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Monoclonal antibodies specific for the *neu* oncogene product directly mediate anti-tumor effects *in vivo*

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We have produced a panel of monoclonal antibodies which bind cell surface domains of the 185 Kd tumor antigen (p185) encoded by the *neu* oncogene. All of these antibodies stain *neu*-transformed cells in immunofluorescence assays and immunoprecipitate p185 from metabolically labeled cell lysates. All of the anti-p185 monoclonal antibodies, regardless of isotype, exert a selective cytostatic effect on the growth of *neu*-transformed cells suspended in soft agar, demonstrating their ability to directly inhibit the transformed phenotype. Anti-p185 antibodies of the IgM, IgG2a, and IgG2b isotypes exert a cytolytic effect on *neu*-transformed cells in the presence of complement. Only one IgG2a monoclonal antibody is also able to mediate minimal levels of antibody-dependent cellular cytotoxicity (ADCC) (Roussel *et al.*, 1984) in the presence of non-immune spleen cells. *In vivo* administration of anti-p185 antibodies of the IgG1, IgG2a, and IgG2b isotypes exerts a profound inhibitory effect on the tumorigenic growth of *neu*-transformed cells. This tumor inhibitory effect is unaffected by depleting tumor bearing animals of complement, and is only minimally affected by depleting tumor bearing animals of macrophages. This suggests that neither complement-mediated killing nor ADCC are necessary for the anti-tumor effects of p185-specific monoclonal antibodies. The results presented here demonstrate that monoclonal antibodies reactive with cell surface domains of an oncogene-encoded protein can directly inhibit tumor growth *in vitro* and *in vivo*. Such antibodies may prove useful in the therapy of certain malignancies.

Introduction

Recent studies of the molecular genetics of cancer have implicated a group of genes, termed oncogenes, in the neoplastic process (Bishop, 1987). Genes closely related to these genes, proto-oncogenes, are found in somatic cells of all eukaryotic species examined and have been highly conserved in evolution; it is thought that proto-oncogenes normally play critical roles in cellular growth and development. Oncogene amplification and chromosomal rearrangements involving oncogenes have been detected in a large number of tumors. Furthermore, some tumors have been shown to contain activated oncogenes which in DNA transfection assays are capable of conferring neoplastic properties upon non-neoplastic rodent fibroblast cell lines (Weinberg, 1985). Collectively these studies suggest that alterations in proto-oncogene structure and function play a critical role in the development of neoplasia.

Although most oncogene-encoded proteins reside in the nucleus or the cytoplasm (Bishop, 1987; Weinberg, 1985), some oncogenes encode proteins that express antigenic sites on the cell surface. For example, the *erbB*, *fms*, and *ros* oncogene products are transmembrane glycoproteins that possess extracellular domains (Berg & Hayman, 1984; Roussel *et al.*, 1984; Neckameyer *et al.*, 1986). The *sis* oncogene product may also exist in a membrane associated form on the surface of transformed cells (Robbins *et al.*, 1985). Another oncogene which encodes a protein that exposes antigenic sites on the surface of transformed cells has been identified by transfection of DNA from ethyl nitrosourea-induced rat neuroblastomas into NIH3T3 cells; we have termed this oncogene *neu* (Schechter *et al.*, 1984). The *neu* gene has been found to be amplified in some human tumors, particularly those of the breast, suggesting that this gene may play a role in the etiology of human cancer (Semba *et al.*, 1985; King *et al.*, 1985; Yokota *et al.*, 1986; Slamon *et al.*, 1987; Kraus *et al.*, 1987).

We have identified a 185 000 dalton transmembrane protein (p185) encoded by the *neu* oncogene (Schechter *et al.*, 1984; Drebin *et al.*, 1984). Recently, we described an IgG2a monoclonal antibody reactive with a domain of p185 expressed on the surface of *neu*-transformed cells, which was able to significantly inhibit the tumorigenic growth of *neu* transformed cells (Drebin *et al.*, 1986). In an effort to elucidate the mechanism(s) by which anti-p185 antibodies inhibit tumor growth, we have produced a number of additional p185 specific monoclonal antibodies of several different isotypes. Here, we describe these p185-specific monoclonal antibodies, examine their cytostatic and cytotoxic effects on *neu*-transformed cells *in vitro*, and compare the *in vitro* anti-tumor effects of p185-specific monoclonal antibodies with their abilities to inhibit tumor growth *in vivo*.

Results

Production of monoclonal antibodies reactive with cell surface antigens on neu*-transformed cells*

Hybridomas secreting monoclonal antibodies reactive with cell surface antigens on *neu*-transformed cells were produced and screened by immunofluorescence as described (Drebin *et al.*, 1984). To date, five such hybridomas have been identified. The names of these hybridomas, their immunoglobulin heavy chain isotypes, and

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representative immunofluorescence flow cytometry profiles of binding to the *neu*-transformed NIH3T3 cell line B104-1-1 and the Ha-*ras*-transformed NIH3T3 cell line XHT-1-1a are shown in Figure 1. It is clear that all five monoclonal antibodies show significant levels of binding to B104-1-1 cells but not XHT-1-1a cells (Figure 1, b–f). In contrast, a control monoclonal antibody (UPC 10, reactive with phosphorylcholine) fails to bind either B104-1-1 cells or XHT-1-1a cells (Figure 1, g). Thus the monoclonal antibodies described here identify a cell surface antigen associated with the presence of the *neu* oncogene in transfected NIH3T3 cells.

Immunoprecipitation of p185 by monoclonal antibodies reactive with cell surface antigens on neu*-transformed cells.*

Initial immunoprecipitation studies to identify antigens reactive with the *neu* specific monoclonal antibodies, conducting anti-mouse immunoglobulin and protein A-bearing *Staph. aureus*, demonstrated that monoclonal antibodies 7.16.4 and 7.21.2 could specifically immunoprecipitate the p185 product of the *neu* oncogene (Figure 2, lanes 2 and 3). Antibodies 7.5.5, 7.9.5, and 7.16.5 were unable to precipitate p185 (or any other antigens) using these experimental conditions (data not shown). However when we purified these monoclonal antibodies and covalently coupled them to sepharose beads using cyanogen bromide, we were able to demonstrate that they also specifically immunopreciptated

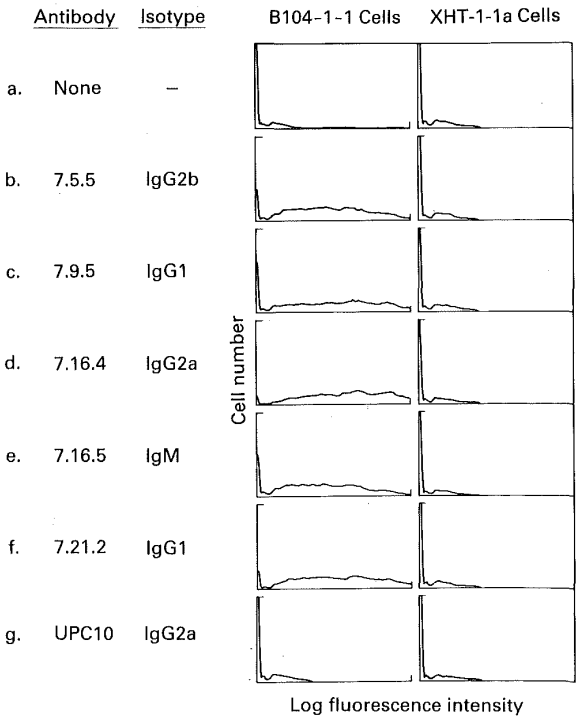

Figure 1 Identification of monoclonal antibodies which specifically bind cell surface determinants on *neu*-transformed NIH3T3 cells. NIH3T3 cells transformed by transfection with an activated *neu* oncogene (cell line B104-1-1) or an activated Ha-*ras* oncogene (cell line XHT-1-1a) were processed for indirect immunofluorescence using saturating amounts of the indicated antibodies, as described in Materials and methods. Antibody isotypes were determined by indirect immunodiffusion according to the method of Ouchterlony (Hudson & Hay, 1980)

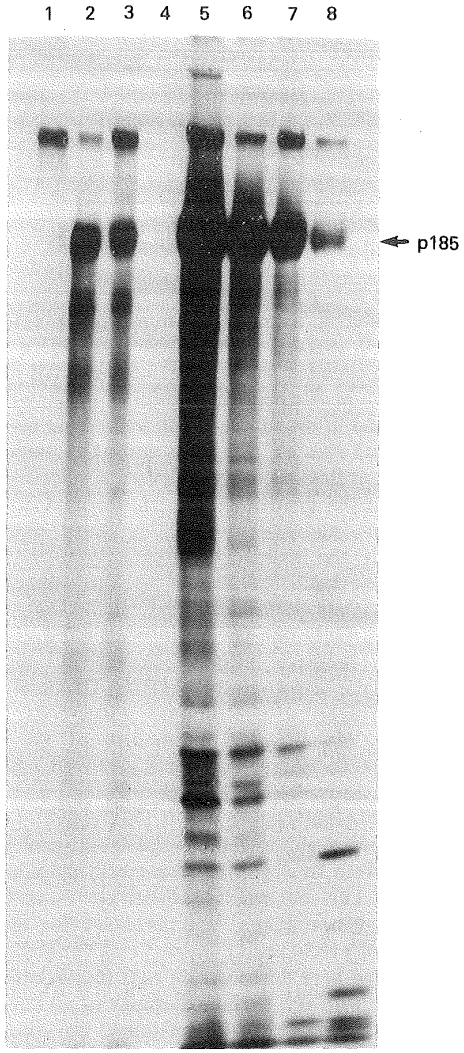

Figure 2 Immunoprecipitation of p185 by monoclonal antibodies reactive with cell surface determinants on *neu*-transformed NIH3T3 cells. Immunoprecipitation and SDS-polyacrylamide gel electrophoresis of [^{35}S]-cysteine labeled B104-1-1 cell lysates were performed as described in Materials and methods. Lanes 1–3: immunoprecipitation with free monoclonal antibodies, anti-mouse immunoglobulin, and protein A-bearing *Staph. aureus*. Lane 1 – normal mouse serum. Lane 2 – antibody 7.16.4. Lane 3 – antibody 7.21.2. Lanes 4–8: immunoprecipitation with antibodies covalently coupled to sepharose beads. Lane 4 – protein A purified normal mouse immunoglobulin. Lane 5 – antibody 7.16.4. Lane 6 – antibody 7.9.5. Lane 7 – antibody 7.5.5. Lane 8 – antibody 7.16.5

p185 from metabolically labeled lysates of *neu*-transformed cells (Figure 2, lanes 6, 7, and 8). Thus all of the monoclonal antibodies which specifically bound the surface of *neu*-transformed cells were reactive with the p185 molecule encoded by the *neu* oncogene.

Effects of anti-p185 monoclonal antibodies on the anchorage-dependent growth of neu*-transformed cells*

One of the most stringent characteristics distinguishing malignant from non-malignant cells is the capacity for anchorage-independent growth. We have recently shown that exposure of *neu*-transformed cells to the p185 specific monoclonal antibody 7.16.4 caused the down-modulation of p185 from the cell surface and

Table 1 Anti-p185 monoclonal antibodies inhibit the anchorage-independent growth of *neu*-transformed cells

Antibody (specificity)	*Anchorage-independent colonies* (percent inhibition)*				
	0	*100 ng*	*1 μg*	*10 μg*	*100 μg*
None	25 ± 2.2	—	—	—	—
7.5.5 (anti-p185)		15 ± 0.3 (40)	10 ± 2.5 (60)	6 ± 1.0 (76)	4 ± 0.9 (84)
7.9.5 (anti-p185)		14 ± 1.2 (44)	9 ± 0.9 (64)	7 ± 0.6 (72)	0.3 ± 0.3 (99)
7.16.4 (anti-p185)		3 ± 3.3 (88)	1 ± 0.6 (96)	1 ± 0.6 (96)	0.7 ± 0.3 (97)
7.16.4 (anti-p185)		16 ± 1.9 (36)	3 ± 0.9 (88)	1 ± 0.3 (96)	0.3 ± 0.3 (99)
7.21.2 (anti-p185)		25 ± 2.0 (0)	11 ± 0.9 (56)	12 ± 1.9 (52)	12 ± 1.2 (52)
9BG5 (IgG2a, anti-reovirus)		21 ± 1.5 (16)	23 ± 2.0 (8)	21 ± 3.2 (16)	22 ± 1.3 (12)
87.92.6 (IgM, anti-beta adrenergic receptor)		22 ± 1.2 (12)	26 ± 2.6 (<0)	29 ± 2.9 (<0)	23 ± 1.2 (8)

* colonies > 0.5 mm were counted using a dissecting microscope after 14 days, described in Materials and methods

resulted in loss of the capacity for anchorage-independent growth (Drebin *et al.*, 1985). We have subsequently examined the ability of each of the p185 specific antibodies described here to inhibit the anchorage-independent growth of *neu*-transformed cells. As shown in Table 1, all of the anti-p185 monoclonal antibodies are able to cause over 50% inhibition of the anchorage-independent growth of B104-1-1 cells at doses of less than 1 μg per dish. The potency of the different anti-p185 antibodies in inhibiting anchorage-independent growth parallels their relative affinity for binding B104-1-1 cells, with antibody 7.16.4 having the highest affinity and antibody 7.21.2 having the lowest affinity. The relative affinity has been deduced by the saturable binding curves of the various purified antibodies for p185 expressed on B104-1-1 cells (data not shown). In addition, in studies to be described elsewhere, the antibodies have been found to identify three distinct domains of p185. Thus, 7.16.4, 7.9.5, and 7.21.2 react with independent epitopes of the extracytoplasmic portions of p185. Hence, the effects observed in this study cannot be attributed to the binding of the different monoclonals to the same site.

In contrast to the effects of anti-p185 antibodies, two control monoclonal antibodies fail to significantly inhibit the anchorage-independent growth of *neu*-transformed cells even at 100 μg per dish (Table 1). It is important to note that one of these control antibodies, 87.92.6, is reactive with a beta-adrenergic like receptor on B104-1-1 cells and shows significant binding to these cells by immunofluorescence (data not shown), but has no effect on their anchorage-independent growth. This demonstrates that the effects of the anti-p185 monoclonal antibodies on the anchorage-independent growth of *neu*-transformed cells does not simply result from antibody binding the cell surface, but reflects a specific cytostatic effect resulting from antibody binding to specific domains of the p185 molecule.

The ability of anti-p185 monoclonal antibodies to inhibit the growth of *neu*-transformed cells occurs exclusively under conditions that are selective for neoplastic behaviour, such as when the cells are suspended in soft agar. In contrast adherent growth in the 10% fetal calf serum, which is a property shared by non-neoplastic cells as well as neoplastic cells, is unaffected by anti-p185 antibodies. As shown in Figure 3, antibody 7.16.4 has no effect on the adherent growth of *neu*-transformed cells in liquid medium, even at concentrations that inhibit the anchorage-independent growth of *neu*-transformed cells by >95%, as shown above. We have examined the density of p185 on cells grown under a variety of conditions and have not discerned any major changes in p185 expression (data not shown). These studies exclude differential expression patterns of p185 as a significant element in the failure to observe effects

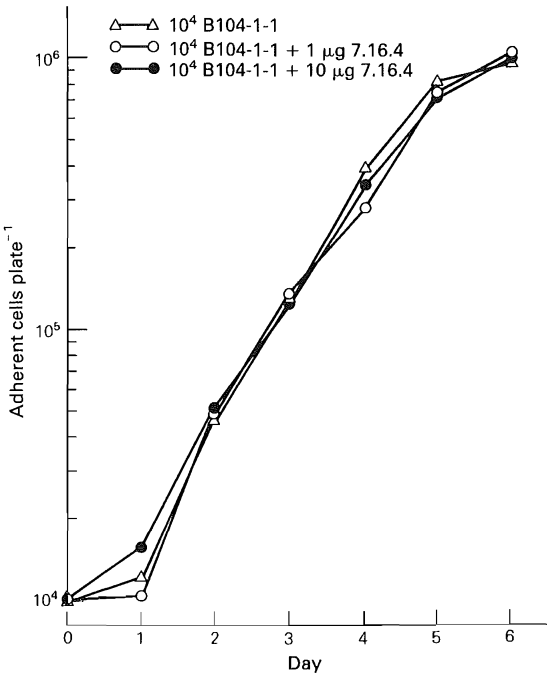

Figure 3 Antibody 7.16.4 has no effect on the adherent growth of B104-1-1 cells in 10% fetal calf serum. B104-1-1 cells were plated at 1×10^4 cells per dish in 60 mm tissue culture dishes containing Dulbecco's Modified Eagle's Medium supplemented with 10% fetal calf serum, antibiotics, and the indicated amounts of purified antibody 7.16.4. At the days indicated, cells from three plates in each experimental group were removed from the culture dish surface with trypsin-versene solution and were counted using a hemocytometer

of anti-p185 antibodies on cells grown in liquid media. Collectively, these studies demonstrate that anti-p185 antibodies selectively inhibit the neoplastic behavior of *neu*-transformed cells, without in any way affecting cell viability.

Complement mediated lysis of neu-*transformed cells by p185-specific monoclonal antibodies*

All of the anti-p185 antibodies described in this report exert a cytostatic effect on the growth of *neu*-transformed cells suspended in soft agar. In order to identify additional mechanisms by which anti-p185 monoclonal antibodies might exert anti-tumor effects, we examined their abilities to kill tumor cells *in vitro* in the presence of rabbit complement. Figure 4 presents data demonstrating that purified immunoglobulin from the 6.16.4 hybridoma is able to lyse *neu*-transformed cells in the presence of complement in one hour [^{51}Cr] release assay at immunoglobulin concentrations as low as 5 ng ml^{-1} (Figure 4a). The ability of antibody 7.16.4 to kill *neu*-transformed cells is completely dependent on the addition of complement since purified immunoglobulin does not exert cytotoxic effects in the absence of complement in either short term *in vitro* [^{51}Cr] release assays (Figure 4b) or longer term cell cultures, as shown previously (Figure 3).

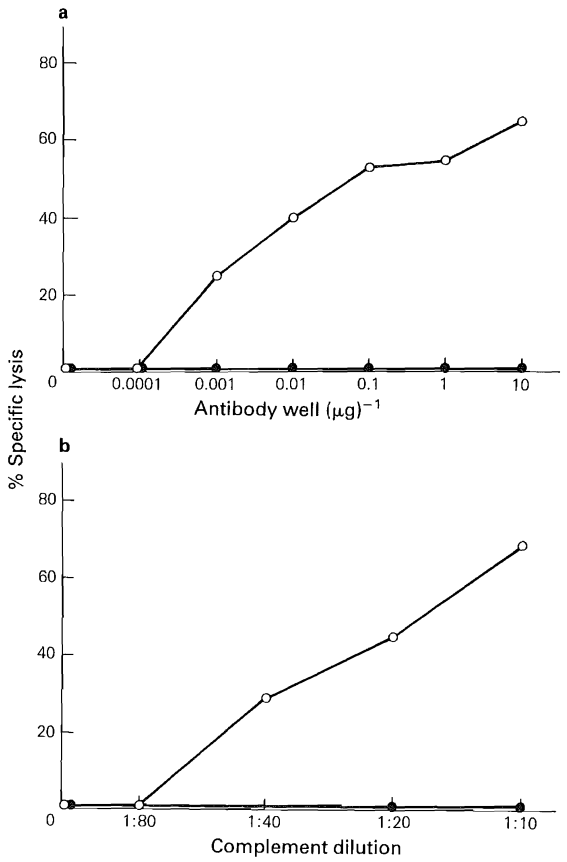

Figure 4 Lysis of *neu*-transformed cells by antibody 7.16.4 and complement. (**a**) [^{51}Cr]-labeled B104-1-1 cells (open circles) or XHT-1-1a cells (solid circles) were incubated in microwells in a total volume of 200 μl with the indicated amounts of purified antibody 7.16.4 and rabbit compliment diluted to a final concentration of 1 : 20. After incubation at 37°C for 1 hour, specific lysis was calculated as described in Materials and methods. (**b**) [^{51}Cr]-labeled B104-1-1 cells (open circles) or XHT-1-1a cells (solid circles) were incubated in microwells in a total volume of 200 μl with 1 μg/well of purified antibody 7.16.4 and rabbit complement diluted to the concentrations indicated. After incubation at 37°C for 1 hour, specific lysis was calculated as described in Materials and methods

Table 2 Complement-mediated lysis of *neu*-transformed cells by anti-p185 monoclonal antibodies

		Specific lysis of tumor targets†	
*Antibody**	*Isotype*	*B104-1-1 cells*	*XHT-1-1a cells*
7.5.5	IgG2b	43%	0%
7.9.5	IgG1	2%	0%
7.16.4	IgG2a	66%	0%
7.16.5	IgM	28%	0%
7.21.2	IgG1	8%	0%

* Antibodies were used at 5 μg ml^{-1} in a total volume of 0.2 ml
† Determined by [^{51}Cr]O_4 release assay with 5% rabbit serum complement source, as described in Materials and methods

Several other anti-p185 antibodies, in addition to antibody 7.16.4, are able to cause significant lysis of the *neu*-transformed NIH3T3 cell line B104-1-1 in the presence of complement (Table 2). There is no killing of the control cell line XHT-1-1a by any of the monoclonal anti-p185 antibodies. In agreement with observations from other laboratories, IgM and IgG2 monoclonal antibodies are effective and IgG1 monoclonal antibodies are ineffective in mediating complement-dependent lysis (Oi *et al.*, 1984).

Antibody dependent cell mediated lysis of neu-*transformed cells by p185-specific monoclonal antibodies*

In contrast to the effective complement-mediated lysis observed with several of the anti-p185 monoclonal antibodies, only the IgG2a anti-p185 antibody 7.16.4 was able to mediate even modest levels of antibody-dependent cell-mediated lysis of *neu*-transformed cells (Table 3). Anti-p185 antibodies of other isotypes had no activity in antibody-dependent cellular toxicity (ADCC) assays (data not shown). The level of killing obtained with antibody 7.16.4 was relatively low regardless of whether spleen cells, complete Freund's adjuvant elicted macrophages or thioglycollate elicited microphages were used as cellular effectors (data not shown). Even enhancing the adherence of anti-p185 antibodies to the surface of effector cells with polyethylene glycol, a procedure that increases ADCC activity (Jones & Segal, 1980), failed to increase the cell-mediated lysis of *neu*-transformed cells (data not shown). We have examined

Table 3 Antibody dependent cell-mediated lysis of *neu*-transformed cells by antibody 7.16.4

	Effector†: *Target Ratio*	*Specific lysis of tumor targets*‡	
*Antibody**		*B104-1-1 cells*	*XHT-1-1a cells*
7.16.4	100 : 1	12%	4%
"	33 : 1	6%	5%
"	11 : 1	7%	3%
Control IgG2a††	100 : 1	1%	—

* Antibodies were used at 5 μg ml^{-1} in a total volume of 0.2 ml
† Nucleated spleen cells from Balb/c nude mice were used as Antibody-Dependent Cellular Toxicity (ADCC) effectors
‡ Determined by [^{51}Cr]O_4 release assay as described in Materials and methods
†† Monoclonal antibody UPC10, specific for phosphorylcholine

several *neu*-transformed cell lines with the same result. Therefore, we conclude, ADCC is not an important effector mechanism in this system.

Mechanisms of in vivo *tumor growth inhibition by p185-specific monoclonal antibodies*

We have recently shown that antibody 7.16.4 is able to exert a profound inhibitory effect on the tumorigenic growth of *neu*-transformed cells implanted into nude mice (Drebin *et al.*, 1986). The present study has identified a number of *in vitro* anti-tumor effects mediated by antibody 7.16.4, including a direct effect on the anchorage-independent growth of *neu*-transformed cells, as well as the targeting of immunologic effectors to mediate complement-dependent cytotoxicity but only negligible levels of ADCC. Evidence that host immunologic factors play only a minimal role in the *in vivo* anti-tumor effects of p185 specific immunotherapy with antibody 7.16.4 comes from studies examining the effects of depleting complement or macrophage functions in the tumor bearing host. As shown in Figure 5, depleting complement with cobra-venom factor does not inhibit the anti-tumor effects of antibody 7.16.4. Independently, we verified that mice treated with cobra venom factor had no discernible complement activity (data not shown). Similarly, depleting macrophage functions with carrageenan, only modestly (although statistically significant on the day of the assay in this experiment) inhibited the antitumor effects of antibody 7.16.4 (Figure 5). Both of these studies have been performed with long periods of observation of greater than 21 days. The absence of any significant effect during this time indicates that complement components and ADCC are not critical to the mechanism of action. These results suggest that a direct effect of antibody 7.16.4 on the neoplastic growth of *neu*-transformed cells is principally responsible for its *in vivo* anti-tumor effects. In recent studies, a role for direct oligomerization followed by internalization of p185 has been shown to affect the malignant phenotype (Drebin *et al.*, 1985). We believe that this might also occur *in vivo*.

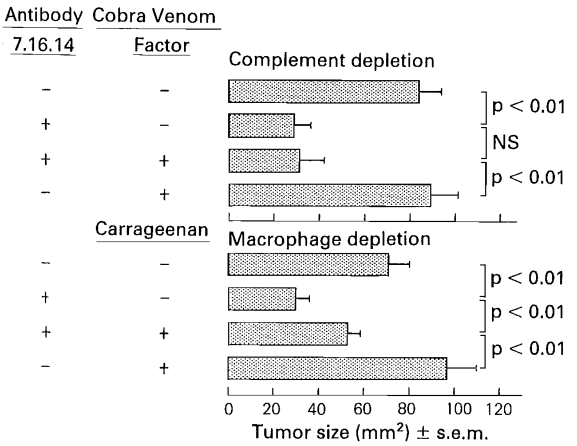

Figure 5 Effects of complement-depletion and macrophage depletion on the anti-tumor activity of antibody 7.16.4. Balb/c nude mice received subcutaneous injections of 1×10^6 B104-1-1 tumor cells on day 0. Some groups of mice received an intravenous injection of 50 μg of purified antibody 7.16.4 within 2 hours of tumor cell implantation. Some groups of mice were treated with cobra venom factor (to deplete serum complement activity) or lambda carrageenan (to deplete macrophage activity) as described in Materials and methods. Tumor size was measured on day 9 following tumor implantation in the complement depletion experiment (upper panel) and on day 10 following tumor implantation in the macrophage depletion (lower panel). NS = not significantly different

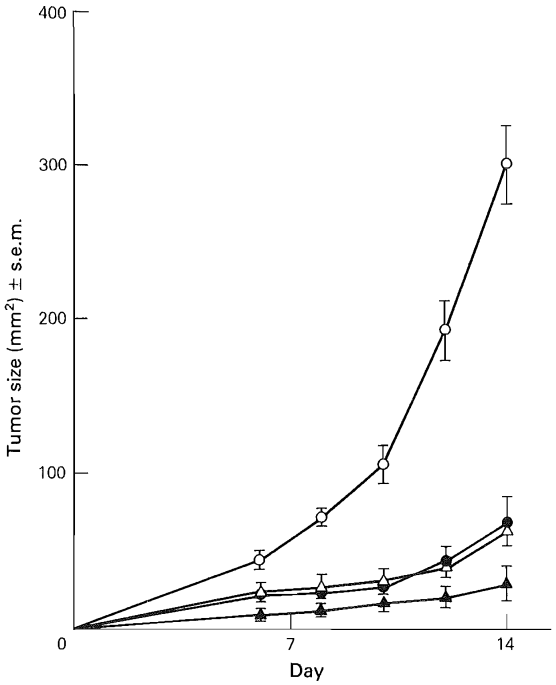

Figure 6 Inhibition of tumor growth by p185-specific monoclonal antibodies of several different isotypes. Balb/c nude mice received subcutaneous injections of 1×10^6 B104-1-1 tumor cells on day 0. Groups of 5 mice received 1 ml intraperitoneal injections of Hank's Balanced Salt Solution (open circles), antibody 7.5.5 ascites fluid (open triangles), antibody 7.9.5 ascites fluid (solid circles), or antibody 7.16.4 ascites fluid (solid triangles) on days 0 and 7. Tumor size was measured as described in Materials and methods

The effects of other p185-specific monoclonal antibodies, which possess a more limited repertoire of *in vitro* anti-tumor activities, on the tumorigenic growth of B104-1-1 cells implanted into nude mice have also been examined. These studies provide additional evidence that a direct effect on the tumorigenic growth of *neu*-transformed cells is responsible for the anti-tumor activity of anti-p185 antibodies. As shown in Figure 6, antibodies 7.5.5 (open triangles), 7.9.5 (solid circles), and 7.16,4 (solid triangles) are all able to profoundly inhibit B104-1-1 tumor growth. Antibody 7.21.2 has a somewhat less striking effect, whereas antibody 7.16.5 has no significant effect on B104-1-1 tumor growth (data not shown). Antibodies 7.9.5 (IgG1) and 7.21.2 (IgG1) are unable to effect complement-mediated cytolysis, and antibodies 7.5.5 (IgG2b) and 7.9.5 are unable to effect any ADCC, as shown above. The potent *in vivo* anti-tumor activity of these antibodies suggest that neither complement-mediated lysis nor ADCC play a critical role in the inhibition of tumor growth by p185-specific monoclonal antibodies. In independent studies not shown, we have also demonstrated that some of these antibodies can be shown to localize directly to the tumor *in vivo* in labeling and homing experiments. It appears therefore that the principle mechanism

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