Proc. Natl. Acad. Sci. USA
Vol. 92, pp. 3353–3357, April 1995
Immunology

Suppression and promotion of tumor growth by monoclonal antibodies to ErbB-2 differentially correlate with cellular uptake

ESTHER HURWITZ, ILANA STANCOVSKI, MICHAEL SELA, AND YOSEF YARDEN

Department of Chemical Immunology, The Weizmann Institute of Science, Rehovot 76100, Israel

Contributed by Michael Sela, December 14, 1994

ABSTRACT **Amplification and overexpression of the *erbB-2/neu* protooncogene are frequently associated with aggressive clinical course of certain human adenocarcinomas, and therefore the encoded surface glycoprotein is considered a candidate target for immunotherapy. We previously generated a series of anti-ErbB-2 monoclonal antibodies (mAbs) that either accelerate or inhibit the tumorigenic growth of *erbB-2*-transformed murine fibroblasts. The present study extended this observation to a human tumor cell line grown as xenografts in athymic mice and addressed the biochemical differences between the two classes of mAbs. We show that the inhibitory effect is dominant in an antibody mixture, and it depends on antibody bivalency. By using radiolabeled mAbs we found that all of three tumor-inhibitory mAbs became rapidly inaccessible to acid treatment when incubated with tumor cells. However, a tumor-stimulatory mAb remained accessible to extracellular treatments, indicating that it did not undergo endocytosis. In addition, intracellular fragments of the inhibitory mAbs, but not of the stimulatory mAb, were observed. Electron microscopy of colloidal gold–antibody conjugates confirmed the absence of endocytosis of the stimulatory mAb but detected endocytic vesicles containing an inhibitory mAb. We conclude that acceleration of cell growth by ErbB-2 correlates with cell surface localization, whereas inhibition of tumor growth is associated with an intrinsic ability of anti-ErbB-2 mAbs to induce endocytosis. These conclusions are relevant to the selection of optimal mAbs for immunotherapy and may have implications for the mechanism of cellular transformation by an overexpressed *erbB-2* gene.**

The ErbB-2 protein (also called Neu and HER-2) belongs to a subgroup of receptor tyrosine kinases (1) whose prototype is the epidermal growth factor (EGF) receptor. However, unlike the EGF receptor, which binds many known ligands, no direct ligand of ErbB-2 has been reported. Nevertheless, both EGF and a series of isoforms of Neu differentiation factor (NDF or heregulin) can transmodulate ErbB-2 through receptor heterodimerization (2–4). ErbB-2 attracted extensive research efforts because of its role in malignant transformation. While a point mutated version of the rodent protein is highly oncogenic (5), overexpression of the wild-type version of the human homolog is sufficient to drive transformation of murine fibroblasts (6, 7). These observations are relevant to human cancer because amplification and/or overexpression of the *erbB-2* gene have been observed in 20–30% of adenocarcinomas of the breast, ovary, lung, and stomach (reviewed in ref. 8). Moreover, overexpression has been linked to poor prognosis in breast (9) and ovarian (10) cancer.

The relatively high oncogenic action of human ErbB-2, as compared with the homologous EGF receptor, has been attributed to a single substitution of an amino acid in the cytoplasmic part of the protein; this substitution results in profound activation of the intrinsic protein-tyrosine kinase activity (11). Despite the lack of understanding how this affects tumor development, the wealth of evidence implicating ErbB-2 in cancer and the cell surface localization of this oncogenic receptor made it an excellent target for immunotherapy. The first monoclonal antibody (mAb) to Neu/ErbB-2 was directed to the rodent homolog and was shown to inhibit growth of *neu*-transformed cells both *in vivo* and *in vitro* (12, 13). This was followed by several studies that described mAbs to the human homolog with potent inhibitory activity toward transformed cells expressing ErbB-2 (14–17).

We previously described a series of anti-ErbB-2 mAbs that either inhibited or accelerated the tumorigenic growth of *erbB-2*-overexpressing murine cells in athymic mice (17). Whereas the stimulatory action was correlated with the ability of a mAb to activate the intrinsic catalytic function of ErbB-2, the tumor-inhibitory effects of certain mAbs may be related to their ability to induce differentiation and growth arrest of certain cultured mammary tumor cells (18). In the present study we extended the observation of opposing growth-regulatory effects to a human tumor cell line that overexpresses *erbB-2* and analyzed the cellular basis of the differential cellular effects. We report that a tumor-stimulatory mAb is unique among other antibodies in its ability to remain at the cell surface with minimal endocytosis and degradation. On the other hand, the tumor-inhibitory potential correlates, to some extent, with the kinetics of cellular uptake of antibodies.

MATERIALS AND METHODS

Cells. The N87 human gastric tumor cell line has been described (19). The 3T3/ErbB-2 cell line has been established by selection for *erbB-2* overexpression (6).

***In Vivo* Assay of Antitumor Effects.** Cultured cells ($0.2–1 \times 10^7$ per mouse) were injected subcutaneously into the back of female CD1 mice (nude, 7–10 weeks old). Four to 6 days later, groups of six to eight mice received antibodies by either intraperitoneal or intravenous injection. The mAbs were administered twice a week.

Antibodies. mAbs were purified from ascites fluid by ammonium sulfate precipitation followed by affinity chromatography on a column of immobilized protein A. $F(ab')_2$ dimers were prepared from mAb N12 by the standard pepsin digestion procedure. F(ab') was prepared from $F(ab')_2$ by reduction for 1 hr at 22°C with 5 mM dithiothreitol (Sigma) and alkylation with 50 mM iodoacetic acid in Tris/HCl buffer at pH 8.2 for 30 min at 22°C. Rabbit anti-mouse $F(ab')_2$ was obtained from Jackson ImmunoResearch.

Radiolabeling of Antibodies. Radiochemicals were purchased from Amersham. For radiolabeling with ^{125}I, we used $Na^{125}I$ [0.5 mCi (18.5 MBq)] and the chloramine-T method (20). The range of specific activity of labeled mAbs was 1–2 $\mu Ci/\mu g$ of protein. For biosynthetic labeling of mAbs, hybridoma cells (9×10^6) were incubated in 8 ml of methionine-free Dulbecco's modified Eagle's medium that was supplemented

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: EGF, epidermal growth factor; mAb, monoclonal antibody.

with 10% dialyzed horse serum and 1.125 mCi of [^{35}S]methionine. After 20 hr at 37°C, the growth medium containing the secreted labeled antibody was dialyzed extensively against phosphate-buffered saline (10 mM sodium phosphate 0.14 M NaCl, pH 7.2) and the mAb was purified by affinity chromatography on a protein A column. The specific activity of biosynthetic labeling was in the range of 0.03–0.05 μCi/μg of antibody.

Internalization of Antibodies. To release cell-bound mAbs, we used either washing with a low-pH solution (21, 22) or proteolysis of surface-exposed molecules. The acidic wash was performed at 4°C by incubation with 50 mM glycine buffer (pH 2.8) containing 0.1 M NaCl or with 0.1 M acetic acid (pH 2.8) containing 0.15 M NaCl. Each acid wash was repeated twice for 10 min. Alternatively, papain (Sigma) was used at 2.5 mg/ml in RPMI 1640 medium that was adjusted to pH 2.5 (23). The analysis of internalized ^{125}I-labeled antibodies was followed by PAGE under nonreducing conditions essentially as described (24). Prior to the experiment, cells (1–5 $\times$ 10^6) were plated in 3.5-cm-diameter dishes for 24–48 hr and ^{125}I-labeled antibodies (6 $\times$ 10^6 cpm per plate) were added. In some experiments a chase was performed with an excess of unlabeled antibody (50 μg/ml).

Electron Microscopy. Colloidal gold (10 nm; Zymed) was conjugated to mAbs N12 and N28, as well as to control antibodies, according to a published protocol (25). The gold-conjugated antibodies were incubated with N87 cells in 6-cm Petri plates for 30 min at 4°C on a shaker. Binding was terminated by washing and further incubation for 30 min at 4°C, followed by 10 min at 22°C. The latter incubation was aimed to simulate the trypsin-mediated detachment treatment. The labeled cells were then fixed with 2% glutaraldehyde (electron microscopy grade) in phosphate-buffered saline at pH 7.2. Thin-section micrographs were prepared and examined at 80 kV on a Phillips 410 microscope.

RESULTS

Effects of mAbs and Their Fragments on Tumorigenic Growth of Cells Overexpressing *erbB-2*. In accordance with our previous report (17), repeated injections of mAbs N12 and N29 into athymic mice that were inoculated with *erbB-2*-transformed murine fibroblasts inhibited their tumorigenic growth, whereas mAb N28 increased the rate of tumor growth (Fig. 1*A*). A combination of the inhibitory antibody N29 and the stimulatory antibody exerted an inhibitory effect. In addition, a bivalent dimer, F(ab')$_2$, of N12 retained part of the inhibitory effect (Fig. 1*A*). By contrast, a monovalent F(ab') fragment of the other inhibitory mAb, N29, completely lost the tumor-inhibitory activity (data not shown). The N87 human cell line was originally cultured from a liver metastasis (19), and it developed subcutaneous tumors upon injection into mice (16). In this model cellular system, mAb N12 was the most effective inhibitor of tumor development (Fig. 1*B*), and its effect persisted for at least 5 months after the last antibody injection (data not shown). Similar to its effect on *erbB-2*-overexpressing 3T3 cells, mAb N28 accelerated the rate of growth of N87 cells (Fig. 1*B*). Likewise, the combination of mAb N12 with mAb N28 did not alter the inhibitory capacity of the first mAb (Fig. 1*B*), whereas the F(ab')$_2$ fragment of N12 retarded tumor growth less efficiently than the intact antibody (Fig. 1*C*). On the basis of the results presented in Fig. 1, we concluded that various mAbs to ErbB-2 can exert distinct biological effects that are independent of cell type and that the inhibitory activity is dominant over the stimulatory action but requires antibody bivalency.

Internalization Kinetics of Anti-ErbB-2 mAbs. To address the possibility that the opposing growth-regulatory effects of anti-ErbB-2 mAbs correlate with their metabolism by target cells, we used antibodies metabolically labeled with [^{35}S]methionine and analyzed their capacity to undergo internalization into N87 human tumor cells. To remove cell-bound mAbs we washed the monolayers under acidic conditions that facilitate ligand dissociation. The results of this experiment are presented in Table 1. Evidently, the tumor-inhibitory mAb N12 underwent rapid uptake into N87 cells, whereas the tumor-stimulatory mAb N28 remained mostly attached to the outer side of the plasma membrane. The partially tumor-inhibitory mAbs N24 and N29 underwent slower cellular uptake than mAb N12, and their cellular accumulation decreased upon

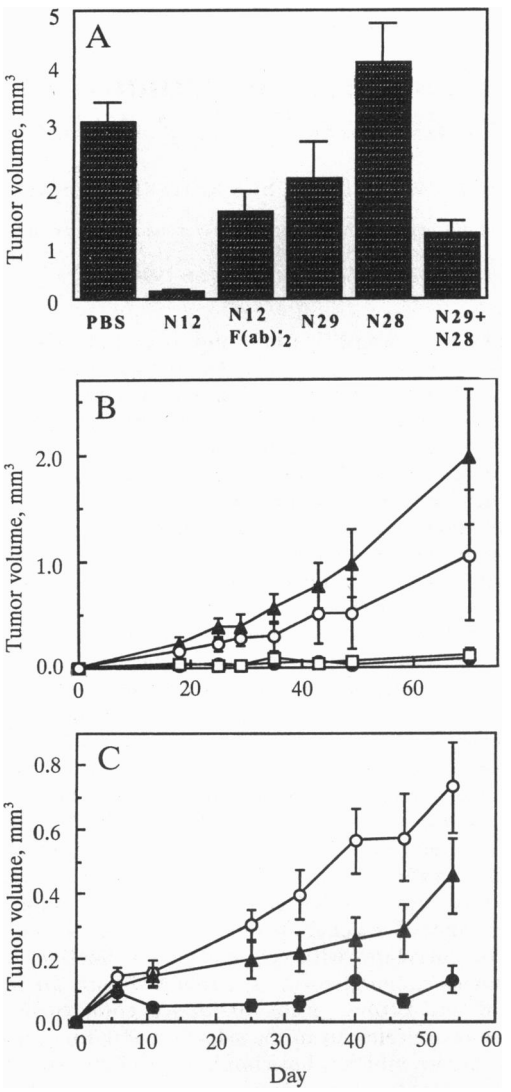

FIG. 1. Inhibition of tumor growth by intact antibodies, their fragments, and a combination of antibodies. (*A*) Athymic mice received a subcutaneous injection of 3 $\times$ 10^6 3T3/ErbB-2 cells. The indicated antibodies, or combinations of them, were injected intraperitoneally after 4, 7, and 11 days, and tumor volumes were measured 36 days later. Mice injected with phosphate-buffered saline (PBS) were used for control. The results obtained were significant ($P < 0.01$) by analysis of variance. Bars represent standard errors. (*B*) N87 human gastric carcinoma cells (5 $\times$ 10^6) were injected subcutaneously into athymic mice, and mAbs were injected intraperitoneally 6, 9, 12, and 15 days later at a total dose of 2 mg per mouse. The antibodies used were N12 (●, partly hidden by squares), N28 (▲), and a mixture of both mAbs (□). Control animals were injected with PBS (○). (*C*) Mice were injected subcutaneously with 10^7 N87 cells and mAbs were injected intravenously 4, 7, 12, and 15 days later at a total dose of 1.8 mg per mouse. The data presented were analyzed statistically and yielded *P* values <0.05. The antibodies used were N12 (●) and F(ab')$_2$ of N12 (▲). Control mice were injected with PBS (○). Each group included six to eight mice.

Table 1. Cellular uptake of [^{35}S]methionine-labeled mAbs to ErbB-2

Time, min	Intracellular radioactivity, % (mean ± range)			
	N12	N24	N29	N28
20	32 ± 1	74 ± 2	35 ± 2	14 ± 1
75	87 ± 3	68 ± 1	63 ± 2	21 ± 1
150	83 ± 2	49 ± 3	63 ± 1	22 ± 2
300	80 ± 1	42 ± 4	31 ± 1	31 ± 2

[^{35}S]Methionine-labeled mAbs were incubated with N87 cells at 37°C for various times. The fractions of endocytosed antibodies were determined by removal of the surface-bound antibody with an acidic solution. Radioactivity (cpm) obtained with an unrelated antibody that does not bind to N87 cells was subtracted from the corresponding radioactivity of anti-ErbB-2 antibodies for each cellular fraction. The assay was performed in duplicate and the ranges are indicated.

long incubation. In experiments that are not presented, the extracellular radioactivity and the exocytosed antibody were analyzed after precipitation with 20% (wt/vol) trichloroacetic acid. This analysis showed that 80–90% of the radioactively labeled molecules in this fraction was acid-precipitable, indicating that secretion of proteolyzed antibodies was limited. To confirm the differences between the antibodies, we used an alternative assay in which radiolabeled antibodies were incubated with N87 cell for 20 min at 4°C, unbound antibody was removed, and incubation was then continued for 20 min at 37°C. At the end of the experiment the medium was collected and its radioactivity was determined, whereas cell-associated radioactivity was fractionated into membrane-bound and intracellular antibody. Quantitative analysis of the results of four experiments showed that 64–76% of the cell-associated mAb N12 was inaccessible to membrane stripping, compared with only 8–10% of mAb N28. Upon longer incubation (2 hr) at 37°C, the tumor-inhibitory antibodies showed no major change in the distribution of radioactivity (Fig. 2). However, mAb N28 exhibited a moderate increase in the amount of internalized antibody and mAbs N24 and N29 showed a distribution similar to that of mAb N12.

Analysis of Cell-Associated Antibodies by Gel Electrophoresis. We next compared the extent of metabolism of the tumor-inhibitory and tumor-stimulatory antibodies by using gel electrophoresis and mAb molecules that were radiolabeled with ^{125}I. The radiolabeled antibodies were incubated with N87 cells for 20 min at 4°C, and then the radioactive molecules were

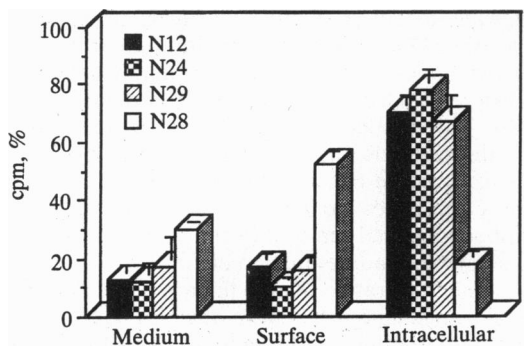

FIG. 2. Subcellular fractionation of radiolabeled antibodies to ErbB-2. The indicated ^{125}I-labeled mAbs were incubated for 20 min at 4°C with N87 gastric carcinoma cells. Unbound antibody was then removed by washing and the cells were further incubated for 2 hr at 37°C. Radioactivity released into the medium (Medium) was collected and the surface-bound antibody was dissociated by an acidic wash (Surface). Cells were detached with trypsin and their radioactivity was determined (Intracellular). Histograms show radioactivity in each cell fraction as a percentage of cell-associated radioactivity. Bars represent ranges of duplicate determinations.

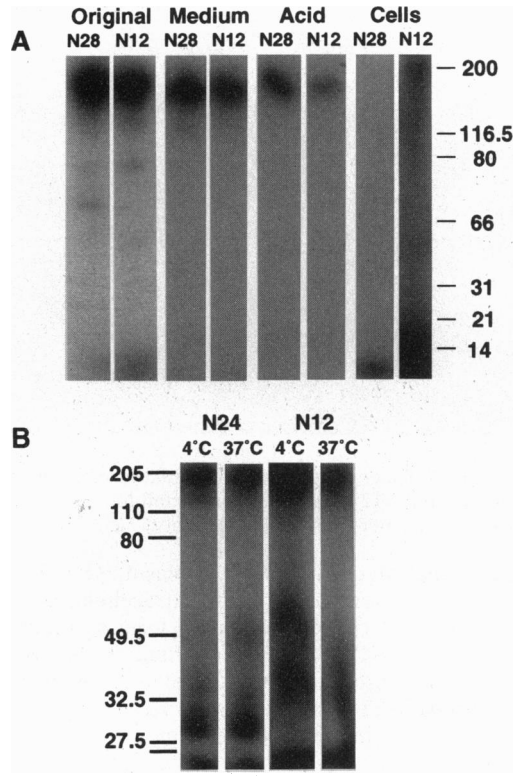

FIG. 3. SDS/PAGE analysis of radiolabeled anti-ErbB-2 mAbs in subcellular fractions. (*A*) The indicated ^{125}I-labeled mAbs were incubated for 20 min at 4°C with N87 cells and unbound radioactivity was removed by washing. The cells were then incubated for 90 min at 4°C in medium that contained unlabeled antibody (50 μg/ml). Finally, the cells were detached at room temperature by incubating them for 20 min in trypsin/EDTA solution. The exocytosed (Medium), membrane-bound (Acid), or intracellular antibodies (Cells) were resolved by SDS/PAGE under nonreducing conditions. For control, the labeled antibodies were analyzed before incubation with the cells (Original). (*B*) Internalized fractions of cells treated with mAb N24 or N12 were analyzed after a 2-hr incubation at either 4°C or 37°C, as indicated. Locations of marker proteins and the corresponding molecular masses (kDa) are indicated.

replaced with unlabeled antibodies and incubation was continued for 90 min at either 4°C (Fig. 3*A*) or 35°C (Fig. 3*B*). SDS/PAGE analysis of the surface-bound and the spontaneously released fractions revealed that both antibodies displayed mostly intact forms in these fractions (Fig. 3). By contrast, multiple proteolytic products were observed when the internalized fraction of mAb N12 was analyzed, but no endocytosed mAb N28 was detectable in cell lysates (Fig. 3*A*). Similar analysis of an endocytosed fraction of mAb N24 revealed that it, like N12, underwent uptake and degradation that were enhanced at an elevated temperature (Fig. 3*B*). In conclusion, N87 cells were able to endocytose both mAb N12 and mAb N24, but mAb N28 underwent minimal endocytosis and proteolysis.

Electron Microscopic Analysis of the Cellular Uptake of Anti-ErbB-2 mAbs. Our biochemical analyses, which were performed with ^{125}I-labeled and [^{35}S]methionine-labeled antibodies, indicated lack of endocytosis and proteolytic degradation of the tumor-stimulatory mAb N28 but rapid endocytosis of the tumor-inhibitory mAb N12. To confirm this difference by ultrastructural analysis, we prepared colloidal gold conjugates of the two mAbs and analyzed their interaction with N87 cells by using electron microscopy (Fig. 4). While short incubation of N87 cells with mAb N12 resulted in the appearance of colloidal gold particles in peripheral large vesicles and tubular structures, only few particles of mAb N28

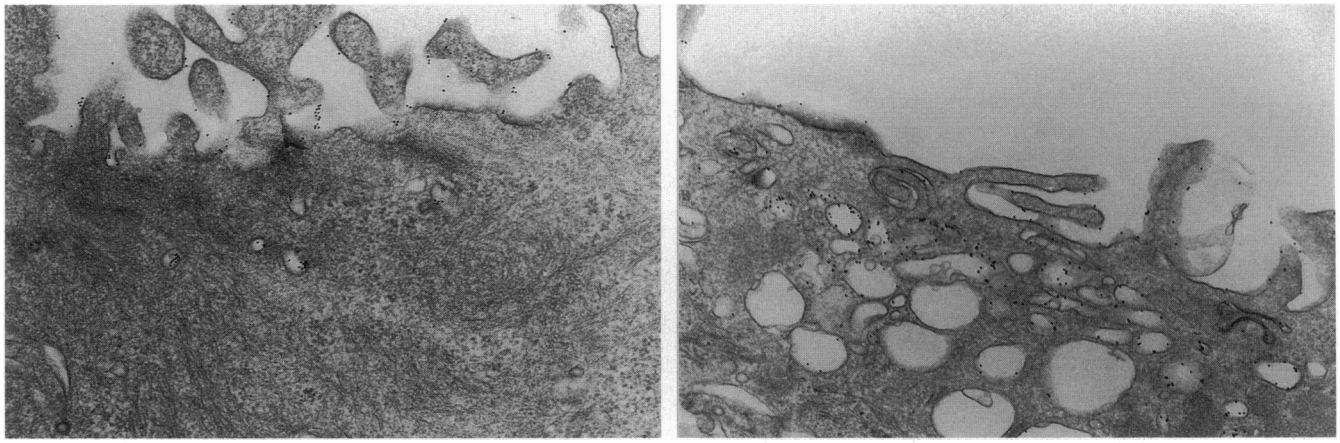

FIG. 4. Electron microscopy of colloidal gold-labeled antibodies to ErbB-2 after incubation with N87 cells. Colloidal gold-conjugated mAbs (10 nM) N28 (*Left*) and N12 (*Right*) were incubated for 30 min at 4°C with N87 human gastric carcinoma cells. The unbound antibody was removed and the cells were further incubated for 10 min at 22°C. Thin-section micrographs were prepared after cell fixation in 2% glutaraldehyde. (×21,500.)

conjugates penetrated into the cytoplasm. Quantitatively, mAb N28 was localized mostly to the extracellular side of the cell membrane (74% of the gold grains), whereas the majority of cell-bound mAb N12 (83%) was confined to the cytoplasm (83%). It is, therefore, conceivable that the observed accumulation of mAb N12 in submembrane vesicles rendered this inhibitory antibody inaccessible to removal by acid or by proteolytic enzymes.

DISCUSSION

As more mAbs to ErbB-2 are generated and their interactions with *erbB-2*-expressing cells are characterized, it becomes clear that differences exist between the intrinsic actions of various antibodies (14–18, 21, 26, 27). Understanding the molecular bases of the differential intrinsic activities of antibodies to ErbB-2 is important for rational selection of specific mAbs for immunotherapy and subsequent improvement of their efficacy by protein engineering, humanization, and conjugation to drugs, toxins, and radioactive isotopes. In addition, because the direct natural ligand of ErbB-2 is still unknown (28), lessons learned with various mAbs may be relevant to the mechanism by which ErbB-2 contributes to malignant transformation. The present study took advantage of the opposing effects of two mAbs on tumorigenic growth *in vivo*. It was first demonstrated that remarkably different activities were displayed by the two mAbs when either *erbB-2*-transfected murine cells or *erbB-2*-overexpressing human tumor cells were examined (Fig. 1). By using radiolabeled mAbs, cellular fractionation analyses (Table 1, Fig. 2, and Fig. 3), and electron microscopy (Fig. 4), we demonstrated that one clear difference between the tumor-stimulatory mAb N28 and the tumor-inhibitory mAb N12 was the ability of the latter to undergo rapid cellular uptake. Two additional mAbs that displayed partial tumor-inhibitory effects *in vivo*, N24 and N29 (17), also underwent endocytosis. However, the rates of uptake of these mAbs and the amount of internalized molecules were low in comparison with N12. Although correlative, these results support the possibility that antibody-mediated internalization of ErbB-2 causes a reduction in the ability of the receptor to maintain a transformed phenotype. A similar conclusion was drawn from experiments with antibodies to an oncogenic mutant of the rodent Neu/ErbB-2—namely, that removal of the oncoprotein from the cell surface inhibited tumor growth (29). Likewise, a combination of two mAbs to human ErbB-2 has been shown to be more efficient than each mAb alone in the induction of tumor regression, as well as in accelerating receptor degradation (16).

mAbs to ErbB-2 appear to share their route of endocytosis with ordinary ligands of growth factor receptors (for review see ref. 30). Initially, the receptor is diffusely distributed on the cell surface and is mostly excluded from endocytic pits (31). Upon binding of certain mAbs, ErbB-2 follows the internalization pathway used by antibodies to transferrin and other receptors (31), and is destined to endosomes and multivesicular bodies. Our results indicate that three tumor-inhibitory mAbs to ErbB-2 follow this pathway, whereas a tumor-stimulatory mAb is excluded from or inefficiently destined to this pathway. Because this mAb acts better than other antibodies as an agonist of tyrosine autophosphorylation (17), it is worthwhile to examine the connection between endocytosis and kinase activation. Experiments that made use of various mutants of ErbB-2 led to the conclusion that neither autophosphorylation sites nor tyrosine kinase activity are required for antibody-mediated endocytosis of ErbB-2 (32). Moreover, two groups reported on mAbs to ErbB-2 that stimulate tyrosine autophosphorylation of the receptor, undergo internalization, and exert growth-inhibitory effects (14, 32). Because the kinetics of internalization of these antibodies were not compared with those of other mAbs, we cannot relate endocytosis to growth inhibition, but it is safe to conclude that the agonist activity of anti-ErbB-2 mAbs is not necessarily coupled to growth effects.

On the basis of our results and other considerations we favor the following model of ErbB-2 involvement in malignant transformation. Due to gene amplification and overexpression, spontaneous dimers of ErbB-2 are frequently formed at the cell surface and a concomitant growth-promoting signal is generated. This can be amplified by an agonist mAb that activates the catalytic function without inducing significant endocytosis. Because ErbB-2 utilizes the Ras–Raf-1 pathway (33), whose substrates are localized to the plasma membrane (34), antibody-induced removal of the active kinase from the plasma membrane and destination of ErbB-2 to the endocytic pathway result in cessation of growth promotion. According to this model, a default mechanism that involves phenotypic cellular differentiation may be induced in certain mammary cells upon antibody- or ligand-induced translocation of ErbB-2 from the plasma membrane (18, 35). In agreement with an oncogenic function of a surface-localized receptor, an internalization-defective EGF receptor is more oncogenic than the wild-type receptor (36), and cytoplasmic localization of ErbB-2 in breast cancer tumors was linked to better prognosis in comparison with tumors that displayed membranous immunostaining (37). The validity of the proposed model may be tested by using a larger battery of mAbs to ErbB-2 and by

examination of mutant receptors that undergo no endocytosis. If confirmed, the internalization kinetics of anti-ErbB-2 mAbs may turn out to be a useful predictor of their clinical potential.

We thank Adi Gazdar and Rick King for N87 cells and David Givol for critical comments. This research was supported in part by the Bristol-Myers Squibb Foundation Cancer Grant Award and by the Israeli Ministry of Health.

1. Yarden, Y. & Ullrich, A. (1988) *Annu. Rev. Biochem.* **57,** 443–478.
2. Tzahar, E., Lefkowitz, G., Karunagaran, D., Yi, L., Peles, E., Lavi, S., Chang, D., Liu, N., Yayon, A., Wen, D. & Yarden, Y. (1994) *J. Biol. Chem.* **269,** 25226–25233.
3. Wada, T., Qian, X. & Greene, M. I. (1990) *Cell* **61,** 1339–1347.
4. Goldman, R., Ben-Levy, R., Peles, E. & Yarden, Y. (1990) *Biochemistry* **29,** 11024–11028.
5. Bargmann, C. I., Hung, M. C. & Weinberg, R. A. (1986) *Cell* **45,** 649–657.
6. Hudziak, R. M., Schlessinger, J. & Ullrich, A. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 7159–7163.
7. Di Fiore, P. P., Pierce, J., Kraus, M. H., Segatto, O., King, C. R. & Aaronson, S. A. (1987) *Science* **237,** 178–182.
8. Stancovski, I., Sela, M. & Yarden, Y. (1994) in *Regulatory Mechanisms of Breast Cancer*, eds. Lippman, M. & Dikson, R. (Kulwer, Boston), pp. 161–191.
9. Slamon, D. J., Clark, G. M., Wong, S. G., Levin, W. J., Ullrich, A. & McGuire, W. L. (1987) *Science* **235,** 177–182.
10. Slamon, D. J., Godolphin, W., Jones, L. A., Holt, J. A., Wong, S. G., Keith, D. E., Levin, W. J., Stuart, S. G., Udove, J., Ullrich, A. & Press, M. F. (1989) *Science* **244,** 707–712.
11. Di Fiore, P. P., Helin, K., Kraus, M. H., Pierce, J. H., Artrip, J., Segatto, O. & Bottaro, D. P. (1992) *EMBO J.* **11,** 3927–3933.
12. Drebin, J. A., Link, V. C. & Greene, M. I. (1988) *Oncogene* **2,** 387–394.
13. Drebin, J. A., Stern, D. F., Link, V. C., Weinberg, R. A. & Greene, M. I. (1984) *Nature (London)* **312,** 579–588.
14. Hudziak, R. M., Lewis, G. D., Winget, M., Fendley, B. M., Shepard, H. M. & Ullrich, A. (1989) *Mol. Cell. Biol.* **9,** 1165–1172.
15. Fendly, B. M., Winget, M., Hudziak, R. M., Lipari, M. T., Napier, M. A. & Ullrich, A. (1990) *Cancer Res.* **50,** 1550–1558.
16. Kasprzyk, P. G., Song, S. U., DiFiore, P. P. & King, C. R. (1992) *Cancer Res.* **52,** 2771–2776.
17. Stancovski, I., Hurwitz, E., Leitner, O., Ullrich, A., Yarden, Y. & Sela, M. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 8691–8695.
18. Bacus, S. S., Stancovski, I., Huberman, E., Chin, D., Hurwitz, E., Mills, G. B., Ullrich, A., Sela, M. & Yarden, Y. (1992) *Cancer Res.* **52,** 2580–2589.
19. Park, J. G., Frucht, H., LaRocca, R. V., Bliss, D. P. J., Kurita, Y., Chen, T. R., Henslee, J. G., Trepel, J. B., Jensen, R. T., Johnson, B. E., Bang, Y.-J., Kim, J.-P. & Gazdar, A. F. (1990) *Cancer Res.* **50,** 2773–2780.
20. Hunter, M. W. & Greenhood, F. C. (1962) *Nature (London)* **194,** 495–496.
21. Tagliabue, E., Centis, F., Campiglio, M., Mastroianni, A., Martignone, S., Pellegrini, R., Casalini, P., Lanzi, C., Menard, S. & Colnaghi, M. I. (1991) *Int. J. Cancer* **47,** 933–937.
22. Yarden, Y., Gabbay, M. & Schlessinger, J. (1981) *Biochim. Biophys. Acta* **674,** 188–203.
23. Press, O. H., Hanse, J. A., Far, A. & Martin, P. J. (1988) *Cancer Res.* **48,** 2249–2257.
24. Garrigues, J., Garrigues, U., Hellstrom, I. & Hellstrom, K. E. (1993) *Am. J. Pathol.* **142,** 607–622.
25. De-Mey, J. R. (1983) *Handbook Series: Methods in the Neurosciences* (Wiley, New York), Vol. 3, pp. 347–369.
26. van Leeuwen, F., van de Vijver, M. J., Lomans, J., van Deemter, L., Jenster, G., Akiyama, T., Yamamoto, T. & Nusse, R. (1990) *Oncogene* **5,** 497–503.
27. McKenzie, S. J., Marks, P. J., Lam, T., Morgan, J., Panicali, D. L., Trimpe, K. L. & Carney, W. P. (1989) *Oncogene* **4,** 543–548.
28. Peles, E. & Yarden, Y. (1993) *BioEssays* **15,** 815–824.
29. Drebin, J. A., Link, V. C., Stern, D. F., Weinberg, R. A. & Greene, M. I. (1985) *Cell* **41,** 697–706.
30. Sorkin, A. & Waters, C. M. (1993) *BioEssays* **15,** 375–382.
31. Lotti, L. V., Di Lazzaro, C., Zompetta, C., Frati, L. & Torrisi, M. S. (1992) *Exp. Cell Res.* **202,** 274–280.
32. Maier, L. A., Xu, F. J., Hester, S., Boyer, C. M., McKenzie, S., Bruskin, A. M., Argon, Y. & Bast, R. C. J. (1991) *Cancer Res.* **51,** 5361–5369.
33. Ben-Levy, R. (1994) *EMBO J.* **13,** 3302–3311.
34. Aronheim, A., Engelberg, D., Li, N., Al-Alawi, N., Schlessinger, J. & Karin, M. (1994) *Cell* **78,** 949–961.
35. Peles, E., Bacus, S. S., Koski, R. A., Lu, H. S., Wen, D., Ogden, S. G., Ben-Levy, R. & Yarden, Y. (1992) *Cell* **69,** 205–216.
36. Wells, A., Welsh, J. B., Lazar, C. S., Wiley, H. S., Gill, G. N. & Rosenfeld, M. G. (1990) *Science* **247,** 962–964.
37. Zschiesche, W., Schonborn, I., Minguillon, C. & Spitzer, E. (1994) *Cancer Lett.* **81,** 89–94.