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Down-Modulation of an Oncogene Protein Product and Reversion of the Transformed Phenotype by Monoclonal Antibodies

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Summary

Exposure of *neu*-oncogene-transformed NIH 3T3 cells to monoclonal antibodies reactive with the *neu* gene product, p185, results in the rapid and reversible loss of both cell-surface and total cellular p185. Although not directly cytotoxic, monoclonal anti-p185 antibody treatment causes *neu*-transformed NIH 3T3 cells to revert to a nontransformed phenotype, as determined by anchorage-independent growth. Isotype matched control antibodies of an unrelated specificity do not affect p185 levels or colony formation in soft agar by *neu*-transformed NIH 3T3 cells. Soft agar colony formation by NIH 3T3 cells transformed by *ras* oncogenes is not affected by anti-p185 antibody treatment. Anchorage-independent growth of cells from the ethylnitrosourea-induced rat neuroblastoma line in which *neu* was originally detected by DNA transfection is also inhibited in the presence of anti-p185 monoclonal antibodies. Collectively, these results suggest that p185 is required to maintain transformation induced by the *neu* oncogene.

Introduction

Malignant cells display a variety of in vitro characteristics that distinguish them from normal cells. These characteristics, collectively known as the transformed phenotype, include anchorage-independent growth, decreased serum requirements, rounded cellular morphology, increased hexose uptake, loss of microfilaments, increased plasminogen activator secretion, decreased cell surface fibronectin, and increased sensitivity to the drug ouabain (Freedman and Shin, 1974; Pollack et al., 1984; Noda et al., 1983). Anchorage-independent growth, as determined by the formation of colonies in soft agar, is the most reliable parameter of the transformed phenotype because it is the phenotypic property most tightly linked with tumorigenic behavior in vivo (Freedman and Shin, 1974).

The study of acutely transforming retroviruses has revealed a class of genes, called oncogenes, capable of rapidly conferring the transformed phenotype on nontransformed cells (Bishop and Varmus, 1982). These retroviral oncogenes originated from cellular genes, called proto-oncogenes, that were transduced by retroviruses (Bishop, 1983). Proto-oncogenes have been highly conserved in evolution (Shilo and Weinberg, 1981), which has led to the suggestion that these genes may play critical roles in normal cellular growth and development. It is thought that linkage to retroviral promoters, mutation of cellular sequences in retroviral genomes, or both, result in activation of the malignant properties of proto-oncogenes transduced by retroviruses (Bishop, 1983).

The malignant properties of cellular proto-oncogenes may also be activated by nonviral means. Within the past several years activated cellular oncogenes, capable of neoplastically transforming NIH 3T3 cells in DNA transfection assays, have been identified in a variety of nonvirally induced tumors and tumor cell lines (Cooper, 1982; Land et al., 1983b). Furthermore, proto-oncogene rearrangements (Taub et al., 1982; de Klein et al., 1982), duplications (Collins and Groudine, 1982), and aberrant expression (Eva et al., 1982; Slamon et al., 1984) have been observed in a substantial fraction of tumors. Collectively these findings have led to the suggestion that genetic alterations involving cellular proto-oncogenes may play a critical role in neoplastic transformation. A corollary of this hypothesis is that proteins encoded by activated cellular oncogenes may be specifically involved in the initiation and maintenance of the neoplastic state. The best evidence in support of this hypothesis is that mutants of Rous sarcoma virus (RSV) that are temperature sensitive for the ability to transform cells encode an aberrant oncogene product, $pp60^{src}$, which is temperature sensitive in its protein kinase activity (Sefton et al., 1980). This suggests that the $pp60^{src}$ protein kinase activity is responsible for the neoplastic state in RSV-transformed cells.

Although many activated cellular oncogenes detected by DNA transfection assays are related to retroviral *ras* oncogenes (Der et al., 1982; Parada et al., 1982; Santos et al., 1982; Shimizu et al., 1983), transfection studies have identified a number of oncogenes that are distinct from those found in retroviruses (Cooper, 1982; Pulciani et al., 1982; Cooper et al., 1984; Lane et al., 1984; Padua et al., 1984). We have previously described one such oncogene, which has been isolated from several independent ethylnitrosourea-induced rat neuroblastomas (Shih et al., 1981). This oncogene, which we have termed *neu*, is related to, but distinct from, the *erbB* oncogene and its normal cellular homolog, the epidermal growth factor receptor gene (Schechter et al., 1984). The putative *neu* oncogene product is a 185 kilodalton (kd) phosphoprotein (p185) that is glycosylated and possesses intrinsic tyrosine kinase activity (Padhy et al., 1982; D. F. S., unpublished data). We have developed monoclonal antibodies reactive with domains of the p185 molecule exposed on the surface of intact cells (Drebin et al., 1984). We describe here the effects of these antibodies on p185 protein levels within, and on the transformed phenotype of, cells transformed by *neu* oncogenes.

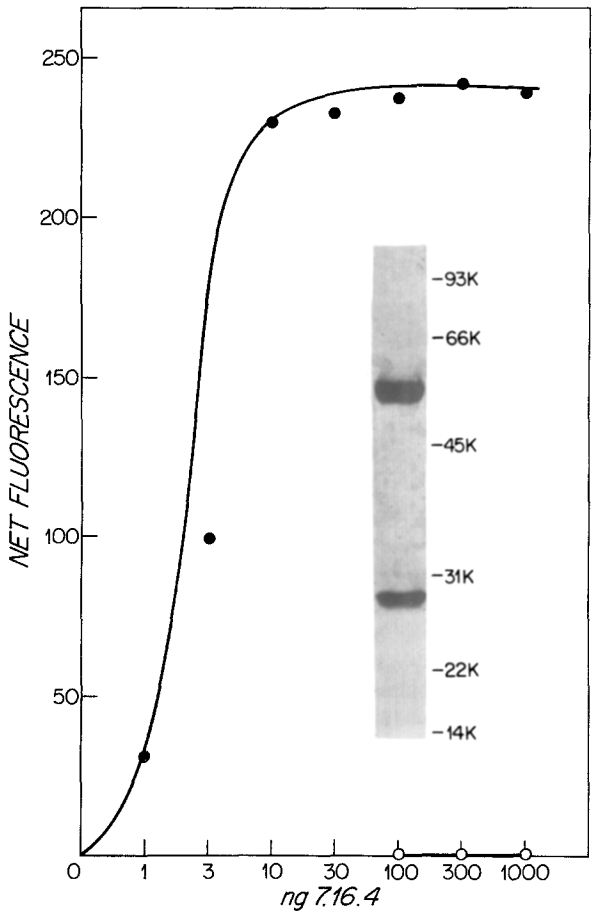

Figure 1. Specific Binding of Purified Monoclonal Antibody 7.16.4 to *neu*-Transformed NIH 3T3 Cells

Samples containing 1 × 10^6 *neu* transformed NIH 3T3 cells (cell line B104-1-1; solid circles) or H-*ras*-transformed NIH 3T3 cells were incubated (cell line XHT-1-1a; open circles) with the indicated amounts of purified antibody 7.16.4 and processed for immunofluorescent flow cytometric analysis as described in Experimental Procedures. (Inset) Coomassie blue staining of monoclonal antibody 7.16.4 (20 μg) electrophoresed on an SDS-polyacrylamide gel under reducing conditions. Only immunoglobulin heavy and light chain peptides are observed.

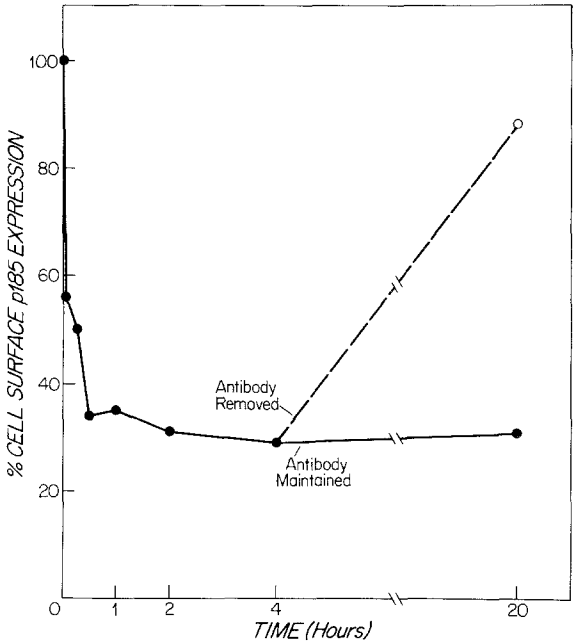

Figure 2. Reversible Down-Modulation of Cell Surface p185 by Monoclonal Antibody 7.16.4

Purified antibody 7.16.4 (50 μg/dish) was added to B104-1-1 cell cultures at time 0. At the time points indicated, cells from antibody-treated dishes and untreated control dishes were processed for immunofluorescent flow cytometry as described in Experimental Procedures. Data are presented as the percentage of cell surface fluorescence of antibody-treated cells as compared with the untreated control. There is clearly a rapid and persistent down-modulation of cell surface p185 in the presence of antibody (solid circles). Removal of antibody from the culture medium results in reexpression of p185 (open circle).

Results

Effects of Purified 7.16.4 Monoclonal Antibody on Cellular p185 Levels

Monoclonal antibody 7.16.4 (IgG2a) was purified from hybridoma ascites fluid by ammonium sulfate precipitation and protein A–Sepharose affinity chromatography as described in Experimental Procedures. This resulted in an immunoglobulin preparation that was more than 95% pure as determined by SDS polyacrylamide gel electrophoresis and Coomassie blue staining (Figure 1, inset). The ability of this antibody preparation to bind specifically cell surface determinants of a *neu*-transfected NIH 3T3 cell line (B104-1-1), as quantitated by immunofluorescent flow cytometry, is shown in Figure 1. The binding of antibody 7.16.4 to B104-1-1 cells saturates at 10-20 ng/10^6 cells (Figure 1, closed circles). This suggests that 2 × 10^5 binding sites exist on the B104-1-1 cell surface if each antibody molecule binds two p185 molecules. In contrast, no binding of antibody 7.16.4 to the Ha-*ras*-transfected NIH 3T3 cell line XHT-1-1a is detectable, even at 1000 ng/10^6 cells (Figure 1, open circles). Untransfected NIH 3T3 cells behave similarly to *ras*-transfected cells in this assay (data not shown). Thus the purified 7.16.4 monoclonal antibody is specific in its binding to cells that contain the p185 product of the *neu* oncogene.

The antibody binding studies described above were carried out on cells maintained at 4°C in order to prevent any reduction of cell surface p185 due to antigenic modulation. To assess whether antibody 7.16.4 could remove p185 from the cell surface, we examined p185 expression on B104-1-1 cells after the addition of antibody 7.16.4 to cell cultures maintained at 37°C. As shown in Figure 2, addition of monoclonal 7.16.4 to cultured B104-1-1 cells causes rapid down-modulation of cell surface p185 expression. This modulation persists as long as cells are cultured in the presence of antibody, and is reversed when antibody is eliminated from the culture media (Figure 2).

The ability of polyvalent and monoclonal antibodies to cause down-modulation of their cognate cell surface antigens has been described in a number of studies (Boyse et al., 1967; Edelman, 1976; Schreiner and Unanue, 1977; Baumann and Doyle, 1980; Ritz et al., 1980; Levy and Miller, 1983; Carroll et al., 1984). Generally, divalent IgG molecules are capable of inducing antigenic modulation,

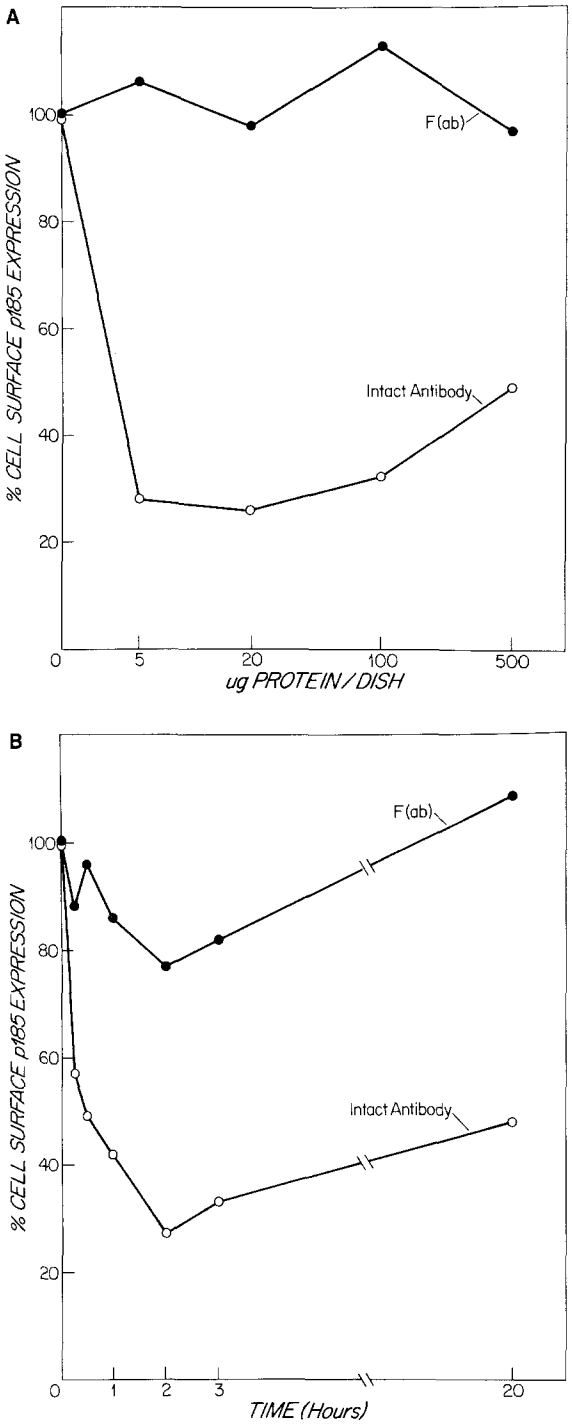

Figure 3. Down-Modulation of p185 by Intact Antibody 7.16.4, but Not by Monovalent F(ab) Fragments

(A) Varying amounts of antibody 7.16.4 or its F(ab) fragment were added to B104-1-1 cultures at time 0. After 3 hr of incubation at 37°C, cells were removed from dishes, restained with saturating amounts of intact 7.16.4 or F(ab) fragments, and processed for flow cytometric analysis as described in Experimental Procedures. Results are presented as the percentage of cellular fluorescence of antibody-7.16.4-treated cells as compared with untreated cells immunofluorescently stained by antibody 7.16.4 (open circles), and the percentage of cellular fluorescence of F(ab)-treated cells as compared with untreated cells immunofluorescently stained with F(ab) fragments (solid circles).

(B) Antibody 7.16.4 or its F(ab) fragment (50 μg/dish) was added to B104-1-1 cultures at time 0. After varying amounts of time at 37°C, cells were removed from dishes and processed for flow cytometry. Results are presented as in A.

whereas their monovalent F(ab) fragments are not. It is thought that down-modulation results from the ability of divalent antibodies to cross-link cell surface structures, which leads in turn to their internalization or shedding from the membrane; monovalent F(ab) fragments cannot cross-link cell surface antigens, and therefore do not induce antigenic modulation. As shown in Figure 3A, exposure of B104-1-1 cells to intact antibody 7.16.4 for 3 hr causes p185 modulation at doses as low as 5 μg/dish. In contrast, monovalent F(ab) fragments of antibody 7.16.4 bind to cell surface p185 but do not cause its down-modulation in this amount of time, even at doses of 500 μg/dish. Figure 3B provides evidence that the lack of an effect of F(ab) fragments on p185 expression does not change with extended exposure to F(ab) fragments. We conclude that down-modulation of p185 by antibody 7.16.4 results from cross-linking of cell surface p185 molecules by the intact antibody molecule.

Incubation of B104-1-1 cells with antibody 7.16.4 causes a significant decrease in cell surface p185 expression; antibody-treated cells display only 20% to 40% as much cell surface p185 as untreated cells. To determine whether this antibody-mediated decrease in cell surface p185 results in lower steady state levels of the protein, we metabolically labeled B104-1-1 cells for 18 hr with ^{35}S-cysteine, and then added antibody to the cells for an additional 3 hr. The presence of labeled p185 was demonstrated by immunoprecipitation of cell lysates with additional antibody followed by SDS-polyacrylamide gel electrophoresis. As shown in Figure 4A, cells incubated with a control IgG2a antibody (lane 4) display comparable amounts of p185 to cells incubated without antibody (lane 2). In contrast, cells incubated with anti-p185 antibody 7.16.4 contain markedly less total p185 (lane 3). The reduction in labeled p185 found in antibody-modulated cells is comparable to the reduction in cell surface p185—a 60% to 80% decrease. It is noteworthy that the major intracellular p185 precursor, which migrates slightly more rapidly than p185 (Figure 4A), is also precipitated by antibody 7.16.4 and does not appear to be affected by antibody-mediated down-modulation of mature p185. This suggests that antibody treatment does not inhibit the synthesis of p185 precursor proteins, and might act by causing loss from the cell of mature p185.

To determine whether the decrease in the steady state p185 level is accompanied by loss of the p185 protein from antibody-treated cells, we measured the effect of antibody treatment on the stability of p185. B104-1-1 cells that had been metabolically labeled for 18 hr with ^{35}S-cysteine were washed free of isotope and incubated for 3 hr in the presence or absence of antibody. The presence of labeled p185 was determined by immunoprecipitation and polyacrylamide gel electrophoresis as described above. Pulse-chase studies, to be presented elsewhere, have shown that the p185 molecule has a half-life of well over 6 hr. As shown in Figure 4B, significant amounts of labeled

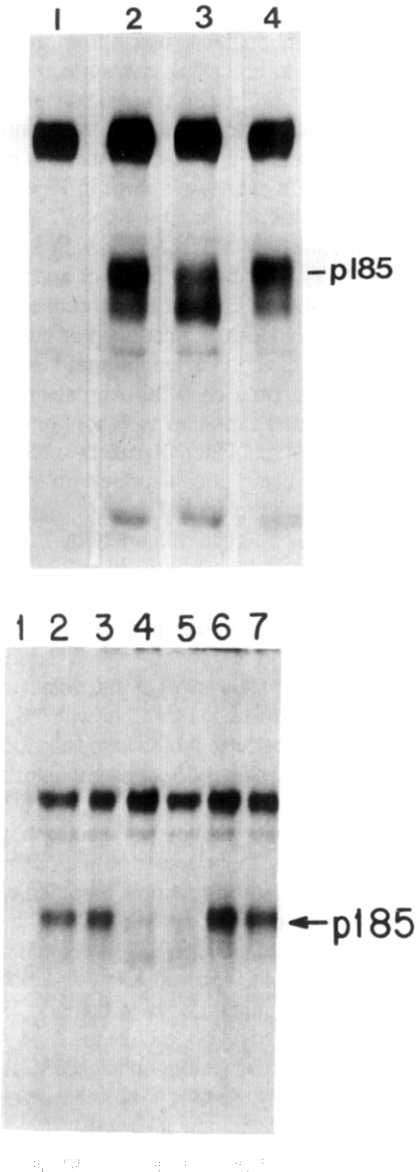

Figure 4. Effect of Antibody 7.16.4 on Steady State p185 Levels and on p185 Stability

(A) B104-1-1 cells were metabolically labeled with ^{35}S-cysteine for 18 hr and then were incubated in the absence or presence of antibody (25 μg/dish) for 3 hr. The level of labeled p185 was analyzed by SDS-polyacrylamide gel electrophoresis following detergent lysis and immunoprecipitation. Equal volumes of ^{35}S-labeled lysate were immunoprecipitated in each group. Lane 1: Cells labeled in the absence of antibody, immunoprecipitated with a mixture of normal mouse and normal rabbit serum. Lane 2: Cells labeled in the absence of antibody, immunoprecipitated with antibody 7.16.4. Lane 3: cells labeled in the presence of antibody 7.16.4, immunoprecipitated with antibody 7.16.4. Lane 4: Cells labeled in the presence of control antibody 9BG5, immunoprecipitated with antibody 7.16.4.

(B) B104-1-1 cells were metabolically labeled with ^{35}S-cysteine for 18 hr and then incubated without label (chased) in the absence or presence of antibody for 3 hr. The level of p185 remaining was analyzed as described above. Equal amounts (counts per minute) of ^{35}S-labeled lysate were immunoprecipitated in each group; experimental groups were done in duplicate. Lane 1: Cells chased in the absence of antibody, immunoprecipitated with a mixture of normal mouse and normal rabbit serum. Lanes 2,3: Cells chased in the absence of antibody, immunoprecipitated with antibody 7.16.4. Lanes 4,5: Cells chased in the presence of 25 μg/dish 7.16.4, immunoprecipitated with 7.16.4. Lanes 6,7: Cells chased in the presence of 25 μg/dish control antibody 9BG5, immunoprecipitated with antibody 7.16.4.

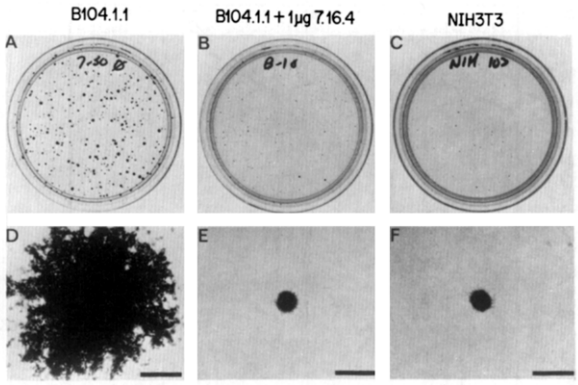

Figure 5. Inhibition of Soft-Agar Colony Formation by B104-1-1 Cells in the Presence of Antibody 7.16.4

We plated 1 × 10^3 cells/dish in soft agar as described in Experimental Procedures. (A-C) Photographs of entire culture plates. (D-F) Representative colonies photographed at 40× magnification. (A,D) untreated B104-1-1 cells, (B,E) B104-1-1 cells cultured with 1 μg 7.16.4, (C,F) untreated NIH 3T3 cells. Solid bars in D-F indicate 200 μm.

p185 are precipitated from untreated B104-1-1 cells (lanes 2, 3) and from B104-1-1 cells incubated during the 3 hr chase with a control IgG2a antibody (lanes 6, 7). In contrast, essentially no labeled p185 is precipitated from B104-1-1 cells incubated with antibody 7.16.4 during the 3 hr chase (lanes 4, 5). The relative lack of detectable p185 precursor in Figure 4B also demonstrates that significant synthesis of new labeled p185 did not occur during the 3 hr chase in the absence of label. Collectively, these results indicate that down-modulation of cell surface p185 is correlated with lower steady state levels of the p185 protein and with an increased rate of destruction of the p185 molecule.

Effect of Monoclonal Antibody 7.16.4 on the Anchorage-Independent Growth of *neu*-Transformed Cells

Neoplastic cells display unusual properties in tissue culture when compared with normal cells (Pollack, 1984). The most definitive in vitro characteristic that distinguishes tumorigenic cells from nontumorigenic cells is their ability to form anchorage-independent colonies (Freedman and Shin, 1974). NIH 3T3 cells transformed by transfection with activated *neu* oncogenes (cell line B104-1-1) grow well in soft agar, with 5% to 10% of the input cells yielding large (>0.5 mm) colonies after 14 days (Figure 5A). In contrast, normal NIH 3T3 cells develop only small clusters of cells, which grow slowly and then stop (Figure 5C). Less than 0.1% of NIH 3T3 cells plated in soft agar give rise to large colonies.

The addition of 1 μg of purified monoclonal 7.16.4 to B104-1-1 soft-agar cultures (0.17 μg/ml) almost completely inhibits the formation of large colonies by *neu* oncogene transformants (Figure 5B) and causes the cells to form colonies having a morphology similar to normal NIH 3T3 cells (compare Figures 5D, 5E, 5F). The inhibition by antibody 7.16.4 of B104-1-1 soft-agar colony formation is dose dependent (Figure 6). As little as 100 ng of antibody inhibits B104-1-1 colony formation by more than 50%; larger amounts inhibit colony formation by more than 99%.

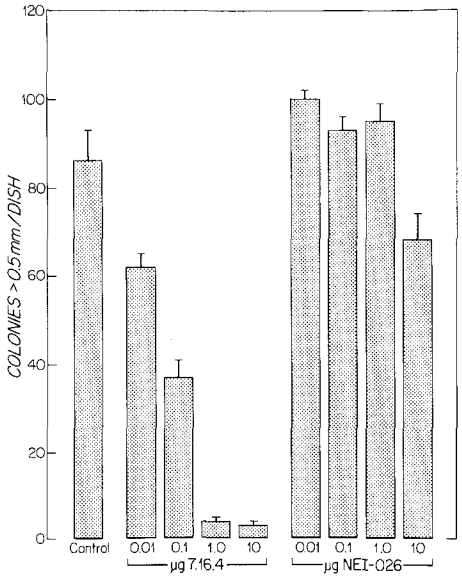

Figure 6. Dose-Dependent Inhibition of B104-1-1 Soft-Agar Colony Formation by Antibody 7.16.4

Soft agar cultures were prepared as described in Experimental Procedures and supplemented with varying amounts of antibody. Each group shows the mean of triplicate samples and the standard error of the mean.

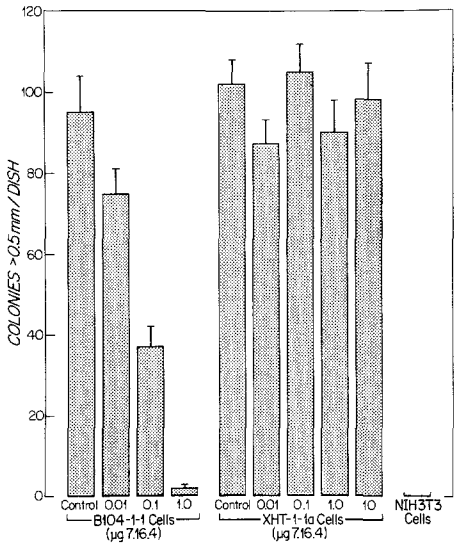

Figure 7. Inhibition of the Anchorage-Independent Growth of *neu* Transfectant B104-1-1 Cells, but Not H-*ras* Transfectant XHT-1-1a Cells, by Antibody 7.16.4

Soft-agar cultures were prepared as described in Experimental Procedures and supplemented with varying amounts of antibody. Each group shows the mean of triplicate samples and the standard error of the mean.

To test the possibility that such inhibition results from toxic effects due to the presence of immunoglobulin in the soft agar, or from nonspecific effects of the binding of immunoglobulin to the cell surface, we examined the effects of a control IgG2a antibody on the anchorage-independent growth of B104-1-1 cells. As shown in Figure 6, a mouse IgG2a monoclonal antibody that binds B104-1-1 cells via cell surface β_2-microglobulin molecules does not inhibit the anchorage-independent growth of B104-1-1 cells. Thus, neither the presence of IgG2a protein in the agar layer, nor its binding to the cell surface, is sufficient to inhibit the anchorage-independent growth of these cells.

The anchorage-independent growth of an independently derived *neu* oncogene transfectant, cell line B104-1-2, is inhibited by antibody 7.16.4 to approximately the same degree as is cell line B104-1-1 (data not shown). In contrast antibody 7.16.4 has no effect on anchorage-independent growth of the H-*ras*-transfected NIH 3T3 line XHT-1-1a, even at a 100-fold higher concentration (10 μg) than that which inhibits B104-1-1 cell anchorage-independent growth by more than 50% (Figure 7). We conclude that the ability of antibody 7.16.4 to inhibit *neu*-transformed NIH 3T3 soft agar colony formation is a function of the p185-specific antibody itself, and not of a toxic contaminant that might copurify with it.

Although repeated feeding of B104-1-1 soft agar cultures with antibody will continue to suppress colony formation, B104-1-1 cell cultures that are inhibited by antibody exposure for 2 weeks will eventually develop large colonies if fed with antibody-free media (data not shown). This suggests that the antibody exerts a cytostatic effect, rather than an irreversible cytotoxic effect, on *neu*-transformed cells in soft agar. Furthermore, the antibody does not affect adherent growth of B104-1-1 cells in medium containing 10% fetal calf serum (data not shown). All experiments were performed using serum depleted of complement by heating at 56°C, and we have been unable to demonstrate lysis of B104-1-1 cells using antibody 7.16.4, or antibody plus complement, in standard ^{51}Cr release microcytotoxicity assays (data not shown). These results indicate that the effect of anti-p185 antibody on the anchorage-independent growth of B104-1-1 cells does not involve antibody-mediated cell killing.

Inhibition of Anchorage-Independent Growth Requires Cross-linking of p185 Molecules by Monoclonal Antibody

The inhibition of anchorage-independent growth by antibody 7.16.4 could result simply from its binding to cell surface p185, this affecting in turn the activity of the p185 molecule. Thus the antibody might block a receptor site for some critical growth factor, or might induce a conformational change in the p185 molecule that renders it unable to impart oncogenic signals to the cell. Alternatively, the ability of the antibody to inhibit anchorage-independent growth might stem from its ability to induce down-modulation of p185. The removal of p185 from its normal site of residence in the plasma membrane and the lowering of cellular p185 levels by antibody might deprive the *neu*-transfected cells of the protein that makes possible their anchorage-independent growth.

These two alternatives can be distinguished by comparing the effects of intact divalent antibody and monovalent F(ab) antibody fragments on the anchorage-independent growth of *neu*-transformed cells. The intact antibody and

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