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Mechanistic aspects of the opposing effects of monoclonal antibodies to the ERBB2 receptor on tumor growth

(growth factors/tyrosine kinase/adenocarcinoma/oncogene)

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ABSTRACT **The *ERBB2* (also called *HER2*, *neu*, and *c-erbB-2*) gene product, which encodes a growth factor receptor, was implicated in the malignancy of human adenocarcinomas. An antibody directed to the rat oncogenic receptor has been previously shown to have an antitumor effect in model systems. In an attempt to extend this observation to the protooncogenic human receptor and also to understand the underlying mechanism, we generated a panel of monoclonal antibodies specific to the extracellular portion of the ERBB2 protein. The effects of the antibodies on tumor growth were compared with their cellular and biochemical actions *in vitro*. Surprisingly, opposing *in vivo* effects were observed: although some antibodies almost completely inhibited the growth in athymic mice of transfected murine fibroblasts that overexpress Erbb-2, other antibodies either accelerated tumor growth or resulted in intermediate responses. When tested on cultured human breast carcinoma cells or ERBB2 transfectants, the tumor-stimulatory antibody was found to induce significant elevation of tyrosine phosphorylation of the ERBB2 protein. In contrast, only partial correlation was observed between the capacity to restrict tumor growth and the effects of the antibodies on receptor degradation and cellular proliferation *in vitro*. This suggests that the antitumor antibodies affect both receptor function and host–tumor interactions. Our results may help establish experimental criteria for the selection of specific antibodies for use either alone or in conjunction with other molecules as pharmacological antitumor agents.**

Evidence has been accumulated in recent years for the involvement of growth factors and their receptors in the process of malignant transformation. The ERBB2 protein is a receptor tyrosine kinase (1), homologous to the epidermal growth factor (EGF) receptor (2, 3). The rat homologue of the gene undergoes oncogenic activation through a single point mutation (4). The ERBB2 protein was found to be overexpressed in several types of human adenocarcinomas, especially in tumors of the breast and the ovary (5–7), and the overexpression was correlated with short time to relapse and poor survival of breast cancer patients (5).

The potential use of monoclonal antibodies (mAbs) in diagnosis and treatment of cancer has been studied extensively (8). Receptors for growth factors constitute an ideal target for this approach because their location on the cell membrane makes them accessible to antibody molecules. Moreover, antibodies directed to growth factor receptors can potentially block biological functions essential for cell proliferation. Previous studies have demonstrated, in model systems, the potential therapeutic effect of mAbs against the epidermal growth factor receptor (9, 10). Likewise, different mAbs to the ERBB2 receptor inhibited the proliferation of a human breast carcinoma cell line in culture (11), and an antibody directed to the rat ERBB2 protein inhibited the tumorigenicity of fibroblasts transformed by the mutated rat *ERBB2* oncogene (12, 13). mAbs that recognize the protein product of the human ERBB2 protooncogene have been raised and used to study the biological function of the presumed receptor (14–16).

Our studies were aimed at the generation of antibodies with potential use in immunotherapy of human cancer, either alone or conjugated with drugs or toxins. To this end we raised a panel of mAbs to the extracellular portion of the ERBB2 receptor. These antibodies induced opposing effects on tumor growth in athymic mice. Our attempts to analyze the mechanism of antibody-mediated tumor enlargement suggest that activation of the tyrosine kinase is involved, but inhibition of tumor growth is not simply correlated with one receptor function.

MATERIALS AND METHODS

Chemicals and Reagents. Affinity-purified goat anti-mouse $F(ab')_2$ was from Jackson ImmunoResearch. It was radiolabeled with $Na^{125}I$ (Amersham) by the chloramine T procedure (17). [^{32}P]Orthophosphate was from the Nuclear Research Center (Negev, Israel); [^{35}S]methionine and [γ-^{32}P]ATP were from Amersham. Sepharose-protein A was purchased from Pharmacia. The anti-phosphotyrosine mAb 1G2 (18) was purified from ascites fluid.

Cell Lines. The HER2 cell line has been described (19). The SKBR3 human breast carcinoma cell line was obtained from the American Type Culture Collection.

Experimental Animals. BALB/c mice, $CB6/F_1$ mice, and CD1/nude mice were obtained from the Experimental Animals Center of the Weizmann Institute of Science.

Generation of mAbs to the ERBB2 Receptor. BALB/c mice (2 mo old) were injected i.p. three times with 3–5 $\times$ 10^6 living SKBR3 human breast carcinoma cells, at intervals of 2 weeks. Antisera were tested by an immunoprecipitation assay using HER2 cells (NIH 3T3 cells transfected with human *ERBB2* gene, ref. 19), labeled metabolically with [^{35}S]methionine. The spleens of mice that developed a strong immune response were selected for fusion. The spleen cells were fused with NSO myeloma cells by using polyethylene glycol (20), and the hybridomas were selected with hypoxanthine/aminopterin/thymidine medium. Supernatants of the growing cells were screened by using an indirect binding assay. CHO cells transfected with the *ERBB2* gene (HCC cell line) were plated on a flexible 94-well plate, previously coated with polylysine (1 mg/ml). The cells were fixed with 3% (wt/vol) paraformaldehyde, and supernatants of hybridomas were incubated for 1 hr at 22°C with the fixed cells. The bound

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Abbreviation: mAb, monoclonal antibody.

antibodies were detected with ^{125}I-labeled goat anti-mouse F(ab')$_2$ antibody. As a negative control we used the parental, untransfected CHO cell line.

The antibodies that specifically bound to the HCC cells were selected for further analysis by using either an immunoprecipitation assay with [^{35}S]methionine-labeled cells or immunoprecipitation followed by autophosphorylation in the presence of $MnCl_2$ and [γ-^{32}P]ATP (21). Positive hybridomas were cloned twice by limiting dilution. Determination of antibody class was done with class-specific second antibodies. Large quantities of specific mAbs were produced by preparation of ascites fluid in CB6/F$_1$ mice. The IgM antibody was separated on a Sephacryl G300 column, and the IgG1 and IgG2a antibodies were purified by affinity chromatography on Sepharose-protein A, using elution conditions specific for each subclass.

Indirect Binding Assay on Living Cells. SKBR3 cells or HER2 cells were plated in 24-well plates and assayed at confluence. The cells were incubated for an hour at 22°C with various concentrations of antibodies in phosphate-buffered saline (PBS)/1% bovine serum albumin. After being washed with the same buffer, the cells were incubated for 90 min with ^{125}I-labeled goat anti-mouse F(ab')$_2$ (10^5 cpm per well). The cells were then washed and solubilized with 0.1 M NaOH; the radioactivity was then determined in a γ counter.

Determination of the *in Vivo* Effect of the mAbs. HER2 cells (3×10^6) were injected s.c. into nude mice, followed by three i.p. injections of the mAbs on days 3, 7, and 10. Tumor parameters were measured twice a week with callipers, and tumor volume was calculated according to the formula: tumor volume equals length × width × height. To validate volume measurements the correlation between the tumor volume and tumor weight was determined on the day of animal killing.

Determination of Tyrosine Phosphorylation in Living Cells. The SKBR3 or HER2 cells were grown in a 24-well plate and labeled for 4 hr in Dulbecco's modified Eagle medium (DMEM) without phosphate but in the presence of 1% dialyzed fetal calf serum and [^{32}P]orthophosphate (0.5 mCi/ml; 1 Ci = 37 GBq). The cells were washed with PBS and incubated for 15 min at 22°C with fresh medium containing antibodies at a concentration of 10 μg/ml. After being washed, the cells were lysed in solubilization buffer (21), and the tyrosine-phosphorylated ERBB2 protein was immunoprecipitated with an agarose-immobilized antibody to phosphotyrosine (18). The immune complexes were eluted with solubilization buffer containing 50 mM *p*-nitrophenylphosphate and subjected to immunoprecipitation with a rabbit polyclonal anti-ERBB2 antibody, directed to the carboxyl terminus of the receptor (21).

Determination of the Effect of the mAbs on Receptor Turnover. SKBR3 or HER2 cells were grown in 24-well plates to 80% confluence and then labeled for 16 hr at 37°C with [^{35}S]methionine (50 μCi/ml). After being washed with PBS, the cells were incubated with fresh medium in the absence or presence of the antibodies (at a concentration of 10 μg/ml) for various periods of time. The cells were then washed, and cell lysates were subjected to immunoprecipitation with a rabbit polyclonal antibody to the ERBB2 protein (21).

Complement-Dependent Cytotoxicity (CDC) Assay. The SKBR3 tumor cells were incubated at 37°C for 2 hr in a volume of 0.1 ml of fetal calf serum, with 300 μCi of Na[^{51}Cr]O$_4$ (DuPont/NEN). At the end of the labeling period the cells were washed three times in PBS, and 1.5×10^4 cells (in 25 μl) were plated in each well of a 96-well microtiter plate. Various concentrations of the mAbs (25 μl) were added and incubated with the cells for 1 hr followed by the addition of human or rabbit complement and incubation for further 3 hr. Appropriate control wells containing cells alone, cells with no antibody or no complement, and cells lysed in 10% SDS were set up in parallel. ^{51}Cr release was determined in a γ counter. The means of triplicate determinations are given.

Antibody-Mediated Cell-Dependent Cytotoxicity (ADCC) Assay. The SKBR3 tumor cells were labeled with Na[^{51}Cr]O$_4$ as described above. Cells (5×10^3) in 25 μl were incubated for 1 hr with various concentrations of the mAbs and then for 5 hr with effector cells, human peripheral blood lymphocytes (0.1 ml, lymphocytes/tumor cells = 140:1), or with mouse splenocytes (120:1). ^{51}Cr release was determined as described above.

RESULTS

Generation of mAbs Directed to the ERBB2 Receptor. Five hybridomas were selected after the fusion of NSO myeloma cells with splenocytes obtained from mice immunized with intact cells of the human breast carcinoma SKBR3 cell line. This immunization procedure elicited specific antibodies to the extracellular portion of the human ERBB2 antigen. The isotypes and subclasses of the resulting mAbs are given in Table 1. Three of these antibodies were found to be of the IgG1 subclass (N12, N24, N28), one was an IgM (N10), and one an IgG2a antibody (N29). As depicted in Fig. 1, all the mAbs specifically bound to cultured cells that express the ERBB2 receptor, yet they bound with different apparent affinities. Antibodies N28 and N24 displayed the highest apparent affinity, whereas N10 mAb exhibited the lowest apparent affinity. All five mAbs immunoprecipitated a single protein of 185 kDa from metabolically labeled HER2 cells, as shown in Fig. 2*A*. This result was also reflected in an *in vitro* kinase assay performed on the immunoprecipitates (Fig. 2*B*). None of these mAbs reacted with the epidermal growth factor receptor or with the rat p185^{neu} (data not shown). Immunoblot analysis of the ERBB2 protein showed that only the N12 and N29 antibodies could react with the denatured form of the receptor (Table 1).

The Effect of mAbs upon Tumor Growth *in Vivo*. The mAbs were assayed for their ability to affect tumor growth of murine fibroblasts transformed by overexpression of the *ERBB2* gene (HER2 cells), in nude mice. The mAbs or a control antibody to dinitrophenol (anti-DNP) were injected i.p., into groups of five mice, on days 3, 7, and 10 after tumor inoculation. Fig. 3*A* depicts tumor volumes of each group of mice, on day 21, postinoculation. The tumorigenic growth of HER2 cells was significantly inhibited ($P < 0.05$, as calculated by using Duncan's multiple comparison test) in nude mice injected with mAbs N29 and N12, when compared with mice that received no antibody or the control anti-dinitrophenyl antibody. As depicted in Fig. 3*B*, the inhibitory

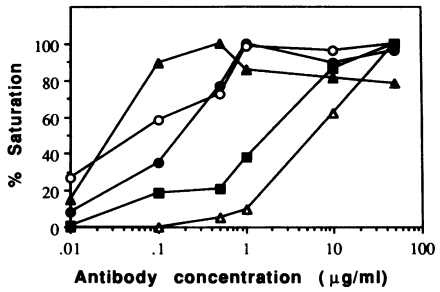

FIG. 1. Binding of monoclonal anti-ERBB2 antibodies to HER2 cells. Confluent monolayers of HER2 cells were incubated for 1 hr at 22°C with various concentrations of mAbs. Bound antibodies were subsequently determined with ^{125}I-labeled goat anti-mouse F(ab')$_2$. Control cells were incubated without the murine antibody, and their background binding was subtracted. △, N10 (IgM); ●, N12 (IgG1); ○, N24 (IgG1); ▲, N28 (IgG1); ■, N29 (IgG2a).

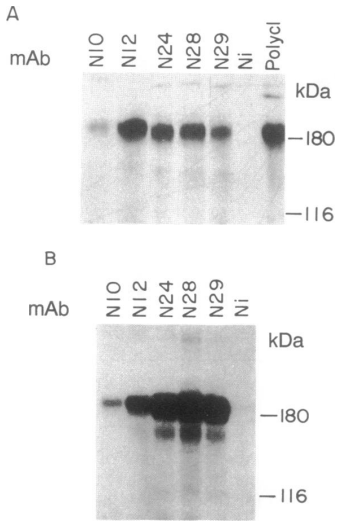

FIG. 2. Immunoprecipitation of the ERBB2 protein by mAbs. (*A*) HER2 cells were metabolically labeled with [^{35}S]methionine, and the cell lysates were separately subjected to an immunoprecipitation assay with 10 μg of each mAb. As a control nonimmune serum (Ni) was used. Proteins were separated on a SDS/7.5% polyacrylamide gel. (*B*) The immunoprecipitation assay was done as described but with unlabeled cells. Before electrophoresis, the proteins from the cell lysate were labeled by autophosphorylation with [γ-^{32}P]ATP and 10 mM $MnCl_2$. Autoradiograms are shown. NI, nonimmune serum; Polycl, polyclonal anti-ERBB2 antibody.

effect persisted over 31 days after tumor injection. Antibodies N10 and N24 exhibited less efficient inhibition of tumor growth. In contrast, mAb N28 consistently stimulated tumor growth. Essentially identical results were obtained in three separate experiments. To test the possibility that the effects seen *in vivo* are reflected *in vitro*, we used cell proliferation assay in culture and cytotoxicity assays on SKBR3 human breast tumor cells. Partial, if any, correlation was found between the results obtained in these assays and the *in vivo* experiments (Table 1).

Stimulation of Tyrosine Phosphorylation of ERBB2. It has been shown (22) that mAbs directed against the rat ERBB2 protein elevated tyrosine phosphorylation of this receptor. Two different assays were used to test the capacity of our mAbs to elevate tyrosine phosphorylation of the ERBB2 protein: HER2 cells were metabolically labeled with [^{32}P]orthophosphate, incubated with the mAbs, and subjected to two consecutive immunoprecipitation steps with anti-phosphotyrosine and anti-ERBB2 antibodies (21). Alternatively, SKBR3 cells were first incubated with the mAbs and then subjected to two consecutive immunoprecipitation steps, followed by an *in vitro* phosphorylation assay in the presence of [γ-^{32}P]ATP and $MnCl_2$. As depicted in Fig. 4, similar results were obtained in both experiments: mAb N28 significantly stimulated phosphorylation of the ERBB2 receptor on tyrosine residues, whereas the other mAbs displayed low (N12, N24, N29 mAbs) or no activity (N10 antibody) in living cells.

The Effects of the mAbs on the Rate of Receptor Turnover. The interaction of receptor tyrosine kinases with their respective ligands is usually coupled to rapid endocytosis. It was further shown that antibodies could induce an analogous effect on the rat ERBB2 receptor (22) and that this activity was associated with disappearance of the transformed phenotype (23). We, therefore, tested the potential of our mAbs to the human ERBB2 protein to accelerate the turnover of the

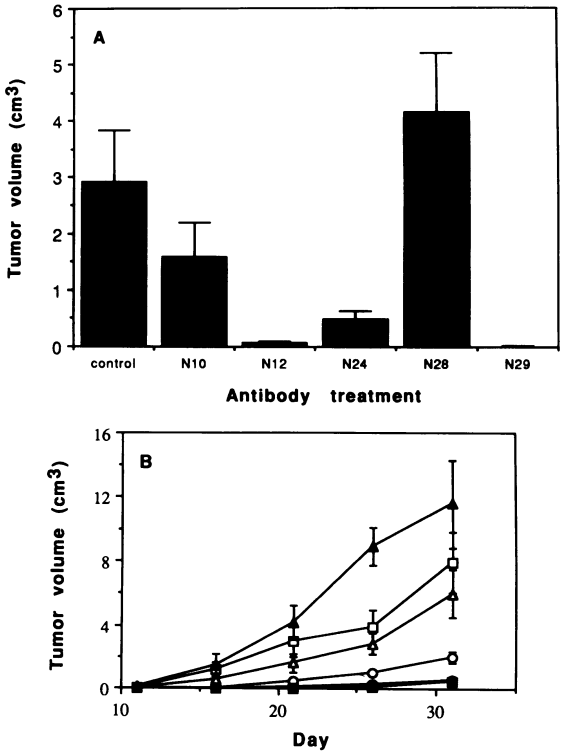

FIG. 3. Effect of mAbs on tumor growth in athymic mice. Cells (3×10^6) were injected, s.c. into CD1/nude mice. Groups of five mice received three i.p. injections on days 3, 7, and 10 at a total mAb dose of 2 mg per mouse. Tumor size was measured as described. As control, an irrelevant antibody anti-dinitrophenol or buffer alone (PBS) was used. (*A*) Effects of antibody treatment after 21 days postinoculation. (*B*) Kinetics of tumor growth in antibody treated mice. □, Control; △, N10; ●, N12; ○, N24; ▲, N28; and ■, N29. Statistical analysis was done by using the analysis of variance and Duncan's multiple comparison test.

receptor. For this purpose, HER2 cells were biosynthetically labeled with radioactive methionine and then chased for various periods of time with fresh medium that contained different mAbs. At the end of the chase period, the residual labeled proteins were immunoprecipitated and analyzed by gel electrophoresis and autoradiography. The results of this experiment are shown in Fig. 5: all the mAbs accelerated, to different extents, the rate of turnover of the receptor, with antibody N29 being the most effective.

DISCUSSION

Overexpression of ERBB2 protein is frequently found in human adenocarcinomas, and it is believed to be involved in the progression of the malignancy state (5–7). This possibility was supported by gene transfer experiments demonstrating that overexpression of the apparently normal gene, driven by heterologous promoters, confers tumorigenicity on murine fibroblasts (19, 24). These observations, together with the tyrosine kinase activity of the ERBB2 protein, have made this human receptor an excellent target candidate for antibody-mediated therapy of human solid tumors. Indeed, many different mAbs to the human protein have been generated (14–16), but their anti-tumor activity was not extensively investigated *in vivo*. On the other hand, a mAb to the rat ERBB2 protein efficiently inhibited the growth of tumori-

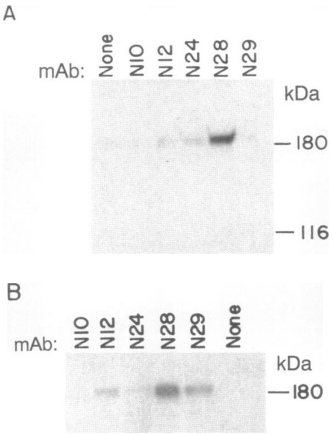

FIG. 4. Antibody-induced stimulation of tyrosine phosphorylation of the ERBB2 receptor. (*A*) Monolayers of HER2 cells were labeled with [^{32}P]orthophosphate and then incubated for 15 min at 22°C with each antibody at 10 μg/ml. Tyrosine-phosphorylated proteins were immunoprecipitated with an anti-phosphotyrosine antibody, followed by specific elution and a second immunoprecipitation step with rabbit anti-ERBB2 polyclonal antibody, directed to the carboxyl terminus of the protein. (*B*) SKBR3 cells were first incubated with the antibodies, immunoprecipitated in two consecutive steps, as described above, and labeled by autophosphorylation with [γ-^{32}P]ATP and Mn^{2+}. The autoradiograms of the SDS/gel-separated proteins are shown.

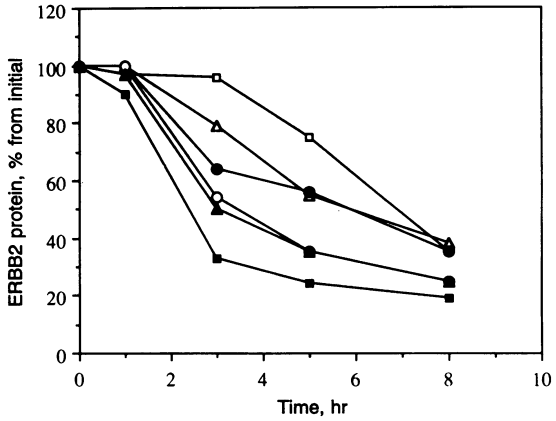

FIG. 5. Effect of mAbs on the rate of turnover of the ERBB2 receptor. HER2 cells were labeled with [^{35}S]methionine in a 24-well plate and then chased for the indicated periods of time with fresh medium that contained the indicated mAbs. Residual ^{35}S-labeled ERBB2 protein was subjected to immunoprecipitation with a rabbit polyclonal antibody directed to the carboxyl terminus of the protein and separated on SDS gel. Quantitative analysis of receptor turnover is shown, as determined by measuring densitometry of the autoradiogram. □, Control cells without antibody treatment; △, N10; ●, N12; ○, N24; ▲, N28; and ■, N29-antibody-treated cells.

genic cells carrying the oncogenic mutated ERBB2 protein (12, 13).

In the present study we used a murine model system to address the potential and diversity of mAbs to ERBB2 as anti-tumor agents. We further attempted to understand the mechanisms of action of the antibodies in the hope that this may constitute an experimental basis for selection of an optimal mAb. Of the five mAbs surveyed in this study, two almost completely inhibited tumor growth, two displayed moderate inhibitory effects, and the last one significantly accelerated the rate of tumor growth (Fig. 3). These differential biological activities can be attributed to different epitopes on the exoplasmic portion of the receptor. The mechanism by which different receptor regions may mediate opposing effects on tumor growth is apparently important for both receptor structure–function relationships and also for the elucidation of the biochemical mechanism underlying tumor inhibition. One simple explanation may be that the ligand-binding site of the putative receptor (25, 26) is involved in the action of the biologically active antibodies. However, in the absence of a well-characterized ligand for the ERBB2 protein this possibility cannot be experimentally tested.

Aware of its limitations, we tried to find a correlation between the *in vivo* effects of the mAbs and their actions on cultured cells. The results of this analysis are summarized in Table 1; in contrast with our inability to correlate the binding

Table 1. Comparison of the biological properties of anti-ERBB2 mAbs

Antibody	Immunoblot*	Tumor growth,† %	Cell proliferation,‡ %	CDC,§ %	ADCC,¶ %	Tyrosine phosphorylation,‖ -fold	Receptor degradation,** $t_{1/2}$ in hr
Anti-DNP	−	100	100	ND	ND	1.0	6.5
N10 IgM	−	54	247	68 ± 3.1	7 ± 1	0.9	6
N12 IgG1	+	2	63	9 ± 0.9	10 ± 0.01	1.8	6
N24 IgG1	−	16	196	60 ± 1.1	9 ± 2.2	2.5	3.5
N28 IgG1	−	141	107	10 ± 1.7	18 ± 0.01	14.0	3
N29 IgG2a	+	0.3	72	9 ± 2.2	12 ± 0.33	1.2	2.5

DNP, dinitrophenol; CDC, complement-dependent cytotoxicity; ADCC, antibody-mediated cell-dependent cytotoxicity, ND, not determined.
*HER2 cell lysates were separated by SDS/gel electrophoresis, transferred to nitrocellulose, and blotted with the mAbs, followed by detection with horseradish peroxidase-conjugated goat anti-mouse F(ab′)$_2$.
†Average tumor volume (as percentage of control; n = 5) measured 21 days after tumor inoculation.
‡SKBR3 breast tumor cells were plated in 24-well plates (10^3 cells per well) and incubated for 48 hr in medium supplemented with 10% fetal calf serum. The amount of serum was then decreased to 5%, and the indicated antibodies were added at 10 μg/ml. Five days later, the numbers of viable cells were determined.
§Complement-dependent cytotoxicity assay of SKBR3 tumor cells was done as described. Values represent [^{51}Cr]O_4 release from cells treated with the indicated mAbs (50 μg/ml) and human complement, as percentages of total cellular content of ^{51}Cr. Corrections were made for spontaneous release, in the absence of antibody and complement. Similar results were obtained by using rabbit complement.
¶Antibody-mediated cell dependent lysis of SKBR3 cells was assayed as described, using each antibody at 50 μg/ml, and human effector cells, and expressed as percentages (see §). Similar results were obtained with mouse splenocytes.
‖Extent of induction of tyrosine phosphorylation of ERBB2 protein by mAbs was determined by densitometry of autoradiograms, according to the assay of Fig. 4*A*.
**Down-regulation of ERBB2 protein was determined with [^{35}S]methionine-labeled HER2 cells, as described in text and Fig. 5, and expressed as half-life of the labeled protein ($t_{1/2}$).

affinities of the various antibodies (Fig. 1) or their effects on cell proliferation in culture with their actions on tumors, an interesting correlation was found with direct responses exhibited by the receptor protein. Thus, our single tumor stimulatory mAb, N28, dramatically stimulated the tyrosine kinase activity of the receptor (Fig. 4). On the other hand, both tumor-inhibitory antibodies were the only mAbs capable of recognizing the fully denatured protein. This may reflect similar characteristics of the epitopes recognized by these antibodies, but the correlation to tumor effect is not readily apparent. Although the most efficient tumor inhibitory antibody, N29, led to the shortest receptor half-life (Fig. 5), this correlation is difficult due to the effects seen with the other antibodies.

The simplest interpretation of these observations is that the positive effect on tumor growth involves stimulation of the tyrosine kinase function of the ERBB2 receptor, whereas tumor inhibition may involve other effects including reduction in the cellular level of intact receptor-kinase molecules. The *in vivo* and *in vitro* effects of mAbs N28 and N29 are in line with the oncogenic role of the overexpressed ERBB2 protein and are also consistent with the tumor-inhibitory function of a mAb directed to the rat ERBB2-transforming protein (12). Nevertheless, tumor inhibition may occur even without a significant effect on receptor down-regulation, as reflected by the action of the N12 mAb. It is, therefore, conceivable that several independent mechanisms may lead to inhibition of tumor growth. Cellular proliferation of either SKBR3 cells (Table 1) or HER2 cells (data not shown) in the presence of the mAbs turned out to be a limited predictor of the anti-tumor potential of each mAb (Table 1). What is the significance then of the lack of reflection *in vitro* of the effects of the various antibodies on tumors *in vivo*? One possible explanation is that the antibodies interfere with a process that occurs only in the living animal. This process may involve changes in tumor invasiveness, attraction of blood vessels (angiogenesis), or a paracrine loop. Interestingly, the differential tumor inhibitory potential of the mAbs also did not correlate with cell lysis *in vitro* (Table 1), suggesting that neither complement- nor antibody-mediated cell lysis significantly contributes to the inhibitory function.

In summary, our results stress the caution with which antibody therapy should be considered, as different mAbs to the same protooncogenic receptor may have opposing effects on tumor growth. Nevertheless, the presented study provides further support to the notion that overexpression of a growth factor receptor leads to oncogenic transformation. It also demonstrates that a carefully selected mAb may be an efficient antitumor agent, at least in an experimental animal system.

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