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Differential responses of human tumor cell lines to anti-p185^{HER2} monoclonal antibodies

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Abstract. The HER2 protooncogene encodes a receptor tyrosine kinase, p185^{HER2}. The overexpression of p185^{HER2} has been associated with a worsened prognosis in certain human cancers. In the present work we have screened a variety of different tumor cell lines for p185^{HER2} expression using both enzyme-linked immunosorbent and fluorescence-activated cell sorting assays employing murine monoclonal antibodies directed against the extracellular domain of the receptor. Increased levels of p185^{HER2} were found in breast (5/9), ovarian (1/6), stomach (2/3) and colorectal (5/16) carcinomas, whereas all kidney and submaxillary adenocarcinoma cell lines tested were negative. Some monoclonal antibodies directed against the extracellular domain of p185^{HER2} inhibited growth in monolayer culture of breast and ovarian tumor cell lines overexpressing p185^{HER2}, but had no effect on the growth of colon or gastric adenocarcinomas expressing increased levels of this receptor. The most potent growth-inhibitory anti-p185^{HER2} monoclonal antibody in monolayer culture, designated mumAb 4D5 (a murine IgG1κ antibody), was also tested in soft-agar growth assays for activity against p185^{HER2}-overexpressing tumor cell lines of each type, with similar results. In order to increase the spectrum of tumor types potentially susceptible to monoclonal antibody-mediated anti-p185^{HER2} therapies, to decrease potential immunogenicity issues with the use of murine monoclonal antibodies for human therapy, and to provide the potential for antibody-mediated cytotoxic activity, a mouse/human chimeric 4D5 (chmAb 4D5) and a "humanized" 4D5 (rhu)mAb 4D5 HER2 antibody were constructed. Both engineered antibodies, in combination with human peripheral blood mononuclear cells, elicited antibody-dependent cytotoxic responses in accordance with the level of p185^{HER2} expression. Since this cytotoxic activity is independent of sensitivity to mumAb 4D5, the engineered monoclonal antibodies expand the potential target population for antibody-mediated therapy of human cancers characterized by the overexpression of p185^{HER2}.

Key words: p185^{HER2} – Monoclonal antibodies – Growth inhibition – Cytotoxicity

Introduction

The pathogenesis of human cancer often involves alteration in the structure and expression of various oncogene products. Although direct causal relationships between oncogene amplification and/or overexpression and certain types of cancer remain ill-defined, there are examples of correlations between the occurrence of particular cancers and oncogene activation. For instance, amplification of the N-*myc* oncogene has been found in neuroblastomas and retinoblastomas [9, 33, 48], while c-*myc* amplification was reported in small-cell lung cancer, as well as in breast and kidney cancer [7, 26, 36, 69]. Activated *ras* oncogenes are known to occur in a variety of tumors and tumor cell lines [54, 64]. Among receptor tyrosine kinases, amplification and overexpression of the epidermal growth factor receptor (EGFR) gene has been observed most consistently in squamous cell carcinomas and glioblastomas [34, 38, 60, 67, 68], although other tumors of epithelial origin, such as breast and kidney tumors, are reported to have elevated levels of this growth factor receptor [18, 41, 43, 69]. The product of the HER2 protooncogene (also known as *neu* and c-*erb*B-2) is a growth factor receptor with extensive homology to the EGFR [12] and to c-*erb*B-3, a third member of the EGFR family [32]. p185^{HER2} can be distinguished from EGFR by differences in chromosomal location [12, 19, 46], transcript size [12, 60], molecular mass [2, 45, 57], ligand activation of the associated tyrosine

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kinase [2, 57], and antigenicity, as determined by interaction with specific monoclonal antibodies [16, 24]. HER2 protooncogene amplification has been demonstrated sporadically in adenocarcinomas of the stomach [19, 28, 40, 70], salivary gland [49], thyroid [1], colon [10, 58, 72], lung [29, 47], pancreas [65], and ovary [4, 56]. Amplification and overexpression of HER2 are found frequently in breast-tumor-derived cell lines [25, 30, 31] and in human mammary carcinomas [6, 61–63]. Investigations of large numbers of tumors from breast cancer patients have revealed that approximately 20%–30% have amplified the HER2 protooncogene [6, 20, 21, 50, 55, 61]. Moreover, analyses of clinical parameters show a correlation between both amplification and overexpression of HER2 and a worsened prognosis in that HER2-overexpressing patients have a shorter disease-free and overall survival period [20, 21, 50, 55, 59, 66]. Similar findings were reported with smaller numbers of tumor samples for ovarian cancer [4, 56], and more recently for lung [29], gastric [71], bladder [39] and endometrial carcinomas [5, 8] as well. Although mechanistic explanations for the aggressiveness of $p185^{HER2}$-overexpressing tumors remain elusive, it has been shown that overexpression correlates with lower levels of estrogen receptor (resistance to tamoxifen therapy) [3] and with tumor cell resistance to immune cell cytotoxicity [23, 24, 35].

In order to begin deciphering the biological functions of $p185^{HER2}$, several groups have developed monoclonal antibodies raised in mice immunized with NIH-3T3 fibroblasts that express large amounts of either $p185^{HER2}$ or $p185^{neu}$. These antibodies were able to inhibit anchorage-independent growth of the transfected 3T3 cells in soft agar, but had no effect on their proliferation in monolayer culture [13, 14, 24]. The anti-*neu* antibodies were also shown to mediate tumor cell cytolysis in vitro in the presence of complement [14], and to inhibit tumor formation by *neu*-transformed NIH 3T3 fibroblasts in nude mice [14, 15]. In addition, we recently reported that anti-$p185^{HER2}$ monoclonal antibodies had antiproliferative effects on breast tumor cells overexpressing $p185^{HER2}$ [24]. In the present report, we have expanded these initial studies in order to determine whether the antiproliferative effects we observed on breast tumor cells overexpressing $p185^{HER2}$ could also be observed on non-breast tumor cell lines that overexpress this growth factor receptor. In addition, we constructed chimeric and humanized monoclonal antibodies derived from murine mAb 4D5, our most potent growth-inhibitory antibody, in order to provide the engineered monoclonal antibody with the ability to direct cytotoxic activity against the overexpressing tumor cells via antibody-dependent cellular cytotoxicity (ADCC). The results presented here demonstrate that the sensitivity of breast tumor cell lines to antibody-mediated growth inhibition correlates well with their level of $p185^{HER2}$, but that this relationship may not apply to other types of tumor cells with elevated levels of this receptor. The chimeric and humanized 4D5 antibodies, however, could mediate ADCC against different types of tumor cells overexpressing $p185^{HER2}$, regardless of their sensitivity to the parent 4D5 antibody.

Materials and methods

Cell lines and materials. The majority of our cell lines were obtained from the American Type Culture Collection (Rockville, Md.). Two ovarian carcinoma cell lines, SK-OV-6 and HEY, were a gift from Dr. Jan Vaage (Roswell Park Cancer Institute, Buffalo, N.Y.). The MKN7 gastric carcinoma cell line was obtained from Mitsubishi Corporation (Tokyo, Japan). The mammary epithelial lines 184, 184A1 and 184B5 were kindly provided by Dr. Martha Stampfer, Lawrence Berkeley Laboratory (Berkeley, Calif.). Tumor cells were cultured in Ham's F-12 medium plus Dulbecco's modified Eagle medium (1:1, v/v) supplemented with 100 units/ml penicillin G, 100 μg/ml streptomycin, 2 mM L-glutamine (all from Grand Island Biochemical Company, Grand Island, N.Y.) and 10% heat-inactivated (56° C, 45 min) fetal bovine serum (FBS; Armour Pharmaceutical Company, Kankakee, Ill.). Normal human mammary epithelial cell lines were maintained in mammary epithelial growth medium plus 0.4% bovine pituitary extract (Clonetics Inc., San Diego, Calif.), supplemented with 5 μg/ml transferrin. Anti-$p185^{HER2}$ and anti-EGFR monoclonal antibodies were prepared and characterized as in [16]. The cloning and expression of chimeric mAb 4D5 and humanized mAb HER2 are described in [11].

FACS analysis of $p185^{HER2}$ and EGFR expression. The procedure used to measure cell-surface levels of $p185^{HER2}$ by FACS (fluorescence-activated cell sorter) analysis is as follows. Cells were detached from T-75 flasks with 25 mM EDTA in 150 mM NaCl, centrifuged at 1000 rpm for 10 min, and resuspended in 1% (v/v) FBS in phosphate-buffered saline (PBS). Cell suspensions were then counted, adjusted to 10^6 cells/ml, and incubated for 60 min on ice with 10 μg either anti-$p185^{HER2}$ mumAb 4D5 or anti-EGFR monoclonal antibody 6C5, or with diluent (PBS). All samples were washed twice, resuspended in 0.1 ml 1% FBS/PBS, and incubated with 12.5 μg fluorescein-isothiocyanate-conjugated $F(ab')_2$ fragment of goat anti-(mouse IgG) (Organon Teknika-Cappel, Malvern, Pen.) for 45 min on ice. Following this incubation period, the cell suspensions were washed twice with 1% FBS/PBS to remove any unbound fluorochrome, resuspended in 1 ml assay buffer and analyzed using a FACScan cell sorter (Becton Dickinson, Mountain View, Calif.). These measurements were repeated three or four times per cell line, giving identical results each time.

Measurement by ELISA. Cell lines were plated in 20×100-mm dishes and allowed to grow to 70% confluence. The monolayers were then washed once with PBS, lysed with 0.5% NP-40 in PBS, and allowed to sit on ice for 60 min. Cell lysates were clarified by centrifugation, and the supernatants were assayed for $p185^{HER2}$ and EGFR levels by corresponding specific ELISA and for protein by the Pierce Micro BCA* assay (Pierce, Rockford, Ill.). The $p185^{HER2}$ ELISA procedure utilized microtiter plates coated with anti-$p185^{HER2}$ mumAB 7F3 to which samples or standards and horseradish-peroxidase-labeled mumAb 4D5 were added for color development [53]. The EGFR ELISA was performed in a similar manner using anti-EGFR mumAb 13A9 for coating the plates, and two horseradish-peroxidase-conjugated mAbs (3G2 and 5G3) for color detection [16]. Results are expressed as concentration of receptor/concentration of total cellular protein.

Cell proliferation assays. Tumor cell lines were plated in 96-well microtiter plates at the following densities: 10^4 cells/well for HBL-100 and SK-OV-3; 2×10^4 cells/well for 184, 184A1, 184B5, SK-BR-3, BT-474, COLO 201, KATO III, MKN7, SW1417, ZR-75-1, MCF7, MDA-MB-231, MDA-MB-436, MDA-MB-453; 4×10^4 cells/well for MDA-MB-175-VII; 8×10^4 cells/well for MDA-MB-361. After the cells had been allowed to adhere for 2 h, medium alone or medium containing anti-$p185^{HER2}$ monoclonal antibodies (final concentration of 10 μg/ml) was added to give a total volume of 0.2 ml. After incubation for 5 days, the monolayers were carefully washed twice with PBS and stained with crystal violet dye (0.5% in methanol) for determination of relative cell proliferation as described previously [24]. Treatment groups consisted of 8–16 replicates, and the coefficient of variation was always less than 12%.

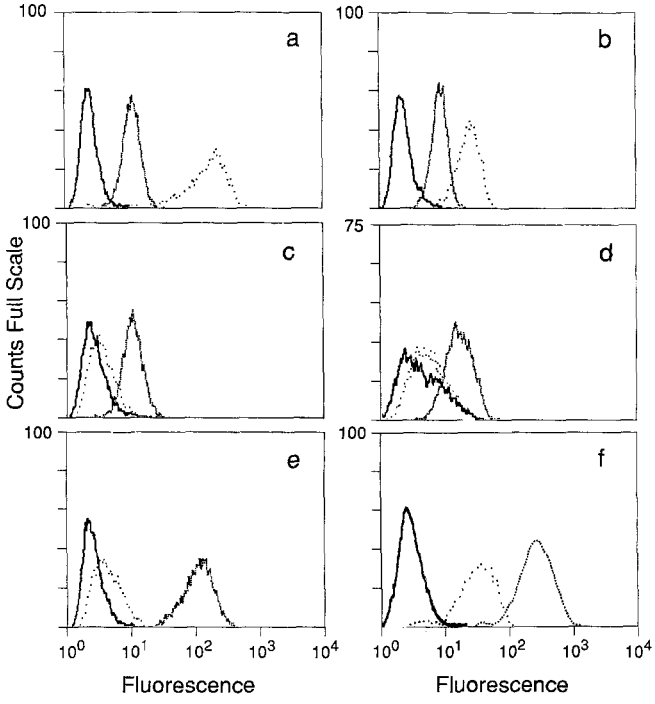

Fig. 1 a–f. Fluorescence-activated cell sorting (FACS) histograms of anti-p185^{HER2} mAb 4D5 (*stippled line*) or anti-(epidermal growth factor receptor) mAb 6C5 (*dotted line*) binding to cell lines. *Solid line* represents background fluorescence [binding of fluorescein-isothiocyanate-labeled goat anti-(mouse Ig) in the absence of murine mAb 4D5 or 6C5]. Cell lines analyzed are: **a** A431 epidermoid carcinoma; **b** HBL-100 mammary epithelial cells; **c** MCF7 breast adenocarcinoma; **d** MDA-MB-175-VII breast ductal carcinoma; **e** MDA-MB-361 breast adenocarcinoma; **f** SK-BR-3 breast adenocarcinoma

Soft agar assays. Assays to determine colony formation in soft agar by tumor cell lines were performed as follows: using 15 × 60-mm tissue-culture dishes, a bottom layer consisting of 4 ml culture medium containing 0.5% purified agar (Difco Laboratories, Detroit, Mich.) was first allowed to solidify. Aliquots of cells (10^5/dish) were added, and 3 ml medium containing 0.25% agar was then layered on top. Experiments were performed in triplicate, with three dishes of each cell type receiving 10 μg 4D5, and three dishes receiving 10 μg irrelevant, isotype-matched antibody, 40.1.H1, directed against the hepatitis B surface antigen [24]. Colonies were counted after a 3- to 6-week incubation using the Omnicon 3600 tumor colony analysis system (Imaging Products International Inc., Chantilly, Va.).

Antibody-dependent cell-mediated cytotoxicity (ADCC). Peripheral blood mononuclear cells (PBMC) were isolated from heparinized whole blood obtained from normal donors by density gradient sedimentation using lymphocyte separation media (Organon Teknika Corp., Durham, N.C.). After three washes with PBS, the cells were resuspended at a density of 3×10^6/ml in culture medium containing 100 units/ml recombinant human interleukin-2 (Boehringer Mannheim, Germany). Following overnight incubation, the PBMC were washed twice with culture medium and serially diluted into 96-well round-bottom microtiter plates to give effector : target ratios of 25 : 1, 12.5 : 1, 6.25 : 1 or 3.13 : 1. Various dilutions of the different antibodies (mumAb 4D5, chmAb 4D5, and (rhu)mAb HER2) or medium were then added. Antibody concentrations used were 1, 10, 100 or 1000 ng/ml. Target cell lines were labeled with 150 μCi $Na^{51}CrO_4$ (Amersham Corp., Arlington Heights, Ill.) for 45 min at 37° C, then washed three times in culture medium. A total of 0.1 ml target cells (10^5/ml) was added per well for a final volume of 0.2 ml/well. The plates were then incubated for 4 h at 37° C, after which the supernatants were harvested and the radioactivity determined in an automatic gamma counter. Percentage specific lysis was calculated as follows: specific lysis (%) = $(A–B/C–B) \times 100$, where A represents ^{51}Cr (cpm) from test supernatants, B represents spontaneous release (^{51}Cr from untreated target cells), and C represents maximum release (^{51}Cr from target cells lysed with 0.4% NP-40). Each treatment was performed in triplicate. Spontaneous release from target cells alone was less than 20% of the maximum for all experiments.

Results

Tumor cell expression of p185^{HER2} as measured by FACS

Cell-surface expression of p185^{HER2} and EGFR was determined for a number of breast tumor cell lines by measuring binding of fluorescein-conjugated anti-(mouse antibody) to cells that had been pretreated with murine monoclonal antibodies specific for the extracellular domains of either p185^{HER2} (mumAb 4D5) or EGFR (mumAb 6C5).

Figure 1 shows histograms representative of the range of p185^{HER2} levels expressed on different breast tumor lines. p185^{HER2} expression is low (arbitrarily designated as 1.0, Table 2) on HBL-100, an immortalized mammary epithelial cell line (Fig. 1 b) and slightly higher on the MCF7 breast tumor cell line (1.2-fold increase, Fig. 1 c). MDA-MB-175-VII breast tumor cells display intermediate levels of p185^{HER2} (4.5-fold higher than HBL-100, Fig. 1 d), whereas both MDA-MB-361 and SK-BR-3 breast tumor cell lines are high overexpressors (16.7-fold and 33.0-fold greater than HBL-100, Fig. 1 e and f respectively). The A431 epidermoid carcinoma line, which overexpresses EGFR (approximately 2×10^6 receptors/cell [17]), was used as a positive control for measuring EGFR (Fig. 1 a). The data obtained for each of the cell lines indicate distinct profiles for the anti-EGFR and anti-p185^{HER2} monoclonal antibodies, and support our earlier conclusion [24] that mumAb 4D5 specifically recognizes p185^{HER2}.

Figure 2 summarizes all the FACS data obtained from cell lines with mumAb 4D5. These data are arranged according to tumor type from which the cell line was derived. One submaxillary (A253), one gastric (Hs746T), two ovarian (Caov-4 and NIH : OVCAR-3), and all renal carcinoma cell lines tested were negative for p185^{HER2} expression. The majority of the colon and ovarian tumor cell lines tested, as well as the two rectal adenocarcinoma lines, displayed low levels of p185^{HER2}. Moderate amounts of p185^{HER2} were exhibited on two other breast tumor cell lines (MDA-MB-436 and ZR-75-1), on several colon lines (e.g. SW948), and on the gastric carcinoma cell line, KATO III. In addition, the colon lines designated COLO 201 and SW1417 demonstrated higher levels of p185^{HER2}. However, striking overexpression of p185^{HER2} was measured on SK-OV-3 ovarian carcinoma cells, MKN-7 gastric carcinoma cells, and four breast tumor cell lines (MDA-MB-453, MDA-MB-361, BT474, and SK-BR-3). The proportion of cell lines overexpressing p185^{HER2} for each tumor type coincides well with the percentages of tumors with HER2 amplification and overexpression reported in a number of different patient populations [4, 28, 40, 56, 59, 63].

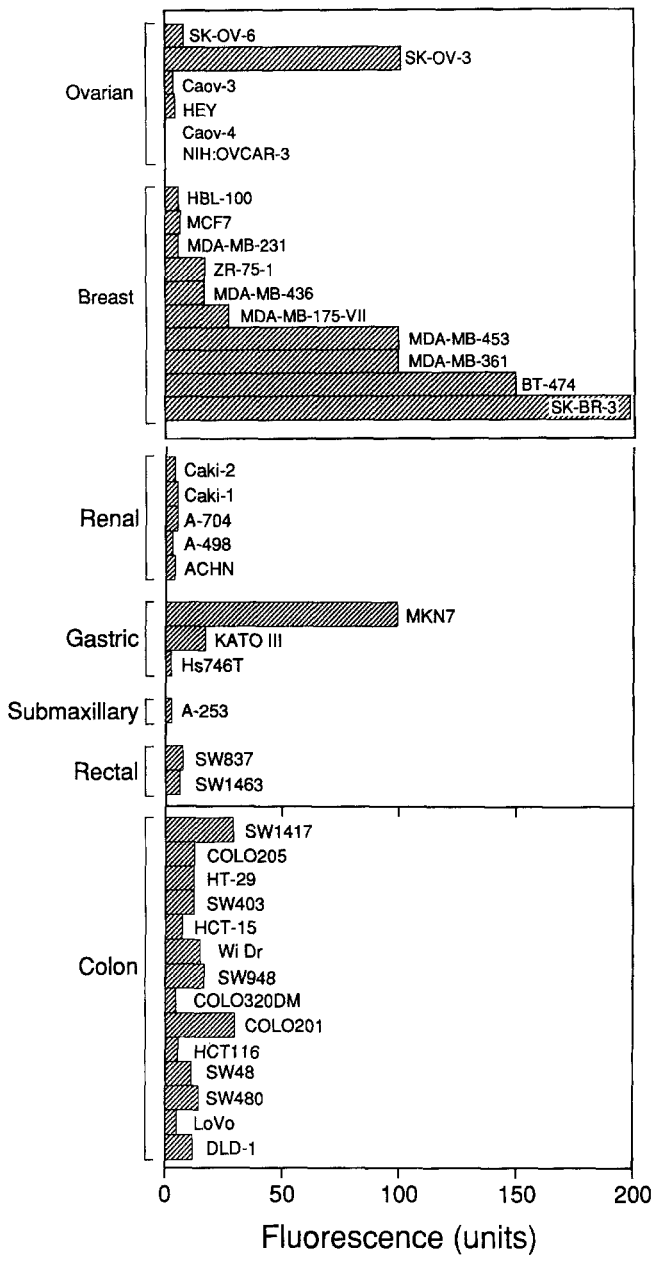

Fig. 2. Summary of p185^{HER2} expression on different types of tumor cell lines as measured by FACS analysis. *Bars* (fluorescence units) represent peak fluorescence intensity in the presence of mumAb 4D5 minus background fluorescence

Table 1. Measurement of p185^{HER2} and epidermal growth factor receptor (EGFR) by enzyme-linked immunosorbent assay (ELISA)[a]

Cell type	Cell line	p185^{HER2} (ng/mg protein)	EGFR (ng/mg protein)
Breast	HBL-100	5.89	242.41
	MCF1	7.27	1.25
	MDA-MB-231	5.40	168.02
	MDA-MB-175-VII	17.48	42.07
	MDA-MB-453	43.73	1.47
	BT474	547.65	116.02
	SK-BR-3	917.27	123.82
	MDA-MB-468	ND[b]	653.55
Ovarian	SK-OV-3	537.26	328.03
Gastric	HS746t	3.31	156.77
	KATO III	10.39	132.31
	MKN7	194.10	340.77
Colon	SW1417	10.12	359.28
	COLO 205	1.87	68.97
	HT-29	1.89	228.08
	SW403	3.11	44.49
	HCT-15	3.81	262.53
	WiDr	1.28	143.31
	SW948	43.23	359.44
	COLO 320DM	1.65	2.93
	COLO 201	11.25	79.69
	HCT116	2.86	227.49
	SW48	12.73	1255.18
	SW480	1.95	184.30
	LoVo	7.65	314.34
	DLD-1	10.96	392.75
Rectal	SW837	5.85	165.95
	SW1463	4.40	307.73

[a] Cells were lysed in 0.5% NP-40 and aliquots were assayed for p185^{HER2} or EGFR levels by specific ELISA, and for protein by the Micro BCA* assay

[b] ND, not detectable

Measurement of p185^{HER2} by ELISA

Levels of p185^{HER2} on these different tumor cell lines were also measured by the ELISA method. These results correspond well with the FACS data, with a few discrepancies. Cell lines overexpressing p185^{HER2}, such as SK-OV-3, BT474, and SK-BR-3, show higher levels of the receptor, relative to HBL-100, in the ELISA compared to the amounts measured by FACS analysis (Table 1). In contrast, relative differences in p185^{HER2} levels in the moderate overexpressors, as described in the previous section, were less pronounced when measured by ELISA. These differences might be due to the types of assays used. The FACS analyses measured surface expression of p185^{HER2} on live cells, whereas the ELISA measured total p185^{HER2} in cell lysates. It is hoped that further work will allow us to distinguish whether different tumor cell types may have varying quantities of intracellular receptor, or if some differences may be due to slight differences in cell growth conditions.

Growth inhibition of tumor cells by anti-p185^{HER2} monoclonal antibodies

We previously reported that anti-p185^{HER2} monoclonal antibodies have antiproliferative activity on the SK-BR-3 breast tumor cell line [24]. From the FACS data shown in Fig. 2, we selected all of the breast carcinoma lines, the SK-OV-3 ovarian carcinoma line, the MKN7 and KATO III gastric carcinoma lines and two colon carcinoma cell lines, SW1417 and COLO 201, for more detailed growth inhibition studies using a variety of different monoclonal antibodies that recognize the extracellular domain of p185^{HER2}. Cell lines were incubated with 10 μg/ml each antibody for 5 days and proliferation was compared to that of untreated control cells.

Table 2. Effect of anti-p185^{HER2} mAb on the growth of human tumor cell lines[a]

Cell line	Relative p185^{HER2} expression[b]	Relative cell proliferation (% of control)					
		4D5	3H4	2C4	7F3	7C2	6E9
184	1.0	116	114	109	116	117	103
184A1	0.3	100	110	103	106	104	110
184B5	0.8	108	107	105	108	108	106
HBL-100	1.0	104	102	103	96	104	105
MCF7	1.2	101	113	100	111	112	105
MDA-MB-231	1.2	91	100	93	98	104	013
ZR-75-1	3.3	102	105	99	97	108	97
MDA-MB-436	3.3	97	91	98	93	92	101
MDA-MB-175	4.5	62	77	29	48	87	96
MDA-MB-453	16.7	61	65	88	80	70	101
MDA-MB-361	16.7	63	67	64	76	105	99
BT474	25.0	27	29	60	21	78	91
SK-BR-3	33.0	33	40	73	51	82	89
SK-OV-3	16.7	77	85	87	91	97	99
MKN7	16.7	99	102	103	111	106	108
KATO III	5.0	91	102	101	98	107	99
COLO201	8.3	107	132	123	125	122	110
SW1417	6.7	98	97	99	100	98	96

[a] Cells were seeded in 96-well microtiter plates and allowed to adhere before the addition of different anti-p185^{HER2} mAb at a final concentration of 10 μg/ml. Monolayers were stained with crystal violet dye after 5 days for determination of relative cell proliferation. Each group consisted of 8–16 replicates, with the coefficient of variation for each group always less than 12%

[b] Levels of p185^{HER2} expression, measured by fluorescence-activated cell sorting, relative to the normal mammary epithelial cell line 184

Table 2 compares the antiproliferative activity of the different antibodies with the relative levels of p185^{HER2} expression as determined by FACS assay. Growth of the normal human mammary epithelial line, 184, was slightly stimulated by the different anti-p185^{HER2} antibodies, whereas the growth of the immortalized mammary epithelial cell lines, 184A1, 184B5, and HBL-100, was unaffected by treatment with these antibodies. For the breast tumor cell lines, there is a clear relationship between the level of HER2 protooncogene expression and sensitivity to the growth-inhibitory effects of the antibodies. It appears that cell lines with more than 4-fold overexpression (relative to 184 or HBL-100 cells) are growth-inhibited. For example, growth of cells showing little expression (e.g., MCF7, MDA-MB-231, ZR-75-1 and MDA-MB-436) is not inhibited by the monoclonal antibodies. Accordingly, cell lines with higher levels of p185^{HER2} (MDA-MB-175-VII, MDA-MB-453, MDA-MB-361) are increasingly more sensitive to antibody-mediated growth inhibition. Hence, SK-BR-3 and BT474 cells, which express abundant amounts of p185^{HER2}, are the most sensitive to the antiproliferative effects (showing approximately 70% growth inhibition).

This work also indicates that coexpression of EGFR and p185^{HER2} may not be required for expression of the growth-inhibitory activity of p185^{HER2} since the MDA-MB-453 and MDA-MB-361 cell lines express very little EGFR (Table 1, Fig. 1). The data in Table 2 also demonstrate that antibodies that share a common epitope have a similar spectrum of activity. Antibodies mumAb 4D5 and mumAb 3H4 compete for binding to the extracellular domain of p185^{HER2} [16], and elicit almost identical responses on each tumor cell line. Similarly, antibodies mumAb 2C4, mumAb 7F3, and mumAb 7C2 show overlapping activities corresponding to their shared epitopes [16]. Furthermore, these results suggest that mumAb 4D5 has the most consistent growth-inhibitory activity toward various breast tumor cell lines.

The apparent correlation between HER2 expression and susceptibility to antibody-induced growth inhibition in monolayer culture does not persist for the MKN7, KATO III, COLO 201 or SW1417 cell lines. SK-OV-3 cells, although somewhat sensitive to the growth-inhibitory effects of mumAb 4D5, appear to be less sensitive than would be expected from their level of p185^{HER2} expression (Table 2). Moreover, the KATO III gastric line and the SW1417 and COLO 201 colon lines, with levels of receptor expression equal to or greater than the MDA-MB-175-VII breast tumor line, are completely resistant to the antiproliferative effects of mumAb 4D5 or mumAb 3H4. In fact, growth of the COLO 201 cells appears to be stimulated by several of the antibodies (e.g., mumAb 3H4 and mumAb 7F3, Table 2). The MKN7 gastric carcinoma line, which has a 17-fold overexpression of surface receptor (similar to the breast tumor cell lines MDA-MB-361, or MDA-MB-453), is also resistant to growth inhibition by the monoclonal antibodies. The reason for this discrepancy is still not understood. Labeling of these tumor cells with [^{35}S]methionine followed by immunoprecipitation showed the receptors to be of the expected size and amount (data not shown). Figure 3 graphically displays the results from Table 2, clearly depicting the relationship between p185^{HER2} expression and growth inhibition by the monoclonal antibodies on the breast tumor cell lines, as well as the resistance of the other tumor cell lines to the different antibodies. In this figure, the cell lines with less than the expected extent of growth inhibition by mumAb 4D5 appear above the "best-fit" line derived from the data obtained using breast tumor cells expressing increasing levels of p185^{HER2}.

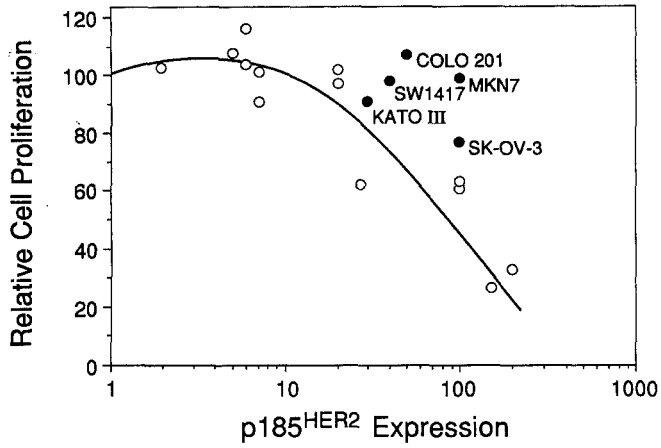

Fig. 3. Relationship between p185^{HER2} expression and growth inhibition mediated by mumAb 4D5 on human breast cell lines, normal and tumor (○), and other types of tumor cell lines overexpressing p185^{HER2} (●)

Several investigators have previously described the inability of anti-*neu* or anti-p185^{HER2} antibodies to inhibit the monolayer growth of NIH 3T3 cells transformed with either the activated c-*neu* or overexpressed HER2 protooncogene [13, 24]. However, anchorage-independent growth in soft agar was abrogated in cultures treated with the appropriate monoclonal antibodies [13, 24]. Therefore, we performed soft-agar growth assays on the same tumor cell lines studied in monolayer assays (Table 3). The mammary epithelial line 184B5 did not form colonies in soft agar. Although the HBL-100 line is also an immortalized mammary epithelial line, anchorage-independent growth was observed (also documented by the American Type Culture Collection). Treatment with mumAb 4D5, however, had no effect on HBL-100 colony formation. Tumor cell lines expressing low levels of p185^{HER2} (MCF7, MDA-MB-231, ZR-75-1, MDA-MB-436) formed equivalent numbers of colonies in soft agar in the presence or absence of mumAb 4D5, as expected. Anchorage-independent growth of breast tumor lines expressing high levels of p185^{HER2} (16.7-fold or greater, relative to the 184 line) was inhibited upon treatment with mumAb 4D5 ($P = 0.06$ for MDA-MB-453, $P = 0.008$ for MDA-MB-361, $P = 0.002$ for BT474, and $P = 0.0001$ for SK-BR-3 compared to the respective controls). Complete suppression of colony formation was observed in the SK-BR-3 and BT474 lines. As with the monolayer growth assays, there is an apparent correlation between p185^{HER2} expression and extent of growth inhibition by the anti-p185^{HER2} antibody. In addition, colony formation by the SK-OV-3 ovarian carcinoma line and the SW1417 colon carcinoma line was significantly inhibited by mumAb 4D5 compared to controls ($P = 0.0001$ and $P = 0.0011$ respectively). However, the gastric carcinoma line KATO III and the colon carcinoma COLO 201 were not inhibited by antibody treatment. The MDA-MB-175-VII and MKN7 lines did not form colonies in soft agar under our standard assay conditions.

Table 3. Colony formation in soft agar[a]

Cell line	Colony count after treatment with:		Percentage of control (%)
	0	4D5	
184B5	0	0	–
HBL-100	546±112	694±95	127
MCF7	293±25	290±25	99
MDA-MB-231	923±20	997±32	108
ZR-75-1	805±30	805±32	100
MDA-MB-436	461±30	457±59	99
MDA-MB-453	778±89	518±47	67
MDA-MB-361	35±5	10±1[b]	30
BT474	321±25	0[b]	0
SK-BR-3	462±17	0[b]	0
SK-OV-3	239±7	107±7[b]	45
KATO III	1449±198	1089±144	75
COLO 201	1489±150	1913±465	128
SW1417	1078±31	705±32[b]	65

[a] Cells were seeded onto a bottom layer of 0.5% agar and covered with a top layer containing 0.25% agar with or without 10 μg mumAb 4D5. Colony formation was determined after 3–6 weeks using the Omnicon 3600 tumor colony analysis system. Numbers represent mean colony counts ± SE

[b] Values significantly different from control group (for P values, see text)

Antibody-dependent cellular cytotoxicity mediated by the chimeric and humanized versions of mumAb 4D5

The issue of mechanism of resistance to mumAb 4D5 in non-breast and ovarian tumor cells remains unresolved. However, we hypothesized that antibody-mediated antitumor effects could also be directed by overexpression of p185^{HER2} based upon grafting of the murine hypervariable domains onto the human immunoglobulin framework variable and constant regions. In order to accomplish this, a chimeric mumAb 4D5 (chmAb 4D5) was prepared and employed as a substrate to generate a family of humanized variants, all of which contained the human IgG1 Fc region [17], which should allow for ADCC.

To test this idea we performed ADCC assays on several different tumor cell lines expressing various levels of p185^{HER2} utilizing each of the monoclonal antibodies, mumAb 4D5, chmAb 4D5 and (rhu)mAb HER2. We selected as targets the SK-BR-3 breast tumor cells (sensitive to mumAb 4D5, with high levels of p185^{HER2}), MKN7 gastric tumor cells (resistant to mumAb 4D5, also with high levels of p185^{HER2}), COLO 201 colon tumor cells (resistant to mumAb 4D5, with moderate p185^{HER2} levels), MCF7 breast tumor cells (resistant to mumAb 4D5, with only slightly elevated membrane expression of p185^{HER2}), and the mammary epithelial cell line 184A1 (resistant to mumAb 4D5, with low receptor expression). The growth response to mumAb 4D5 and levels of p185^{HER2} expression on each cell type are summarized in Table 2.

The chimeric and humanized 4D5 molecules have similar antiproliferative activities on SK-BR-3 cells (57% and 60% inhibition of growth respectively), as compared to mumAb 4D5 (G. D. Lewis, unpublished data). However, the combination of human PBMC and (rhu)mAb HER2 caused lysis of the tumor cells as a function of their level of p185^{HER2} surface expression (Fig. 4). The normal mam-

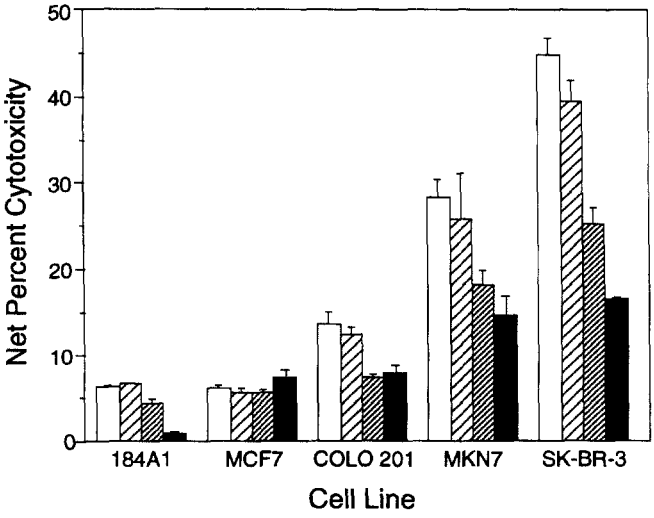

Fig. 4. Antibody-dependent cellular cytotoxicity directed against selected tumor cell lines by recombinant human (rhu)mAb HER2 in the presence of human peripheral blood mononuclear cells. ^{51}Cr-labeled target cells (10^4/well) were incubated for 4 h with different dilutions of effector cells to give effector:target ratios of 25:1 (*open bars*), 12.5:1 (*wide hatched bars*), 6.25:1 (*small hatched bars*) and 3.13:1 (*solid bars*). Incubations were carried out in the presence or absence of 0.1 μg/ml (rhu)mAb HER2. Net percentage cytotoxicity represents cytotoxicity in the presence of humAb HER2 minus nonspecific cytotoxicity (no antibody). Values shown are means ± SE

mary epithelial line 184A1 and MCF7 breast tumor cells are relatively resistant to cytolytic attack, while the tumor cells with increasing levels of p185^{HER2} demonstrate increasing sensitivity to ADCC mediated by (rhu)mAb HER2 as measured by release of chromium-51. A statistically significant relationship was observed between the net percentage cytotoxicity and level of HER2 expression at all four effector:target ratios. The slope of the fitted line was significantly different (P <0.001) from zero in all cases (r^2 values of 0.93, 0.92, 0.87, and 0.66 for effector:target ratios 25:1, 12.5:1, 6.25:1, and 3.13:1 respectively). The chmAb 4D5 antibody appeared equivalent in cytolytic activity with humAb HER2 on the different cell lines, while the mumAb 4D5 control, which lacks effector function in this assay, is unable to mediate cytotoxicity (data not shown).

Discussion

Amplification and overexpression of the HER2 protooncogene has been demonstrated in a number of human malignancies and human tumor cell lines. Shortened time to relapse and decreased overall survival are correlated with HER2 protooncogene amplification and overexpression in subsets of breast, ovarian and several other cancers [4, 5, 20, 21, 29, 50, 55, 56, 59, 66, 71]. Because p185^{HER2} is a membrane-associated growth factor receptor with an extracellular ligand-binding domain, it represents an attractive therapeutic target for monoclonal antibody therapy of human cancers characterized by its overexpression.

We have previously shown that anti-p185^{HER2} monoclonal antibodies inhibit monolayer growth of several breast tumor cell lines that overexpress p185^{HER2} [24]. In the present report, we have extended work in this area to include a variety of different tumor cell lines. For the breast tumor lines tested, the level of p185^{HER2} overexpression was a predictor of the degree of growth inhibition elicited by the anti-p185^{HER2} monoclonal antibodies. This relationship between overexpression and antibody-mediated antiproliferative activity was not found on the other tumor cell lines examined. Assays for anchorage-independent growth were also performed, as this technique presents a more rigorous method for studying neoplastic behavior in cell culture. Previous studies revealed that NIH 3T3 cells transfected with either the HER2 protooncogene or the *neu* oncogene were not growth-inhibited in monolayer culture by the tested monoclonal antibodies, although anchorage-independent growth in soft agar of the same cells was profoundly inhibited by the anti-p185^{HER2} or anti-*neu* antibodies [13, 14, 24]. In the experiments reported here mumAb 4D5 inhibited colony formation in soft agar of breast and ovarian tumor cell lines that overexpress p185^{HER2}, but had no effect on soft agar growth of either a colon or gastric carcinoma cell line also expressing this receptor.

Our experiments in soft agar or monolayer culture demonstrate growth-inhibitory activity of the antibodies, especially mumAb 4D5, on breast and ovarian adenocarcinoma tumor cell lines that overexpress p185^{HER2}. The sensitivity of breast tumor cell lines to antibody-mediated growth inhibition correlates well with their level of p185^{HER2} overexpression. MumAb 4D5 has also been shown to enhance sensitivity to tumor necrosis factor on breast tumor cells overexpressing this receptor [24]. However, their application appears to be limited on other tumor cell types. It was our hope that chimerizing or humanizing mumAb 4D5 with a human IgG1 constant region would enable the engineered monoclonal antibody to mediate immune cell killing of tumor targets overexpressing p185^{HER2}, and that this activity would be dependent upon overexpression of the receptor. Both humanized and chimeric versions of other antibodies have been utilized successfully in cytolytic assays on appropriate target cells [27, 37, 42]. Chimerization or humanization of murine antibodies is also desirable for reasons related to immunogenicity of the murine parent antibody in human patients [22, 52]. Cytotoxicity assays were performed with human PBMC using either humanized or chimeric monoclonal 4D5. Our ADCC assays showed cytotoxic activity on tumor cell lines tested in relation to their levels of p185^{HER2} expression, including the gastric and colon carcinoma cell lines that were resistant to direct growth inhibition by mumAb 4D5. The chimeric and humanized 4D5 antibodies could also distinguish between normal cells expressing low levels of the receptor and tumor cells expressing only moderately elevated levels of p185^{HER2}. These results suggest that antibody-mediating killing of tumor cells may depend upon increased levels of receptor expression and spontaneous clustering of p185^{HER2}, which subsequently triggers effector cell cytotoxic mechanisms.

It is unclear at this time why particular types of p185^{HER2}-overexpressing tumor cells are unresponsive to growth inhibition by the anti-p185^{HER2} monoclonal anti-

bodies. Because our FACS analyses measured only the ability of cells to bind mumAb 4D5 to their surface, whole-cell lysates were also analyzed by ELISA. The relative levels of $p185^{HER2}$ measured in this way were similar to our FACS data. We have previously investigated binding of iodinated mumAb 4D5 to obtain more accurate measurements of receptor number as well as receptor affinity [44]. Of the cell lines tested, the number of mumAb 4D5 binding sites agreed well with the FACS analyses. Furthermore, there appeared to be no alterations in receptor affinity that could account for the lack of biological response in these cell lines. Future studies will be aimed at deciphering the mechanism(s) by which $p185^{HER2}$-overexpressing tumor cells respond or display resistance to mumAb 4D5.

The application of molecular engineering to the humanization of murine monoclonal antibodies allows for the large-scale production of antigen-specific targeting molecules that can be designed to deliver therapeutic agents with the specificity of the parent molecule, but with unique properties distinct from those of the murine parent. In this work, we have described the ability of humanized variants of mumAb 4D5 to mediate antibody-dependent cytotoxicity against tumor cells that overexpress $p185^{HER2}$ protooncogene, regardless of their ability to be growth-inhibited by the parent monoclonal antibody. The engineered versions of the antibodies may, therefore, offer the opportunity of treating cancers expressing a wide range of $p185^{HER2}$. The high level expression of other unique variants of mumAb 4D5 in prokaryotic systems, including the possibility of constructing bispecific monoclonal antibodies [51], will add to our repertoire of possible therapeutic agents directed against human cancers characterized by amplification of the HER2 protooncogene.

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References

1. Aasland R, Lillehaug JR, Hale R, et al (1988) Expression of oncogenes in thyroid tumors: coexpression of c-*erb*B2/*neu* and c-*erb*B. Br J Cancer 57: 358–363
2. Akiyama T, Sudo C, Ogawara H, et al (1986) The product of the human c-*erb*B-2 gene: a 185-kilodalton glycoprotein with tyrosine kinase activity. Science 232: 1644–1646
3. Benz CC, Scott GK, Sarup JC, et al (1991) Tamoxifen resistance associated with $p185^{HER2}$ overexpression in human breast cancer cells transfected with HER2/*neu*. Proc Am Assoc Cancer Res 32: 211
4. Berchuck A, Kamel A, Whitaker R, et al (1990) Overexpression of HER-2/*neu* is associated with poor survival in advanced epithelial ovarian cancer. Cancer Res 50: 4087–4091
5. Berchuck A, Rodriguez G, Kinney RB, et al (1991) Overexpression of HER-2/*neu* in endometrial cancer is associated with advanced stage disease. Am J Obstet Gynecol 164: 15–21
6. Berger MS, Locher GW, Saurer S, et al (1988) Correlation of c-*erb*B-2 gene amplification and protein expression in human breast carcinoma with nodal status and nuclear grading. Cancer Res 48: 1238–1243
7. Bonilla M, Ramirez M, Lopez-Cueto J, et al (1988) In vivo amplification and rearrangement of c-*myc* oncogene in human breast tumors. JNCI 80: 665–671
8. Borst MP, Baker VV, Dixon D, et al (1990) Oncogene alterations in endometrial cancer. Gynecol Oncol 38: 364–366
9. Brodeur GM, Seeger RC, Schwab M, et al (1984) Amplification of N-*myc* in untreated human neuroblastomas correlates with advanced disease stage. Science 224: 1121–1124
10. Brüderlein S, van der Bosch K, Schlag P, et al (1990) Cytogenetics and DNA amplification in colorectal cancers. Genes Chromosomes Cancer 2: 63–70
11. Carter P, Presta L, Gorman CM, et al (1993) Humanization of an anti-$p185^{HER2}$ antibody for human cancer therapy. Proc Natl Acad Sci USA (in press)
12. Coussens L, Yang-Feng TL, Liao Y-C, et al (1985) Tyrosine kinase receptor with extensive homology to EGF receptor shares chromosomal location with *neu* oncogene. Science 230: 1132–1139
13. Drebin JA, Link VC, Stern DF, et al (1985) Down-modulation of an oncogene protein product and reversion of the transformed phenotype by monoclonal antibodies. Cell 41: 695–706
14. Drebin JA, Link VC, Greene MI (1988) Monoclonal antibodies specific for the *neu* oncogene product directly mediate anti-tumor effects in vivo. Oncogene 2: 387–394
15. Drebin JA, Link VC, Greene MI (1988) Monoclonal antibodies reactive with distinct domains of the *neu* oncogene-encoded p185 molecule exert synergistic antitumor effects in vivo. Oncogene 2: 273–277
16. Fendly BF, Winget M, Hudziak RM, et al (1990) Characterization of monoclonal antibodies reactive to either the human epidermal growth factor receptor or HER2/*neu* gene product. Cancer Res 50: 1550–1558
17. Fernandez-Pol JA (1985) Epidermal growth factor receptor of A431 cells. Characterization of a monoclonal anti-receptor antibody noncompetitive agonist of epidermal growth factor action. J Biol Chem 260: 5003–5011
18. Freeman MR, Washecka R, Chung LWK (1989) Aberrant expression of epidermal growth factor receptor and HER-2 (*erb*B-2) messenger RNAs in human renal cancers. Cancer Res 49: 6221–6225
19. Fukushige S-I, Matsubara K-I, Yoshida M, et al (1986) Localization of a novel v-*erb*B-related gene, c-*erb*B-2, on human chromosome 17 and its amplification in a gastric cancer cell line. Mol Cell Biol 6: 955–958
20. Guérin M, Barrois M, Terrier M-J, et al (1988) Overexpression of either c-*myc* or c-*erb*B-2/*neu* protooncogenes in human breast carcinomas: correlation with poor prognosis. Oncogene Res 3: 21–31
21. Guérin M, Gabillot M, Mathieu M-C, et al (1989) Structure and expression of c-*erb*B-2 and EGF receptor genes in inflammatory and non-inflammatory breast cancer: prognostic significance. Int J Cancer 43: 201–208
22. Hama-Schroff RW, Foon KA, Beatty SM, et al (1985) Human anti-murine immunoglobulin responses in patients receiving monoclonal antibody therapy. Cancer Res 45: 879–885
23. Hudziak RM, Lewis GD, Shalaby MR, et al (1988) Amplified expression of the HER2/*erb*B-2 oncogene induces resistance to tumor necrosis factor α in NIH 3T3 cells. Proc Natl Acad Sci USA 85: 5102–5106
24. Hudziak RM Lewis GD, Winget M, et al (1989) $p185^{HER2}$ monoclonal antibody has antiproliferative effects in vitro and sensitizes human breast tumor cells to tumor necrosis factor. Mol Cell Biol 9: 1165–1172
25. Hynes NE, Gerber HA, Saurer S, et al (1989) Overexpression of the c-*erb*B-2 protein in human breast tumor cell lines. J Cell Biochem 39: 167–173
26. Johnson BE, Makuch RW, Simmons AD, et al (1988) *myc* family DNA amplification in small cell lung cancer patients tumors and corresponding cell lines. Cancer Res 48: 5163–5166
27. Junghans RP, Walmann TA, Landolfi NF, et al (1990) Anti-Tac-H, a humanized antibody to the interleukin 2 receptor with new features for immunotherapy in malignant and immune disorders. Cancer Res 50: 1495–1502

28. Kameda T, Yasui W, Yoshida K, et al (1990) Expression of *erb*B2 in human gastric carcinomas: relationship between $p185^{erbB2}$ expression and gene amplification. Cancer Res 50: 8002–8009
29. Kern JA, Schwartz DA, Nordberg JE, et al (1990) $p185^{neu}$ expression in human lung adenocarcinomas predicts shortened survival. Cancer Res 50: 5184–5191
30. King CR, Kraus MN, Aaronson SA (1985) Amplification of a novel v-*erb*B-related gene in a human mammary carcinoma. Science 229: 974–976
31. Krauss MH, Popescu NC, Amsbaugh SC, et al (1987) Overexpression of the EGF receptor-related proto-oncogene *erb*B-2 in human mammary tumor cell lines by different molecular mechanisms. EMBO J 6: 605–610
32. Kraus MH, Issing W, Miki T, et al (1989) Isolation and characterization of *ERBB3*, a third member of the "*ERBB*"/epidermal growth factor receptor family: evidence for overexpression in a subset of human mammary tumors. Proc Natl Acad Sci USA 86: 9193–9197
33. Lee W-H, Murphree AL, Benedict WF (1984) Expression of the N-*myc* gene in primary retinoblastoma. Nature 309: 458–460
34. Liberman TA, Nusbaum HR, Razon N, et al (1985) Amplification, enhanced expression and possible rearrangement of EGF receptor gene in primary human tumors of glial origin. Nature 313: 144–147
35. Lichtenstein A, Berenson J, Gera JF, et al (1990) Resistance of human ovarian cancer cells to tumor necrosis factor and lymphokine-activated killer cells: correlation with expression of HER2/*neu* oncogenes. Cancer Res 50: 7364–7370
36. Little CD, Nau MM, Carney DN, et al (1983) Amplification and expression of the c-*myc* oncogene in human lung cancer cell lines. Nature 306: 194–196
37. Liu AY, Robinson RR, Hellström KE, et al (1987) Chimeric mouse-human IgG1 antibody that can mediate lysis of cancer cells. Proc Natl Acad Sci USA 84: 3439–3443
38. Merlino GT, Xu Y-H, Ishii S, et al (1984) Amplified and enhanced expression of the epidermal growth factor receptor gene in A431 human carcinoma cells. Science 224: 417–419
39. Moriyama M, Akiyama T, Yamamoto T, et al (1991) Expression of C-ERBB-2 gene product in urinary bladder cancer. J Urol 145: 423–427
40. Park J-B, Rhim JS, Park S-C, et al (1989) Amplification, overexpression and rearrangement of the *erb*B-2 protooncogene in primary human stomach carcinomas. Cancer Res 49: 6605–6609
41. Perez R, Pascual M, Macias A, et al (1984) Epidermal growth factor receptors in human breast cancer. Breast Cancer Res Treat 4: 189–193
42. Reichmann L, Clark M, Waldmann H, et al (1988) Reshaping human antibodies for therapy. Nature 332: 323–329
43. Ro J, North SM, Gallick GE, et al (1988) Amplified and overexpressed epidermal growth factor receptor gene in uncultured primary human breast carcinoma. Cancer Res 48: 161–164
44. Sarup JC, Johnson RM, King KL, et al (1991) Characterization of an anti-$p185^{HER2}$ monoclonal antibody that stimulates receptor function and inhibits tumor cell growth. Growth Regul 1: 72–82
45. Schechter AL, Stern DF, Vaidyanathan L, et al (1984) The *neu* oncogene: an *erb*B-related gene encoding a 185,000-M_r tumor antigen. Nature 312: 513–516
46. Schechter AL, Hung M-C, Vaidyanathan L, et al (1985) The *neu* gene: an *erb*B-homologous gene distinct from and unlinked to the gene encoding the EGF receptor. Science 229: 976–978
47. Schneider PM, Hung M-C, Chiocca SM, et al (1989) Differential expression of the c-*erb*B-2 gene in human small cell and non-small cell lung cancer. Cancer Res 49: 4968–4971
48. Seeger RC, Brodeur GH, Sather H, et al (1985) Association of multiple copies of the N-*myc* oncogene with rapid progression of neuroblastomas. N Engl J Med 313: 1111–1116
49. Semba K, Kamato N, Toyoshima K, et al (1985) A v-*erb*B-related protooncogene, c-*erb*B-2 is distinct from the c-*erb*B-1/epidermal growth factor receptor gene and is amplified in a human salivary gland adenocarcinoma. Proc Natl Acad Sci USA 82: 6497–6501
50. Seshadri R, Matthews C, Dobrovic A, et al (1989) The significance of oncogene amplification in primary breast cancer. Int J Cancer 43: 270–272
51. Shalaby MR, Shepard HM, Presta L, et al (1992) The development of humanized bispecific antibodies reactive with cytotoxic lymphocytes and tumor cells overexpressing the *HER2* protooncogene. J Exp Med 175: 217–225
52. Shawler DL, Bartholomew RM, Smith LM, et al (1985) Human immune response to multiple injections of murine monoclonal IgG. J Immunol 135: 1530–1535
53. Sias PE, Kotts CE, Vetterlein D, et al (1990) ELISA for quantitation of the extracellular domain of $p185^{HER2}$ in biological fluids. J Immunol Methods 132: 73–80
54. Slamon DJ, DeKernion JB, Verma IM, et al (1984) Expression of cellular oncogenes in human malignancies. Science 224: 256–262
55. Slamon DJ, Clark GM, Wong SG, et al (1987) Human breast cancer: correlation of relapse and survival with amplification of the HER-2/*neu* oncogene. Science 235: 177–182
56. Slamon DJ, Godolphin W, Jones LA, et al (1989) Studies of the HER-2/*neu* protooncogene in human breast and ovarian cancer. Science 244: 707–712
57. Stern DF, Heffernan PA, Weinberg RA (1986) p185, a product of the *neu* protooncogene, is a receptorlike protein associated with tyrosine kinase activity. Mol Cell Biol 6: 1729–1740
58. Tal M, Wetzler M, Josefberg Z, et al (1988) Sporadic amplification of the HER2/*neu* protooncogene in adenocarcinomas of various tissues. Cancer Res 48: 1517–1520
59. Tandon AK, Clark GM, Cuamness GC, et al (1989) HER2/*neu* oncogene protein and prognosis in breast cancer. J Clin Oncol 7: 1120–1128
60. Ullrich A, Coussens L, Hayflick JS, et al (1984) Human epidermal growth factor receptor cDNA sequence and aberrant expression of the amplified gene in A431 epidermoid carcinoma cells. Nature 309: 418–425
61. van de Vijver M, van de Bersselaar R, Devilee P, et al (1987) Amplification of the *neu* (c-*erb*B-2) oncogene in human mammary tumors is relatively frequent and is often accompanied by amplification of the linked c-*erb*A oncogene. Mol Cell Biol 7: 2019–2023
62. van de Vijver MJ, Peterse JL, Mool WJ, et al (1988) *Neu* protein overexpression in breast cancer. Association with comedo-type ductal carcinoma in situ and limited prognostic value in stage II breast cancer. N Engl J Med 319: 1239–1245
63. Venter DJ, Kumar S, Tuzi NL, et al (1987) Overexpression of the c-*erb*B-2 oncoprotein in human breast carcinomas: immunohistological assessment correlates with gene amplification. Lancet 2: 69–72
64. Weinberg RA (1984) *ras* oncogenes and the molecular mechanisms of carcinogenesis. Blood 64: 1143–1145
65. Williams TN, Weiner DB, Greene MI, et al (1991) Expression of c-erbB-2 in human pancreatic adenocarcinomas. Pathobiology 59: 46–52
66. Wright C, Angus B, Nicholson S, et al (1989) Expression of c-*erb*B-2 oncoprotein: a prognostic indicator in human breast cancer. Cancer Res 49: 2087–2090
67. Yamamoto T, Kamato N, Kawano N, et al (1986) High incidence of amplification of epidermal growth factor receptor gene in human squamous carcinoma cell line. Cancer Res 46: 414–416
68. Yamazaki H, Fukui Y, Ueyama Y, et al (1988) Amplification of the structurally and functionally altered epidermal growth factor receptor gene (c-*erb*) in human brain tumors. Mol Cell Biol 8: 1816–1820
69. Yao M, Shuin T, Misaki H, et al. (1988) Enhanced expression of c-*myc* and epidermal growth factor receptor (c-*erb*B1) genes in primary human renal cancer. Cancer Res 48: 6753–6757
70. Yokota J, Yamamoto T, Miyajima N, et al (1988) Genetic alterations of the c-*erb*B-2 oncogene occur frequently in tubular adenocarcinoma of the stomach and are often accompanied by amplification of the v-*erb*A homologue. Oncogene 2: 283–287
71. Yonemura Y, Ninomiya I, Yamaguchi A, et al (1991) Evaluation of immunoreactivity for erbB-2 protein as a marker of poor short term prognosis in gastric cancer. Cancer Res 51: 1034–1038
72. Zhou D, Battifora H, Yokota J, et al (1987) Association of multiple copies of the c-*erb*B-2 oncogene with spread of breast cancer. Cancer Res 47: 6123–6125