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Identification of Epitope Regions Recognized by Tumor Inhibitory and Stimulatory Anti-ErbB-2 Monoclonal Antibodies: Implications for Vaccine Design

Yum L. Yip,*† Glenn Smith,‡ Joachim Koch,‡ Stefan Dübel,‡ and Robyn L. Ward2*†

The self-oncoprotein ErbB-2 is overexpressed in a number of malignancies. The presence of endogenous anti-ErbB-2 Ab and T cell immune responses to this protein in cancer patients has made ErbB-2 an attractive target for active immunization. However, the finding that murine anti-ErbB-2 Abs can have stimulatory, inhibitory, or no effects on cancer cell growth suggests that an inappropriately induced immune response may have an adverse effect. To ensure the induction of a beneficial Ab response, it is important to identify the epitopes recognized by these Abs. In this study we have used phage-displayed ErbB-2 gene fragment libraries and synthetic peptides to epitope-map a panel of anti-ErbB-2 mAbs. The epitopes of three mAbs, N12, N28, and L87, were successfully located to C531-A586, T216-C235, and C220-C235 of ErbB-2, respectively. It was found that while N12 inhibited tumor cell proliferation, N28 stimulated the proliferation of a subset of breast cancer cell lines overexpressing ErbB-2. The peptide region recognized by N12, (C531-A586; EP531), was used as an immunogen to selectively induce an inhibitory immune response in mice. Mice immunized with the GST fusion peptide (GST-EP531) recognized the peptide region EP531 as well as native ErbB-2. More importantly, Igs purified from mouse sera were able to inhibit up to 85% of tumor cell proliferation. In conclusion, our study provides direct evidence of the function-epitope relationship of anti-ErbB-2 Abs and also emphasizes the value of inducing a potent tumor inhibitory polyclonal Ab response by rationally selecting regions of ErbB-2 used for immunization. The Journal of Immunology, 2001, 166: 5271–5278.

The c-erbB2/neu protooncogene encodes a 185-kDa protein that belongs to the epidermal growth factor receptor (EGFR) family. The protein ErbB-2 consists of three domains: a glycosylated extracellular domain (ECD) with two cysteine-rich regions, a hydrophobic transmembrane domain, and an intracellular tyrosine kinase domain (1). With no direct ligand, ErbB-2 functions as the preferred partner for heterodimerization with other members of the EGFR family (namely EGFR, ErbB-3, and ErbB-4) (2, 3) and thus plays an important role in coordinating the complex ErbB signaling network that is responsible for regulating cell growth and differentiation (4). For a decade, ErbB-2 has been considered an attractive target of cancer immunotherapy, because it is overexpressed in a number of malignancies, and this overexpression is associated with malignant transformation and clinically aggressive disease (5, 6).

A number of anti-ErbB-2 ECD mAbs have been isolated (7–10), and one such Ab, 4D5 or Herceptin, has demonstrated efficacy in the treatment of metastatic breast cancer (11, 12). Although many anti-ErbB-2 Abs inhibit the growth of cancer cells, it is well established that some Abs have no effect on cell growth, while others actively stimulate cancer growth. It has been proposed that this wide spectrum of biological effects is related to the epitope specificity of the Abs and to consequent changes in receptor signaling (13–16).

Active immunization (vaccination) against ErbB-2 represents an alternative to the use of passive mAb therapy. This strategy is based on data that clearly show that immune tolerance to ErbB-2 is not absolute (17–22). It is proposed that boosting existing Ab and T cell immune responses or eliciting a de novo immune response will result in a therapeutic benefit. However, the diverse biological effects of murine mAbs (stimulatory and inhibitory) also suggest that an inappropriately induced immune response may have detrimental effects. To better manipulate this Ab response for therapeutic advantage, it is thus crucial to identify epitopes on ErbB-2 that are targeted by stimulatory and inhibitory mAbs.

In this study we have constructed c-erbB2 gene fragment phage display libraries to epitope-map a number of anti-ErbB-2 mAbs with different biological effects on tumor cell growth. Regions that were responsible for opposing effects of mAbs were identified and were used to immunize mice. The B cell epitope region that was recognized by the tumor inhibitory Ab was immunogenic, and the resultant immune response was capable of inhibiting cancer cell growth in vitro. The peptide could serve as a potential lead for vaccine design.

Materials and Methods

Bacterial strains, expression vectors, and anti-ErbB-2 Abs

Escherichia coli strains MC1061 and K91Kan, the phage display vector fUSE5, and random peptide libraries (X6, X15, and CX5C) were gifts from Prof. George Smith (University of Missouri, Columbia, MO). The GST gene fusion system was purchased from Amersham Pharmacia Biotech (Castle Hill, Australia).

The anti-ErbB-2 ECD mAbs used in this study were L26, L87, N24, and N29, (Neomarkers, Union City, CA); N12 and N28 (gift from Prof. Y.
Yarden, Weizmann Institute of Science, Rehovot, Israel); 9G6 (Santa Cruz Biotechnology, Santa Cruz, CA); and 4D5 (gift from W. Lee, Genentech, South San Francisco, CA). N12 and N28 were provided as ascites fluid and were purified by protein A affinity column (Zymed, San Francisco, CA). Biotinylated N12 used for competitive assay was prepared using EZ-link sulfo-NHS-LC-Biotin (Pierce, Rockford, IL).

The human breast cancer cell lines BT474, SK-BR-3, T47D, and MDA-MB-231 (American Type Culture Collection, Manassas, VA) were cultured in RPMI 1640 medium (Trace, Castle Hill, Australia) supplemented with 10% FCS (Life Technologies, Gaithersburg, MD). Herceptin was a gift from Rodney Fiddes (Garvan Institute of Medical Research, Sydney, Australia).

Construction of c-erbB2 gene fragment libraries and affinity selection

The c-erbB2 gene fragment libraries were constructed in the phage vector fUSEF following Smith’s (23) and Peterson et al.’s (24) protocols with modifications. In short, the c-erbB2 ECD DNA was excised from the c-erbB2/CMVLAG vector (25) with HindIII and BspEI and digested with 0.5 U/ml of Dnase I. c-erbB2 gene fragments between 100 and 300 bp were purified, end-repaired with T4 DNA polymerase, and blunt end ligated to the phosphorylated linkers, either FUSP or FUSC, which allowed the peptide to be displayed in a linear or a cyclic form, respectively (24). Linker-attachment-PCR was used to specifically amplify gene fragments containing linkers on both ends (26). The primers used were 5’-ATATCC AGACCTGGCGGTG-3’ and 5’-ATATCCACACGTGGCGGTG-3’ for FUSP linked fragments and 5’-ATATCCACACGTGGCGGTG-3’ and 5’-ATATCCACACGTGGCGGTG-3’ for FUSC linked fragments. The PCR-amplified fragments were gel-purified, digested with PflMl enzyme, and ligated to SfeI-digested fUSEF vector DNA. The ligation mixture was electrotransformed into MC1061, and phage were amplified and titrated (23). A diagnostic PCR was used to determine the number of clones with a gene fragment insert.

For panning, wells of a microtiter plate were coated overnight at 4°C with 100 μl of mAb (10 μg/ml) in PBS and blocked with 0.05% Tween2% skim milk/PBS. c-erbB2 gene fragment libraries diluted to 10^12 transforming units/100 μl in 0.05% Tween/2% skim milk/PBS were added to the wells, and the plate was incubated at 37°C for 1 h. Unbound phage were removed by washing five times in 0.05% Tween/PBS and five times in 0.5% Tween/PBS. Bound phage were eluted with 100 μl of 0.1 M glycine, pH 3.0, for 10 min at room temperature and neutralized with 10 μl of 1 M Tris-HCl, pH 9.5. Eluted phage were used to infect 1 ml of mid-log K91/Kan plate and were amplified for the next round of panning (23, 24). Aliquots of culture were plated onto a Luria Broth/tet20/kan100 plate containing phage was added to wells of a microtiter plate coated with 100 μl of mAb (10 μg/ml) in PBS. The plate was incubated at 37°C for 1 h. After washing three times, Ab binding was detected with mouse anti-mouse IgG (Dako). Mice were boosted four times with the Ag in PBS every 2 wk. Sera were taken on day 0 (preimmune) and 10 days after the third and fifth immunizations.

Screening for positive clones by phage ELISA and sequence analysis

Single colonies were grown overnight in 1 ml of 2YTet/Kan medium with shaking at 37°C. Bacterial cells were spun down, and the supernatant containing phage was added to wells of a microtiter plate coated with 100 μl of an mAb solution (1 μg/ml in PBS) and preblocked with 2% skim milk/PBS. The plate was incubated at 37°C for 1 h and washed three times with 0.05% Tween/PBS. The Ab-phage complexes were detected with mouse anti-mouse IgG (Dako). The Ab levels in the sera of immunized mice were assayed by ELISA, using the appropriate Ags.

Groups of female BALB/c mice, 6–8 wk old, were immunized i.p. with 100 μl of GST-ErbB3, GST-ErbB2 and GST-ErbB1, were performed following the manufacturer’s instructions.

Immunassays

Ab levels in the sera of immunized mice were assayed by ELISA, using the appropriate Ags. To assess the anti-epitope response, wells of a microtiter plate were coated overnight with 10 μg/ml of phage pIII-displayed peptides (10^-12 phage particles/ml) in 100 μl of PBS. The wells were blocked with 2% skim milk/PBS and washed three times with 0.1% Tween/PBS. Serial dilutions of mouse sera in 2% skim milk/PBS were added to wells and incubated for 1 h at 37°C. After washing three times, Ab binding was detected using rabbit anti-mouse peroxidase conjugates (0.5 μg/ml in 2% skim milk/PBS, Dako, Carpenteria, CA). Wells coated with phage displaying an unrelated 11-aa-long peptide were used to determine the background of nonspecific Ab binding.

To determine the anti-erbB2 response, wells of a microtiter plate were coated overnight at 4°C with 5 μg/ml rabbit anti-ErbB2 Ab (Dako), washed four times with 0.1% Tween/PBS, and blocked with 1% BSA/PBS for 6 h at room temperature with gentle shaking. Wells were then coated overnight at 4°C with either 1% BSA/PBS or SK-BR-3 cell lysate, which was prepared by lysing the cells in lysis buffer containing 1% Triton X-100, 10% glycerol, 150 mM NaCl, 50 mM HEPES (pH 7.5), 1.5 mM MgCl2, 1 mM EDTA, 10 mM pyrophosphate, 100 mM NaF, 0.2 mM Na2VO4, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 mM PMSF. Wells were then washed four times the next day, serial dilutions of mouse sera in 1% BSA/PBS were added, and the plate was incubated for 2 h at room temperature with gentle shaking. Ab binding was detected using rabbit anti-mouse IgG Ab-peroxidase conjugates (Dako).

For each of the ELISAs described above, a standard curve was constructed using serial dilutions of the mAb N12 of known concentration in blocking solution. The serum Ab titer was calculated with reference to this standard curve and was expressed as microliters per milliliter.
Competitive ELISA

For competitive inhibition assays, mouse sera obtained 10 days after the final boost were selected on the basis of producing anti-peptide as well as anti-ErbB-2 Abs. Serial dilutions of these sera were performed in 2% BSA/PBS with 5 ng/ml biotinylated mAb N12, added to preblocked microtiter plates coated with 10 μg/ml GST-EP531, and incubated for 2 h at 37°C. After washing in 0.1% Tween/PBS, peroxidase-conjugated strepavidin was used to determine Ab binding. Concomitantly, serial dilutions of sera from mice immunized with GST alone were performed in 2% BSA/PBS with 5 ng/ml biotinylated mAb N12 and were used as negative controls for non-specific protein inhibition. The percentage of inhibition was calculated by using the following formula: (1 - A/B) × 100%, where A is the absorbance produced by positive mouse sera, and B is the absorbance produced by negative control sera.

Flow cytometry

Breast cancer cells SK-BR-3 (5 × 10^5) were incubated with 10 μl of mouse serum samples at 4°C for 30 min and then washed twice with 200 μl of 1% BSA/PBS. Goat anti-mouse Ig FITC-conjugated Abs were then added, and the samples were incubated for 30 min at 4°C. After washing, the cells were resuspended in 1% BSA/PBS. The flow cytometric analysis was performed using a MoFlo MLS flow cytometer (Cytomation, Fort Collins, CO). The fluorescence intensity of each cell was measured, and the percentage of positivity was established with respect to an isotype control sample using mouse IgG. A forward scatter vs side scatter gate was used to exclude debris and dead cells.

Results

Epitope mapping of anti-ErbB-2 mAbs

Two c-erbB2 gene fragment libraries of 7 × 10^6 (ErbB2-FUSP) and 1 × 10^7 (ErbB2-FUSC) clones were constructed. PCR analysis revealed inserts in 90 and 100% clones of ErbB2-FUSP and ErbB2-FUSC, respectively. The sizes of the inserts ranged from 50 to 150 bp. Because there were three possible reading frames and two possible insertion orientations, only 1 of 18 of the insert sequences produced a productive phage with ErbB-2 peptides correctly displayed on the surface (23). Therefore, the total number of productive clones was estimated to be 4 × 10^5 for the ErbB2-FUSP library and 6 × 10^5 for the ErbB2-FUSC library. Titration after overnight growth yielded 1 × 10^12 TU for each library.

Panning of these libraries against the mAbs L26, L87, N12, N24, N29, N28, and 4D5 allowed the identification of phage clones that were reactive with N12, N28, and L87. For mAb L87, the percentage of positive clones increased from 33% (20 of 60) after round 2 to 95% (19 of 20) after round 3. All the clones (60 total) analyzed after rounds 2 and 3 for mAb N28 were positive. As for mAb N12, 9% (8 of 86) of clones analyzed after round 3 were reactive.

Sequence analysis revealed that all eight clones reactive to mAb N12 contained a unique 55-aa insert flanked by FUSP linkers. The insert was mapped to amino acids C531-A586 within the second cysteine-rich subdomain of ErbB-2 ECD. The epitope was relatively large and contained eight cysteine, four glycine, and four proline residues. For mAb L87, sequencing of 39 clones showed the presence of five inserts of different lengths all flanked by FUSC linkers. Sequence alignment mapped the epitope to amino acids T216-C235 within the first cysteine-rich subregion of ErbB-2 ECD. Similarly, three inserts of different lengths flanked by FUSC linkers were identified from sequencing 40 clones reactive to mAb N28. The epitope of N28 overlapped that of L87, namely T216-C235. The two regions, C531-A586 and T216-C235, were denoted EP531 and EP216, respectively. The specificity of the phage clones binding to their respective selector Abs is shown in Fig. 1.

To further delineate the epitopes recognized by the three mAbs, a set of overlapping 15-residue peptides covering the two regions, EP531 and EP216, was tested for binding to mAb N12, L87, and N28. mAb N12 and N28 failed to bind to any of the synthetic peptides covering the region EP531 or EP216, respectively, suggesting that both N12 and N28 recognize a conformational epitope. The mAb L87 was reactive to peptides 5 and 6 (Fig. 2). The epitope is thus within the region C220-C235. The strong reactivity of L87 to peptide 5 and the weak reactivity to peptide 6 suggest that D234 represents a critical residue, and L87 recognizes either a long linear or an α-helical epitope (28). The results obtained with the synthetic peptides thus indicate that L87 and N28 do not recognize the same epitope.

In an attempt to localize critical residues, random peptide libraries (X_0, X_15, and X_25) were used to pan against mAb N12 and N28. No clones were isolated from these libraries (results not shown).

Effects of mAbs N12 and N28 on the proliferation of breast cancer cells

The effects of mAb N12 and N28 on the proliferation of a range of breast cancer cell lines expressing different levels of ErbB-2 were studied and compared with those of the mAb 4D5 and Heregulin, a ligand of ErbB-3 and ErbB-4 (29–31). It was found that N12 inhibited the growth of breast cancer cells at a level comparable to that of 4D5 (Fig. 3). In particular, the inhibitory effect of N12 appeared to correlate with the level of ErbB-2 expression. Cell lines expressing a high level of ErbB-2 (e.g., BT474) were more growth inhibited by N12 than cell lines expressing a low level of ErbB-2, such as MDA-MB-231. Both N28 and N12 inhibited the growth of cell line T47D, which expressed only moderate levels of
ErbB-2. However, in contrast to the effect of N12, N28 consistently stimulated the growth of both BT474 and SK-BR-3 cells. Although both cell lines expressed high levels of ErbB-2, N28 had a greater proliferative effect on BT474 than SK-BR-3 cells. The effect of L87 on tumor cell growth was not assessed in this study, as it has been reported that this Ab does not have an effect on tumor cell growth (32).

Immune response induced by the peptide regions EP531 and EP216

The peptide regions EP531 and EP216 were expressed as a GST fusion peptide (GST-EP531 and GST-EP216, respectively), and their immunogenicities were evaluated in BALB/c mice. Five of six mice (83%) immunized with GST-EP531 showed an Ab response to peptide EP531 as well as to whole ErbB-2. The other mouse immunized with GST-EP531 showed an anti-peptide response, but not an anti-ErbB-2 response. Although variability between mice was observed, the anti-ErbB-2 response was 6- to 7-fold lower than the anti-EP531 response (Fig. 4). Both anti-EP531 and anti-ErbB-2 responses could be detected after the third immunization, and the responses increased significantly after the fifth boost (Fig. 4). The induced response was epitope region EP531 specific and did not cross-react with EP216. No anti-peptide or anti-ErbB-2 response was observed for mice immunized with GST-EP216 or GST alone. Flow cytometric analysis showed that the sera of mice immunized with GST-EP531 that had an anti-ErbB-2 response also reacted with SK-BR-3 breast cancer cells (Fig. 5).

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To define the fine specificity of the polyclonal Ab response, the sera of three mice immunized with GST-EP531 (GST-1, GST-2,
and GST-3) were subjected to epitope mapping using overlapping 15-mer peptides covering the region EP531 (Fig. 6). As might be expected with antisera, no precise epitopes could be defined, but general trends of reactivity showed that sera recognized two separate groups of peptides located at the N- and C-terminal regions of EP531. Little or no reactivity was seen with the peptides corresponding to the central region of EP531. Competitive ELISA (Fig. 7) showed that at a dilution of between 1/20 and 1/80, immunized sera were able to inhibit the binding of mAb N12 to the peptide EP531, indicating the presence of serum Abs recognizing the same or a similar determinant as that of the mAb N12.

Murine IgG from serum that showed the highest anti-ErbB-2 titer (GST-2) were purified using protein A, and their effect on in vitro tumor cell growth was tested by MTT cell proliferation assay. Fig. 8 showed that at a concentration of 1.25 μg/ml anti-ErbB-2 Abs (150 μg/ml total IgG), polyclonal IgG were able to inhibit tumor cell growth up to 85%. The inhibitory effect of polyclonal IgG was significantly greater than that of N12 (p = 0.014). While the inhibition was also greater than that observed for 4D5, this difference was not significant (p = 0.169).

**Discussion**

The aim of most cancer vaccines is to induce a powerful anti-tumor immune response largely through the induction of T cell immunity. However, the clinical efficacy of anti-ErbB2 Abs and the observation that some patients with cancer (11) demonstrate an endogenous B cell response to this protein (17, 21, 33) suggest that the induction of a strong and targeted humoral immune response may be biologically important.

**FIGURE 6.** Epitope mapping of sera from mice immunized with GST-EP531. The membrane containing a series of 15-residue peptides overlapping by one amino acid and covering amino acids C531-A586 was probed with dilutions of sera (1/100) from three mice immunized with GST-531: GST-1, GST-2, and GST-3. The binding was detected with a peroxidase-conjugated goat anti-mouse IgG.

**FIGURE 7.** Competitive ELISA showing the inhibition of binding of biotinylated mAb N12 to the fusion peptide GST-EP531 in the presence of immunized mouse sera. Serial dilutions of sera from mice immunized with GST-EP531 or GST alone (negative control) were mixed with 5 ng/ml biotinylated mAb N12 and added to plates coated with 10 μg/ml GST fusion peptide (GST-EP531). Binding of mAb N12 was detected using peroxidase-conjugated streptavidin. The percentage of inhibition was calculated using the formula: (1 − A/B) × 100%, where A is the absorbance produced by positive mouse sera, and B is the absorbance produced by negative control sera.

**FIGURE 8.** MTT cell proliferation assay showing the effects of purified murine IgG, 4D5, and N12 on the growth of BT474 cells. Cells were incubated with 4D5 (5 μg/ml); N12 (5 μg/ml); purified IgG from GST-EP531-immunized sera, Ig GST-EP531 (total IgG, 150 μg/ml; anti-ErbB-2 IgG, 1.25 μg/ml); or purified IgG from GST-immunized sera, Ig GST (total IgG, 150 μg/ml). Relative cell numbers were measured indirectly using a nonradioactive MTT cell proliferation assay after 4 days of incubation. All treatments were performed in triplicate, and the percentage of inhibition was calculated by comparing the reading of treated cells to that of untreated control cells. Bars represent the SD of two experiments. *, p = 0.169; **, p = 0.014 (compared with Ig GST-EP531 treatment, using one-way ANOVA with a Bonferroni post test).
In this study we have demonstrated that tumor cell growth can be inhibited by the use of rationally selected ErbB-2 immunogens capable of inducing an anti-ErbB-2 Ab response. The importance of selecting the appropriate immunogens is clearly illustrated by the finding that the epitope specificity of the Ab dictates its biological effect. For instance, we found that mAbs N12 and N28 exert either an inhibitory or a stimulatory effect on tumor cell growth, respectively, by binding to their own specific regions, EP531 and EP216. To avoid possible detrimental effects caused by immunization with the whole ErbB-2 molecule, we chose to use the region EP531, recognized by the tumor inhibitory mAb N12, to ensure the induction of an N12-like inhibitory polyclonal immune response. The result site-directed polyclonal Ab response was capable of strongly inhibiting tumor cell proliferation, and the effect was superior to that of 4D5 or N12. While this finding clearly suggests that polyclonal anti-ErbB-2 Abs could produce a more effective anti-tumor response than equivalent doses of an mAb, it should be noted that it may be difficult to directly compare the effects of mAbs with those of Abs containing multiple binding specificities. The efficacy and protective effects of a polyclonal Ab response targeting inhibitory or neutralizing B cell epitopes or regions have been demonstrated repeatedly in the setting of infectious diseases (34–36). In cancer, few studies have described the induction of epitope-specific B cell immune responses. Our result suggests that this strategy is particularly relevant for ErbB-2, and EP531 is likely to be an important region to target. It is interesting to note that the epitope of 4D5 is reported to be within aa 529–627 of the ECD (37). Lewis-Philips et al. also suggested that anti-ErbB-2 mAbs that bind near the transmembrane region of the ECD have a potent anti-proliferative effect (38). It is thus possible that the region EP531 contains, in addition to the epitope recognized by N12, other B cell epitopes that are important for the inhibitory effects of Abs. In fact, previous studies have shown that certain combinations of anti-ErbB-2 mAbs will have an additive anti-proliferative effect on tumor cell growth (15). When compared with a single-epitope peptide, a peptide that contains multiple epitopes may induce a synergistic, and thus more effective, response.

There are a number of distinct clinical advantages associated with the induction of an anti-ErbB2 inhibitory Ab response. Firstly, it represents an alternative to the injection of large amounts of expensive mAbs. Vaccination using peptidic constructs could also provide a cost-effective means of producing a long term polyclonal immune response. Secondly, as inhibitory anti-ErbB-2 Abs can sensitize tumors to the actions of conventional DNA-damaging drugs (39, 40), inducing such a response may also enhance the efficacy of conventional chemotherapy. Lastly, this vaccination strategy might complement current methods using whole cells (41), ECD (42), DNA (43, 44), or T cell peptide epitopes (45). Although protective immunity against ErbB-2 was demonstrated in these studies, most of them relied on the induction of a CTL response to achieve the desired effect. While CTL are crucial effector cells against cancer, the overwhelmingly high frequency of HLA class I down-regulation in primary breast cancer (up to 88%) (46) and other cancers (47, 48) may limit the potential effectiveness of these vaccines (49, 50).

It is important to note that recent clinical trials have clearly shown that the tolerance against self proteins, such as ErbB-2, can be overcome (51–54). In the case of ErbB-2, while the use of the whole molecule fails to induce an immune response (55), alternative forms of constructs using ECD (42), a hydrophobized polysaccharide/oncoprotein complex consisting of 147 N-terminal amino acids of ErbB-2 (56), or peptides (54) have successfully been used as immunogens in transgenic mouse models or in humans. If peptide EP531 is immunogenic in humans, it would make an interesting potential vaccine for eliciting a specific inhibitory Ab response in patients.

Clearly, a more thorough understanding of the structure of ErbB-2 as well as its B cell epitopes and the mechanism of action of anti-ErbB-2 Abs will aid in the development of B cell epitope-based immunization strategies. Our study has identified a number of important characteristics related to the epitopes of anti-ErbB-2 Abs. In particular, we found that most Abs recognized large and conformational epitopes. The epitopes of N12 and N28 are conformational. The failure to map the other four mAbs also suggests that the epitopes may consist of carbohydrate residues or amino acid residues that are far apart (>100 aa). Indeed, the mAb 4D5 was reported to recognize a carbohydrate epitope (7). Our observation is in agreement with that of Orlandi et al. (57), who defined a linear subregion of a large conformational epitope of an anti-ErbB-2 mAb using random peptide libraries. In this regard, gene fragment libraries, by displaying on the surface of phage a collection of ErbB-2 peptides of different sizes (up to 100 aa) in their native structural context (58), were shown to be superior to synthetic peptides and random peptide libraries for identifying large and conformational epitope regions. The fine epitope mapping of anti-ErbB-2 Abs will be greatly facilitated once the three-dimensional structure of ErbB-2 is available.

The mechanism by which two Abs with different epitope specificities for ErbB-2 can mediate directly opposing effects remains unknown. The ECD of ErbB-2 plays several important roles in ErbB-2-mediated signaling. Although there is probably no direct ligand for ErbB-2, the ECD of ErbB-2 contains low affinity binding sites for Heregulin and other EGF-like ligands (59), and it is sufficient for mediating heterodimerization with other ErbB receptors (60). These observations suggest the Abs may exert their effects by mimicking or blocking ligand binding or by interfering with heterodimer formation. Alternatively, as bivalency has been reported to be necessary for the action of some anti-ErbB-2 Abs (9), it is possible that they may act by altering the signaling properties of the ErbB-2 homodimer. A recent report showed that the relative geometry of ErbB-2 receptors within the dimer is an important determinant of its signaling properties, and there is a particular ErbB-2 dimer interface that is responsible for its transforming activity (61). It is possible that N12 and N28 may favor the formation of an inactive and active dimer conformation, which results in negative and positive signaling, respectively. Indeed, N28 is reported to induce strong ErbB-2 phosphorylation (8). It is also worth noting that all three mAbs mapped to the two cysteine-rich regions. With the extensive disulfide bond formation, the two cysteine-rich regions could represent important domains for dimerization and thus mediate the diverse effects of anti-ErbB-2 Abs.

In conclusion, our study provides direct evidence of the function-epitope relationship of anti-ErbB-2 Abs and thus emphasizes the need to induce tumor inhibitory Ab responses. The use of a peptide region that contains multiple inhibitory B cell epitopes is likely to be superior to the use of a single epitope immunogen. The use of large peptide vaccines may overcome the tolerance associated with use of the whole ErbB2 molecule, allowing the production of an anti-tumor polyclonal humoral immune response. Further information about the structural properties of ErbB-2 and the mechanism of action of truly protective Abs will no doubt further facilitate the design of effective vaccines against this important receptor.

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