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Special Article

Monoclonal Antibody Therapy of Human Cancer: Taking the HER2 Protooncogene to the Clinic

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The HER2 protooncogene encodes a 185-kDa transmembrane protein ($p185^{HER2}$) with extensive homology to the epidermal growth factor (EGF) receptor. Clinical and experimental evidence supports a role for overexpression of the HER2 protooncogene in the progression of human breast, ovarian, and non-small cell lung carcinoma. These data also support the hypothesis that $p185^{HER2}$ present on the surface of overexpressing tumor cells may be a good target for receptor-targeted therapeutics. The anti-$p185^{HER2}$ murine monoclonal antibody (muMAb) 4D5 is one of over 100 monoclonals that was derived following immunization of mice with cells overexpressing $p185^{HER2}$. The monoclonal antibody is directed at the extracellular (ligand binding) domain of this receptor tyrosine kinase and presumably has its effect as a result of modulating receptor function. *In vitro* assays have shown that muMAb 4D5 can specifically inhibit the growth of tumor cells only when they overexpress the HER2 protooncogene. MuMAb 4D5 has also been shown to enhance the TNF-α sensitivity of breast tumor cells that overexpress this protooncogene. Relevant to its clinical application, muMAb 4D5 may enhance the sensitivity of $p185^{HER2}$-overexpressing tumor cells to cisplatin, a chemotherapeutic drug often used in the treatment of ovarian cancer. *In vivo* assays with a nude mouse model have shown that the monoclonal antibody can localize at the tumor site and can inhibit the growth of human tumor xenografts which overexpress $p185^{HER2}$. Modulation of $p185^{HER2}$ activity by muMAb 4D5 can therefore reverse many of the properties associated with tumor progression mediated by this putative growth factor receptor. Together with the demonstrated activity of muMAb 4D5 in nude mouse models, these results support the clinical application of muMAb 4D5 for therapy of human cancers characterized by the overexpression of $p185^{HER2}$.

KEY WORDS: HER2; *neu*; TNF-α; monoclonal antibody therapy.

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BACKGROUND: THE HER2 PROTOONCOGENE AND HUMAN CANCER

Cellular protooncogenes encode proteins that are thought to regulate normal cellular proliferation and differentiation. Alterations in their structure or amplification of their expression lead to abnormal cellular growth and have been associated with carcinogenesis (1–4). Protooncogenes were first identified by either of two approaches. First, molecular characterization of the genomes of transforming retroviruses showed that the genes responsible for the transforming ability of the virus in many cases were altered versions of genes found in the genomes of normal cells. The normal version is the protooncogene, which is altered by mutation to give rise to the oncogene. An example of such a gene pair is represented by the EGF receptor and the v-erbB gene product. The virally encoded v-erbB gene product has suffered truncation and other alter-

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ations that render it constitutively active and endow it with the ability to induce cellular transformation (5).

The second method for detecting cellular transforming genes that behave in a dominant fashion involves transfection of cellular DNA from tumor cells of various species into nontransformed target cells of a heterologous species. Most often this was done by transfection of human, avian, or rat DNAs into the murine NIH 3T3 cell line (1–5). Following several cycles of genomic DNA isolation and retransfection, the human or other species DNA was molecularly cloned from the murine background and subsequently characterized. In some cases, the same genes were isolated following transfection and cloning as those identified by the direct characterization of transforming viruses. In other cases, novel oncogenes were identified. An example of a novel oncogene identified by this transfection assay is the *neu* oncogene. It was discovered by Weinberg and colleagues in a transfection experiment in which the initial DNA was derived from a carcinogen-induced rat neuroblastoma (6,7). Characterization of the *neu* oncogene revealed that it had the structure of a growth factor receptor tyrosine kinase, had homology to the EGF receptor, and differed from its normal counterpart, the *neu* protooncogene, by an activating mutation in its transmembrane domain (8).

The association of the HER2 protooncogene with cancer was established by yet a third approach, that is, its association with human breast cancer. The HER2 protooncogene was first discovered in cDNA libraries by virtue of its homology with the EGF receptor, with which it shares structural similarities throughout (5). When radioactive probes derived from the cDNA sequence encoding $p185^{HER2}$ were used to screen DNA samples derived from breast cancer patients, amplification of the HER2 protooncogene was observed in about 30% of patient samples (9). Further studies have confirmed this original observation and extended it to suggest an important correlation between HER2 protooncogene amplification and/or overexpression and worsened prognosis in ovarian cancer and non-small cell lung cancer (10–14).

The association of HER2 amplification/overexpression with aggressive malignancy, as described above, implies that it may have an important role in progression of human cancer; however, many tumor-related cell surface antigens have been described in the past, few of which appear to have a direct role in the genesis or progression of disease (15,16). The data which support a role of HER2 overexpression in the basic mechanisms of human cancer are summarized below.

Amplified expression of $p185^{HER2}$ can lead to cellular transformation as assessed by morphological alterations and growth of $p185^{HER2}$-overexpressing cells in soft agar and in nude mice (17,18). In addition, NIH 3T3 fibroblasts overexpressing $p185^{HER2}$ have an increased resistance to cytotoxicity mediated by activated macrophages or recombinant human TNF-α (19), the cytokine that appears to be mainly responsible for macrophage-mediated tumor cell cytotoxicity (20). This observation extends also to breast tumor cells, which overexpress $p185^{HER2}$ (19), and suggests that high levels of $p185^{HER2}$ expression may be related to tumor cell resistance to at least one component of the host's antitumor surveillance armamentarium, the activated macrophage. This work has been reviewed previously (21), and similar data have recently been reported for ovarian tumor cell lines which overexpress $p185^{HER2}$ (22). Further support for a role of $p185^{HER2}$ or the related *neu* oncogene-encoded tyrosine kinase in tumorigenesis comes from work with transgenic mice that have been manipulated to overexpress one or the other of these two related genes. Transgenic mice expressing the activated form of the rat *neu* protooncogene, under the control of a steroid inducible promoter, uniformly develop mammary carcinoma (23). In another transgenic mouse model the HER2 protooncogene product, "activated" by point mutation analogous to the rat *neu* oncogene product, or an unaltered form of the HER2 protooncogene, has been expressed in mice (24). The main malignancies induced in this model were either lung adenocarcinoma or lymphoma but not mammary carcinoma. While it is not known why the different transgenic mouse models give such distinct results, the latter model may be of particular significance given the recent report of an association between $p185^{HER2}$ overexpression and poor prognosis in nonsmall cell lung cancer (14). These differing results suggest some difference in the activity of activated *neu* and HER2-encoded tyrosine kianses, although effects due to mouse strain differences cannot be excluded.

The structural similarities between $p185^{HER2}$ and the EGF receptor suggest that function of $p185^{HER2}$ may be regulated similarly to the EGF receptor. In particular, one expects that the tyrosine kinase activity associated with the cytoplasmic domain of

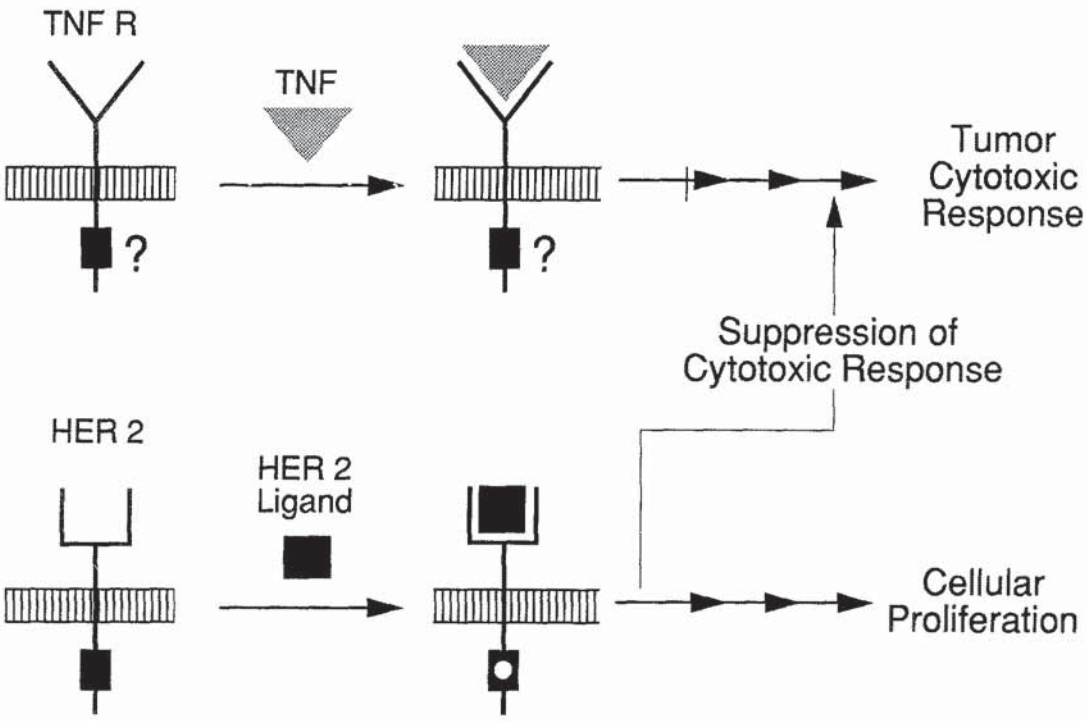

Fig. 1. Suppression of the TNF cytotoxic response by activation of $p185^{HER2}$. The schematic shows both the TNF cytotoxic pathway (top) and the $p185^{HER2}$-stimulated cell proliferation/transformation pathway (bottom). Signaling from the TNF receptor following interaction with TNF has not been characterized. Binding of ligand to $p185^{HER2}$ is shown to activate the receptor-associated tyrosine kinase activity, resulting in stimulation of cellular proliferation and suppression of the tumor cell cytotoxic response to TNF.

the receptor would be ligand activated. This proposal receives support from recent work describing a ligand for $p185^{HER2}$ (25). These data lead to a model (Fig. 1) wherein antagonists that downregulate the function of $p185^{HER2}$ should have the effect of inhibiting growth of tumor cells dependent upon $p185^{HER2}$ function and of increasing the sensitivity of such tumor cells to TNF-α. By analogy with previous work done with two related tyrosine kinases, the EGF receptor (26) and the activated *neu* protooncogene product (27), we hypothesized that monoclonal antibodies targeted to the extracellular domain of $p185^{HER2}$ may have the desired properties.

DERIVATION OF muMAb 4D5

A family of monoclonal antibodies focused against the extracellular domain of $p185^{HER2}$ were prepared (28). To do this, an NIH 3T3 fibroblast cell line that overexpresses $p185^{HER2}$ [NIH 3T3/HER2-3-400 (18)] was used to immunize BALB/c mice. The mice were subsequently boosted with NIH 3T3/HER2-3-400 and, finally, with a preparation enriched for $p185^{HER2}$ by wheat germ agglutinin chromatography of membrane extracts of this cell line. Following splenocyte fusion with a mouse myeloma partner, the hybridomas were cultured in 96-well microtiter plates. Hybridomas positive for anti-$p185^{HER2}$ activity, but with little or no anti-EGFR activity, were detected by ELISA (Fig. 2). A critical property of an anti-$p185^{HER2}$ monoclonal antibody with potential for therapy would be its lack of cross-reactivity with the closely related EGF receptor, which is expressed at elevated levels in multiple tissues (29). To select further monoclonal antibodies with this characteristic, a number of assays were performed, including immunoprecipitation assays utilizing *in vivo* labeled EGF receptor and $p185^{HER2}$ (Fig. 3A) and FACS analysis of antibody binding to tumor cells overexpressing either $p185^{HER2}$ or the EGFR (Fig. 3B). The screening results are summarized in Table I. Based upon these results, nine of the $p185^{HER2}$ monoclonal antibodies were chosen for further characterization, including a cell growth inhibition assay utilizing the SK-BR3 human breast adenocarcinoma cell line, which greatly overexpress $p185^{HER2}$. The monoclo-

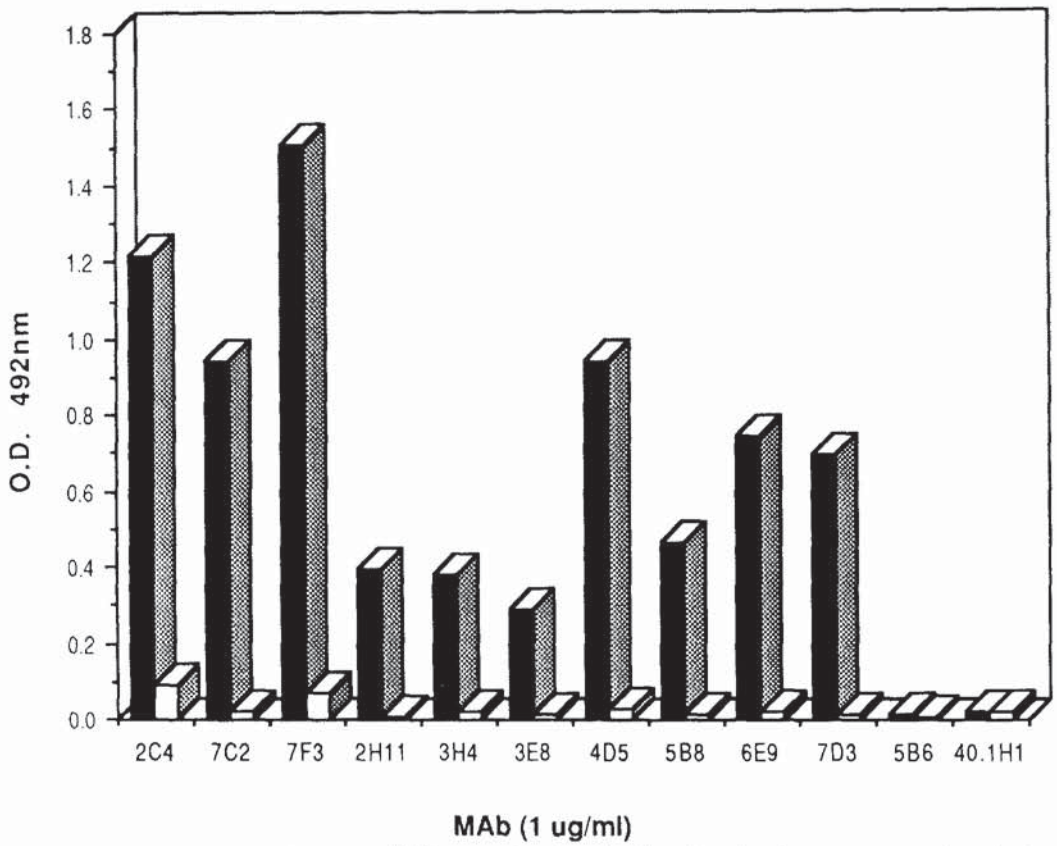

Fig. 2. ELISA screening of anti-p185^{HER2} monoclonal antibodies. Results shown measure the relative reactivities of the purified anti-p185^{HER2} monoclonal antibodies (added to 1 μg/ml) with membrane extracts enriched in EGF receptor (open bars; from A431 squamous carcinoma cells) or enriched in p185^{HER2} (filled bars; from NIH 3T3/HER2-3-400).

nal antibody, muMAb 4D5, was clearly the most effective of the group in this assay (Table II).

The initial results characterizing the growth inhibitory activity of these monoclonal antibodies were extended by comparing them for activity against a battery of human breast and ovarian tumor cell lines that expressed varying levels of p185^{HER2}. These results reveal that the monoclonal antibodies can be growth inhibitory, they may have no affect on cell proliferation, or they may stimulate the proliferation of breast tumor cells. Growth inhibition appears to depend upon overexpression (Table III). This property, in particular, is shared by the monoclonal antibodies 4D5 and 3H4. These monoclonal antibodies may exert their effects on cell growth by similar mechanisms since they compete for binding to the receptor (Tables I and III) (28) and, therefore, may recognize the same or overlapping epitopes. The other monoclonal antibodies vary in their ability to inhibit proliferation, but 7C2 and 6E9 are consistently less active in this respect. The potent growth inhibitory activity of 2C4 for MDA-MB-175 breast tumor cells is not understood at present but may represent cross-reactivity with another receptor expressed on these cells. Similarly, the properties that distinguish 7C2 from the other antibodies with regard to its ability to stimulate the proliferation of several of the tumor cell lines shown in Table III has not been determined. The 6E9 monoclonal antibody has been shown to bind to the extracellular domain of p185^{HER2}, although only to a subset of receptors on the surface of SK-BR-3 tumor cells (30). The functional significance of this subset of receptors is unclear. In addition to its activity on breast tumor cells, which overexpress p185^{HER2}, muMAb 4D5 is also clearly the most active of the monoclonal antibodies with respect to its ability to inhibit growth of SKOV-3, a human ovarian adenocarcinoma cell line that overexpresses p185^{HER2} (Table III). Experiments are currently planned to try to understand in more detail how these monoclonal antibodies may exert

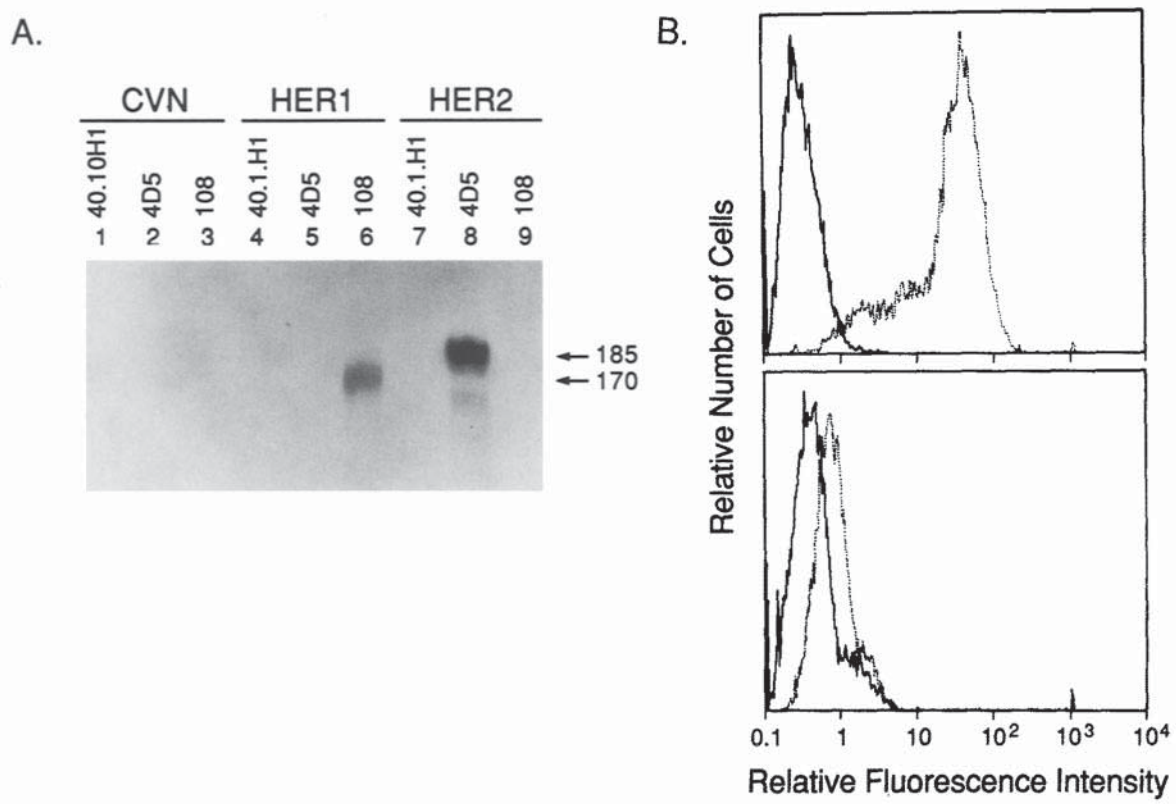

Fig. 3. MuMAb 4D5 does not cross-react with the EGFR. (A) Immunoprecipitation of metabolically labelelled NIH 3T3 cells transfected by control plasmid (CVN), by a plasmid encoding the EGFR (HER1) or a plasmid encoding $p185^{HER2}$ (HER2). MuMAb 40.1.H1 is directed against hepatitis B surface antigen (lanes 1, 4, 7); muMAb 4D5 is directed against $p185^{HER2}$ (lanes 2, 5, 8); muMAb 108 is directed against the EGFR (lanes 3, 6, 9). (B) Fluorescence-activated cell sorter histograms of muMAb 40.1.H1 (solid lines) or muMAb 4D5 (dotted lines) reacted with SK-BR-3 tumor cells (approx. 2×10^6 receptors per cell; upper panel) or the same antibodies reacted with the A431 squamous carcinoma cell line (approx. 2×10^6 EGFR per cell; lower panel).

distinct effects on tumor cell proliferation. The *in vitro* results summarized in Table III clearly show that when the monoclonal antibodies are compared for efficacy, as measured by their abilities to inhibit growth of breast and ovarian tumor cells overexpressing $p185^{HER2}$, muMAb 4D5 is usually the most potent and is therefore a good candidate for further characterization in other models that may be predictive of its efficacy in human clinical trials. Interestingly, the most dramatic activity of the antibody is seen in cell lines that overexpress greater than fivefold the level observed in MCF-7 breast tumor cell lines [a low expressor control cell line; Table III (19)]. Patients who overexpress greater than fivefold the normal level of $p185^{HER2}$ have been shown to have a very poor prognosis (10). These results will aid in choosing patients who are most likely to respond in clinical trials.

The model depicted in Fig. 1 predicts that downregulation of $p185^{HER2}$ by a monoclonal antibody or other reagent should result in decreased cellular proliferation, as shown in Table III, but also increased sensitivity to TNF-α. Results of experiments in which tumor cells overexpressing $p185^{HER2}$ were treated with muMAb 4D5 or a control monoclonal antibody, alone and in combination with TNF-α, suggest the validity of this model (Fig. 4) (31). MuMAb 4D5 treatment of breast tumor cells overexpressing $p185^{HER2}$ resulted in enhanced sensitivity of these cells to TNF-α. The growth and the TNF-α sensitivity of normal cells or tumor cells that do not overexpress the receptor were unaltered.

In addition to the relationship between TNF-α resistance and $p185^{HER2}$ overexpression, a possible relationship between protoncogene expression and resistance to the chemotherapeutic drug cisplatin has been investigated. A correlation between HER2 protooncogene overexpression and resistance to chemotherapeutic drugs rests on the grounds that

Table I. Summary Table of Monoclonal Antibodies Described

MAb	Isotype	ELISA[a]		RIP[b]		Epitope[c]	FACS[d]
		EGFR	$p185^{HER2}$	EGFR	$p185^{HER2}$		
4D5	IgG1,k	–	++	–	++	I(p/c)	+++
2C4	IgG1,k	–	+++	–	++	F(p/c)	+++
2Hll	IgG2a,k	–	+	–	++	H(p/c)	++
3E8	IgG2a,k	–	+	–	+++	H(p/c)	+++
3H4	IgG1,k	–	+	–	+	I(p)	+
SB8	IgG1,k	–	+	–	++	nd(p)	+
6E9	IgG1,k	–	++	–	+	nd(p)	–
7C2	IgG1,k	–	++	–	++	G(p)	+++
7D3	IgG1,k	–	++	–	++	F(p/c)	+++
7F3	IgG1,k	–	+++	–	+++	G/F(p/c)	+++

[a]Summary of OD 492 nm: (–) <0.1; (+) 0.11–0.50; (++) 0.51–1.0; (+++) >1.0.
[b]Summary of autoradiography from immunoprecipitations: (–) bands equal to negative control; (+) weak bands but darker than negative control; (++) moderately exposed bands; (+++) strongly exposed bands.
[c]Letters were assigned to represent individual epitopes A through I (nd, not done). MAbs were considered to share an epitope if each blocked binding of the other by 50% or greater in comparison to an irrelevant MAb control. The epitope composition recognized by immunoprecipitations with each MAb from tunicamycin-treated cells is shown. The letters p, c, or p/c in parentheses indicate that the monoclonal antibody binds only to the polypeptide (p), the carbohydrate (c), or both (p/c) moieties in the extracellular domain of $p185^{HER2}$.
[d]Fluorescence staining of SK-BR-3 cells by the anti-$p185^{HER2}$ monoclonal antibodies: (–) MAbs equal to the negative control MAbs; (+) 1- to 9-fold higher than the negative controls; (++) 10- to 99-fold higher than the negative controls; (+++) >100-fold higher than the negative controls.

patients exhibiting overexpression appear to have a worsened prognosis, especially in ovarian cancer (10, 13). In addition, recent work with the EGF receptor (32) has indicated that when the anti-EGFR monoclonal antibody 108.4 was added together with cisplatin, the antitumor effect of the antibody was greatly enhanced. Because the 108.4 monoclonal antibody and muMAb 4D5 appear to share the ability to inhibit soft agar growth of tumor cells overexpressing their respective receptors, it seemed possible that such an interaction may also occur in the HER2 protooncogene system. The *in vitro* results (Fig. 5) show that treatment of SK-BR-3 breast tumor cells with muMAb 4D5 enhances their sensitivity to cisplatin.

Table II. Inhibition of SK-BR-3 Proliferation by Anti-$p185^{HER2}$ Monoclonal Antibodies[a]

Monoclonal antibody	Relative cell proliferation[b]
4D5	44.2 ± 4.4
7C2	79.3 ± 2.2
2C4	79.5 ± 4.4
7D3	83.8 ± 5.9
3E8	66.2 ± 2.4
6E9	98.9 ± 3.6
7F3	62.1 ± 1.4
3H4	66.5 ± 3.9
2H11	92.9 ± 4.8
40.1 H1[c]	105.8 ± 3.8
4F4	94.7 ± 2.8

[a]SK-BR-3 breast tumor cells were plated at a density of 4×10^4 cells per well into 96-well microdilution plates, allowed to adhere, and then treated with monoclonal antibody (10 μg/ml).
[b]Relative cell proliferation was determined by crystal violet staining of the monolayers after 72hr. Values are expressed as a percentage of results with untreated control cultures (100%).
[c]Control monoclonal antibodies 40.1H1 and 4F4 are directed against hepatitis B surface antigen and human interferon-γ, respectively (27).

IN VIVO PRECLINICAL EFFICACY

A critical part of the rationale supporting the application of muMAb 4D5 to human cancer therapy is its ability to inhibit the growth of tumor cells overexpressing $p185^{HER2}$ *in vivo*. A human tumor xenograft model was used to test this property of muMAb 4D5 and to compare its activities with those of the other monoclonal antibodies in a relevant model of human disease. In this model, a human breast tumor, characterized with respect to HER2 protooncogene amplification and expression, was grafted into the subrenal capsules of nude mice. Therapy was initiated 1 week postimplantation. In order to be active in this model, the monoclonal antibody must be able to localize to the overexpressing tumor cells in the lesion and subsequently exert a growth regulatory effect mediated through $p185^{HER2}$. Growth inhibition occurs only with tumors that overexpress the receptor. Heterotransplants (approximately 1 mg) of Murray breast tumor [a high expresser of the HER2 gene product (10)] were implanted into the subrenal capsule of 48

Table III. Inhibition of Human Breast and Ovarian Tumor Cell Growth by Monoclonal Antibodies Directed Against the Extracellular Domain of $p185^{HER2}$

Cell line	Relative $p185^{HER2}$ expression[a]	Cell proliferation (% control)[b]					
		4D5[c]	3H4[c]	2C4[d]	7F3[d]	7C2[e]	6E9[f]
MCF7	1	94	101	101	97	106	110
ZR-75-1	3	106	113	104	100	149	113
MDA-MB-175	4	61	84	24	48	87	103
MDA-MB-453	7	62	68	91	84	78	101
MDA-MB-361	17	60	68	65	73	113	113
BT474	20	23	25	53	20	74	94
SK-BR-3	33	42	56	66	64	92	105
SK-OV-3	17	77	85	87	91	97	99

[a]Based on FACS assay using muMAb 4D5 and fluorescence-labeled goat anti-murine IgG1 polyclonal antibody.
[b]Five-day assay with 10 μg/ml of indicated monoclonal antibody (SE, ~10%). Other methods as described in the footnotes to Table II.
[c]4D5 and 3H4 define epitope "I."
[d]2C4 and 7F3 will partially block one another, 2C4 is assigned epitope "F," and 7F3 is assigned epitope "F/G."
[e]7C2 defines epitope "G" and will partially block 7F3 binding.
[f]6E9 epitope determination not done.

athymic mice on day 0. Groups of eight animals were injected intravenously with tissue culture-derived muMAb 4D5 (36.4 mg/kg), PBS, or control monoclonal antibody, muMAb 5B6 (directed against gp120; 36.4 mg/kg), as single agents in equally divided doses on days 7, 10, and 13. Four mice from each group were sacrificed on day 20, and the remainder of the animals were sacrificed on day 34. Tumor sizes were measured using ocular micrometer and gravimetric techniques. A summary of the tumor weights (mean ± SD) from animals sacrificed on days 20 and 34 is shown in Table IV. On day 20, average tumor weights of animals receiving muMAb 4D5 were significantly less than those receiving the same dose of the control antibody muMAb 5B6. Interactive effects between muMAb 4D5 and cisplatin have also been observed in this model (33). These studies in athymic mice bearing human breast tumor xenografts have demonstrated efficacy and suggested an enhanced effect when muMAb 4D5 is given in combination with cisplatin.

MECHANISM OF ACTION

The results described above are consistent with muMAb 4D5 having receptor antagonist activity. Surprisingly, however, muMAb 4D5 treatment of SK-BR3 tumor cells stimulates receptor tyrosine kinase activity (Table V) (30, 34). In addition, it can mediate the phosphorylation of intracellular substrates by $p185^{HER2}$ (34). Consistent with its ability to stimulate receptor activity, muMAb 4D5 treatment of SK-BR-3 or SK-OV-3 tumor cells results in a modulation of intracellular second messengers, including diacylglycerol. Diacylglycerol (DAG) is a product of phospholipase C breakdown of phosphatidylinositol-4,5-bisphosphate. It is a cofactor

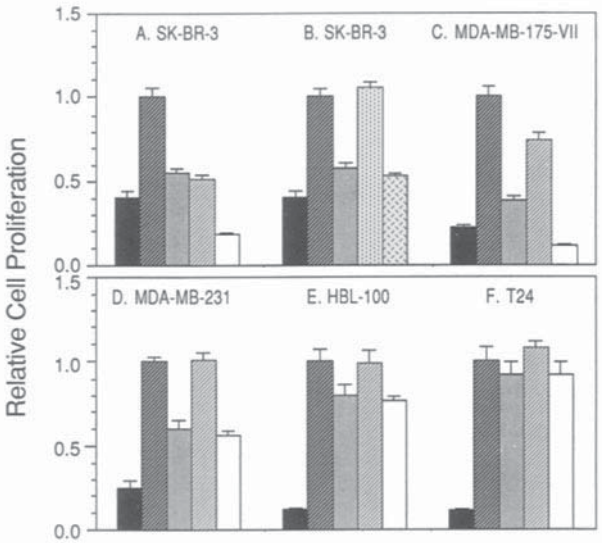

Fig. 4. Monoclonal antibody 4D5 sensitizes breast tumor cells to the cytotoxic effects of TNF-α. Filled bars, cell number at initiation of the assay; dark cross-hatching, untreated control; dark stipples, TNF-α alone; light cross-hatching, MuMAb 4D5; open bars, MuMAb 4D5 combined with TNF-α. (B) Lack of growth inhibition of SK-BR3 tumor cells by muMAb 40.1 H1 (anti-hepatitis B antigen; light stipples) and failure of the 40.1 H 1 to enhance SK-BR-3 tumor cell sensitivity to TNF-α (broken cross-hatching). SK-BR-3 and MDA-MB-175-VII overexpress p185HER2 (see Table III). MDA-MB-231 and HBL-100 are breast cell lines which do not overexpress $p185^{HER2}$, and T24 is a nonoverexpressing human bladder carcinoma cell line. The assay was performed as described in Ref. 31.

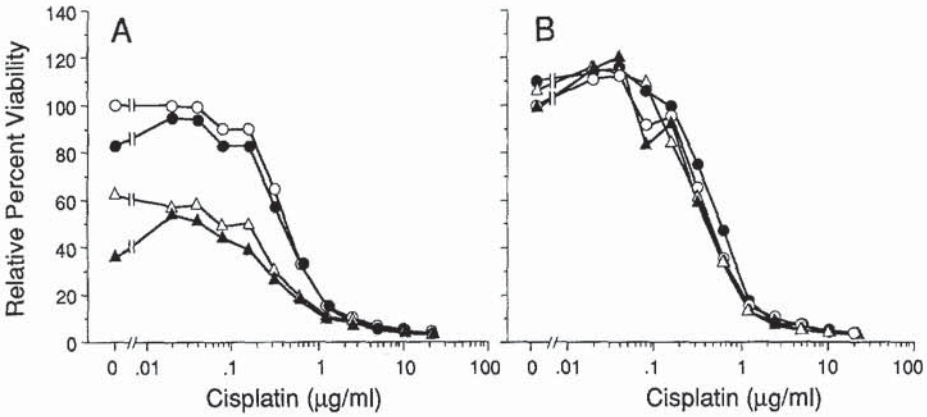

Fig. 5. Treatment of SK-BR-3 breast tumor cells with muMAb 4D5 enhances sensitivity to cisplatin. MuMAb 4D5 (A) or muMAb 6E9 (control; B) and cisplatin were added at the indicated concentrations to SK-BR-3 breast tumor cells. The plate cultures were incubated for 3 days and relative cell proliferation was determined as described (31). No antibody (○); 0.156 μg/ml muMAb 4D5 or muMAb 6E9 (●); 0.625 μg/ml muMAb 4D5 or muMAb 6E9 (△); 2.5 μg/ml muMAb 4D5 or muMAb 6E9 (▲).

for activation of protein kinase C and has been closely associated with growth factor activity (35). As may be predicted from its effect on cell proliferation, muMAb 4D5 treatment of SK-BR-3 tumor cells results in downregulation of intracellular pools of DAG (Table VI) (30). This result is consistent with overall antagonist activity, as is inhibition of tumor cell proliferation. Other data suggest that muMAb 4D5 may inhibit association of ligand with the receptor (25). Similar monoclonal antibodies have been reported for the EGFR system (26, 28). Further work is under way to characterize the ligand(s) that binds $p185^{HER2}$ and the mechanism of action of muMAb 4D5.

While the ability of muMAb 4D5 to stimulate phosphorylation of $p185^{HER2}$ is consistent with an agonist of receptor function, it is important to note that our current data suggest that it does not behave as an agonist in our cell growth assays *in vitro* or in nude mice. The results of an experiment that compares the effects of muMAb 4D5 on the growth of MCF-7 and SK-BR-3 breast tumor cells are shown in Fig. 6. These data demonstrate that muMAb 4D5 has no effect on the growth of nonoverexpressing tumor cells (MCF-7; Tables III, VII) at any of the doses tested between 0.7 p*M* and 67 n*M*. Also, whatever allows the muMAb 4D5 to have differential effects on overexpressing tumor cells, this difference does not lie in different receptor affinities for the monoclonal antibody. Table VII clearly shows that SK-BR-3 and SK-OV-3, both $p185^{HER2}$ overexpressors, which are growth inhibited by 4D5, and MCF-7, which is not, all have similar affinities for muMAb 4D5. The clearest difference between these cell lines is the number of binding sites per tumor cell. These data are consistent with other work that has been previously reported with tumor cells in monolayer culture or in soft agar (18, 19, 25).

A possible mechanistic explanation, which takes many of our experimental observations into account, is a model in which muMAb 4D5 binds

Table IV. MuMAb 4D5 Inhibits the Growth of a Human Breast Tumor (Murray) in Athymic Mice[a]

Group (n = 4)	Tumor weight (mg)[b]	
	Day 20	Day 34
PBS	6.79 ± 9.79	36.0 ± 30.7
Control IgG (muMAb5B6)	7.11 ± 5.48	88.1 ± 91.4
muMAb4D5	1.48 ± 1.10	6.5 ± 6.4

[a]Administered as equally-divided intravenous doses on days 7, 10, and 13 post tumor implantation.
[b]Data are mean ± standard deviation (SD) (n = 4).

Table V. Effect of muMAb 4D5 on Phosphoamino Acid Content of $p185^{HER2}$ in SK-BR-3 Cells

Treatment	Phospho-tyrosine		Phospho-serine		Phospho-threonine	
	cpm[a]	%[b]	cpm	%	cpm	%
None	11	1.5	564	75	176	23.5
muMAb4D5	827	14.0	3,658	62.0	1,429	24.0

[a]Phosphoamino acids as cpm are expressed following background subtraction (17 cpm for none, 21 cpm for muMAb 4D5).
[b]Percentage of total phosphoamino acids.

Table VI. Effect of muMAb 4D5 or 6E9 Monoclonal Antibodies on *sn*-1,2-diacylglycerol levels in SK-BR-3 Cells

Time	Treatment	pmol *sn*-1,2-DAG/10^6 cells	% change
5 min	Vehicle	111.0 ± 10	0
5 min	muMAb4D5	133.2 ± 11.3	+20
5 min	muMAb6E9	133.3 ± 12.9	+20
24 hr	Vehicle	98.6 ± 9.6	0
24 hr	muMAb4D5	62.1 ± 7.4	−37
24 hr	muMAb6E9	92.0 ± 12.7	−7

[a]Following incubation with monoclonal antibody (33 n*M*) or vehicle (PBS) control, the reactions were terminated by aspirating the media and adding 1 ml of ice-cold 100% MeOH. Cells were scraped from the plates and transferred to 13 × 100-mm glass tubes containing 1 ml 100% chloroform. Plates were rinsed with an additional 1 ml of cold methanol, and the rinses combined and mixed thoroughly. Following phase separation at room temperature for 30 min, 1 ml methanol and 1 ml NaCl were added, the samples were centrifuged at 3000 rpm for 5 min, and the top aqueous layer was aspirated. The remaining organic phase was assayed for *sn*-1,2-diacylglycerol by standard procedures.

tightly to $p185^{HER2}$, excludes ligand binding, stimulates receptor internalization, and downregulates receptor signaling pathways as a result of constitutive activation of tyrosine kinase activity that results from nondissociation of the muMAB 4D5/$p185^{HER2}$ complex during receptor cycling. This hypothesis has additional support from our observations that the monoclonal antibody is not degraded following internalization (30) and, in the nude mouse experiments, localizes to and remains at the tumor site for more than 7 days following a single administration of antibody (D.M., personal communication). Such downregulation could result from activation of the serine–threonine protein kinase C, which is known to downregulate the function of other receptor tyrosine kinases (5). The mechanism of action of muMAb 4D5 remains a subject for continuing work.

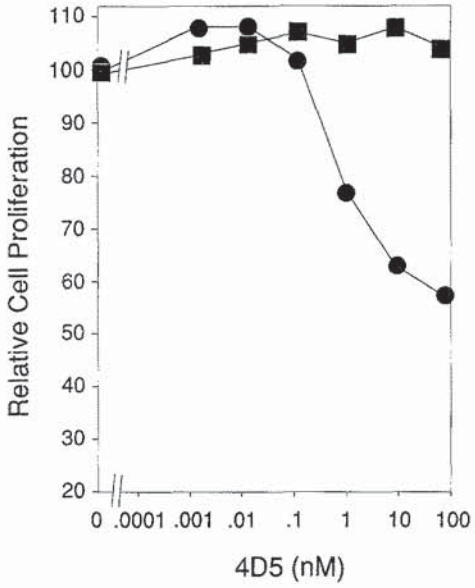

Fig. 6. MuMAb 4D5 does not stimulate proliferation of breast tumor cells. Cells were plated and the assay performed as described (31). MCF-7 breast tumor cells (squares) are compared with the SK-BR-3 tumor cell line (circles). MCF-7 expresses a low amount of $p185^{HER2}$, while SK-BR-3 expresses about 33-fold more (Table III). Coefficient of variation was less than 10%.

Table VII. Monoclonal Antibody Binding to Cultured Human Adenocarcinoma Cells

Cell line	Antibody	K_d (n*M*)	Receptor no. (sites/cell)	Growth inhibition[a]
SK-BR-3	muMAb4D5	6.0	926,650	—*
SK-OV-3	muMAb4D5	5.0	428,930	—*
MCF7	muMAb4D5	1.2	5,525	–

[a]Growth inhibition was measured as described in the footnotes to Table II.
*Statistically significant inhibition of growth ($P<0.05$) as compared to an untreated control.

SUMMARY AND CONCLUSIONS

A convincing body of clinical and experimental evidence supports the role of $p185^{HER2}$ in the progression of human cancers characterized by the overexpression of this protooncogene product. Important aspects of this evidence include the worsened prognosis of breast, ovarian, and non-small cell lung carcinoma patients whose tumors overexpress $p185^{HER2}$, as well as observations that indicate that modulation of $p185^{HER2}$ activity by muMAb 4D5 can reverse many of the properties associated with tumor progression mediated by a growth factor receptor. The properties of muMAb 4D5 that indicate its potential usefulness for the therapy of human cancers characterized by the overexpression of $p185^{HER2}$ are as follows: (i) downregulation of receptors from the cell surface (30); (ii) reversal of the transformed phenotype [as measured by inhibition of colony formation in soft agar of $p185^{HER2}$ overexpressing tumor cells (25, 31)]; (iii) inhibition of the proliferation of overexpressing breast and ovarian tumor cells in monolayer culture (Tables II, III); (iv) reversal of the TNF-α resistant phenotype of breast tumor cells overexpressing $p185^{HER2}$ (Fig. 4) (31); (v) enhancement of the sensitivity to cisplatin of the SK-BR-3 breast tumor cell line *in vitro* (Fig. 5); and (vi)

inhibition of the growth of breast tumor xenografts in a nude mouse model, which may be enhanced when the animals bearing human breast xenografts are also treated with cisplatin (Tables IV, V).

The evidence supporting a role for $p185^{HER2}$ overexpression in human cancer makes this receptor an attractive target for development of cancer therapeutics. Our first exploration of this system with muMAb 4D5 will allow us to obtain information regarding antibody localization and possibly efficacy in combination with cisplatin or as a result of induction of macrophage sensitivity. The muMAb 4D5 also serves as a template for antibody engineering efforts to construct humanized versions more suitable for chronic therapy or other molecules which may be directly cytotoxic for tumor cells overexpressing the HER2 protooncogene.

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