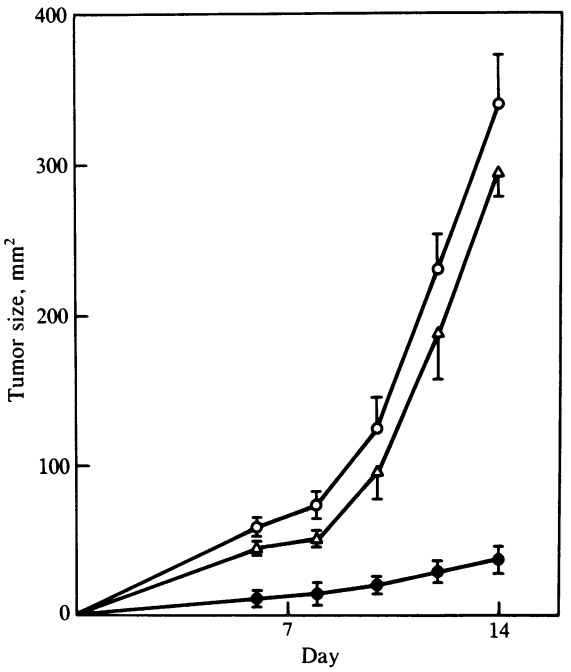

FIG. 1. Inhibition of the tumorigenic growth of *neu*-transformed NIH 3T3 cells by monoclonal antibody 7.16.4. BALB/c nude mice were injected subcutaneously in the mid-dorsum with 10^6 B104-1-1 tumor cells on day 0. Groups of five mice received 1-ml intraperitoneal injections of HBSS (△), control ascites fluid containing anti-reovirus antibody 9BG5 (○), or ascites fluid containing anti-p185 antibody 7.16.4 (●) on days 0 and 1. Growing tumors were measured and statistical calculations were performed as described in *Materials and Methods*.

Fig. 2 demonstrates that purified IgG2a immunoglobulin from 7.16.4 ascites fluid is able to inhibit the tumorigenic growth of *neu*-transformed cells in a dose-dependent manner. Intravenous injection of 1 mg of purified antibody on the day of tumor cell implantation markedly inhibited tumor growth (Fig. 2, filled circles, $P < 0.05$ at all days measured). Treatment with 100 μg (open triangles) or 10 μg (filled triangles) of antibody had a lesser but still significant ($P < 0.05$ at all days measured) effect on tumor growth when compared to that of the saline-treated control group. We have also found that infusion of purified 7.16.4 immunoglobulin can have a significant antitumor effect in mice inoculated with B104-1-1 tumor cells 7 days prior to antibody treatment (data not shown). Thus, antibody 7.16.4 treatment can inhibit the growth of both fresh tumor inocula and established tumors composed of *neu*-transformed cells.

Specificity of Anti-p185 Antibody Treatment. To determine whether the antitumor activity of monoclonal antibody 7.16.4 injection was specific for *neu*-transformed cells, we examined the effect of antibody 7.16.4 on growth of an NIH 3T3 cell line transformed by an oncogene unrelated to the *neu* gene. For this experiment we utilized the Ha-*ras*-transformed NIH 3T3 cell line XHT-1-1a. We have previously established that this cell line does not display p185 reactive with antibody 7.16.4 in either quantitative immunofluorescence or immunoprecipitation assays (18). Treatment with 7.16.4 ascites fluid on days 0 and 7 following tumor cell inoculation had no effect on the tumorigenic growth of Ha-*ras*-transformed NIH 3T3 cells (Fig. 3 *Right*). In contrast, the tumorigenic growth of *neu*-transformed NIH 3T3 cells was significantly inhibited by identical doses of 7.16.4 ascites fluid in an experiment conducted in parallel (Fig. 3 *Left*, $P < 0.05$ at all days

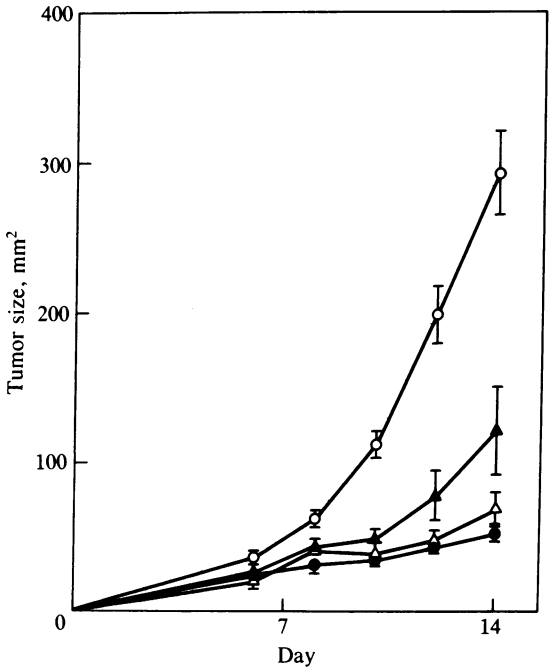

FIG. 2. Purified immunoglobulin from the 7.16.4 hybridoma inhibits the tumorigenic growth of *neu*-transformed NIH 3T3 cells in a dose-dependent manner. Groups of five BALB/c nude mice received subcutaneous injections of 10^6 B104-1-1 cells and 0.4-ml intravenous injections of either HBSS (○) or HBSS containing 1 mg (●), 100 μg (△), or 10 μg (▲) of protein A-purified antibody 7.16.4 on day 0.

measured). Thus, the ability of antibody 7.16.4 to inhibit tumor growth is specific for tumor cells that display p185 on their surface.

Analysis of Cell-Surface p185 on *neu*-Transformed Cells from Tumors That Have Escaped Antibody Inhibition. Although treatment with antibody 7.16.4 was able to significantly inhibit the tumorigenic growth of *neu*-transformed cells, tumors eventually progressed in all treated animals. To determine whether tumor progression was due to selection for tumor cells that no longer expressed cell surface p185, we explanted fragments from tumors progressing despite antibody treatment and examined cell surface p185 expression after a single passage *in vitro*. As shown in Table 1, cells from two independent B104-1-1 tumors that had progressed in the face of continued administration of antibody 7.16.4 express levels of p185 comparable to that expressed by B104-1-1 cells maintained *in vitro*. Thus, the eventual progression of tumor growth despite anti-p185 antibody treatment is not due to the selection of variants that have ceased to express p185 on their surface.

Anti-p185 Antibody Treatment Inhibits the Growth of Rat Neuroblastoma Cells Implanted in Nude Mice. The rat neuroblastoma cells in which the activated *neu* oncogene arose express much less cell surface p185 than do NIH 3T3 transfectants (17, 18). Studies by Herlyn *et al.* (28) have suggested that high antigen density is strongly correlated with the success of monoclonal antibody-mediated tumor therapy. It was therefore of interest to determine whether anti-p185 antibody therapy could interfere with the tumorigenic growth of the rat neuroblastoma cells. Fig. 4 shows that the tumorigenic growth of rat neuroblastoma cells (cell line B104) was indeed significantly inhibited in nude mice that received injections of purified antibody 7.16.4 (open circles) when compared to mice that received injections of control monoclonal antibody 9BG5 (filled circles, $P < 0.05$ at all days measured) or normal saline (unpublished data). It is also

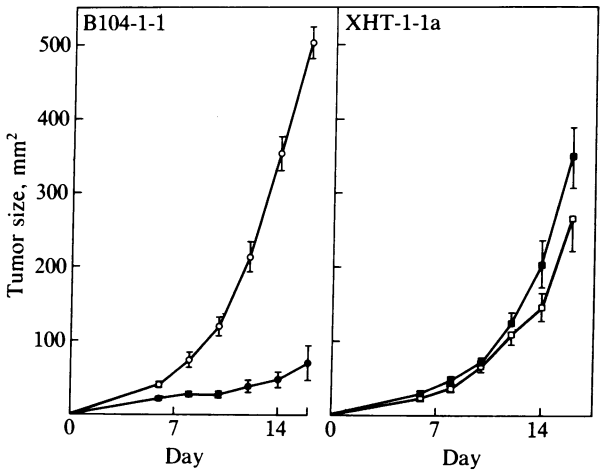

FIG. 3. Antibody 7.16.4 inhibits the tumorigenic growth of *neu*-transformed NIH 3T3 cells but not Ha-*ras*-transformed NIH 3T3 cells. (*Left*) Groups of five BALB/c nude mice were injected subcutaneously with 10^6 *neu*-transformed B104-1-1 tumor cells and received intraperitoneal injections with 1 ml of either HBSS (○) or 7.16.4 ascites fluid (●) on days 0 and 7 after tumor implantation. (*Right*) In an experiment conducted in parallel, groups of five mice were injected subcutaneously with 10^6 Ha-*ras*-transformed XHT-1-1a tumor cells and received intraperitoneal injections with 1 ml of either HBSS (□) or 7.16.4 ascites fluid (■).

noteworthy that B104 tumors were metastatic to regional lymph nodes in 3/6 and 2/6 of the B104-bearing nude mice treated with control antibody or saline, respectively. In contrast, lymph node metastases were not observed in B104-bearing nude mice treated with the anti-p185 antibody.

Anti-p185 Antibody Treatment Inhibits the Growth of Rat Neuroblastoma Cells in Syngeneic Rats. Antibody 7.16.4 is not reactive with the product of the mouse *neu* gene, but it does react with the products of the activated and normal rat *neu* genes (17, 18). The experiments presented above used monoclonal antibodies to inhibit the growth of tumor cells that express a tumor antigen (rat p185) that is antigenically distinct from any related proteins expressed in other tissues of the tumor-bearing nude mice. Although antibody 7.16.4 was able to significantly inhibit the tumorigenic growth of *neu*-transformed cells implanted into nude mice, it was

Table 1. Cell-surface p185 levels on tumors progressively growing despite anti-p185 antibody treatment

Cell line	Immunofluorescence* with antibody 7.16.4
B104-1-1	38
B104-1-1/Nu1	39
B104-1-1/Nu2	34
NIH 3T3	<1
XHT-1-1a	<1

B104-1-1 cell tumors (B104-1-1/Nu1 and -/Nu2) that were progressively growing in nude mice despite treatment with 1 ml of 7.16.4 ascites fluid on days 0, 3, 7, 10, and 14 after implantation were excised, minced, and placed in tissue culture dishes containing culture medium; the medium was changed every 3 days until the cells had formed a dense monolayer. The cells were then processed for immunofluorescence staining of cell-surface p185 as described in *Materials and Methods*.

*Values represent specific immunofluorescence, calculated by subtracting the median fluorescence channel number of populations stained with fluorescein isothiocyanate-conjugated rabbit anti-mouse IgG alone (negative control) from the median fluorescence channel of populations stained with saturating amounts of antibody 7.16.4 followed by fluorescein isothiocyanate-conjugated rabbit anti-mouse IgG (positive staining).

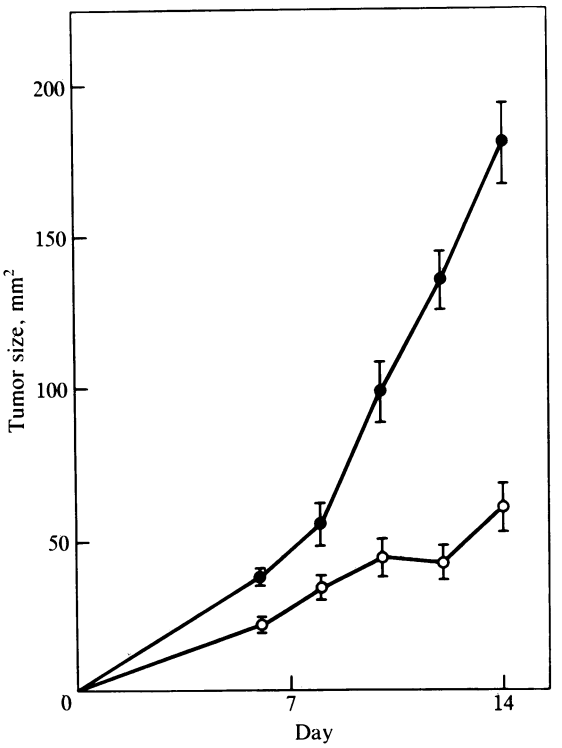

FIG. 4. Antibody 7.16.4 inhibits the growth of B104 rat neuroblastoma cells in nude mice. Groups of six BALB/c nude mice received subcutaneous injections on day 0 with 10^6 B104 neuroblastoma cells and daily intravenous injections for 10 days with 20 μg (per day) of protein A-purified anti-p185 antibody 7.16.4 (○) or control antibody 9BG5 (●) in 0.4 ml of HBSS.

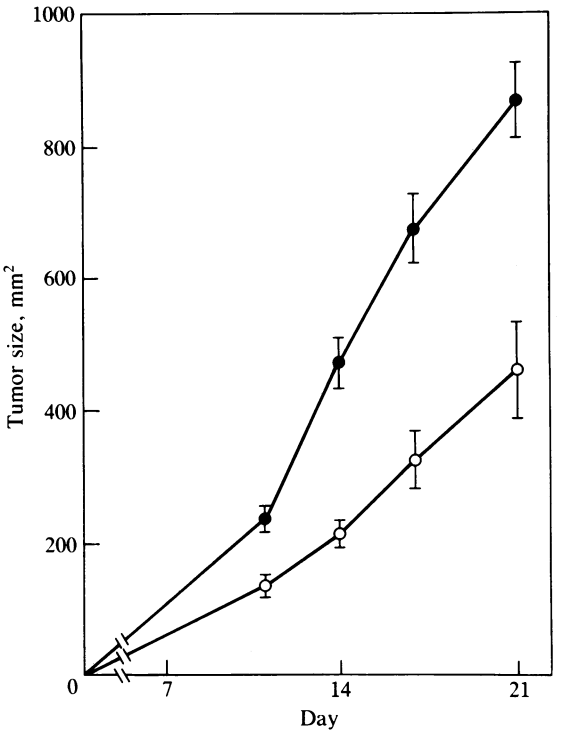

FIG. 5. Antibody 7.16.4 inhibits the growth of B104 rat neuroblastoma cells in syngeneic BDIX rats. Groups of four BDIX rats received subcutaneous injections of 10^6 B104 tumor cells on day 0 and received intravenous injections of 100 μg of antibody 7.16.4 in 0.4 ml of HBSS (○) or 0.4 ml of HBSS alone (●) on days 0, 1, 3, 7, 10, and 14 following tumor cell implantation. Tumor growth was measured and statistical calculations were performed as described in *Materials and Methods* except that rats were anesthetized with ether prior to tumor measurement.

important to address the question of whether such therapy would also affect the growth of B104 neuroblastoma tumors in syngeneic BDIX rats. Since the *neu* protooncogene is expressed at low levels in a variety of normal tissues (29), there was a possibility that injecting antibody 7.16.4 into rats might prove toxic. However, preliminary studies showed that intravenous injection of up to 4 mg of purified antibody 7.16.4 per rat had no discernible toxic effects (unpublished data).

We therefore injected B104 neuroblastoma cells subcutaneously into syngeneic BDIX rats and examined the effect of intravenous antibody 7.16.4 infusion on subsequent tumor growth. As shown in Fig. 5, treatment with small doses of anti-p185 antibody was able to significantly inhibit the growth of B104 neuroblastoma cells in syngeneic immunocompetent rats compared with saline-treated controls ($P < 0.05$ at all days measured). Injections of a control IgG2a mouse monoclonal antibody into BDIX rats had no significant effect on the growth of B104 tumors (unpublished data). These results demonstrate that it is possible to inhibit the growth of a tumor by the administration of a monoclonal antibody specific for an oncogene-encoded tumor antigen that is also displayed by the normal tissues of the tumor-bearing host.

DISCUSSION

Alterations in cellular oncogenes, resulting from gene amplification, chromosomal translocation, or point mutations, have been identified in a significant fraction of tumors (14–16). While the proteins encoded by most of the known oncogenes normally reside within the nucleus or cytoplasm, some oncogene-encoded proteins are found on the external surface of the plasma membrane. Among human tumors that have been examined, tumors of several histologic types express high levels of the *erbB* oncogene product (30–32), and some adenocarcinomas express high levels of the *neu* oncogene product (23–25). Such oncogene-encoded cell-surface proteins represent particularly attractive targets towards which to direct antitumor immunotherapy, since they are expressed at high levels and may play an important role in the neoplastic transformation of the cells that display them. In this report we have shown that a monoclonal antibody reactive with the rat *neu* oncogene product, p185, can mediate antitumor effects *in vivo*.

Systemic injection of a p185-specific monoclonal antibody, of the IgG2a isotype, profoundly inhibits the tumorigenic growth in nude mice of NIH 3T3 cells transformed by an activated *neu* oncogene. Injection of this monoclonal anti-p185 antibody has no effect on the growth of NIH 3T3 cells transformed by a Ha-*ras* oncogene. The inhibition of tumor growth which occurs following the administration of anti-p185 monoclonal antibodies is dose-dependent, with as little as 10 μg of antibody causing a significant effect. Both fresh tumor inocula and established tumors are inhibited by the administration of the appropriate monoclonal antibodies. Injections of p185-specific monoclonal antibodies also inhibit the tumorigenic growth of the *neu* oncogene donor rat neuroblastoma cell line B104 in both nude mice and syngeneic immunocompetent BDIX rats. Collectively, these findings demonstrate that treatment with an anti-oncogene product monoclonal antibody can result in significant antitumor effects *in vivo*.

Our studies have not defined the mechanism(s) by which antibody 7.16.4 inhibits the tumorigenic growth of *neu*-transformed cells. This antibody mediates multiple antitumor effects *in vitro*, including direct reversion of the transformed phenotype, complement-dependent lysis of antibody-coated

tumor cells, and targeting of murine lymphoid cells to mediate modest levels of antibody-dependent cellular cytotoxicity (ref. 26 and unpublished data). It is possible that the antitumor effects of antibody 7.16.4 reflect both the direct cytostatic effects of p185 crosslinking by anti-p185 antibodies and the cytolytic effects resulting from the recognition of antibody-coated tumor cells by immunologic mediators in the tumor-bearing host.

Although profound inhibition of tumor growth and prolonged survival resulted from treatment with anti-p185 monoclonal antibodies, all of the treated animals eventually died from tumor progression. Examination of cells from explanted tumors that were progressing despite continued antibody treatment revealed normal levels of p185. This suggests that selection of variants expressing reduced levels of p185 was not the mechanism responsible for treatment failure.

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