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RELATIVE CYTOTOXIC ACTIVITY OF IMMUNOTOXINS REACTIVE WITH DIFFERENT EPITOPES ON THE EXTRACELLULAR DOMAIN OF THE c-*erb*B-2 (HER-2/*neu*) GENE PRODUCT p185

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Different epitopes on the extracellular domain of the HER-2 receptor can serve as distinct targets for immunotoxins. To determine the optimal epitope target for immunotoxin therapy, 7 anti-HER-2 ricin A chain murine monoclonal immunotoxins, each reactive with different epitopes of HER-2 receptor, were tested for cytotoxic activity. The immunotoxins produced 1.2–4.6 logs of cytotoxicity in limiting dilution clonogenic assays with 2 breast cancer cell lines that overexpressed HER-2. Cytotoxicity did not correlate with immunoglobulin isotype, binding affinity, relative position of epitopes or internalization of the anti-HER-2 immunotoxins. Interestingly, the most and least effective immunotoxins bound to epitopes in very close proximity. Competitive binding assays with unconjugated antibodies have previously indicated that our antibodies recognized epitopes that are arranged in a linear array. To orient this relative epitope map, deletions were prepared in the HER-2/*neu* gene and these mutant constructs were expressed in NIH3T3 cells. Epitope expression was determined by antibody binding and radioimmunoassay. Epitopes targeted by the PB3, 454C11 and NB3 antibodies are localized N-terminal to the epitopes recognized by ID5, BD5, 741F8 and 520C9 antibodies. The 2 non-conformational epitopes PB3 and NB3 were localized to regions corresponding to amino acides 78–242 of the p185^{HER-2} protein. *Int. J. Cancer* 82:525–531, 1999.
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The protein product of the HER-2/c-*erb*B-2 oncogene (p185), a tyrosine kinase receptor, is an appealing target for serotherapy. Approximately one third of breast and ovarian cancers overexpress p185 and overexpression is associated with poor prognosis in these patients, whereas normal tissues express relatively low levels of this receptor (Slamon *et al.,* 1989; Berchuck *et al.,* 1990). Antibodies against HER-2 either alone or in combination with chemotherapy have shown promising results in clinical studies (Cobleigh *et al.,* 1998; Slamon *et al.,* 1998). Identification and functional analysis of epitopes recognized by distinct anti-HER-2 antibodies may be important for optimizing this therapeutic strategy. Characterization of immunogenic regions of the HER-2 protein is also important to design strategies to enhance immune response against this tumor-associated antigen, and functional analysis of epitopes may reveal important relationship between HER-2 protein structure and function.

Various anti-HER-2 monoclonal antibodies (MAbs) inhibit cell growth *in vitro* with varying efficiency (Drebin *et al.,* 1988; Hudziak *et al.,* 1989; Tagliabue *et al.,* 1991). The functional activity of an anti-HER-2 antibody is more related to the epitope that it recognizes than to its antigen binding affinity or ability to block ligand binding (Xu *et al.,* 1993). Antibodies that bind near the transmembrane region of the HER-2 extracellular domain may have more potent antiproliferative activity than those binding the N-terminus (Lewis-Phillips *et al.,* 1998). On the other hand, antibodies that bind close to the N-terminus exert more cytotoxic effects than antibodies that bind near the transmembrane region (Lewis-Phillips *et al.,* 1998). Immunotoxins directed against distinct p185 epitopes may also exert different levels of cytotoxicity (Rodriguez *et al.,* 1993; Tecce *et al.,* 1993). Such differential cytotoxicity has been demonstrated for immunotoxins reactive with the CD2 (Press *et al.,* 1988) and IgD molecules (May *et al.,* 1990). Conjugates that recognize epitopes on the C-terminus of the CD2 molecule are more effective than those that bind the N-terminus. For IgD, anti-Fc immunotoxins have greater cytotoxicity than anti-Fd immunotoxins. For both molecules, epitopes more proximal to the cell membrane provide the most effective targets, possibly permitting more efficient translocation of toxin moieties.

Overexpression of p185 on breast and ovarian cancer cell lines was required for effective kill with TA1-RTA (Rodriguez *et al.,* 1993). As only the TA1 immunotoxin was evaluated, it was not clear whether antibodies that bound to different p185 epitopes would provide more potent immunotoxins. In this study, we have characterized the cytotoxic activity of 7 distinct anti-HER-2 immunotoxins directed against different epitopes on the extracellular domain of p185^{HER-2}. We attempted to determine whether cytotoxicity is related to antigen binding affinity, epitope location or efficiency of internalization of the immunotoxin. By expressing deletion mutants of the extracellular domain of HER-2 protein in NIH3T3 cells, we investigated if amino acid sequence information could be linked to an epitope map previously generated by competitive binding assays.

MATERIAL AND METHODS

Cell lines

SKBr3 and BT474 human breast cancer cell lines were maintained in RPMI-1640 medium supplemented with 15% heat-inactivated fetal bovine serum (FBS) and 2 mM L-glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin. The NIH3T3 murine fibroblast cell line and the 17313 subclone of this cell line (McKenzie *et al.,* 1989) were kindly provided by Dr. S. McKenzie (Cambridge, MA). The 17313 clone was produced by transfection of the full-length human *neu* gene into NIH3T3 cells. Both murine cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS and 2 mM L-glutamine. Medium for 17313 was additionally supplemented with 400 μg/ml G418 (GIBCO BRL, Grand Island, NY). The additional transfectants of NIH3T3 described in this report were maintained in medium identical to that described for the 17313 cell line. The LTR-1/ΔN*erb*B2 transfectant and its parental NIH3T3 line were kindly provided by Dr. S. Aaronson (National Cancer Institute, Bethesda, MD). The LTR-1/ΔN*erb*B2 transfectant contains a construct of c-*erb*B-2 that lacks most of the extracellular domain (Di Fiore *et al.,* 1987). The LTR-1/ΔN*erb*B2 transfectant and its parental NIH3T3 cells were cultured in DMEM supplemented with 10% FBS and 2 mM L-glutamine.

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MAbs

Murine MAbs, that react with the extracellular domain of p185 were kindly provided by Dr. S. McKenzie (TA1, BD5, ID5, NB3, RC1, RC6, PB3 and OD3) ABT-Oncogene Science, Cambridge, MA and by Dr. D. Ring (520C9, 741F8 and 454C11) (Ring *et al.,* 1991), Emeryville, CA. The 225 MAb recognizes the extracellular domain of the epidermal growth factor receptor and was kindly provided by Dr. J. Mendelsohn (Masui *et al.,* 1989). All the murine MAbs were of the IgG_1 isotype except PB3 (IgG_{2a}), 454C11 (IgG_{2a}), and OD3 (IgM). MOPC21 (IgG_1), H16-L10-4R5 (IgG_{2a}) and NS.4.1 (IgM) were obtained from the ATCC (Rockerville, MD) and used as isotype-matched controls that did not bind to p185. Purified MAbs were prepared from hybridoma-induced ascites fluid as described previously (Xu *et al.,* 1993). Competitive binding assays reported previously suggest that these different antibodies recognize a linear array of epitopes on the extracellular domain of p185 (Xu *et al.,* 1993).

Immunotoxin preparation

Immunotoxins were prepared by conjugation with recombinantly derived RTA kindly provided by Dr. L. Houston (Emeryville, CA) using 2-iminothiolane (Wawrzynaczak and Thorpe, 1987). The ratio of toxin/antibody was 3 to 5. Immunotoxin preparations were characterized by SDS-PAGE. Purified immunotoxins contained no free antibody or ricin A chain by gel analysis. Activity of RTA and antibody-RTA conjugates was determined by inhibition of translation in a rabbit reticulocyte lysate *in vitro* translation system (Promega, Madison, WI).

Clonogenic assay

To study the effect of immunotoxins on growth of breast cancer cell lines, an *in vitro* limiting dilution clonogenic assay was used. SKBr3 or BT474 breast cancer cells (10^6) were incubated at 37°C for 3 hr in an atmosphere of 5% CO_2 and humidified air on a rotator with different concentrations of the immunotoxin conjugates in growth medium. The cells were washed twice with growth medium and serially diluted 5-fold. A 100 µl portion of each dilution was plated in each of the 6 wells within a 96-well flat-bottomed microtiter plate. An additional 100 µl aliquot of tissue culture medium was added to each well. The cells were incubated for 14 days at 37°C. Clonogenic growth was determined using an inverted phase microscope, scoring the number of wells with at least 1 tumor colony that contained at least 10 cells. Estimates of the surviving clonogenic units were calculated according to a modification of the method of Spearman and Karber. Clonogenic elimination was calculated by subtracting the log clonogenic units of the medium-treated cultures from the log clonogenic units of the treated cell culture for each individual cell line.

*Deletion constructs of c-*erb*B-2*

Deletion constructs of the c-*erb*B-2 gene were prepared in a shuttle vector, excised and re-ligated into an expression vector for transfection into NIH3T3 cells (Fig. 1). A pUC19-plasmid shuttle vector (pUC-HER) was prepared which contained the full-length HER-2/c-*erb*B-2 cDNA in a unique Xho1 restriction site kindly provided by Dr. S. Aaronson. The shuttle vector was restricted with Msc1 to remove a fragment of 630 bp from the intracellular domain of the c-*erb*B-2 cDNA. This excision was required to remove sequences containing restriction sites from the cDNA which would have interfered with construction of the deletions in the extracellular domain. This deletion does not interfere with the protein reading frame, with expression of protein in transfected cells or with conformation of the extracellular domain of the expressed c-*erb*B-2 protein. Mutations were then generated within the c-*erb*B-2 cDNA as described below, followed by excision with Xho1, and insertion by ligation into the appropriate expression vector.

Construct MMTneo Mro1. The pUC-HER/Msc vector was restricted with Mro1 at cDNA position 2219. The 3′ overhangs were filled in with Klenow and the blunt ends re-ligated. This construct created an out-of-frame sequence beginning at amino acid 684 and ending with a termination codon at amino acid position 700.

Construct PMx1112 Eco 2-20. The pUC-HER/Msc vector was restricted with EcoR5 and Nae1, at positions 408 and 900, respectively. The vector containing the remaining c-*erb*B-2 sequences was then re-ligated. Since both enzymes cleaved between codons, the reading frame was maintained with the deletion of 492 bp, or 164 amino acids.

Construct MMTneo Hinc 6-17. The pUC-HER/Msc vector was restricted with Nae1 and Hinc2 at positions 900 and 1764, respectively, removing a fragment of 864 bp. The vector containing

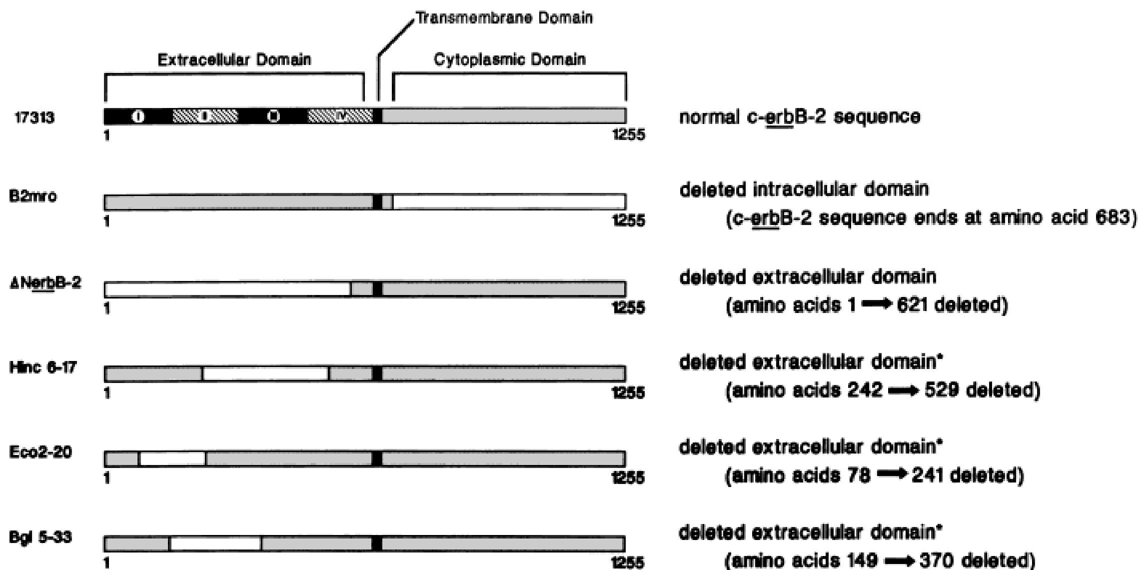

FIGURE 1. Deletion constructs of the c-*erb*B-2 gene. Deletions were prepared as described in Material and Methods.

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the remaining c-*erb*B-2 sequences was re-ligated, resulting in the maintenance of the reading frame and the loss of 288 amino acids.

Construct MMTneo Bgl 5-33. The pUC-HER/Msc vector was restricted with Bgl2, which cleaves at cDNA positions 615 and 1280, removing a fragment of 666 bp. The vector containing the remaining c-*erb*B-2 sequences was re-ligated, resulting in the maintenance of the reading frame and the loss of 222 amino acids. The constructs were excised from the shuttle vector with Xho1, ligated into the MMTneo or PMx1112 expression vectors, and determined to have the correct orientation by use of the unique Kpn1 site at position 3250. Verification of the deletions in the different constructs was performed by sequence analysis.

Transfection

Deletion constructs of c-*erb*B-2 were transfected into NIH3T3 cells using calcium phosphate. In the case of the PMx1112 expression vector, NIH3T3 cells were cotransfected with the PMx1112 vector and the SV2neo vector containing the neomycin resistance gene (Dr. R. Kaufman, Durham, NC) at a 5 to 1 ratio of PMX112 DNA to SV2neo DNA. Transfectants were selected in medium containing 400 µg/ml neomycin sulfate (G418; GIBCO BRL).

Immunohistochemical staining

Transfectants containing constructs with deletions in the extracellular domain of p185 were screened initially for the presence of the intracellular domain with the 135C6EE and 145WWA-1 MAbs kindly provided by Dr. C. Nolan (Chicago, IL) which were produced against a peptide containing amino acid residues 1056 through 1072 of the p185 sequence. Transfectants were grown in Lab-Tek chamber slides (Nunc, Naperville, IL), and monolayers rinsed with PBS, air dried and fixed in cold acetone. Binding of 135C6EE, 145WWA-1 and MOPC21 (non-specific control) to the different transfectants was assessed with the Vectastain ABC Kit (Vector, Burlingame, CA). MAbs were diluted to 5 µg/ml for immunohistochemical staining. Slides were incubated with diluted antibody for 1 hr at room temperature and washed with PBS. Slides were then incubated with biotinylated anti-mouse IgG (Vectastain ABC kit), washed with PBS and incubated with ABC regent (Vectastain ABC kit) according to the manufacturer's instructions. Slides were developed with the enzymatic substrate diaminobenzidine, washed in tap water and counterstained with methyl green. Transfectants in which expression of the construct was documented by positive staining with 135C6EE and 145WWA-1 were further screened for binding of the different antibodies to epitopes on the extracellular domain of p185 by indirect live cell radioimmunoassay.

Indirect live cell radioimmunoassay

The indirect live cell radioimmunoassay was performed as described previously (Boyer *et al.,* 1989). Briefly, 1–2 $\times 10^4$ cells were plated in flat-bottomed RemovaCell microtiter plates (Dynatech, Alexandria, VA) and incubated overnight at 37°C in an atmosphere of 5% CO_2 and 95% humidified air. Assay wells were blocked with RPMI-1640 medium supplemented with 10% BSA and 0.08% sodium azide for 1 hr at 37°C. Blocking medium was removed and 50 µl MAb diluted to 5 µg/ml in assay medium (RPMI-1640 medium supplemented with 1% BSA and 0.08% sodium azide) was added to quadruplicate wells. Plates were incubated for 1 hr at 37°C. Following 3 washes with assay medium, ^{125}I-labeled sheep antimouse F(ab′)$_2$ fragments (10^5 cpm) (NEN, Boston, MA) were added in 50 µl of assay medium and plates were incubated for 1 hr at 37°C. Plates were washed 3 times with assay medium and RemovaCell wells separated and counted for 1 min in a gamma counter. Binding ratios were calculated for each antibody by dividing the mean cpm with antibody divided by the mean cpm for assay medium. The average binding ratio obtained in multiple experiments for the non-specific isotype control antibody was compared to the average binding ratio for each anti-p185 antibody by the Student's *t*-test. An epitope was considered retained on a construct if the average binding ratio of the anti-p185 antibody was statistically different ($p < 0.05$) from the average binding ratio of the control and the amount of binding was at least 2-fold above the binding of the control antibody.

Radioiodination of MAbs and immunoconjugates

MAbs were labeled with Na^{125}I using the iodogen method. In brief, 50 µl of phosphate buffer (0.5 M, pH 7.4) were added to a 15 $\times$ 75 mm borosilicate tube coated with 10 µg of iodogen (Pierce, Rockford, IL). MAb (50 µg) was added in a volume of 95 µl PBS (50 mM phosphate buffer, 0.15 M NaCl). Radioiodination was initiated by the addition of 0.5 mCi of Na^{125}I (5 µl) and the mixture was incubated for 30 min on ice. The protein-bound iodine was separated from free ^{125}I by gel filtration on a PD-10 column (Pharmacia, Pleasant Hill, CA) that had been equilibrated with PBS. A sample of 3 µl from each fraction was counted in a gamma counter to measure protein-bound radioactivity. Iodination efficiency was calculated using the following formula:

$$\text{Iodination efficiency} = \frac{\text{protein bound cpm}}{\text{total cpm}} \times 100\%.$$

Direct live cell radioimmunoassay and Scatchard analysis

Cells were plated as if indirect live cell radioimmunoassay were to be performed. After overnight incubation, monolayers were washed with RPMI-1640 medium supplemented with 1% FBS and 0.1% sodium azide. Different amounts of ^{125}I-labeled MAb or immunotoxin were added in 50 µl aliquots to triplicate cell monolayers. Non-specific binding was determined by adding different amounts of ^{125}I-labeled MAbs or immunotoxins to empty wells. After incubation on ice for 4 hr, unbound antibodies were removed by washing the wells 4 times with ice-cold assay medium supplemented with 5% FBS and 0.1% sodium azide. Individual wells were then detached, and radioactivity was determined in a gamma counter. The EBDA program was used to calculate the number of binding sites (McPherson, 1985):

$$\text{Binding sites} = \frac{\text{maximum binding (mol)} \times 6.23 \times 10^{23} \times \text{volume (L)}}{\text{cell number}}.$$

Internalization of antibodies and immunotoxins

^{125}I-labeled antibody or immunotoxin (0.25 µg/ml) was added to aliquots of 10^6 SKBr3 cells. After incubation for 1 hr at 4°C to allow binding, cells were washed 3 times with RPMI-1640 medium with 1% BSA and then either counted immediately to determine the total amount of antibody or immunotoxin bound or incubated at 4°C or 37°C for 1 hr to permit internalization of immunoglobulin. To remove antibody still bound to the cell surface, 2.5 mg/ml of proteinase K (Sigma, St. Louis, MO) was added to the cells for 1 hr at 37°C. The cells were washed 3 times in RPMI-1640 medium supplemented with 1% BSA and 0.1% sodium azide. Radioactivity associated with cell pellets was counted in a gamma counter. The amount of antibody internalized was determined by subtracting the cpm obtained after incubation at 4°C followed by protease stripping from the cpm obtained after incubation at 37°C for the same time interval, followed by protease stripping. The percentage internalized was then calculated by dividing the cpm of the antibody internalized by the total cpm initially bound to the cell surface.

Western blot

SKBr3 cells (20 $\times 10^6$) were removed from a tissue culture flask by scraping into cold PBS, pelleted at 1,000*g* for 10 min at 4°C and incubated in 0.5 ml of lysis buffer (150 mM NaCl, 5 mM EDTA, 50 mM Tris, pH 8, containing 4 mM benzamidine, 10 µg/ml leupeptin, 10 µg/ml pepstatin A, and 1 mM phenylmethylsulfonyl fluoride, and 1% Nonidet P-40) on ice for 30 min. The lysate was centrifuged at 1,000*g* for 10 min at 4°C and the supernatant was

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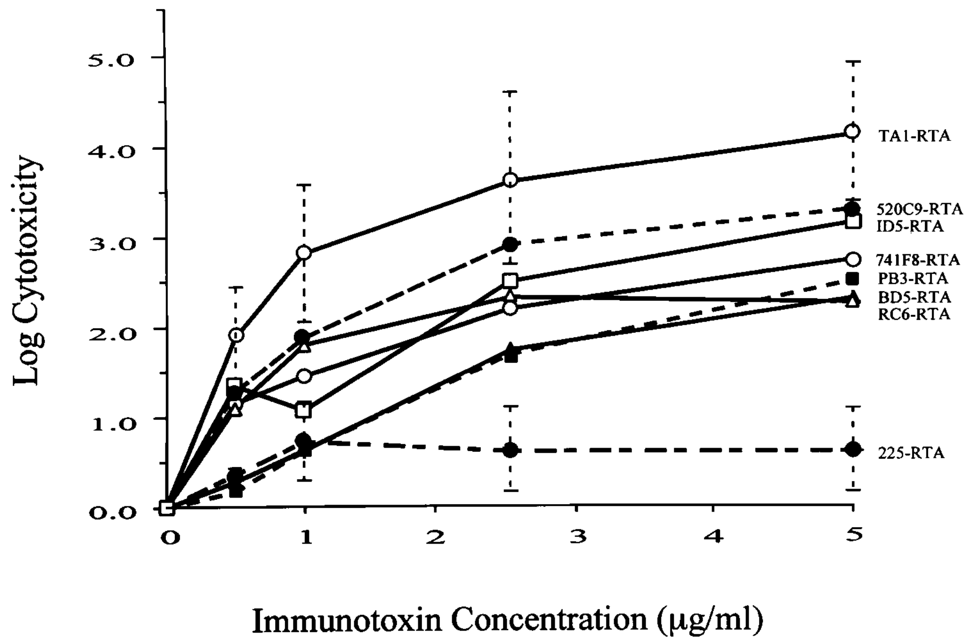

FIGURE 2 – Cytotoxic activity of RTA immunotoxins reactive with different epitopes of p185$^{c\text{-}erbB\text{-}2}$ and the epidermal growth factor receptor in SKBr3 breast cancer cells. Data are expressed as mean log kill for 2–4 experiments at a given concentration for each immunotoxin.

collected. Proteins in the SKBr3 lysate were separated by SDS-PAGE in 7.5% polyacrylamide gels under reducing conditions (PhastSystem; Pharmacia LKB, Piscataway, NJ). Separated proteins were electrophoretically transferred to nitrocellulose using the PhastTransfer semidry protein transfer kit (Pharmacia). Remaining protein binding sites on the nitrocellulose membranes were blocked by incubation in Blotto (3% non-fat dry milk powder, 2% normal goat serum and 0.1% Tween-20 in PBS) for 1 hr at room temperature. Membranes were then incubated for 3 hr with anti-p185 antibody diluted to 20 µg/ml in Blotto at room temperature with gentle agitation. Membranes were washed 3 times in wash buffer (20 mM Tris, 1 M NaCl, 0.05% Tween-20, pH 7.6) for 10 min with gentle agitation followed by incubation with phosphatase-labeled goat anti-mouse IgG + IgA + IgM diluted 1:500 in Blotto (Kirkegaard and Perry, Gaithersburg, MD) for 1 hr at room temperature with gentle agitation. Membranes were washed 3 times as described above and developed with the BCIP/NBT phosphatase substrate system.

Statistical analysis

Limiting dilution analysis was performed using a Spearman estimate. The mean of the dose-response function for each treatment was estimated by

$$m = X_0 + \frac{d}{2} - \frac{d}{n}\sum_{i=0}^{k} r_i$$

where X_0 = ln initial dose = ln 10^{-1}; d = ln dilution factor = ln 5; n = number of wells at each dilution = 6; k = number of 5-fold dilutions; and r_i = the number of wells with observed growth at the ith dilution. The estimated number of clonogenic units per ml was then calculated as $0 = \exp(-0.57722 - m)$. The initial dose (10^{-1} ml) and the number of dilutions (k = 9) were chosen to have a high probability (>0.99) that r_0 = n and r_k = 0. Tests of significance to compare 2 treatments were based on the asymptotic normality of the estimates of the means (m), each with variance closely approximated by d $\ln^2$/n. A "Z" statistic was used to estimate the level of significance. A formal comparison was performed in which the means and variances were obtained from the Spearman estimator; 5-fold dilutions with 6 wells/dilution permitted the detection (at $p = 0.05$, 2-sided) of a 10-fold difference in clonogenic units between 2 treatment groups with probability 0.90. Corrections for multiple comparisons were made when appropriate.

TABLE I – CYTOTOXIC ACTIVITY (LOG KILL, MEAN ± SE) OF RTA IMMUNOTOXINS REACTIVE WITH DIFFERENT EPITOPES OF p185$^{c\text{-}erbB\text{-}2}$ AND THE EPIDERMAL GROWTH FACTOR RECEPTOR IN SKBr3 BREAST CANCER CELLS

Cell lines	Cytotoxic activity			
	5 µg/ml	2.5 µg/ml	1 µg/ml	0.5 µg/ml
TA1-RTA	4.12 ± 0.77	3.61 ± 0.90	2.82 ± 0.76	1.89 ± 0.53
BD5-RTA	2.61 ± 0.05	1.75 ± 0.47	0.64 ± 0.06	0.29 ± 0.06
RC6-RTA	2.51 ± 1.27	1.28 ± 0.82	0.64 ± 0.06	0.17 ± 0.06
PB3-RTA	2.27 ± 0.41	2.33 ± 0.24	1.81 ± 0.41	1.11 ± 0.30
520C9-RTA	3.30 ± 1.14	2.91 ± 0.51	1.98 ± 0.66	1.28 ± 0.50
ID5-RTA	3.15 ± 0.69	2.50 ± 0.29	1.28 ± 0.35	1.34 ± 0.18
741F8-RTA	2.74 ± 0.53	2.21 ± 0.58	1.46 ± 0.65	1.16 ± 0.35
225-RTA	0.62 ± 0.47	0.62 ± 0.47	0.73 ± 0.40	0.36 ± 0.08

RESULTS

Differential cytotoxicity of immunotoxins reactive with distinct p185 epitopes

To compare the activity of immunotoxins reactive with different p185 epitopes, ricin A chain (RTA) conjugates were prepared from 7 antibodies, each recognizing different epitopes on p185 (Xu *et al.,* 1993), and 1 antibody, 225, was directed against an epitope on the extracellular domain of the epidermal growth factor receptor. Cytotoxicity was measured in limiting dilution clonogenic assays. Both SKBr3 and BT474 breast cancer cells overexpress p185 and also express low levels of the epidermal growth factor receptor. The cytotoxic effect of the different immunotoxins was similar on BT474 and SKBr3 cells. TA1-RTA was the most effective immunotoxin, whereas BD5-RTA and RC6-RTA were the least effective Anti-epidermal growth factor receptor immunotoxin 225-RTA also had low cytotoxic activity. Differences were particularly marked at immunotoxin concentrations of 0.5 and 1 µg/ml. Unconjugated anti-p185 antibodies are ineffective in limiting dilution clonogenic assays (data not shown). Both BD5 and ID5 antibodies react with

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TABLE II – BINDING CHARACTERISTICS OF ANTI-p185 ANTIBODIES BEFORE AND AFTER CONJUGATION TO RICIN A CHAIN

Antibody/immunotoxin	Relative affinity[1] (M − 1)	Binding sites/cell ($\times 10^5$)
TAI	$(2.56 \pm 0.68) \times 10^{-9}$	8.0
TAI-RTA	$(2.93 \pm 1.94) \times 10^{-9}$	11.0
BD5	$(1.09 \pm 0.18) \times 10^{-8}$	14.8
BD5-RTA	$(1.23 \pm 0.16) \times 10^{-8}$	41.9
RC6	$(8.15 \pm 0.64) \times 10^{-9}$	5.8
RC6-RTA	$(5.95 \pm 1.40) \times 10^{-8}$	11.0

[1]Mean ± SD.

TABLE III – INHIBITION OF PROTEIN SYNTHESIS IN AN *IN VITRO* TRANSLATION SYSTEM BY DIFFERENT RICIN A CHAIN IMMUNOTOXINS AND RICIN A CHAIN ALONE (RTA)[1]

RTA or antibody-RTA	5.6×10^{-10} M	5.6×10^{-11} M	5.6×10^{-12} M	5.6×10^{-13} M
RTA	3	3	16	55
TAI-RTA	4	4	14	51
RC6-RTA	5	5	35	55
BD5-RTA	5	5	16	40
PB3-RTA	5	3	5	18
520C9-RTA	2	3	5	16
741F8-RTA	3	3	6	21
225-RTA	7	5	11	32

[1]Percent inhibition of ^{35}S-methionine incorporation into proteins in rabbit reticulocyte lysate *in vitro* translation system. Inhibition is expressed as percentage of control (treatment with corresponding unconjugated antibody alone) measured at 4 different immunotoxin and RTA concentrations).

TABLE IV – INTERNALIZATION OF ^{125}I-LABELED ANTI-p185 ANTIBODIES AND IMMUNOTOXINS[1]

Antibodies/immunotoxins	% internalization[2]
TAI	13 ± 3
TAI-RTA	7 ± 2
BD5	12 ± 4
BD5-RTA	11 ± 1
RC6	8 ± 1
RC6-RTA	8 ± 1

[1]The percentage of internalization after one hr incubation with antibody/immunotoxin at 37°C was calculated as described in Material and Methods.–[2]Mean ± SE.

the extracellular domain of p185 at or near the ligand binding site, since these antibodies competitively inhibit ligand binding to HER-2 receptor and paradoxically also induce growth simulation in soft agar (Xu *et al.,* 1993).

Cytotoxicity of immunotoxins is not related to isotype, different p185 binding affinity or differential ricin activity

Six of the 7 antibodies used in these studies are of the IgG_1 isotype and 1, PB3, is of the IgG_{2a} isotype, indicating that cytotoxic activity *in vitro* is not related to antibody isotype. Similarly, the relative binding affinity of the different anti-p185 antibodies did not correlate with the cytotoxicity of the corresponding immunotoxin. Relative binding affinities of 11 different anti-p185 antibodies including the 7 utilized in our study have been determined on 2 cell lines with differing levels of p185 and reported previously (Xu *et al.,* 1993). TA1 demonstrated the highest relative binding affinity and the relative binding affinities for the remaining 6 antibodies were similar.

To determine whether the RTA conjugation affected binding affinity, Scatchard analysis was performed for the most effective (TA1) and least effective (BD5 and RC6) immunotoxins before and after conjugation to RTA (Table II). The relative binding affinities for TA1 and TA1-RTA, BD5 and BD5-RTA were identical before and after conjugation. However, for RC6 the conjugation process decreased the relative binding affinity by about 10-fold. The binding sites/cell were within a 2-fold range for both RC6 and RC6-RTA.

To establish that each immunotoxin inhibited protein synthesis at a comparable level and that the ricin A chain remained active after the conjugation process, immunotoxins were tested in a rabbit reticulocyte lysate *in vitro* translation system (Table III).

Internalization levels of different anti-p185 antibodies and immunotoxins are similar

Ricin-conjugated and unconjugated forms of TA1, BD5 and RC6 were tested for efficiency of internalization in SKBr3 cells. After 1 hr of incubation at 37°C with antibodies, followed by protease treatment to remove cell surface bound antibodies, the amount of cell-associated radioactivity representing internalized antibodies was determined. Results are presented in Table IV. RTA conjugation did not affect the amount of internalization observed when immunotoxins were compared to unconjugated antibody. All 3 immunotoxins, each recognizing a different epitope of p185 protein, produced similar levels of internalization.

Position and orientation of the relative epitope map on the extracellular domain of p185

Previous competitive inhibition studies with radiolabeled antibodies have defined an epitope map for the extracellular domain in which most of the epitopes were arranged in a linear array (Xu *et al.,* 1993). The results, however, could not indicate the location of epitopes with respect to the peptide sequence of p185. To determine the position and orientation of the relative epitope map on the p185 sequence, deletion mutants of c-*erb*B-2 were prepared, transfected into NIH3T3 cells and evaluated for expression of the different p185 epitopes. NIH3T3 cells that expressed full-length p185 (17313) (McKenzie *et al.,* 1989) and NIH3T3 cells that expressed a deletion construct lacking most of the extracellular domain (LTR-1/ΔN*erb*B-2) (Di Fiore *et al.,* 1987) were obtained for study (Fig. 1). In the B2mro construct, most of the intracellular domain was deleted. In the Hinc 6–17, Eco 2–20 and Bgl 5–33 constructs, portions of the extracellular domain were deleted. The deletions in the Hinc 6–17 (codons 242–529) and Eco 2–20 (codons 78–241) constructs share a common border between codons 241 and 242. The deletion in the Bgl 5–33 (codons 149–370) construct spans the common border shared by the Hinc 6–17 and Eco 2–20 constructs.

NIH3T3 transfectants that contained deletions in the extracellular domain were screened for expression of the construct by immunohistochemical staining with antibodies that recognized the intracellular domain of p185. Only those transfectants that were positive for expression were further evaluated in indirect live cell radioimmunoassays with antibodies that recognized different epitopes on the extracellular domain of p185. The 17313 cells that expressed full-length p185 and the extracellular domain epitopes were used as a positive control. As expected, all 10 of the anti-p185 antibodies bound to 17313 cells (Fig. 3). Deletion of the intracellular domain had no effect on the binding of antibodies to the extracellular domain. The Hinc 6–17 construct retained binding for the 454C11, PB3, NB3 and RC6 antibodies. The complementary Eco 2–20 construct retained binding for the RC6, ID5, BD5, 741F8 and 520C9 antibodies. Although the Bgl 5–33 construct was expressed based on binding of antibodies to the intracellular domain, it did not retain any of the epitopes recognized by the 10 antibodies to the extracellular domain. Since the Eco 2–20 deletion was N-terminal to the Hinc 6–17 deletion, this oriented the PB3, 454C11 and NB3 epitopes N-terminal to the ID5, BD5, 741F8 and 520C9 epitopes.

When epitopes were retained by a construct, both the conformation and sequence information for the epitope were maintained. When epitopes were lost it is possible that the conformation was destroyed or that the sequence was deleted. In the case of the PB3 and NB3 epitopes, antibody binding did not require native p185 conformation since these antibodies bound p185 after electrophore-

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