Oncogene (1997) 14, 2099–2109
© 1997 Stockton Press All rights reserved 0950–9232/97 $12.00

A subclass of tumor-inhibitory monoclonal antibodies to ErbB-2/HER2 blocks crosstalk with growth factor receptors

Leah N Klapper[1], Nora Vaisman[1], Esther Hurwitz[1], Ronit Pinkas-Kramarski[2], Yosef Yarden[2] and Michael Sela[1]

Departments of [1]Immunology and [2]Molecular Cell Biology, The Weizmann Institute of Science, Rehovot 76100, Israel

ErbB-2 is an orphan receptor that belongs to a family of tyrosine kinase receptors for either epidermal growth factor (EGF) or Neu differentiation factor (NDF/neuregulin). Because overexpression of the *erbB*-2 proto-oncogene is frequently associated with an aggressive clinical course of certain human adenocarcinomas, the encoded protein is an attractive target for immunotherapy. Indeed, certain monoclonal antibodies (mAbs) to ErbB-2 effectively inhibit tumor growth in animal models and in clinical trials, but the underlying mechanism is incompletely understood. To study this question, we generated a large battery of mAbs to ErbB-2, that were classified epitopically. Whereas most antibodies stimulated tyrosine phosphorylation of ErbB-2, their anti-tumor effect correlated with its accelerated endocytic degradation. One group of tumor-inhibitory mAbs (Class II mAbs) was elicited by the most antigenic site of ErbB-2, and inhibited *in trans* binding of NDF and EGF to their direct receptors. The inhibitory effect was due to acceleration of ligand dissociation, and it resulted in the reduction of the ability of ErbB-2 to transactivate the mitogenic signals of NDF and EGF. These results identify two potential mechanisms of antibody-induced therapy: acceleration of ErbB-2 endocytosis by homodimerization and blocking of heterodimerization between ErbB-2 and growth factor receptors.

Keywords: signal transduction; tyrosine kinase; oncogene; Neu differentiation factor; epidermal growth factor; adenocarcinoma

Introduction

The identification of tumor associated antigens (TAA) accessible on human cancer cells, hailed immunotherapeutic approaches relying on specific recognition of neoplasms (Hellstrom and Hellstrom, 1989). Extensive efforts have indeed been invested in examining the plausibility of anti-TAA monoclonal antibodies (mAbs) in the treatment of human malignancies proving promising in laboratory and clinic (Goldenberg, 1993). Protooncogene-encoded growth factor receptors are putative targets for such recognition-dependent therapy, due to their suggested role in pathological proliferation of cells (Aaronson, 1991). ErbB-2, a receptor-like tyrosine kinase, has been repeatedly implicated in cell transformation (Hynes and Stern, 1994; Stancovski *et al*., 1994). Amplification of the corresponding gene and overexpression of the protein itself were observed in 20% to 30% of adenocarcinomas of the breast (King *et al*., 1985; Slamon *et al*., 1987, 1989), ovary (Slamon *et al*., 1989), lung (Kern *et al*., 1990) and stomach (Park *et al*., 1989). Causative relationships between the cellular ErbB-2 content and the tumor's proliferative capacity and aggressiveness have been supported by different lines of evidence. When overexpressed in mouse fibroblasts, the human gene conferred a transformed phenotype *in vitro* and tumorigenesis *in vivo* (Di Fiore *et al*., 1987; Hudziak *et al*., 1987). Consistently, receptor overexpression is considered a predictor of poor survival and short time to relapse (Slamon *et al*., 1987, 1989). Direct interference with the transforming potential of ErbB-2 has thus become a subject of great interest. Antibodies directed against the extracellular domain of either a mutated version of the rodent homolog of this receptor-like molecule, or against the human wild-type protein have been shown to confer inhibitory, as well as stimulatory, effects on tumor growth *in vivo* (Drebin *et al*, 1986; Fendly *et al*., 1990; Hudziak *et al*., 1989; Stancovski *et al*., 1991). Moreover, a murine antibody capable of such growth inhibition has been recently humanized and tested in a phase II clinical trial, resulting in anti-tumor activity in patients with ErbB-2-overexpressing metastatic breast cancers (Baselga *et al*., 1996).

Although the potential therapeutic use of anti-ErbB-2 mAbs is presently acknowledged and intensely examined, the molecular mechanisms underlying these effects are not well understood. Accelerated down-regulation of the receptor has been suggested to mediate antibody inhibition of cell transformation (Hudziak *et al*., 1989; van Leeuwen *et al*., 1990). However, the ability of mAbs to induce receptor internalization showed only partial correlation with anti-tumorigenic activity (Harwerth *et al*., 1992; Hurwitz *et al*., 1995). An obstacle in the understanding of mAb-mediated effects is the possibility that ErbB-2 has a direct ligand, that has not yet been completely characterized (Dougall *et al*., 1994). Activation of receptor tyrosine kinases is dependent on receptor dimerization induced by the binding of specific ligands (Yarden and Schlessinger, 1987). However, ErbB-2 may participate in signal transduction even in the absence of a direct ligand, because it forms heterodimeric complexes with its family members, namely ErbB-1 (EGF receptor) and the two NDF/neuregulin receptors, ErbB-3 and ErbB-4 (Goldman *et al*., 1990; Pinkas-Kramarski *et al*., 1996; Riese *et al*., 1995; Wada *et al*., 1990). Hierarchical

Correspondence: M Sela

DOCKET ALARM
Find authenticated court documents without watermarks at docketalarm.com.

signaling and resultant cellular fate (Tzahar *et al.*, 1996). The importance of ErbB-2 as a transregulator expands in light of reported coexpression of ErbB family receptors in malignant cells (Gullick, 1990; Lemoine *et al.*, 1992), as well as by its ability to reconstitute the aberrant tyrosine kinase activity characteristic of ErbB-3 (Pinkas-Kramarski *et al.*, 1996; Riese *et al.*, 1995; Sliwkowski *et al.*, 1994). Heterodimer formation between ErbB-2 and ErbB-1 is responsible for synergistic growth signals in cells that co-overexpress the two receptors (Kokai *et al.*, 1989). A similar synergy was observed upon co-overexpression of ErbB-2 and ErbB-3 (Alimandi *et al.*, 1995; Wallasch *et al.*, 1995), probably due to the extremely high mitogenic potential of the corresponding receptor heterodimer (Pinkas-Kramarski *et al.*, 1996). Selective suppression of ErbB-2 expression at the cell surface by means of retention in the endoplasmic reticulum (Beerli *et al.*, 1994), demonstrated that this molecule can act as a shared signaling subunit of both EGF- and NDF-receptors (Graus-Porta *et al.*, 1995; Karunagaran *et al.*, 1996) that augments and prolongs signaling by deceleration of the rate of ligand dissociation (Karunagaran *et al.*, 1996).

The ability of ErbB-2 to serve as a pan ErbB auxiliary receptor subunit implies a versatility of mechanisms by which the receptor is involved in transformation. Hence, attempts to inhibit malignancies should consider the transacting potential of ErbB-2, in addition to its presumed ability to act through homodimer formation. Our study has addressed the possibility that mAbs directed against ErbB-2 might exert at least part of their tumor-inhibitory effects via interference with receptor-receptor interactions. A battery of antibodies directed against the extracellular domain of ErbB-2 has been generated and classified into groups according to specific epitope recognition. Several classes of tumor-inhibitory mAbs that accelerate cellular degradation of ErbB-2 were identified. Interestingly, one class of tumor-inhibitory antibodies partially reduced cellular binding of both NDF and EGF. Consistent with an ability to interfere with receptor crosstalk, these mAbs also reduced the trans-stimulatory effect of ErbB-2 on growth signals. We suggest that anti-ErbB-2 antibodies can inhibit cancer not only by impeding the homodimer-dependent activity, but also by blocking heterodimer formation and receptor crosstalk. This implies wider than currently accounted-for mechanisms that can be utilized for the designing of therapeutically efficient anti-ErbB-2 inhibitors. In addition, our results may be relevant to the mechanism by which EGF-like ligands recruit ErbB-2 into receptor heterodimers.

Results

Classification of anti-ErbB-2 monoclonal antibodies

To study the mechanistic basis of tumor inhibition by certain mAbs to ErbB-2, we extended our antibody repertoire by employing an exhaustive immunization protocol. Essentially, mice were immunized with a

for antibody binding to a cell surface-expressed ErbB-2. A dozen of new mAbs was generated, and analysed together with a panel of five mAbs that we previously described (Stancovski *et al.*, 1991). The new mAbs were assayed for their ability to affect the tumorigenic growth in nude mice of the N87 human gastric carcinoma cell line overexpressing the ErbB-2 protein. Nine different mAbs, or saline as control, were injected intraperitoneally into groups of six mice, on days 3, 7 and 10 after tumor inoculation. Figure 1 depicts tumor progression in the presence of four representative mAbs. The tumorigenic growth of N87 cells was inhibited by 85%, 61% and 82% in nude mice injected with mAbs L26, L140 and L431, respectively. These values correspond to mean of inhibition measured at five time points throughout a period of 46 days post inoculation. Antibody L87 showed no effect on the growth of tumors *in vivo*, presumably due to its relatively low affinity (data not shown). Because previous studies suggested that different regions on the extracellular domain of ErbB-2 mediate mAb-dependent effects on tumor growth (Bacus *et al.*, 1992) we examined the dependency of tumor inhibition on specific immunogenic determinants by performing a reciprocal binding assay (Figure 2). Several mAbs were radiolabeled and their binding to N87 cells was determined in the presence of all other antibodies to enable classification of the mAbs. Thus, antibody L431 was efficiently displaced by the strong tumor-inhibitory mAb N12 (Stancovski *et al.*, 1991), but not by any other antibody (Figure 2A). Therefore, these mAbs were classified into the same group, denoted Class I. In a similar manner we could categorize mAbs L26, L96, and L288 into a second group, denoted Class II (Figure 2B) and mAb L140 into Class III, that comprises only a single antibody. Antibody L87 could be displaced by antibodies from several groups, although it could not fully displace its own binding

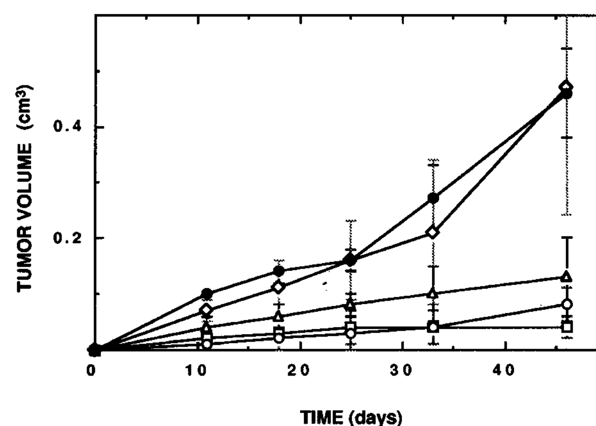

Figure 1 Inhibition of tumor growth by representative mAbs to ErbB-2. Athymic mice received a subcutaneous injection of 3×10^6 N87 human gastric cancer cells that overexpress ErbB-2. Three, 7 and 10 days later monoclonal antibodies (a total dose of 1 mg per animal) were injected intraperitoneally, and tumor volumes were measured at the end of the indicated time periods. Phosphate-buffered saline- (PBS-) injected mice were used for control (closed circles). The following mAbs were used: L26 (circles), L87 (rhombuses), L140 (triangles) and L431 (squares). Bars represent standard deviations for groups of five mice. The

DOCKET
ALARM
Find authenticated court documents without watermarks at docketalarm.com.

(Figure 2D). This characteristic of mAb L87 is in accordance with its weak precipitating ability of the native ErbB-2 protein and is reinforced by the ability of mAb L87 to recognize the denatured protein (data not shown). The low affinity of mAb L87 to conformationally-intact ErbB-2 could underlie the observed pattern of displacement by a wide variety of mAbs. Several additional mAbs (e.g., L151, L242, L219, N28 and N29) were found to react with distinct antigenic determinants of ErbB-2, indicating multiplicity of antigenic sites, of which site II is apparently the most efficient. Anti-ErbB-2 antibody classification is summarized in Table 1.

Tumor inhibition correlates with mAb-induced receptor internalization but not with kinase activation

Upon binding of certain mAbs, ErbB-2 has been shown to undergo internalization (Drebin *et al*., 1985) in a pathway shared by other growth factor receptors, when induced by ligands and antibodies (Sorkin and Waters, 1993). Several lines of evidence indicate a correlation between tumor-inhibitory activity of mAbs and their ability to accelerate ErbB-2 uptake and down-regulation (Hurwitz *et al*., 1995; Tagliabue *et al*., 1991). To test the applicability of such a correlation to the new inhibitory mAbs, we studied

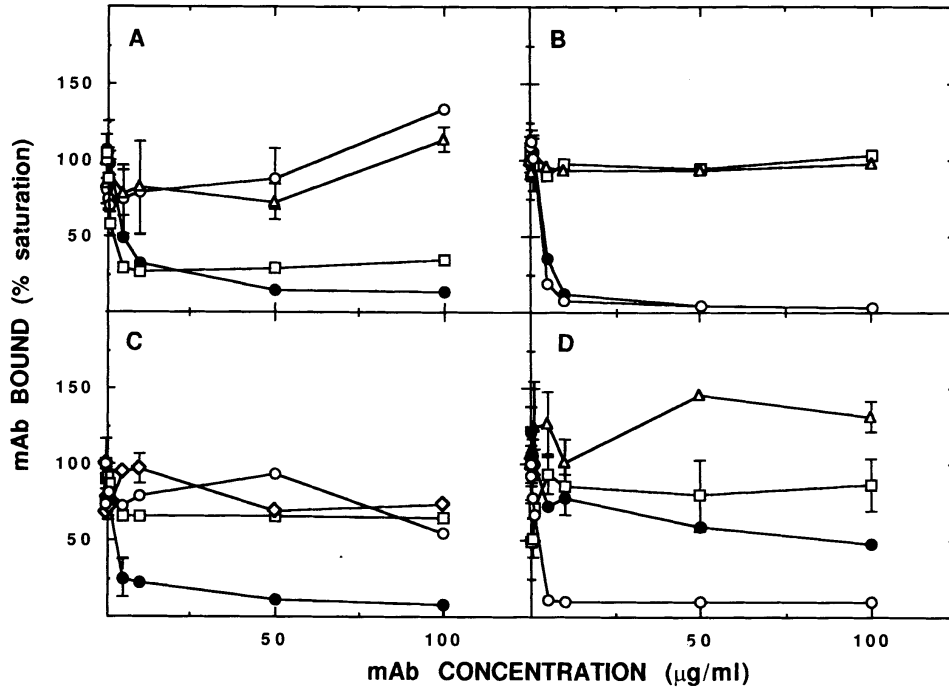

Figure 2 Analyses of competitive antibody binding to ErbB-2. The ability of unlabeled mAbs to displace a cell surface-bound ^{125}I-mAb was used as a measure for the degree of antigenic overlap. The following radiolabeled mAbs were used: L431 (**A**), L288 (**B**), L140 (**C**) and L87 (**D**). The labeled mAbs were added to the medium of N87 cells growing in 96-well culture dishes, in the presence of the same unlabeled antibody (closed circles). Alternatively, unlabeled mAbs representing the different classes were used: Class I (squares, N12 in **A** and **C**, L431 in **B** and **D**), II (circles, L26), III (triangles, L140) and IV (rhombuses, L87 in **C**). Following 1 h of incubation at 22°C, the monolayers of cells were washed three times with PBS and solubilized in an alkaline solution. The results are presented as the mean ± s.d. of duplicates. The experiments were repeated thrice

Table 1

Antibody class	*Antibody*	*Tumor growth inhibition (%)*[a]	*Receptor internalization*[b]	*Receptor phosphorylation*[c]	*Ligand binding inhibition*[d] *EGF*	*NDF*
I	L431	82	+ + +	+	–	–
I	N12	86	+ + +	ND	ND	–
II	L26	85	+ +	+	+	+
II	L96	60	+	+	+	+
II	L288	74	+ +	+	+	ND
III	L87	–	–	–	–	–
IV	L140	61	+	+ +	–	–
V	L151	40	–	+	ND	ND
VI	L219	49	ND	ND	ND	–
VII	L242	61	+ + +	+	–	–
VIII	N28	−40*	–	+	–	–
IX	N29	88	ND	+	–	–

[a]Average tumor volume inhibition of five time points, as percentage of control. [b]Receptor internalization was determined by cell surface protein labeling, as described in text and Figure 3 and its strength is expressed in correlation of the appearance of early (+), intermediate (+ +), and

DOCKET ALARM
Find authenticated court documents without watermarks at docketalarm.com.

their effect on the internalization of membrane-bound ErbB-2. The internalization assay used has not been previously applied to ErbB-2, and it included biotinylation of the surface-exposed protein, followed by exposure to the various mAbs. Molecules that underwent internalization escaped a subsequent digestion with pronase, that was applied extracellularily and were thus visualized by streptavidin detection. A 20 min-long incubation in the presence of mAbs from Class I (represented by L431) revealed a band of approximately 85 kDa, that represents a relatively late degradation product of ErbB-2 (Figure 3). A similar result was obtained upon incubation with the tumor-inhibitory mAb L242 (comprising a single-mAb group), whereas antibodies from Class II caused the appearance of the apparently early proteolytic product of 120 kDa, implying a slower degradation pathway. Internalization of ErbB-2 by mAb L140 (Class III), a moderate inhibitory mAb, resulted in both proteolytic products and a residual intact receptor (185 kDa protein band). Antibody L87 (Class VI), a non-inhibitory antibody, induced no detectable internalization of ErbB-2, an effect that was shared with the control IID2 antibody against α-fetoprotein. Because the size of the protein recovered by the internalization assay presumably reflects the rate of internalization, the results presented in Figure 3 suggest a dependency of tumor inhibition on the ability of the mAbs to internalize the receptor (Table 1). Of note, however, is the relatively slow endocytic processing that was induced by Class II mAbs (L26 and L288), implying that their strong anti-tumor effect depends on additional activities.

It has been previously reported that tumor-inhibitory effects of anti-ErbB-2 mAbs only partially correlate with modulations of the phosphotyrosine content of the receptor (Kumar *et al*., 1991; Stancovski *et al*., 1992). To examine the relationship between tumor inhibition and stimulation of ErbB-2 phosphorylation, we selected a model cellular system of 32D myeloid cells that ectopically express ErbB-2 in the absence of other ErbB proteins, thereby excluding transphosphorylation effects that widely occur within the ErbB family (Pinkas-Kramarski *et al*., 1996). Lysates of antibody-treated cells, immunoblotted for the detection of proteins phosphorylated on tyrosine residues, demonstrated the dependency of ErbB-2 phosphorylation on mAb bivalency (Figure 4, upper panel). Tumor-inhibitory mAbs from both Class I and Class II (L431 and L26, respectively) caused a comparable extent of phosphorylation, that was absent when the monovalent antibody fragments (Fab) were used. Antibody L140, a moderate cancer effector, exerted maximal elevation of receptor phosphorylation, that was higher than the effect of the tumor-stimulatory mAb, N28 (Stancovski *et al*., 1991). This suggests that the ability of antibodies to affect the extent of ErbB-2 phosphorylation reflects only their capacity to form ErbB-2 homodimers and as implied by Table 1 it may be independent of the long-term biological activity *in vivo*. Consistent with its low binding affinity, mAb L87 could not mediate a phosphorylation signal upon the receptor.

Class II mAbs inhibit ErbB-2 interactions with ErbB-familiy counterparts

The engagement of ErbB-2 in hetero-complexes with other ErbB-family tyrosine kinases, has been shown to augment both binding and signaling of EGF and NDF when associated with their respective receptors (Graus-Porta *et al*., 1995; Karunagaran *et al*., 1996; Kokai *et al*., 1989; Sliwkowski *et al*., 1994). To examine whether anti-ErbB-2 mAbs can interfere with heterodimer formation, we used ligand affinity as an indicator of ErbB-2 involvement in ligand binding complexes. This

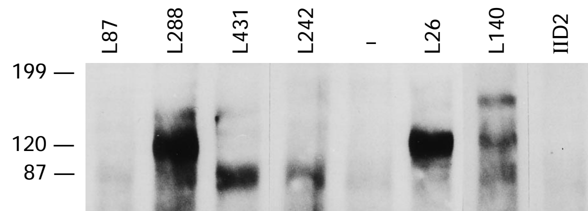

Figure 3 Effect of mAbs on degradation of ErbB-2. Cell surface proteins of confluent monolayers of N87 cells (10 cm dishes) were labeled with biotin as described under Materials and methods. The cells were then exposed for 30 min at 4°C to the indicated mAbs (at 40 μg/ml). The monolayers were thereafter incubated for 20 min at 37°C in order to allow receptor internalization and degradation. At the end of this incubation, the monolayers were transferred back to 4°C, treated for 30 min with pronase to digest surface-exposed proteins and cell lysates were prepared. ErbB-2 proteins that escaped hydrolysis by pronase were visualized by immunoprecipitation with the NCT antibody, that was followed by gel electrophoresis, transfer to nitrocellulose filter and detection with horseradish peroxidase-labeled streptavidin. Note that only internalized receptor is visualized. For control we incubated the cells in the absence of mAb (lane labeled -) or in the presence of an irrelevant mAb (antibody IID2). The locations of

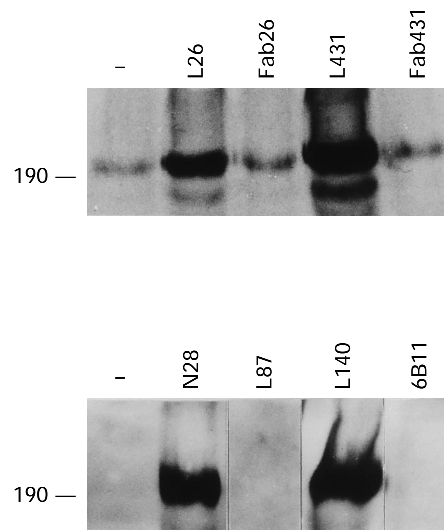

Figure 4 Antibody-induced stimulation of ErbB-2 phosphorylation on tyrosine residues. ErbB-2-expressing 32D cells (denoted D2 cells) were incubated for 15 min at 37°C with the indicated mAbs at 20 μg/ml, or with their respective monovalent fragments (Fab, 20 μg/ml). Whole cell lysates were then prepared and subjected to gel electrophoresis. The gel-resolved proteins were transferred to a nitrocellulose filter that was blotted with an antibody to phosphotyrosine and detected with a secondary antibody. Incubation in the absence of antibody (lane labeled –) or with an isotope matched control mAb (antibody 6B11) were

DOCKET ALARM
Find authenticated court documents without watermarks at docketalarm.com.

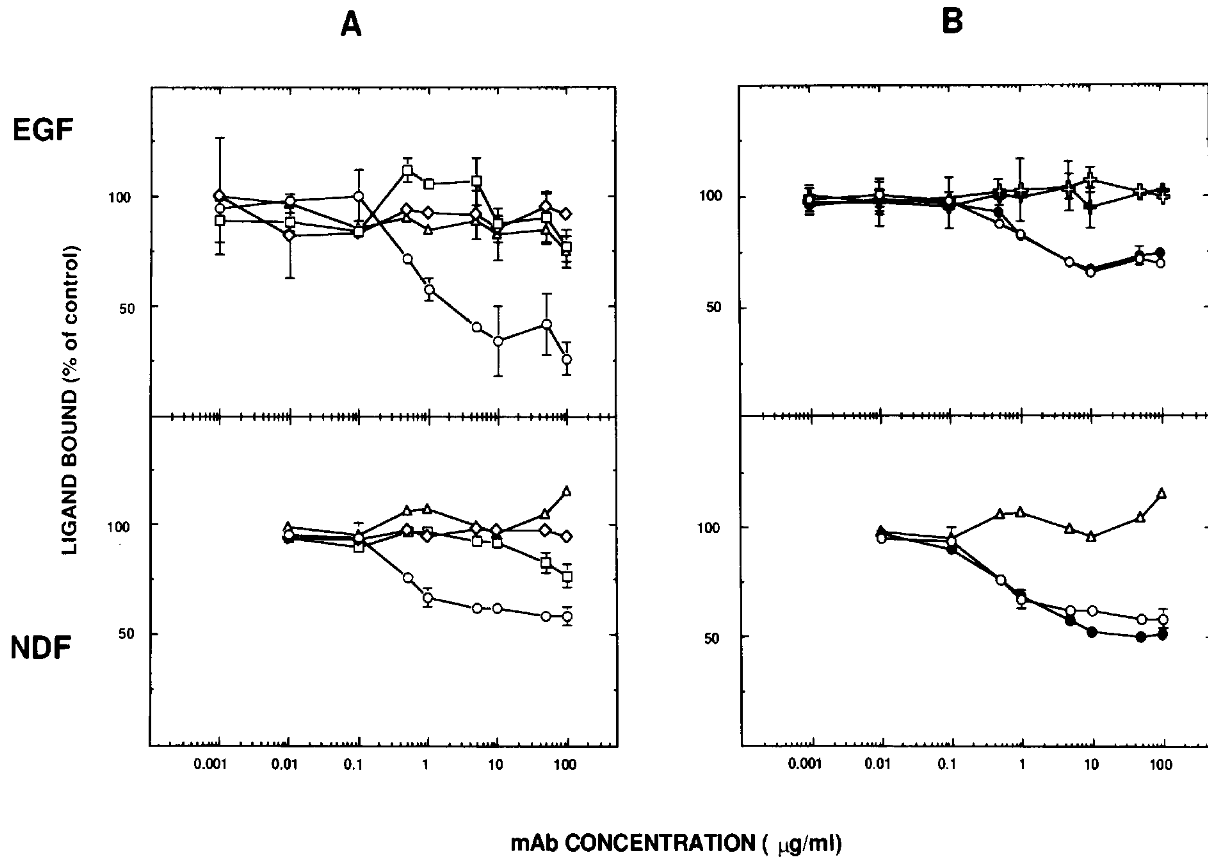

Figure 5 Effect of mAbs to ErbB-2 and their Fab fragments on receptor binding of NDF and EGF. (**A**) Monolayers of T47D cells growing in 48-well dishes were incubated for 2 h at 4°C with either ^{125}I-EGF or ^{125}I-NDF (each at 10 ng/ml) in the presence of increasing concentrations of the following mAbs that represent four different classes of mAbs: L431 (squares), L26 (circles), L140 (triangles), and L87 (rhombuses). Unbound radiolabeled ligand was then removed by washing and cell-associated radioactivity determined. (**B**) Experiments were conducted as in (**A**) to compare ligand binding in the presence of the Fab fragment of mAb L26 (closed circles) to the binding in the presence of the whole mAb (open circles). Antibody N28 (open crosses) and its Fab fragment (closed crosses) and antibody L140 (open triangles) served as controls. Each data point represents the average and standard deviation (bars) of duplicate determinations after subtraction of the non-specific ligand binding. The experiment was performed thrice

assay was performed on several cell lines, including N87 that expresses ErbB-1, ErbB-2 and ErbB-3 receptors and the human T47D breast cancer cell line, that expresses all four ErbB proteins. Figure 5A depicts the results of binding analyses of radiolabeled NDF and EGF, in the presence of representative mAbs directed against different ErbB-2 epitopes. It is important to note that none of the antibodies cross-reacted with other ErbB family members. Antibody L26, as well as other Class II mAbs, were able to displace up to 74% and 42% of cell-bound EGF and NDF, respectively. This phenomenon was not characteristic of mAbs capable of recognizing other receptor determinants (e.g., mAbs L431, L87 and L140, Figure 5A), suggesting that the epitope bound by mAbs from Class II is involved in the formation of heterodimers. This hypothesis was further supported by the inhibition of EGF and NDF binding to T47D cells by monovalent fragments of antibody L26 (Figure 5B). Fab fragments of L26 could inhibit the binding of both ligands, to an extent similar to that of the whole mAb, whereas the Fab of an antibody incapable of ligand binding inhibition (N28) could not. It is worth noting

internalization. Similar results were obtained with the N87 cell line (data not shown). To directly test the prediction that Class II mAbs inhibit ligand binding by interfering with the formation of ErbB-2-containing heterodimers, we covalently labeled each receptor with a radiolabeled ligand, and analysed coprecipitation of the affinity labeled receptor with ErbB-2. The results of this experiment, that was performed with N87 cells, are shown in Figure 6. Evidently, both monomeric and dimeric receptor species were precipitated by anti-ErbB-2 antibodies. However, the presence of mAb L26 during the affinity labeling reaction significantly reduced coprecipitation with ErbB-2. The effect was larger with EGF than with NDF, consistent with the results of the ligand displacement assay (Figure 5A), and it was not induced by a control non-relevant antibody (6B11). However, all mAbs to ErbB-2 slightly reduced the efficiency of affinity labeling, especially with EGF, probably due to aspecific masking of primary amino groups.

To further study the mechanism underlying antibody-induced inhibition of ligand binding we measured the effect of mAb L26 on the affinity of EGF and NDF

DOCKET ALARM Find authenticated court documents without watermarks at docketalarm.com.

DOCKET ALARM

Explore Litigation Insights

Docket Alarm provides insights to develop a more informed litigation strategy and the peace of mind of knowing you're on top of things.

Real-Time Litigation Alerts

Keep your litigation team up-to-date with **real-time alerts** and advanced team management tools built for the enterprise, all while greatly reducing PACER spend.

Our comprehensive service means we can handle Federal, State, and Administrative courts across the country.

Advanced Docket Research

With over 230 million records, Docket Alarm's cloud-native docket research platform finds what other services can't. Coverage includes Federal, State, plus PTAB, TTAB, ITC and NLRB decisions, all in one place.

Identify arguments that have been successful in the past with full text, pinpoint searching. Link to case law cited within any court document via Fastcase.

Analytics At Your Fingertips

Learn what happened the last time a particular judge, opposing counsel or company faced cases similar to yours.

Advanced out-of-the-box PTAB and TTAB analytics are always at your fingertips.

API

Docket Alarm offers a powerful API (application programming interface) to developers that want to integrate case filings into their apps.

LAW FIRMS

Build custom dashboards for your attorneys and clients with live data direct from the court.

Automate many repetitive legal tasks like conflict checks, document management, and marketing.

FINANCIAL INSTITUTIONS

Litigation and bankruptcy checks for companies and debtors.

E-DISCOVERY AND LEGAL VENDORS

Sync your system to PACER to automate legal marketing.

WHAT WILL YOU BUILD? | **sales@docketalarm.com** | **1-866-77-FASTCASE**

fastcase® Smarter legal research.