Characterization of Murine Monoclonal Antibodies Reactive to Either the Human Epidermal Growth Factor Receptor or HER2/neu Gene Product

Brian M. Fendly, Marcy Winget, Robert M. Hudziak, Michael T. Lipari, Mary Anna Napier, and Axel Ullrich

ABSTRACT

High levels of expression of either the epidermal growth factor receptor or the receptor-like HER2/neu gene product p185HER2 have been observed in a variety of human malignancies. Because of the association of this high level expression with certain human tumors, we have generated a panel of monoclonal antibodies specific for either the epidermal growth factor receptor or p185HER2 to study their structure, function, and antigenic domains in the normal and neoplastic states. We used the epidermoid carcinoma line A431 to generate five monoclonal antibodies which immunoprecipitate the epidermal growth factor receptor. These monoclonal antibodies bind to the extracellular domain of the epidermal growth factor receptor and demonstrate variable effects on epidermal growth factor binding. We used a stably transfected NIH 3T3 cell line expressing the HER2/neu gene to produce and characterize 10 monoclonal antibodies which immunoprecipitate p185HER2. These monoclonal antibodies bind to the extracellular domain of p185HER2 and do not cross-react with the epidermal growth factor receptor. The characteristics and potential applications of these monoclonal antibodies will be discussed.

INTRODUCTION

Alterations in growth factor receptor structure and level of expression have been implicated in the process of abnormal growth control, transformation, and oncogenesis. Some of these receptors share homology with retroviral oncogenes, have intrinsic tyrosine kinase activity, and are generally referred to as protooncogenes (for review see Ref. 1). Included in this group of receptors is the epidermal growth factor receptor which is the cellular homologue of the avian retrovirus oncogene v-erbB.

The p185HER2 product of the human EGFR related gene (HER2/neu) is a receptor-like tyrosine kinase without any oncogene homologue identified in a retrovirus. The EGFR and p185HER2 are similar but distinct glycoproteins encoded by genes located on human chromosomes 7 (2, 3) and 17 (4), respectively. The EGFR and p185HER2 have approximately 40% homology in their extracellular ligand binding domains and 78% homology in their intracellular ATP binding and tyrosine kinase regions as determined by their predicted primary amino acid sequences (4). The EGFR is a M, 170,000 single chain glycoprotein of 1,186 amino acids with a 621-amino acid, extracellular, NH2-terminal domain (5, 6). This extracellular domain of the EGFR binds the 53-amino acid, M, 6,025 EGF (7) as well as the 50-amino acid, M, 6,000 transforming growth factor α (8). In both cases binding of the growth factor results in activation of the receptor associated tyrosine kinase (9, 10). The p185HER2 receptor is also a single chain, M, 185,000 glycoprotein of 1,255 amino acids with a 632-amino acid, extracellular domain which is thought to bind an unknown ligand (1, 4).

Amplification of expression of the EGFR is associated with human cervical and ovarian carcinomas (11), epidermal squamous cell carcinomas (12), and malignant gliomas (13). Amplification of the HER2/neu gene has been found in mammary gland carcinomas (14–16), and ovarian cancer (17) and is associated with the most aggressive forms of breast and ovarian tumors and poor prognosis from these cancers (17, 18). In addition, amplification of HER2/neu has also been shown in other human adenocarcinomas including salivary gland (19), stomach, and kidney (20). The MAbs to these cell surface glycoproteins and MAbs which are specific to phosphorylated tyrosines were generated to investigate expression of these protooncogenes in tumor cell lines and to study the structure/function relationships and antigenic domains of these receptors. We performed extensive specificity studies on the MAbs raised to either the EGFR or p185HER2 to evaluate shared antigenic sites on these related structures since these MAbs are candidates for the development of receptor based tumor therapies, in vivo and in vitro diagnostics, as well as important reagents for the development of assays to monitor the in vitro effects of receptor modulation.

MATERIALS AND METHODS

Cell Lines and Isolation of Receptors from Cells. The human epidermoid carcinoma A431 cell line (21) was used for isolation of the EGFR (22), since this cell line has been shown to express 2–3 × 107 copies/cell of the EGFR (21, 22–25). A431 cells were grown to confluence at 37°C and 10% CO2 atmosphere in medium consisting of DMEM/Ham’s F-12 (50/50, v/v), 5% fetal calf serum, 15 mM HEPES (pH 7.2), 50 units/ml penicillin, and 10 µg/ml streptomycin. The cells were solubilized in 1% Triton X-100 (26), and the EGFR was partially purified by WGA affinity chromatography. Elution of the receptor was achieved with 40 mM HEPES, pH 7.5, containing 0.3 M N-acetylglucosamine, 0.15 M NaCl, 0.2% Triton X-100, and 10% glycerol. Protein concentration was determined by the method of Bradford (27). The affinity (approximately 20 nmol) of the solubilized receptor and the receptor concentration (approximately 5 pmol receptor/mg protein) were determined by Scatchard analysis of 125I-EGF binding (28). EGF (Sigma, St. Louis, MO) was iodinated by the standard chloramine-T method (29). Tyrosine kinase activity was measured in the presence of 80 µCi/ml [γ-32P]ATP, 10 µM ATP, 4 mM MgCl2, in 2 mM MnCl2 in 20 mM HEPES, pH 7.5, containing 0.1% Triton X-100, and 10% glycerol. HER2/neu transfected NIH 3T3 cells, NIH 3T3/HER2-3400 (30), expressing approximately 1 × 107 p185HER2 molecules/cell (data not shown) were grown to confluence in the above media. The cells were extracted with 1% Triton X-100 and the membrane protein p185HER2 was partially purified by WGA affinity chromatography as described above. The tyrosine kinase activity was measured as above except the concentration of [γ-32P]ATP was 100 µCi/ml and there was no unlabeled ATP in the kinase solution.
Generation of Monoclonal Antibodies Specific for the EGFR. BALB/c mice were immunized with 2–4 × 10⁶ A431 cells in PBS, i.p. on weeks 0, 2, 4, and 6. The immunized mice were tested for an antibody response by immunoprecipitation of [32P]-labeled EGFR. The mice with the highest serum titers were given i.v. injections of a WGA purified A431 membrane extract during week 18. Three days later their spleenocytes were fused with mouse myeloma line X63-Ag8.653 (31), using 50% polyethylene glycol 4000 by the procedure of Oi and Herzenberg (32). Fused cells were plated at a density of 2 × 10⁶ cells/well in 96-well microtiter plates (day 0) and hybridoma selection was begun on day 1 with 10% fetal calf serum in DMEM media containing hypoxanthine/azaserine (33). Beginning on day 10 hybridoma supernatants were screened for the presence of EGFR specific antibodies as described below.

Generation of Monoclonal Antibodies Specific for p185HER2. NIH 3T3/HER2-3400 cells were harvested with PBS containing 25 mm EDTA and were used to immunize BALB/c mice. The mice were given injections i.p. of 10⁶ cells in 0.5 ml PBS on weeks 0, 2, 4, and 6. The mice with antisera that immunoprecipitated [32P]-labeled p185HER2 were given i.v. injections of a WGA purified p185HER2 membrane extract on weeks 9 and 13. This was followed by an i.v. injection of 0.1 ml of the p185HER2 preparation and the spleenocytes were fused as described for the anti-EGFR fusions. The hybridoma supernatants were screened by ELISA as described below.

Enzyme-linked Immunosorbent Assays. ELISAs were performed as initial screens of fusions, to monitor the purification of the anti-p185HER2 and anti-EGFR MAbs, initial screens of the phosphorylatable fusions, and as one approach to determine the cross-reactivity between anti-EGFR and anti-p185HER2 MAbs. Immuno II (NUNC Roskilde, Denmark) microtiter plates were coated with 1 μg/ml of either the WGA affinity purified p185HER2, WGA affinity purified EGFR membrane preparations, or phosphorytamine coupled to soybean trypsin inhibitor. ELISA positive parental culture supernatants were further assayed by immunoprecipitation with [32P]-phosphorylated EGFR as described below.

Radioimmunoprecipitation of [γ-32P]ATP Phosphorylated Receptors. Radioimmunoprecipitations were performed after the first autophosphorylation of the appropriate Triton X-100 cell lysates with [γ-32P]ATP in kinase buffer containing 180 μCi/ml [γ-32P]ATP, 5 μM ATP, 2 mm MnCl₂, 4 mm MgCl₂, and the WGA purified extracts of either the p185HER2 (35 μg/ml) or the EGFR (17.5 μg/ml) in HTG buffer for 30 min at room temperature. The kinase mixture, 50 μl, was then mixed with 2 μg of the appropriate MAb, diluted to 50 μl with HTG buffer, and incubated for 1 h at room temperature. Protein A-Sepharose CL-4B, 50 μl, (Pharmacia, Uppsala Sweden), coated with 1 mg/ml rabbit anti-mouse IgG to enhance binding of all IgG subclasses, was added and incubated for 1 h at room temperature. Samples were washed twice with HTG and once with PBS containing 0.2% deoxycholate and 0.2% Tween 20. Reducing SDS sample buffer containing 30 μM β-mercaptoethanol-2% SDS in 14 mm Tris, pH 6.8, 50 μl, was added to the pelleted immunocomplexes and the samples were heated to 90°C for 5 min. The RAM-SPA was separated by centrifuging for 2 min at 10,000 × g and the supernatant was loaded on a 7.5% SDS polyacrylamide gel (37), electrophoresed at constant 30-mA current and followed by autoradiography to determine the relative molecular weights of the bound autophosphorylated proteins.

Radioimmunoprecipitation of [35S]Cysteine Labeled Receptors. Subconfluent cell cultures in 100-mm plates were washed once with cysteine-free DMEM and incubated overnight in 2 ml of cysteine-free media containing 10% dialyzed fetal bovine serum and 0.5 μCi [35S]-cysteine (Amersham, Arlington, IL). The labeling medium was aspirated and the cells were rinsed with HNEG buffer at pH 7.6. The cells were lysed with 0.5 ml of HNEG buffer with 1% Triton X-100 and 1.5 mm MgCl₂ (lysis buffer), diluted with 1 ml HNEG and 1% BSA (dilution buffer), and the cell membrane fragments were removed by centrifugation at 10,000 × g for 1 h. The labeled cell supernatant was precipitated with RAM-SPA for 30 min at room temperature and centrifuged to remove above labeled proteins which bound nonspecifically. This preabsorbed extract was divided into 4–6 samples and incubated with 2 μg of the appropriate MAb for 30–60 min. The washing, immunoprecipitation, and electrophoresis steps were performed as described above.

Isotopic Labeling of MAbs. Isotyping of the MAbs was performed by using the Pandex Screen Machine and anti-mouse IgG coated particles. Polyethylene glycol, 1 ml of a 5% solution, was incubated in 1.0 mg of affinity purified anti-mouse IgG (PelFreez, Rogers, AZ) and incubated on a shaker at 4°C for 1 h. The beads were washed twice with PBS and resuspended in 20 ml of PBS at 0.25% particles. Coated particles, 20 μl, were added to 20 μl of the appropriate MAb supernatants in wells of Pandex microtiter plates and incubated on a shaker at room temperature for 30 min. The plates were washed and FITC-conjugated rat monoclonal anti-mouse isotype specific reagents (generous gift of David Buck, Becton-Dickinson Monoclonal Center) were added at 1–2 μg/ml in PBST. Samples were incubated 15 min, washed, and the fluorescence was quantitated by the Pandex software.

Epitope Determination by MAb Competitive Binding Analysis. Cross-blocking studies were done on both panels of MAbs by direct fluorescence on intact cells by using the Pandex Screen Machine to quantitate fluorescence. Each MAb was conjugated with FITC, using established procedures (39). Confluent monolayers of A431 or NIH 3T3/HER2-3400 cells were trypsinized, washed once, and resuspended at 1.75 × 10⁶ cells/ml in cold PBS containing 0.5% BSA (BSA/PBS) and 0.1% NaN₃. A final concentration of 1% latex particles (IDC, Portland, OR) was added to reduce clogging of the Pandex plate membranes. Cells in suspension, 20 μl, and 20 μl of purified MAbs (100 μg/ml to 0.1 μg/ml) were added to the Pandex plate wells and incubated on ice for 30 min. A predetermined dilution of FITC-labeled MAbs in 20 μl was
MONOCLONAL ANTIBODIES; EGFR, HER2/neu/GENE AND p185HER2

added to each well, incubated for 30 min, washed, and the fluorescence was quantitated by the Pandyx.

Blocking of EGF Binding by Anti-EGFR MAbs. To determine if any of the anti-EGFR MAbs blocked EGF binding to the EGFR on intact cells, 200 µl of A431 cells (5 x 10⁶ cells/ml) were mixed with 200 µl of various concentrations of either purified MAbs or unlabeled murine EGF (Sigma), in triplicate, starting at 6 nM, 1.0 mg/ml or 100 µg/ml, respectively, and incubated 30 min on ice. Murine [³²P]-EGF, 6 x 10⁶ cpm in 100 µl, was added to each sample and incubated an additional 30 min on ice. Cells were washed once, counted on a gamma counter, and the percentage of bound counts was calculated and the average of triplicates plotted.

Immunochemistry Staining of Cells. The cell lines A431 and the breast adenocarcinoma cell line SK-BR-3, previously shown to express amplified levels of p185HER2 (40, 41), were used to characterize the EGFR and p185HER2 specific panels of MAbs by FACS techniques. Confluent monolayers of cells were harvested with 25 mM EDTA in PBS, washed, and resuspended at 1 x 10⁶ cells/ml in PBS containing 1% FBS (1% FBS/PBS). An aliquot, 1 ml, of each cell line was added to 10 µg of the appropriate MAb and incubated for 1 h on ice. After 7 days the cells were removed from the plates by trypsinization and the total cell number was determined by Coulter counter. The difference in growth of cells treated with the experimental and control MAbs was expressed as the ratio of their respective cell numbers. Each MAb was assayed in duplicate and the cell counts were averaged. As an additional positive control, EGF at 40 ng/ml (8 nM) was included; previous work has shown this to be growth inhibitory for A431 cells (42, 43).

RESULTS

ELISA Characterization of MAbs. Since these anti-EGFR and anti-p185HER2 MAbs could be used to develop important diagnostic reagents, a variety of immunological techniques were used to determine their reactivity and specificity. The ability of the purified MAbs to bind to immobilized WGA purified EGFR or p185HER2 was determined by ELISA. Relative activities of the antibodies at various concentrations, measured as A₄₅₀ nm, were determined and the results at 1-µg/ml MAb concentrations are presented (Fig. 1). Four of the five anti-EGFR MAbs (5G3, 6C5, 13A9, and 19C5) were specific for the EGFR, with their reactivity with p185HER2 at background levels. In contrast, MAb 3G2 appeared to bind to both the EGFR and p185HER2. Our MAb 13A9 had reactivity to EGFR equivalent to the well characterized anti-EGFR MAb 108 (generous gift of Joseph Schlessinger, Rorer Biotechnology, Inc.) described earlier (44). The anti-p185HER2 MAbs exhibited positive but variable reactivity against immobilized p185HER2 in ELISA and none of these MAbs reacted with immobilized EGFR.

MAb Immunoprecipitation of [³²P]Labeled Receptors. The specificity of the MAbs was also evaluated by utilizing radioligand immunoprecipitation on [³²P]labeled proteins. Purified MAbs were reacted with [γ-³²P]ATP autophosphorylated EGFR and p185HER2 receptor preparations (Fig. 2). All the MAbs raised to A431 cells immunoprecipitated EGFR, with MAbs 13A9 and 108 being the most efficient. MAb 3G2 also precipitated a strong p185HER2 band by comparison to the EGFR band precipitated by this antibody (Figs. 2A and 3). These precipitation results are consistent with the ELISA results showing cross-reactivity of MAb 3G2 with both EGFR and p185HER2 coated plates. The MAbs against p185HER2 precipitated p185HER2 and not the EGFR (Fig. 2). None of the anti-p185HER2 MAb precipitations of the EGFR were above background levels of the negative MAbs 5B6 (anti-gpl20 of human immunodeficiency virus) or 40.1.H1 (anti-human B surface antigen of hepatitis B virus), which gave some weak background bands due to slight overexposure of the autoradiography film prior to photographing. The MAbs specific for phosphorylase were also generated to investigate growth factor receptor structure and function including the associated tyrosine kinase activity. The anti-phosphotyrosine MAb 5E2 was pursued since it efficiently immunoprecipitated both the EGFR and p185HER2 (Fig. 3). MAb 5E2 did not react with either phosphoserine or phosphothreonine (data not shown) and has also been useful in immunoblotting of phosphorylated EGFR receptors (45).

MAb Immunoprecipitation of [³⁵S]Cysteine Labeled Receptors. The antigen specificity of the MAbs was further confirmed by immunoprecipitation of the EGFR and p185HER2 from extracts of A431 and SK-BR-3 cells metabolically labeled with [³⁵S]-
Fig. 2. Autoradiography of 7.5% SDS-polyacrylamide gel electrophoresis of [γ-32P]ATP labeled proteins immunoprecipitated by either anti-EGFR MAbs (A, Lanes 1-12) or anti-p185HER2 MAbs (A, Lanes 13-18; B, Lanes 1-14) from either the A431 (odd numbered lanes) or the NIH 3T3/HER2-3400 (even numbered lanes) WGA purified extracts. Negative control MAbs: 5B6 (anti-gpl20 of human immunodeficiency virus, IgG1; Lanes 15 and 16) and 40.1.111 (anti-surface antigen B of hepatitis, IgG2a; Lanes 17 and 18) are included as isotype controls.

Fig. 3. Autoradiography of 7.5% SDS-polyacrylamide gel electrophoresis of [γ-32P]ATP autophosphorylated labeled EGFR and p185HER2 comparing anti-EGFR, anti-p185HER2, and anti-phosphotyrosine MAbs. The pl85HER2 extracts are run in the odd numbered lanes and the EGFR extracts are run in the even numbered lanes. Anti-EGFR MAbs (Lanes 1-4), anti-p185HER2 MAb (Lanes 5 and 6) anti-phosphotyrosine MAb (Lanes 7 and 8) and control MAb (Lanes 9 and 10).

cysteine (Fig. 4). All the anti-EGFR MAbs precipitated the EGFR from the extract from SK-BR-3 cells (Fig. 4B). None of these EGFR MAbs, including MAb 3G2, precipitated p185HER2 from the [35S]cysteine labeled SK-BR-3 extracts. The lack of detectable p185HER2 in the immunoprecipitation of [35S]cysteine labeled p185HER2 by the anti-EGFR MAb 3G2 may be due to the relative lower specific activity of the 35S versus 32P labeled receptors.

The anti-p185HER2 MAbs 3E8 and 7F3 were the most efficient in precipitating [35S]cysteine labeled p185HER2; 2C4, 2H11, 4D5, 5B8, 7C2, and 7D3 were intermediate, while 3H4 and 6E9 were the least efficient (Fig. 4B). None of the p185HER2 MAbs immunoprecipitated the EGFR. These data are consistent with the rank order observed in the immunoprecipitations with autophosphorylated p185HER2.

Mab Immunoprecipitation of Native and Deglycosylated Receptors. To determine whether individual MAB binding epitopes are protein or carbohydrate in composition, immunoprecipitations were performed on 32P labeled, tunicamycin treated, and native EGFR from A431 cells and p185HER2 from NIH 3T3/HER2-3400. The autoradiography bands from the MAB immunoprecipitations were compared with those from appropriate polyclonal antisera, following the rationale that those antisera bound both glycosylated and deglycosylated receptors. Anti-EGFR MAbs 3G2, 5G3, 6C5, 13A9, 19C5, and 108 immunoprecipitated both the native (M, 170,000) and the lower molecular weight (approximately M, 140,000), deglycosylated forms of the EGFR (Fig. 5A). The p140 band appears only in the tunicamycin treated A431 cells and not in the untreated cells prepared by the same method. The anti-hepatitis MAB 40.1H1 reproducibly bound to a deglycosylated determinant on the
EGFR. This cross-reactivity was not seen with the anti-gp120 MAb 9F6. The anti-p185HER2 MAbs 3H4, 5B8, 6E9, 7C2, and 7F3 bound preferentially to the lower molecular weight, deglycosylated p185HER2 (approximately M, 170,000), whereas 2C4, 2H11, 4D5, and 7D3 bound preferentially to the glycosylated form of p185HER2, and 3E8 bound equally to the native and deglycosylated forms of p185HER2 by comparison to the polyclonal antiserum G-H2C17 (Fig. 5B). As with the deglycosylated EGFR, the lower molecular weight band appears only in the tunicamycin treated NIH 3T3/HER2-3400 cells and not in the untreated cells prepared by the same method. The immunological characteristics of the MAbs described including isotype, immunogen, ELISA reactivity, immunoprecipitation results, and epitope analysis and composition are summarized in Table 1.

Anti-EGFR MAbs Blocking of EGF Binding. The anti-EGFR MAbs were evaluated for their ability to block the ligand binding to the EGFR on A431 cells (Fig. 6). The anti-EGFR MAbs 5G3 and 6C5 inhibited 125I-murine EGF ligand binding to receptor on A431 cells by 40-50% at an IgG concentration of 0.6 μM. MAbs 5G3 and 6C5 bound preferentially to the glycosylated form of p185HER2, and 3E8 bound equally to the native and deglycosylated forms of p185HER2 by comparison to the polyclonal antiserum G-H2C17 (Fig. 5B). As with the deglycosylated EGFR, the lower molecular weight band appears only in the tunicamycin treated NIH 3T3/HER2-3400 cells and not in the untreated cells prepared by the same method. The immunological characteristics of the MAbs described including isotype, immunogen, ELISA reactivity, immunoprecipitation results, and epitope analysis and composition are summarized in Table 1.

FACS Analysis of MAbs Binding to Cells. To determine whether the MAbs could bind to and distinguish EGFR and p185HER2 on the surface of viable cells, FACS analysis was used with A431 and SK-BR-3 cells and the RMF of the molecules was calculated for each MAb (Fig. 7). The anti-EGFR MAbs bound to A431 cells with RMF values at least 100-fold greater than those measured on SK-BR-3 cells. This trend is consistent with the EGFR immunoprecipitation results from A431 and SK-BR-3 with the anti-EGFR MAbs (Fig. 4B). Although weak, all the EGFR MAbs, except 13A9, bound to the EGFR present on SK-BR-3 cells. The inconsistency with anti-EGFR MAb 13A9 in immunoprecipitation and FACS analysis may reflect an EGFR epitope which is unexpressed on intact A431 cells. With the exception of MAbs 5B8 and 6E9, the anti-p185HER2 MAbs bound to SK-BR-3 cell with RMF values ranging 10- to 100-fold greater than the A431 cell values. These results are consistent with observed EGFR copy numbers of 1.2 × 10^6 and 8.7 × 10^6 for A431 and SK-BR-3 cells, respectively, and a relative p185HER2 expression of approximately 20-fold lower on A431 and 10-fold higher on SK-BR-3 cells, by comparison to the EGFR expression on these cell lines. The NIH 3T3/HER2-3400 cells express 1.0 × 10^7 copies of p185HER2. We have not determined the number of murine EGFR expressed by NIH 3T3/HER2-3400 cells. Fig. 3 demonstrates that the anti-EGFR MAb 13A9 immunoprecipitates a weak p170 band from this cell line. Until we determine whether the entire anti-EGFR MAb panel binds to murine EGFR, the specificity of either the EGFR or p185HER2 specific MAbs should be concluded from the reactivity data generated with the human A431 and SK-BR-3 cell lines.

Growth Inhibitory Effects of Anti-EGFR MAbs on A431 Cells. We also investigated whether any of the anti-EGFR MAbs had growth inhibitory effects on A431 cells in vitro. Anti-EGFR MAbs 5G3 and 6C5, at final concentration of 16.7 nM, inhibited A431 cell proliferation by approximately 85% when compared to the A431 cell proliferation in the presence of anti-p185HER2 MAb 4D5 control (Fig. 8). This level of growth inhibition was equivalent to that of the EGF control at 8 nM. Anti-EGFR MAbs 13A9, 108, 3G2, and 19C5 had A431 growth inhibitory effects ranging from 50 to 30%, respectively. Hudziak et al. (38) performed similar in vitro growth inhibition experiments with the anti-p185HER2 MAbs and demonstrated that 4D5 was the most effective growth inhibitor of SK-BR-3.

DISCUSSION

We have used the established human epidermoid tumor line, A431, and a stably transfected murine cell line expressing the HER2/neu gene, NIH-3T3/HER2-3400, as immunogens to produce MAbs which bind to the EGFR or p185HER2 on the surface of human cells. The resultant MAbs were used to investigate the expression and compare antigenic determinants on the extracellular domains of the EGFR and the related p185HER2. Several groups have produced EGFR specific MAbs [(43, 46–49) partial list], some of which recognize noncarbohydrate determinants and react with the EGFR on a variety of cells. However, there has been little published antigenic characterization of these antibodies with respect to their cross-reactivity with p185HER2. Schechter et al. (50) has shown that rabbit antiserum raised to EGFR from A431 cells will immunoprecipitate p185HER2 from NIH 3T3 cells transfected with the rat, ethylnitrosourea-induced neu oncogene. In contrast, the anti-rat p185HER2 MAb 7.16.4 described by Drebin et al. (51) does not react with either the human p185HER2 or the human EGFR (50). The previous description of p185HER2 specific MAbs, reported by van de Vijver et al. (52), did not address the question of cross-reactive determinants present on both the EGFR and p185HER2. Results from immunoprecipitation experiments with immune perfusion sera (data not shown) from our BALB/c
Table 1  Summary table of monoclonal antibodies described

<table>
<thead>
<tr>
<th>MAbs</th>
<th>Isotype</th>
<th>EGFR ELISA*</th>
<th>p185HER2 ELISA</th>
<th>EGFR RIP</th>
<th>p185HER2 RIP</th>
<th>Epitope</th>
<th>FACS</th>
</tr>
</thead>
<tbody>
<tr>
<td>3G2</td>
<td>IgG1x</td>
<td>++</td>
<td>+</td>
<td>++</td>
<td>-</td>
<td>(p)</td>
<td>+++</td>
</tr>
<tr>
<td>5G3</td>
<td>IgG1x</td>
<td>++</td>
<td>-</td>
<td>++</td>
<td>-</td>
<td>(p)</td>
<td>+++</td>
</tr>
<tr>
<td>6C5</td>
<td>IgG1x</td>
<td>++</td>
<td>-</td>
<td>++</td>
<td>-</td>
<td>(p)</td>
<td>+++</td>
</tr>
<tr>
<td>13A9</td>
<td>IgG1x</td>
<td>+++</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>(p)</td>
<td>+++</td>
</tr>
<tr>
<td>19C5</td>
<td>IgG1x</td>
<td>+</td>
<td>-</td>
<td>++</td>
<td>-</td>
<td>(p)</td>
<td>+++</td>
</tr>
<tr>
<td>108</td>
<td>IgG2a</td>
<td>++</td>
<td>-</td>
<td>++</td>
<td>-</td>
<td>(p)</td>
<td>+++</td>
</tr>
<tr>
<td>2C4</td>
<td>IgG1x</td>
<td>-</td>
<td>+++</td>
<td>-</td>
<td>++</td>
<td>(c)</td>
<td>+++</td>
</tr>
<tr>
<td>2H11</td>
<td>IgG2a</td>
<td>+</td>
<td>-</td>
<td>++</td>
<td>-</td>
<td>(c)</td>
<td>+++</td>
</tr>
<tr>
<td>5B8</td>
<td>IgG1x</td>
<td>+</td>
<td>-</td>
<td>++</td>
<td>-</td>
<td>(c)</td>
<td>+++</td>
</tr>
<tr>
<td>6E9</td>
<td>IgG1x</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>nd(p)</td>
<td>(c)</td>
<td>+++</td>
</tr>
<tr>
<td>7D7</td>
<td>IgG1x</td>
<td>-</td>
<td>++</td>
<td>-</td>
<td>F(c)</td>
<td>(c)</td>
<td>+++</td>
</tr>
<tr>
<td>7F3</td>
<td>IgG1x</td>
<td>++</td>
<td>-</td>
<td>++</td>
<td>-</td>
<td>(c)</td>
<td>+++</td>
</tr>
<tr>
<td>5E2</td>
<td>IgG1x</td>
<td>nd</td>
<td>nd</td>
<td>+++</td>
<td>nd</td>
<td>G/F(p)</td>
<td>+++</td>
</tr>
</tbody>
</table>

* ELISA data columns (summary of $A_{450}$, nmol/mg): <0.1 = —¿; 0.11-0.50 = +; 0.51-1.0 = ++; >1.0 = +++; RIP data columns (summary of autoradiography from immunoprecipitations): bands equal to negative control = —¿; weak bands but darker than negative control = +; moderately exposed bands = ++; strongly exposed bands = +++; Epitope data columns: letters assigned to represent individual epitopes A through I. MAbs were considered to share an epitope if each blocked binding to the negative control MAbs = —¿; 1-9-fold higher than the negative controls = +; 10-99-fold higher than the negative controls = ++: >100-fold higher than the negative controls = +++.

**Generous gift from J. Schlessinger.

** nd, not done.
MONOCLONAL ANTIBODIES; EGFR, HER2/new/GENE AND p185HER2

Fig. 7. RMF binding of purified anti-EGFR MAbs (upper) or the anti-p185HER2 MAbs (lower) to A431 (□) and SK-BR-3 (■). Included are the negative isotype controls 5B6 (IgG1) and 40.1H1 (IgG2a). RMF was calculated as

\[ RMF = \frac{N}{e^{x C}} \]

where \( N \) is the "linearized fluorescence value", \( x \) is the log to linear transforming factor, and \( C \) is the channel number.

the EGF binding (45% inhibition), while 19C5 and 108 show weaker EGF blocking (25-40% inhibition), and 13A9 and 3G2 did not block murine EGF binding to A431 cells. These results could reflect the mechanism by which MAbs to the EGFR inhibit A431 cell division in vitro. Similar receptor blocking experiments with the anti-p185HER2 MAbs await identification of the putative ligand.

It appears that in mice the major immunodominant determinants on the EGFR and p185HER2 are not in the homologous regions comprising 40% of the extracellular domains of these receptors. It has previously been shown that a membrane structure sharing homologous extracellular regions to the EGFR and p185HER2 exists in Drosophila (53, 54). A high degree of conservation throughout evolution, in certain extracellular regions of the EGFR and p185HER2, could be accompanied by a lack of immunogenicity in these regions. Since two of the EGFR specific MAbs described here partially block EGF binding, it is likely that at least one of the immunogenic, nonhomologous epitopes on the EGFR is located in or near the ligand binding site. The possibility does exist that we have broken immunological tolerance to the murine EGFR, and future experiments, pending identification of murine cell line(s) which overexpress the EGFR, will include evaluation of these EGFR MAbs for species specificity. We have observed by FACS analysis (data not shown) that our p185HER2 MAbs do not bind to the endogenous neu on the surface of RAT-1 cells indicating that, like MAb 7.16.4, they bind to p185HER2 epitopes that may be species specific. This result might be predicted since the anti-p185HER2 MAbs resulted from fusions in the murine system and mice may be tolerant to conserved determinants on p185HER2.

Whether the immunogenic, nonhomologous regions on p185HER2 are in the putative ligand binding pocket will require further investigation.

Overexpression or altered forms of the EGFR and/or p185HER2 are found in many forms of human cancers including cervical, ovarian, squamous, adenocarcinomas, and primary breast tumors. In the case of breast and ovarian cancer, amplification of HER2/new expression is associated with approximately 30% of the primary tumors tested and is correlated with metastatic disease and poor prognosis (17, 18). The location of these receptors on the surface of cells, prognostic significance and probable causal role makes them very attractive targets for production of monoclonal antibodies for immunodiagnostics and immunotherapy. Immunohistochemistry data on tumor biopsy tissue could be useful in determining the aggressiveness of therapy depending on the level of p185HER2 expression. These MAbs would also be candidates for in vivo radioimaging for detection of relevant primary tumors and micrometastases. The receptor nature of these antigens, including surface location, ligand binding, and receptor/ligand complex internalization, makes tumors expressing these receptors potential targets for immunotherapeutic intervention utilizing antibodies as radioligand, drug carriers, or immunotoxins. An alternate approach would be to exploit the ligand blocking characteristics of some receptor specific MAbs and deprive transformed cells of their intracellular signal transduction. This has been demonstrated with EGFR specific MAbs where EGF blocking is the mechanism for growth inhibition on EGF dependent cells (43, 55). The anti-p185HER2 MAbs should be useful reagents to investigate similar receptor/ligand studies on p185HER2 and may facilitate identification of the putative ligand.

We are optimistic that diagnostic and therapeutic applica-
tions of MABs specific for receptor related molecules associated with human malignancy will allow exploitation of this probable causal relationship in transformation and the process of onco-
genesis.

ACKNOWLEDGMENTS

The authors wish to thank Bill Lagrimas for performing our immunization protocols, K. C. McFarland for providing phosphotyramine

REFERENCES


Characterization of Murine Monoclonal Antibodies Reactive to Either the Human Epidermal Growth Factor Receptor or HER2/neu Gene Product

Brian M. Fendly, Marcy Winget, Robert M. Hudziak, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/50/5/1550

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.