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PRINCIPAL INVESTIGATOR: Jinha M. Park
Michael Press, Ph.D.

CONTRACTING ORGANIZATION: University of Southern California
Los Angeles, California 90033

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Jinha M. Park
Michael Press, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

University of Southern California
Los Angeles, California 90033

park@hsu.usc.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

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13. ABSTRACT *(Maximum 200 words)*

Immunotherapy has proven to be safe and effective in targeting human cancers in a clinical setting. However, durable disease-free remissions have yet to be realized in the majority of treated patients. Unconjugated antibodies can be improved by the attachment of effector molecules to the cancer-specific antibody. Improvements in the design of antibody based therapeutics will require specific delivery of cytotoxic agents such as toxin molecules or toxin-encoding genes. Two monoclonal antibodies with selective binding to HER-2/*neu* overexpressing breast cancer cells have been miniaturized by fusing variable chain genes with the dimerization domain of the human Fc constant region. First, the genes were isolated using low stringency conditions and degenerate oligonucleotides to consensus regions in the framework of the variable chain genes and the constant region of the gamma heavy and kappa light chain regions of mouse immunoglobulin (IgG) mRNAs. Confirmation of cloning was achieved by sequencing of variable regions and comparing framework regions to a database of IgG sequences. The fusion of the variable heavy (VH) gene with the variable light (VL) chain gene was accomplished by the technique of splice overlap extension PCR. The scFv was fused to the human IgG1 Fc domain 3 which dimerizes the heavy chains in an IgG. This expression cassette was introduced by electroporation into NS0 (non-secretor) myeloma cells for antibody secretion. Positive secretor clones were identified by enzyme-linked immunosorbent assay against recombinant $p185^{HER-2}$ extracellular domain. Thus, the engineering of the proper reconfiguration of divalent "minibodies" was confirmed by binding to ECD.

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Julie Park 8/10/99

PI - Signature Date

Michael J. Jones 8/10/99

ANNUAL SUMMARY
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INTRODUCTION

Novel therapies which require internalization of effector domains may be improved by assessing the efficiency of post-binding receptor-mediated endocytosis. To achieve targeted gene therapy or immunotoxin therapy, natural vector-host tropisms must be altered. Recent improvements in monoclonal antibody (mAb) engineering have expanded the potential range of host cells which can be targeted for therapeutic intervention. However, relatively little is known about cellular responses after binding of a vector construct. We have tested the utility of four novel monoclonal antibodies recognizing the extracellular domain of $p185^{HER-2}$, a membrane receptor protein, for use in internalization-dependent therapies. All four antibodies bound to $p185^{HER-2}$ in a number of immunoassays. Two antibodies recognized accessible epitopes of $p185^{HER-2}$ on viable cells. Radioimmunoassay demonstrated that antibody-membrane receptor complexes formed by two antibodies were internalized and trafficked through an endolysosomal degradative pathway. Two of the four antibodies evaluated were found to have favorable internalization characteristics suitable for incorporation in a targeting vector. This analytical approach could be applied to antibodies prior to and after fusion with various vectors or toxins to determine the potential utility of the antibodies for targeted therapy.

The anti-$p185^{HER-2}$ mAbs 8H11 and 10H8 were engineered for therapeutic uses. We sought to make ideal sized constructs for *in vivo* tumor penetration with rapid clearance. In addition, by genetically cloning the variable genes, we could also attach effector molecules to enhance the delivery of toxin proteins or DNA to $p185^{HER-2}$ overexpressing cells. While initially in its early stages, the minibody is the most likely format to reach the goals of targeted therapies to $p185^{HER-2}$ overexpressing breast cancers.

YEAR 2 ANNUAL SUMMARY:

BODY

Isolation and cloning of variable fragments of heavy and light immunoglobulin chains

PCR reactions using degenerate oligonucleotides optimized from the variable regions of mouse heavy and light leader sequences amplified the variable heavy and light chain genes for the 8H11 and 10H8 mAbs (Table 1). After RNA isolation for hybridoma cell lines secreting the 8H11 and 10H8 mAbs, reverse transcription reactions resulted in copying of cDNA. Figure 1 depicts the scheme for isolation and cloning of the VH and VL chain gene of the mAbs. PCR reactions using the VHBi3d and HCRegi2 primers yielded the VH chains of both 8H11 and 10H8 (Figure 2). Likewise, the VkBi7 and LCRegi2 primers yielded the VL chains of both mAbs. PCR products with deoxyadenosine overhangs were ligated into a "TA Cloning" vector plasmid with deoxythymidine overhangs (Invitrogen, Carlsbad, CA). Competent cells were transformed and colonies harboring plasmids with PCR products were selected by blue-white screening according to the manufacturer's protocols. Positive clones were sequenced by the Sanger dideoxy method to confirm that PCR products cloned were indeed bona fide mouse immunoglobulin genes. Using predicted amino acid sequences derived from nucleotide sequneces, we confirmed that all four cDNAs were indeed mouse VH and VL genes (Figure 3). Based on Kabat's database of mouse immunoglobulin genes, each variable region belongs to a known family of heavy or light immunoglobulin types. VH8H11 belongs to Subgroup IA while VH10H8 belongs to Subgroup IIID of the variable region heavy chain sequences. For mouse variable region light chain sequences, VL8H11 matched as a family member of Subgroup IV. VL10H8 matched sequences similar to Subgroup II.

Splice overlap extension (SOE) PCR to generate single-chain variable fragments (scFv)

In order to generate antigen-binding protein conformation by apposition of the variable light and variable chains, we used a technique to SOE ("sew") two cDNA fragments together using a novel application of PCR. The scheme for SOE is depicted in Figure 4. In both 8H11 and 10H8, we synthesized primers with overlapping ends that would provide the basis of complementarity between two distinct cDNAs. Tables 2 and 3 list the primers used in two sets of SOE PCR reactions to generate the scFv of 8H11 and 10H8. In fact, three distinct templates were amplified using these extended primers to generate two SOE PCR reactions per mAb. The first template to be used was the anti-CEA mAb heavy chain leader signal peptide and its endogenous Kozak sequence with ribosome binding site. This sequence is necessary for the expression and secretion of the engineered forms of 8H11 and 10H8 in the Glutamine Synthetase Protein Expression system described below.

The first PCR reactions were straightforward amplifications of the three template components needed for eventual fusion into one cDNA. All three templates were amplified using primer pairs A+B, C+D, and E+F. Then the first two templates to be SOEed were the products AB and CD. Under the conditions of overlap extension, a new template was formed from separated sense and antisense strands of both AB and CD which, upon low temperature conditions at 37°C, were allowed to re-anneal at the primer overlap sequences between the B primer and the C primer. Extension using Taq polymerase created a new fusion between AB and CD depicted as AD. Since there is so little product, ethidium bromide staining of agarose electrophoresed PCR procuts is undetectable. The next step relies on the presence of this AD product for amplification now using primer pairs of A and D. Once enough of this template is amplified, another round of SOE PCR is performed, this time with purifed EF template. And finally, amplification by standard PCR of the AF product yields the scFv. The cloned sequences were again ligated into a TA cloning vector. These sequences were checked by automated sequencing for expected splice success and presence of mutations from Taq PCR. In our two cases, there was only a silent mutation in the VL of the 8H11 that had no effect on the serine encoded by either degenerate codon.

Cloning into the minibody expression vector for production of a divalent, engineered antibody

Two forms of the linker between the scFv and the CH3 human constant domain were tested. The Flex linker, which has the human constant hinge domain sequence, is made up of synthetic sequences to add flexibility to the eventual dimer of scFvs formed. The VE form of the minibody is tested to see if a constrained conformation of the divalent scFvs enhances the avidity of the eventual minibody. Instead of 17 amino acids joining scFv to the CH3, the VE minibody only has a linker of valine and glutamic acid residues. Successful in-frame ligation of the 10H8 scFv into both minibody expression vectors was confirmed by automated sequencing.

Expression of Minibody 10H8 Flex and 10H8 VE in the Glutamine Synthetase Amplification Protein Expression System

Expression constructs were electroporated into NS0 mouse myeloma cells. Supernatants of surviving colonies that survived gluamine withdrawal showed that 10 clones out of 200 selectable clones were positive by protein ELISA for the presence of CH3 containing antibodies that recognized the HER-2/*neu* ECD. Follow-up work on characterization and confirmation of binding of endogeneous an overexpressed full length HER-2/*neu in vitro* and *in vivo* will be assessed.

APPENDICES

A. Figures and Tables

Figure 1. Cloning of variable genes from hybridoma cells. As depicted in the diagram, hybridoma cells were lysed and RNA extracted. RNA was subjected to first strand cDNA synthesis using an oligo-dT primer and MMLV-RT. cDNA was used to isolate the variable heavy and variable light chain gene regions by low stringency conditions of PCR. Optimized degenerate oligonucleotides used to isolate 8H11 and 10H8 genes are listed in Table 1. PCR products were ligated into cloning vectors. Multiple PCR confirmations were performed and several independent clones were manually sequenced to confirm authentic variable mouse IgG genes based on the Kabat database.

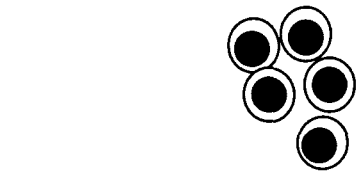
Figure 2. Agarose gel electrophoresis of amplified PCR products of VH and VL genes of 8H11 and 10H8 mAbs. Two independent PCR reactions for each set of variable genes were run to confirm the reproducibility of low stringency PCR. All PCR reactions primarily isolated the variable region genes except for VL8H11 which showed an additional band at 900 bp. Specific products were excised from the gels (370 bp products) and purified for ligation into cloning vectors for manual sequencing confirmation.

Figure 3. Predicted amino acid sequences from cloned variable heavy (VH) and variable light (VL) antibody cDNA sequences. Each predicted polypeptide contains three hypervariable regions (CDR: complementarity determining region) flanked by the four framework regions of more conserved variable region sequences. Using these sequences, the cDNA sequences were authenticated as mouse IgG genes from the Kabat database which has each family of antibodies catalogued. Hypervariable regions are underlined and typed in boldface.

PROPRIETARY AND UNPUBLISHED DATA, PLEASE PROTECT THIS FIGURE 3 LEGEND AND FIGURE ON FOLLOWING PAGE.

Figure 4. Splice overlap extension PCR to join antibody gene fragments. Diagram showing the sequence of PCR reactions necessary to fuse three DNA templates together while simultaneously adding intervening artificial peptide sequences (Whitlow 218 scFv linker). Step (1) All three templates are amplified using unique primers with annealable overlapping sequences (see Tables 2 and 3). Step (2) First SOE reaction was the ligation of the anti-CEA signal sequence to the VL of 8H11 and 10H8. Then the newly formed template was itself amplifed using primers flanking the 5' of the signal sequence and the 3' end of the VL. Step (3) The SOE is repeated in order to ligate the newly formed signal sequence-VL to the VH gene using the primers to insert the 218 linker between the ligated templates. Again, final amplification of the new template formed the scFv for the antibody.

Figure 1. Cloning of Variable Genes from Hybridoma Cells



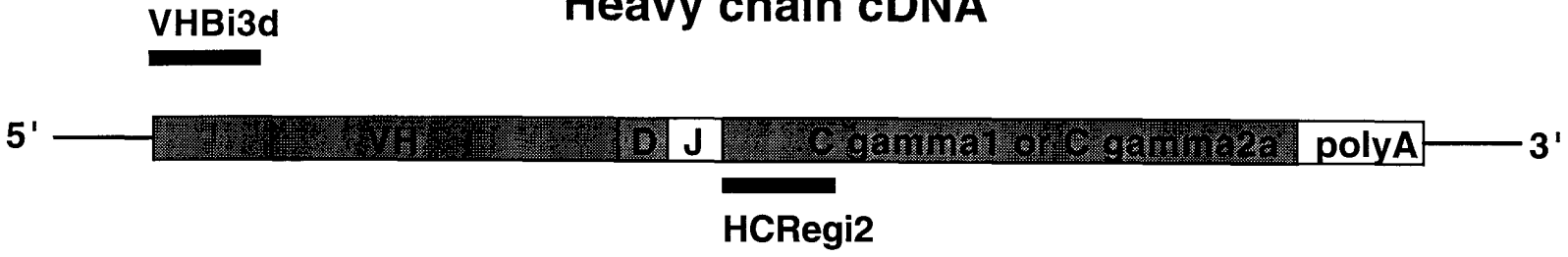
Hybridoma cell line (8H11 or 10H8)

Total RNA extraction → rRNA, mRNA, tRNA

MMLV-RT, oligo-dT primers → oligo-dT-enriched cDNA

RT-PCR:

Heavy chain cDNA



Light chain cDNA

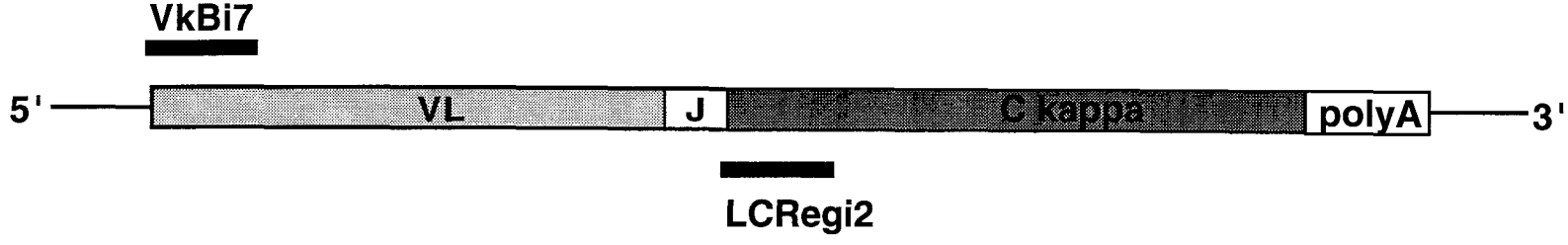


Figure 2.

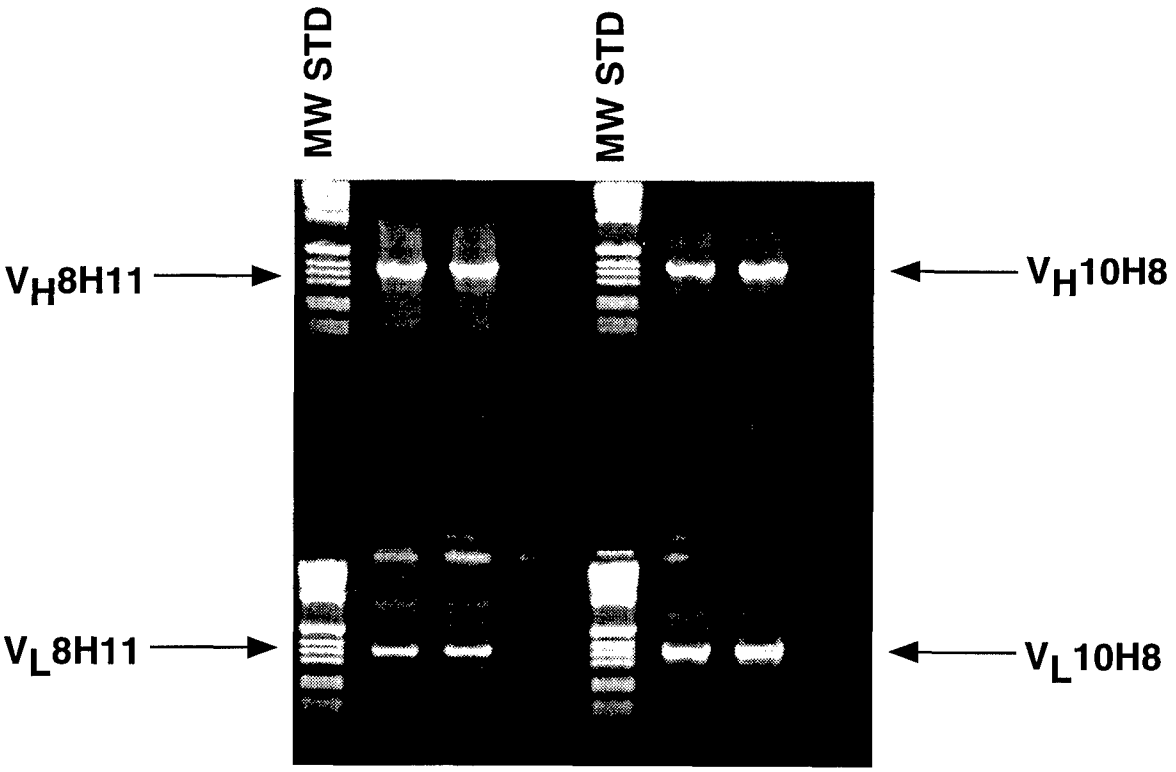


Figure 3. Predicted Amino Acid Sequences from Cloned Variable Heavy (VH) and Variable Light (VL) Antibody cDNA Sequences

VH 8H11

EVQLQESGPELKKPGETVKISCKASGYTFT**NYGMN**WVKQAPGKGLKWMG**WINTNIGEPTYTEEFKG**RFAFSLGTSASTAFLQINNL
KNEDTATYFCAR**DDGYGNRVSY**WGQGTLVTVSA

CDR1: NYGMN
CDR2: WINTNIGEPTYTEEFKG
CDR3: DDGYGNRVSY

VL 8H11

DILMTQSPLSMYTSLGERVTITC**KASQDINSYLS**WFQQKPGKSPKTLIY**RANRLVD**GVPSRFSGSGSGQDYSLTISSLEYEDMGIYYC**LQ**
HDEFPWTFGGGTKLEIK

CDR 1: KASQDINSYLS
CDR 2: RANRLVD
CDR 3: LQHDEFPWT

VH 10H8

EVQLQESGGDLVSPGGSLKLSCAASGFTFS**SNGMS**WVRQTPDKRLEWIA**TISGGGYYIYYPDSVKG**RFTISRDNAKNTLYLQMRSLK
SEDTAMYYCAR**HGDDNSSYLDV**WGAGTTVTVSS

CDR1: SNGMS
CDR2: TISGGGYYIYYPDSVKG
CDR3: HGDDNSSYLDV

VL 10H8

DILMTQTPLSLPVSLGDQASISC**RSSQSLVHSNGNTYLH**WYLQRPGQSPKLLIY**KVSNRFS**GVPDRFSGSGSGTDFTLKISRVEAEDLG
VYFC**SQSTHVPPWT**FGGGTKLEIK

CDR1: RSSQSLVHSNGNTYLH
CDR2: KVSNRFS
CDR3: SQSTHVPPWT

Figure 4. Splice Overlap Extension PCR to Join Antibody Gene Fragments

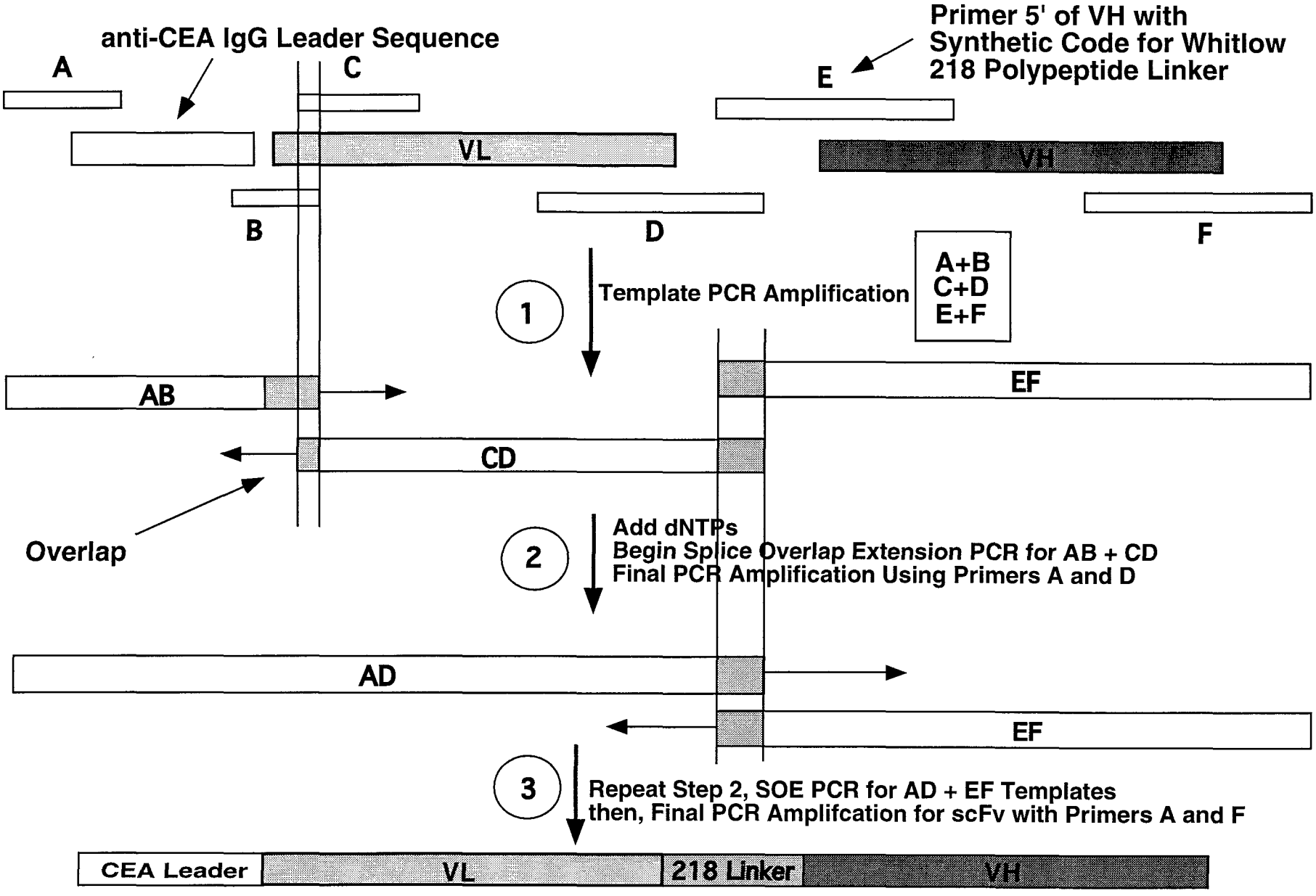


Table 4-1. Degenerate Oligonucleotides Used for Isolation of Variable Heavy and Variable Light Chains of mAb 8H11 and mAb 10H8

Primer	Description	Oligonucleotide Sequence
VHBi3	Heavy chain variable domain	5'-GAGGTGAAGCTGCAGGAGTCAGGACCTAGCCTGGTG-3' (PstI site)
VHBi3c	Heavy chain variable domain	5'-AGGT(C/G)(A/C)AGCTGCAG(C/G)AGTC(A/T)GG-3' (PstI site)
VHBi3d*	Heavy chain variable domain	5'-AGGT(C/G)CAGCTGCAG(C/G)AGTC(A/T)GG-3' (PvuII site)
HCRegi2*	Gamma chain constant domain	5'-CGGAATTCAGGGGCCAGTGGATAGAC-3' (EcoRI site)
VkBi6	Light chain variable domain	5'-GGTGATATCGTGAT(A/G)AT(C/A)CA(G/A)GATGAACTCTC-3' (EcoRV site)
VkBi7*	Light chain variable domain	5'-GGTGATATC(A/T)TG(A/C)TGACCCAA(A/T)CTCCACTCTC-3' (EcoRVsite)
VkBi8	Light chain variable domain	5'-GGTGATATCGT(G/T)CTCAC(C/T)CA(A/G)TCTCCAGCAAT-3' (EcoRV site)
LCRegi2*	Kappa chain constant domain	5'-CGGAATTCGGATGGTGGGAAGATGGA-3' (EcoRI site)

* Actual primers used in cloning VH8H11, VH10H8, VL8H11, and VL10H8.

Table 4-2. Overlapping Oligonucleotides Used for Splicing Together Variable Light and Variable Heavy Chains of mAb 8H11

Primer	Description	Oligonucleotide Sequence
8H11/A (KO255A) *Sense*	anti-CEA IgG heavy leader with signal peptide, Kozak sequence, and restriction enzyme sites	5'-GCG**GAATTC***TCTAGA*GCCGCCACCATGGAGACAGACACACTCCTG-3' **EcoRI** *XbaI*
8H11/B *Antisense*	Light chain Framework 1 with 6-mer binding to anti-CEA heavy leader	5'-TAGAGATGTATACATGGAGAGTGGAGATTGGGTCATCAAGATATCACCTGT-3' Overlaps with 8H11/C
8H11/C *Sense*	Light chain internal	5'-TCCATGTATACATCTCTAGGAGAGAGAGTCACTATCACT-3' Overlaps with 8H11/B
8H11/D *Antisense*	Whitlow 218 linker with 22-mer binding to light chain Framework 4	5'-AGATCCGGGCTTGCCGGATCCAGAGGTGGAGCCTTTGATTTCCAACTTGGTGCCT-3' Overlaps with 8H11/E
8H11/E *Sense*	Whitlow 218 linker with 32-mer binding to heavy chain Framework 1	5'-TCCGGCAAGCCCGGATCTGGCGAGGGATCCACCAAGGGCGAAGTGCAGCTGCA GGAGTCTGGACCTGAGCT-3' Overlaps with 8H11/D
8H11/F *Antisense*	Heavy chain Framework 4 with 6xHis tag and restriction enzyme sites	5'-GATC**GAATTC***AAGCTT*GCGGCCGC**CCCGGG**TCAGTGATGGTGATGGTGATGAGC **EcoRI** *HindIII* NotI **SmaI** ***Stop*** GCTGACAGTGACCAGAGTCCCTT-3'

Table 4-3. Overlapping Oligonucleotides Used for Splicing Together Variable Light and Variable Heavy Chains of mAb 10H8

Primer	**Description**	**Oligonucleotide Sequence**
10H8/A (KO255A) *Sense*	anti-CEA IgG heavy leader for signal peptide, Kozak sequence, and restriction enzyme sites	5'-GCG**GAATTC***TCTAGA*GCCGCCACCATGGAGACAGACACACTCCTG-3' **EcoRI** *XbaI*
10H8/B *Antisense*	Light chain Framework 1 with 6-mer binding to anti-CEA heavy leader	5'-CAAGACTGACAGGCAGGGAGAGTGGAGTTTGGGTCATCAAGATATCACCTGT-3' Overlaps with 10H8/C
10H8/C *Sense*	Light chain internal	5'-TCCCTGCCTGTCAGTCTTGGAGATCAAGCCTCCATCTCTT -3' Overlaps with 10H8/B
10H8/D *Antisense*	Whitlow 218 linker with 24-mer binding to light chain Framework 4	5'-AGATCCGGGCTTGCCGGATCCAGAGGTGGAGCCTTTGATTTCCAGCTTGGTGCC TCC -3' Overlaps with 10H8/E
10H8/E *Sense*	Whitlow 218 linker with 24-mer binding to heavy chain Framework 1	5'-TCCGGCAAGCCCGGATCTGGCGAGGGATCCACCAAGGGCGAAGTCCAGCTGCAGG AGTCTGGG-3' Overlaps with 10H8/D
10H8/F *Antisense*	Heavy chain Framework 4 with 6xHis tag and restriction enzyme sites	5'-GATC**GAATTC***AAGCTT*GCGGCCGC**CCCGGG**TCAGTGATGGTGATGGTGATGTGA **EcoRI** *HindIII* NotI **SmaI** ***Stop*** GCTCACGGTGACCGTGGTCCC-3'

B. Additional Information

1) Key Research Accomplishments:

- variable region genes for both heavy and light chain regions for monoclonal antibodies 8H11 and 10H8 were isolated and cloned

- corresponding VL and VH regions were spliced together to form a single chain antibody chimeric gene (scFv)

- scFv of 10H8 was spliced to a human IgG constant region gene to produce dimer of scFvs: the minibody bound to the extracellular domain of the HER-2/*neu* protein by enzyme-linked immunosorbent assay

2) Reportable Outcomes:

Manuscript submitted and in review:

1. **Park, J.M.**, Yang, X., Park, J.J., Press, O.W., and Press, M.F. Assessment of Novel Anti-p185^{HER-2} Monoclonal Antibodies for Internalization-Dependent Therapies (submitted to *Hybridoma*).
(Draft of manuscript enclosed)

Poster presentation:

1. **Park, J.M.**, Yang, X., Press, O.W., and Press, M.F. "Internalization of Monoclonal Antibodies by HER-2/*neu* Overexpressing Cells." Poster presented at the American Association for Cancer Research 89th Annual Meeting in New Orleans, LA, on March 31, 1998. Abstract in the *Proceedings of the American Association for Cancer Research, 39:* 439, 1998.
(enclosed)

Assessment of Novel anti-p185^{HER-2} Monoclonal Antibodies for Internalization-Dependent Therapies

Jinha M. Park[1], Xiaowei Yang[1], John J. Park[1], Oliver W. Press[2], and Michael F. Press[1]*

[1]Breast Cancer Research Program and the Department of Pathology
USC/Norris Comprehensive Cancer Center
University of Southern California School of Medicine
1441 Eastlake Avenue, Mailstop 73
Los Angeles, California 90033

[2] Departments of Biological Structure and Medicine
University of Washington School of Medicine
and the Fred Hutchinson Cancer Research Center
Seattle, Washington 98195

* To whom correspondence should be addressed:

Michael F. Press
USC/Norris Comprehensive Cancer Center
1441 Eastlake Avenue, Mailstop 73
Los Angeles, California 90033

Voice (323) 865-0563
FAX (323) 865-0122
e-mail: villalob@hsc.usc.edu

Running Title: Internalizable Monoclonal Antibodies

ABSTRACT

Novel therapies which require internalization of effector domains may be improved by assessing the efficiency of post-binding receptor-mediated endocytosis. To achieve targeted gene therapy or immunotoxin therapy, natural vector-host tropisms must be altered. Recent improvements in monoclonal antibody engineering have expanded the potential range of host cells which can be targeted for therapeutic intervention. However, relatively little is known about cellular responses after binding of a vector construct. We have tested the utility of four novel monoclonal antibodies recognizing the extracellular domain of $p185^{HER-2}$, a membrane receptor protein, for use in internalization-dependent therapies. All four antibodies bound to $p185^{HER-2}$ in a number of immunoassays. Two antibodies recognized accessible epitopes of $p185^{HER-2}$ on viable cells. Radioimmunoassay demonstrated that antibody-membrane receptor complexes formed by two antibodies were internalized and trafficked through an endolysosomal degradative pathway. Two of the four antibodies evaluated were found to have favorable internalization characteristics suitable for incorporation in a targeting vector. This analytical approach could be applied to antibodies prior to and after fusion with various vectors or toxins to determine the potential utility of the antibodies for targeted therapy.

INTRODUCTION

Targeted therapy for human cancers requires the use of well-characterized reagents that specifically recognize tumor cells in the host background. Recent identification of differences in the genotype and molecular stucture of cancer cells has provided an opportunity to develop targetable reagents for the specific therapy of selected human cancers (1-4). Amplification of the HER-2/*neu* oncogene in human breast cancers, endometrial cancers, ovarian cancers, gastric cancers, and salivary gland carcinomas provides a potential molecular target for these cancers (5-9). HER-2/*neu* gene amplification is associated with overexpression of the $p185^{HER-2}$ protein. Amplification and overexpression is correlated with a shorter time to relapse and a shorter overall patient survival (8, 9). A humanized monoclonal antibody, Herceptin™, has been used to successfully treat some women with HER-2/*neu* overexpressing breast and ovarian cancers, demonstrating that $p185^{HER-2}$ can be used to selectively target human cancers (10, 11). While outcomes have improved for patients treated with Herceptin™, more effective classes of antibody-based therapies need to be developed to capitalize on cancer specific antigen expression.

Several groups have tested $p185^{HER-2}$ overexpressing cells as a target for gene therapy (12-15). Targeted delivery of genes has been attempted by altering cognate receptors on virions with cellular receptor ligands and single-chain antibodies (16-19). Although these vectors have successfully bound $p185^{HER-2}$ overexpressing cells, none have resulted in significant rates of gene transduction.

Because the successful gene therapy vector will require not only cell targeting, but internalization of the vector and expression of a therapeutic gene, it is important to evaluate as many of these steps as possible prior to construction of the vector. To this end, we isolated several anti-$p185^{HER-2}$ monoclonal antibodies and characterized their internalization potential using a rapid, quantifiable radioimmunoassay. Two of the four $p185^{HER-2}$ monoclonal antibodies bound to viable, intact target cells, were internalized and then trafficked through an endolysosomal pathway. These antibodies are expected to be useful in targeting treatment vectors that require internalization for therapeutic effect.

MATERIALS AND METHODS

Production and isolation of monoclonal antibodies to the extracellular domain (ECD) of $p185^{HER-2}$ involved the use of recombinant ECD^{HER-2} protein or viable HER-2/*neu* overexpressing cell lines for immunization of BALB/c mice, and screening of hybridomas with ELISA and immunocytochemical assays. The monoclonal antibodies were purified by affinity chromatography. The specificity of the antibodies was determined with Western immunoblot analyses, immunoprecipitation assays, and immunohistochemistry. The ability of monoclonal antibodies to specifically recognize viable HER-2/*neu* overexpressing cell lines was evaluated with fluorescence microscopy and flow cytometry. Cellular trafficking of monoclonal antibodies was characterized with radioimmunoassay methods.

Protein production and purification

A truncated $p185^{HER-2}$ protein was synthesized and injected into mice as the initial immunogen for generating anti-$p185^{HER-2}$ monoclonal antibodies. The cDNA of the HER-2/*neu* gene was cut between the NcoI and SphI sites, yielding a portion of the open reading frame which includes the initiation codon extending to the transmembrane domain (20). The 2-kilobase DNA insert was ligated into the multiple cloning site immediately downstream of an inducible *trp-lac* promoter of a pTrcHisA expression vector (Invitrogen, San Diego, CA). Successful in-frame subcloning of the HER-2/*neu* gene was confirmed by restriction fragment analysis and direct DNA sequencing (Amersham Pharmacia Biotech, Piscataway, NJ). TOP10 *E. coli* (Invitrogen) were transformed using the HER-2/*neu* expression

vector and induced to produce a fusion protein containing a leader peptide with six contiguous histidine residues followed by the ECD^{HER-2}. Proteins were purified by conditions using metal chelate affinity chromatography on Ni^{2+}-NTA-agarose as suggested by the manufacturer (QIAGEN, Valencia, CA).

Cell culture

In addition to recombinant ECD^{HER-2} protein, $p185^{HER-2}$ overexpressing intact cells (NIH/189 and SKBR-3 cells) were used to immunize BALB/c mice. NIH/189 and NIH 3T3 cells were used as sources of intact or solubilized $p185^{HER-2}$ protein for immunologic assays. The NIH/189 cell line, which has been described previously as a $p185^{HER-2}$ overexpressor, was a generous gift from C. Richter King (21).

NIH 3T3, NIH/189, and A431 human epidermoid carcinoma cell lines were grown in DMEM (Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS, Hyclone, Logan, UT) and penicillin/streptomycin (Life Technologies). The SKBR-3 human breast adenocarcinoma cell line was grown in McCoy's 5A media supplemented with 10% FBS and penicillin/streptomycin. Normal HMEC (human mammary epithelial cell) cells were grown in media formulated by the supplier of the cells (Clonetics, San Diego, CA). These cells were used as non-$p185^{HER-2}$ expressing controls for Western blots and immunoprecipitations. Sp2/0-Ag14 mouse myeloma cells were grown in RPMI-1640 media supplemented with sodium pyruvate, L-glutamine, penicillin/streptomycin, and 15% FBS prior to cell fusion.

Immunization

Two sets of 8-9 week-old female BALB/c mice were immunized with either protein or live cells as immunogens. Three female BALB/c mice received three sequential immunizations of 100 μg, 50 μg, and 50 μg each of the truncated HER-2/*neu* protein. The first immunization of ECD^{HER-2} was an intraperitoneal injection of protein emulsified in Freund's complete adjuvant (Sigma, St. Louis, MO). At weeks 3 and 5, mice were given two immunizations of 50 μg of protein mixed with Freund's incomplete adjuvant (Sigma). The mouse that yielded two monoclonal antibodies (5A7 and 11F11) in this work was given three further boosts of 50 μg ECD^{HER-2}.

Mice were also inoculated with live $p185^{HER-2}$-overexpressing cells in order to ensure the widest range of monoclonal antibodies against all potential ECD^{HER-2} epitopes, including glycosylated regions. Initial immunizations were intraperitoneal injections of 2-5 $x10^6$ SKBR-3 human breast cancer cells in serum-free RPMI-1640 media over a 7 month period. The mouse that yielded monoclonal antibodies 8H11 and 10H8 had another boost of $5x10^6$ NIH/189 cells in Freund's incomplete adjuvant injected intraperitoneally and $1x10^6$ cells injected subcutaneously. The final immunizations before fusion were an intraperitoneal boost of $10x10^6$ NIH/189 cells in PBS and an intravenous injection of 50 μg of ECD^{HER-2} protein in a buffer containing 0.1M NaH_2PO_4 and 10 mM TrisHCl, pH 8.0.

Hybridoma production and monoclonal antibody screening

Spleen cells from BALB/c mice immunized with either protein or cells were fused to Sp2/0-Ag14 mouse myeloma cells to generate hybridoma cells (22). Cells were fused with polyethylene glycol in a method modified from Köhler and Milstein (23). Hybridoma cells were selected for anti-p185^{HER-2} antibody production by enzyme-linked immunosorbent assay (ELISA) and immunohistochemistry.

Protein and cell ELISA

ELISA was used as the primary method to screen hybridoma cell clone supernatants for secretion of antibodies. ECD^{HER-2} (250 ng/well) protein was coated onto 96-well plates in PBS. After blocking in 3% bovine serum albumin (BSA), 50 μL of supernatant were incubated from 1-2 hours at room temperature. After washing 3 times with PBS, a 1:2000 dilution (in 3% BSA) of horseradish peroxidase-conjugated goat anti-mouse secondary antibody (Bio-Rad, Hercules, CA) was incubated for 30 minutes at room temperature. Positive wells were visualized by *o*-phenylene diamine (OPD) substrate (Sigma) and read at O.D. 490 nm on an ELISA plate reader (Bio-Tek Instruments, Inc., Winooski, VT).

To ensure detection of antibodies recognizing conformational and polysaccharide epitopes, cell ELISA using NIH3T3 and NIH/189 cells was employed to detect such clones. Cells ($1x10^4$/well) were fixed using 100% methanol onto 96-well Linbro Titertek (ICN, Irvine, CA) plates after overnight attachment onto 1.5% gelatin (Difco, Detroit, MI). The protocol for protein ELISA was used for cell ELISA.

Immunohistochemistry

Frozen and paraffin-embedded surgical biopsies of both known low and high $p185^{HER-2}$ expressor breast cancers were tested for binding of $p185^{HER-2}$ by these four monoclonal antibodies. The use of human tissue was approved prior to this work by the Institutional Review Board of the USC School of Medicine.

Frozen tissue section immunohistochemistry was used as a secondary screening method to detect positive hybridomas that secreted antibodies to intact $p185^{HER-2}$ from human tissue. $p185^{HER-2}$ in tissue sections were detected by the peroxidase-anti-peroxidase technique as previously described (24).

Breast cancer cases embedded in paraffin as a single multi-tumor block were also tested for $p185^{HER-2}$ recognition. Formalin-fixed breast cancer biopsies with known low and high expression of $p185^{HER-2}$ protein were embedded in a specified matrix in a single paraffin block (25). Monoclonal antibodies purified from ascites were used at equal concentrations to compare localization in a single specimen.

Western blots and immunoprecipitation

Monoclonal antibodies were used to probe for full-length $p185^{HER-2}$ in total protein lysates of both mouse and human cell lines loaded equally in SDS-PAGE. The method used has been previously described (26).

Immunoprecipitations demonstrated the ability of monoclonal antibodies to bind to full length HER-2/*neu* in soluble detergent lysates of mouse and human cell lines. The immunoprecipitation method used has been previously described (27). A rabbit polyclonal antibody (R60) previously reported for use in $p185^{HER-2}$ Western blots was used (9). A 1:2000 dilution of R60 polyclonal sera in 10% goat serum

was used to detect the C-terminus of immunoprecipitated $p185^{HER-2}$ protein in the blot.

Flow cytometry

A prerequisite for the determination of cellular trafficking of monoclonal antibody-antigen complexes is the binding of monoclonal antibody to its target on the surface of $p185^{HER-2}$ overexpressing cells. Fluorescence-activated cell sorting (FACS) was used to determine ligation of monoclonal antibodies to native $p185^{HER-2}$ on the surface of SKBR-3 cells. The flow cytometry method has been previously described (28). Cells were analyzed on a Coulter Elite ESP Cell Sorter (Beckman Coulter, Miami Lakes, FL). Mouse IgG isotype control was used as a negative control for FACS (Zymed).

Radioimmunoassay

A method to detect internalization and intracellular trafficking of monoclonal antibodies directed against cell-surface antigens was used as previously described to characterize anti-$p185^{HER-2}$ monoclonal antibodies with this potential (29, 30). Monoclonal antibodies 8H11 and 10H8 were labeled with ^{125}I (Amersham Pharmacia) using the Iodo-Gen method (Pierce). NIH 3T3 and NIH/189 cells were pulsed with labeled monoclonal antibodies and chased with 1% BSA/RPMI-1640. Supernatant, acid-releasable surface-bound, and intracellular label were collected and counted on a Cobra Automated Gamma Counter (Packard, Meriden, CT). The supernatant fraction was further divided between 25% TCA precipitable and 25% TCA soluble components representing shed, intact monoclonal antibodies and

degraded monoclonal antibodies, respectively. These fractions were collected at times of 0, 1, 4, 19, and 24 hours. All timepoint determinations were corrected for non-specific initial binding (T=0 hours) by subtracting counts in fractions after surface blocking by unlabeled monoclonal antibodies.

RESULTS

Four monoclonal antibodies have been isolated and characterized for their individual characteristics in standard immunoassays and in an internalization assay to determine their potential for use with internalization-dependent therapies.

Production and characterization of monoclonal antibodies. A 70 kDa, truncated ECD^{HER-2} protein was isolated from inclusion bodies of bacteria which had been transformed by an inducible expression plasmid (Fig. 1). This ECD^{HER-2} protein solution was used as the immunogen in mice producing hybridoma clones expressing 5A7 and 11F11 monoclonal antibodies recognizing $p185^{HER-2}$ in ELISA assays, Western immunoblot and immunohistochemistry of tissue sections but not in viable cells (see below). BALB/c mice were also immunized with viable cells overexpressing $p185^{HER-2}$ as well as the recombinant ECD^{HER-2} protein in order to isolate hybridoma clones 8H11 and 10H8.

Both protein and cell ELISA were used as the primary screening methods for detection of antibodies to $p185^{HER-2}$ from hybridoma supernatants. Supernatants positive in either of these screens were further tested for the presence of antibodies which could detect full-length endogenous $p185^{HER-2}$ in frozen breast cancer tissue from a patient with HER-2/*neu* gene overexpression. The monoclonal antibodies from hybridomas which bound $p185^{HER-2}$ in these screens were 5A7, 11F11, 8H11, and 10H8. The hybridoma cells were isolated as monoclonally derived cell lines after cloning twice by limiting dilution. 5A7, 11F11, and 8H11 were of the IgG_1/κ class and isotype. 10H8 was of the IgG_{2a}/κ class and isotype.

The specificity of the monoclonal antibodies was tested in Western blots, immunoprecipitation assays, immunohistochemistry, and immunofluorescence. By ELISA, all four monoclonal antibodies bound to ECD^{HER-2} purified from bacterial cell lysates. Cell ELISA performed with whole cells either overexpressing or not expressing HER-2/*neu* showed that all four antibodies bound $p185^{HER-2}$ on NIH/189 cells but did not bind to antigens on the surface of wild-type NIH 3T3 cells (Table 1). In Western blots, three of the monoclonal antibodies, 5A7, 11F11, and 10H8, recognized human $p185^{HER-2}$ from either SKBR-3 cells (endogenous) or NIH/189 cells (transfected) (Fig. 2). There was no cross-reactivity identified with other proteins in these cell lines or with EGF-R expressed in A431 cells. The 8H11 monoclonal antibody did not detect denatured $p185^{HER-2}$ on Western blots. Both 10H8 and 8H11 were able to bind to soluble $p185^{HER-2}$ in immunoprecipitation reactions while 5A7 and 11F11 were not able to bind to soluble $p185^{HER-2}$ (Fig. 3). The presence of additional bands in the immunoblots reflects either alternatively spliced variants or proteolytically degraded fragments of $p185^{HER-2}$.

Recognition of endogenous $p185^{HER-2}$ in membranes of human tissue. The ability to bind endogenous forms of $p185^{HER-2}$ in breast cancer tissue was tested with known low- and high-expression tissue samples by immunohistochemistry. All four monoclonal antibodies recognized endogenous $p185^{HER-2}$ on the cell membranes of tumor cells from a frozen human breast cancer biopsy (Table 1). As a control, the monoclonal antibodies were tested on a known low-expressor of $p185^{HER-2}$ and did not show localization in the membranes (data not shown). The utility of these monoclonal antibodies for measuring $p185^{HER-2}$ status in archival

paraffin-embedded biopsies was also tested. Similar to the findings in Western blots, 8H11 was unable to recognize $p185^{HER-2}$ while 5A7, 11F11, and 10H8 were able to recognize $p185^{HER-2}$ in the formalin-fixed tissue sections (Fig. 4).

Monoclonal antibody binding to viable intact $p185^{HER-2}$ overexpressing cells. While the utility of these monoclonal antibodies in standard immunoassays was demonstrated, the potential therapeutic utility of these monoclonal antibodies needed to be assessed on viable cells. The binding of monoclonal antibodies to $p185^{HER-2}$ was assessed in viable, intact cells by flow cytometric assays using SKBR-3 human breast cancer cells. Only two monoclonal antibodies, 8H11 and 10H8, were able to bind to $p185^{HER-2}$ expressed on the surface of these cells (Fig. 5). Both 8H11 and 10H8 shifted the population of cells indicating a high level of fluorescence labeling. 5A7 and 11F11 did not bind to SKBR-3 cells and exhibited a fluorescence equivalent to that of control IgG antibody. Similar observations were made by fluorescence microscopy (data not shown).

Internalization and cellular trafficking of anti-$p185^{HER-2}$ antibodies in $p185^{HER-2}$ overexpressing cells. Most targeted therapies require antibodies to be able to target cell-specific antigens and trigger internalization. To determine the subcellular distribution of bound monoclonal antibodies, radioimmunoassays were performed to determine the relative amounts of labeled antibodies in each compartment of the cultured NIH/189 cells. The relative percentage of ^{125}I labeled 8H11 and 10H8 in different cellular fractions was found to be similar for both 8H11 and 10H8 (Fig. 6). In these pulse-chase experiments, 8H11 and 10H8 were

found to be almost exclusively surface-bound at the beginning of the experiment (T=0 hour), and to be internalized subsequently. Surface label decreased to 35% and 25% of total cpm for 8H11 and 10H8, respectively, over a 24 hour period when maintained at 37°C (Fig. 6A and 6C). At 1 hour of incubation, intracellular ^{125}I rose to a peak and then leveled off througout the assay. The net loss of surface-bound labeled monoclonal antibody was offset by a net increase in supernatant radioactivity. This supernatant fraction was further divided between TCA precipitable and TCA soluble fractions representing intact monoclonal antibody and degraded monoclonal antibody fragments. The net increase of TCA soluble counts rose faster than the TCA precipitable fractions reflecting a higher proportion of internalized, lysosomally degraded, and exocytosed monoclonal antibody fragments compared with passively shed monoclonal antibody (Fig. 6B and 6D). After 24 hours, 45% of monoclonal antibody 8H11 was internalized, degraded, and exocytosed (Fig. 6A). A similar amount of monoclonal antibody 10H8 was internalized but a higher fraction of the monoclonal antibody was shed (Fig. 6C). As a control to test non-specific internalization, labeled 8H11 and 10H8 were both found exclusively in the supernatant fraction of NIH 3T3 cells. Separation of TCA precipitable from TCA soluble fractions showed that both 8H11 and 10H8 were found exclusively as intact proteins (data not shown). Thus, both 8H11 and 10H8 neither bound to proteins on the surface of NIH 3T3 cells nor were internalized by endocytosis, trafficked to lysosomes, and exocytosed.

DISCUSSION

Targeted gene therapy and immunotherapy capitalize on the strategies of viruses and biological toxins for cellular internalization after docking to surface receptors. Receptor-mediated endocytosis is the most common mode of vector and toxin internalization (31, 32). Rational cell-specific vector design for gene therapy and immunotherapy thus requires development of monoclonal antibodies directed to cell surface antigens, such as $p185^{HER-2}$, expressed by target cells (16, 18, 33). Furthermore, the internalization potential of these monoclonal antibodies should be assessed prior to retargeting of gene therapy or immunotherapy vectors to ensure efficient delivery of genes or toxins to subcellular sites of action. Here we used a radioimmunoassay to identify two novel monoclonal antibodies which were internalized after binding a cell surface receptor and, therefore, may be useful for delivery of genes and/or toxins into target cells via an endolysosomal pathway.

Some monoclonal antibodies mimic the effects of ligands in experimental systems. Monoclonal antibodies directed against the rat p185 receptor trigger receptor dimerization, phosphorylation, and downregulation of rat p185, whereas monovalent Fab' have not (34). Another $p185^{HER-2}$ monoclonal antibody was shown to undergo receptor-mediated endocytosis by electron microscopy following capping at membranes of $p185^{HER-2}$ overexpressing NIH 3T3 cells (30). Intracellular trafficking was previously documented by immunogold detection of other monoclonal antibodies in membrane-bound organelles (29). Although this assay has been used to determine the subcellular fate of the murine precursor to the therapeutic monoclonal antibody Herceptin™, only a few other monoclonal

antibodies in experimental protocols for immunotoxin therapies or targeted gene therapies have been tested for internalization (30, 35, 36). Furthermore, the radioimmunoassays used did not identify evidence of endolysosomal trafficking. Here, a rapid radioimmunoassay has been tested for its utility in determining the post-binding subcellular fates of novel monoclonal antibodies to the $p185^{HER-2}$ receptor.

Evidence of 8H11 and 10H8 antibody trafficking through receptor-mediated endocytosis and degradation by lysosomal sorting was demonstrated. TCA soluble fractions of supernatant counts were found in 8H11 and 10H8 assays. No TCA soluble counts were found in the supernatants of NIH 3T3 pulsed control cells. The radioimmunoassay therefore delineated the fates of antibodies to be assessed for receptor-mediated endocytosis.

Future retargeting of vectors and toxins can be improved by characterizing the post-binding fates of the engineered constructs. In particular, non-viral antibody vectors which lack viral endosome escape domains have been fused with viral or toxin polypeptides known to disrupt endosomal membrane integrity (15, 32, 37-39). Endosomal routing is critical for these added domains to mediate cytoplasmic entry of molecular conjugates. Since divalency is required for antibodies to trigger receptor dimerization and internalization, divalent binding sites may be needed in at least some engineered vectors to ensure receptor-mediated endocytosis (34, 40). Therefore, the radioimmunoassay described may be useful in predetermining antibodies and antibody-based vectors for dimerization and subsequent receptor-mediated endocytosis.

To develop effective monoclonal antibody-based therapies, determining the internalization potential and, particularly, the trafficking of monoclonal antibody-antigen to an endolysosomal pathway is important. After altering targeting specificities, prokaryotic and plant toxin-monoclonal antibody conjugates and targeted viral and non-viral vector conjugates should be assessed for retention of internalization potential. Hence, the radioimmunoassay which determined the internalization of monoclonal antibodies 8H11 and 10H8 could be used to assess the subcellular fates of newly developed monoclonal antibodies against cancer specific surface antigens to improve internalization-dependent therapies.

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Address reprint requests to:

Michael F. Press, M.D., Ph.D.

USC/Norris Comprehensive Cancer Center

1441 Eastlake Avenue, Mailstop 73

Los Angeles, California 90033

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Table 1. Characteristics of anti-p185^{HER-2} Monoclonal Antibodies

Name of mAb	Antigen	Mouse mAb isotype and sublcass	ECD protein ELISA[a]	NIH 3T3 wild-type cell ELISA[b]	NIH/189 cell ELISA[c]	Western blot detection of p185^{HER-2}	IP of p185^{HER-2}	IHC on frozen biopsies	IHC on paraffin biopsies	Indirect immuno-fluorescence detection of SKBR-3 cells
5A7	ECD protein	IgG_1/κ	+	-	+	+	-	+	+	-
11F11	ECD protein	IgG_1/κ	+	-	+	+	-	+	+	-
8H11	Live cells	IgG_1/κ	+	-	+	-	+	+	-	+
10H8	Live cells	IgG_{2a}/κ	+	-	+	+	+	+	+	+

[a]HER-2/*neu* receptor extracellular domain (ECD) polypeptide; ~70 kDa.
[b]NIH 3T3 cells lack p185^{HER-2} expression.
[c]NIH/189 cells have p185^{HER-2} overexpression.
Abbreviations used: mAb, monoclonal antibody; ELISA, enzyme-linked immunosorbent assay; IP, immunoprecipitation; IHC, immunohistochemistry.

Figure Legends

Figure 1. Purification of recombinant HER-2/*neu* extracellular domain (ECD) protein with poly-His tag. The cDNA of HER-2/*neu* was cut between the NcoI and SphI sites and subcloned into an expression vector for production of a 70 kDa epitope-tagged protein. TM=transmembrane domain. Purified protein was resolved by SDS-PAGE, transferred to nitrocellulose and detected by an antibody to the poly-His tag.

Figure 2. Western blots of total protein lysates from human and mouse cell lines. Monoclonal antibodies 5A7, 11F11, 8H11, and 10H8 were tested for binding to $p185^{HER-2}$ in total protein lysates from normal human mammary epithelial cells (HMEC), human breast carcinoma cells overexpressing HER-2/*neu* (SKBR-3), human epidermoid carcinoma cells overexpressing EGF receptor (A431), NIH3T3 cells which lack HER-2/*neu* expression, and NIH/189 cells engineered to overexpress HER-2/*neu*.

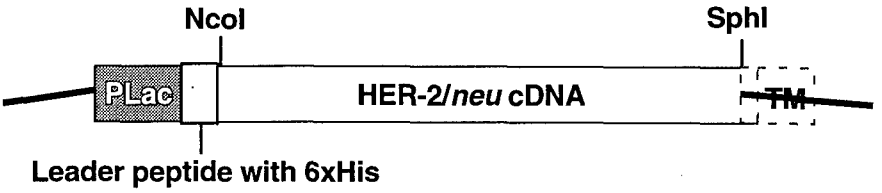
Figure 3. Immunoprecipitation of $p185^{HER-2}$ by monoclonal antibody 8H11. Total protein lysates were incubated with the 8H11 monoclonal antibody. Immune complexes were resolved by SDS-PAGE and transferred to nitrocellulose. $p185^{HER-2}$ was detected by a rabbit polyclonal antibody recognizing a carboxy-terminal epitope of $p185^{HER-2}$.

Figure 4. Immunohistochemical localization of $p185^{HER-2}$. Tissue sections from a single archival paraffin-embedded breast tumor were analyzed for monoclonal antibody localization to membranes of carcinoma cells. Membrane localization of anti-$p185^{HER-2}$ monoclonal antibodies were detected by the peroxidase-anti-peroxidase method.

Figure 5. Flow cytometry using novel monoclonal antibodies to detect $p185^{HER-2}$ on SKBR-3 cells. SKBR-3 cells overexpressing $p185^{HER-2}$ were incubated with indicated monoclonal antibodies and then tagged with secondary FITC-labeled antibodies. 8H11 and 10H8 monoclonal antibodies detected $p185^{HER-2}$ on the surface of viable SKBR-3 cells. IgG isotype control, 5A7, and 11F11 show no fluorescence.

Figure 6. Receptor-mediated endocytosis assays showing internalization and catabolism of monoclonal antibodies 8H11 and 10H8 by NIH/189 cells. (Top panels) Monoclonal antibodies labeled with ^{125}I were incubated on ice with NIH/189 cells. % Total cpm indicate fractions of label on the surface, within the cells, and in the supernatant at various incubation times at 37°C. Mean cpm were calculated for triplicate wells. (Bottom panels) Supernatant cpm was treated with 25% TCA and divided between TCA precipitable cpm (representing intact antibodies) and TCA soluble cpm (representing small MW metabolites of antibodies). Increased TCA soluble cpm fractions shows degradation and exocytosis of internalized monoclonal antibodies. (A and B) 8H11; (C and D) 10H8.

Figure 1.


NcoI
SphI
PLac
HER-2/neu cDNA
TM
Leader peptide with 6xHis

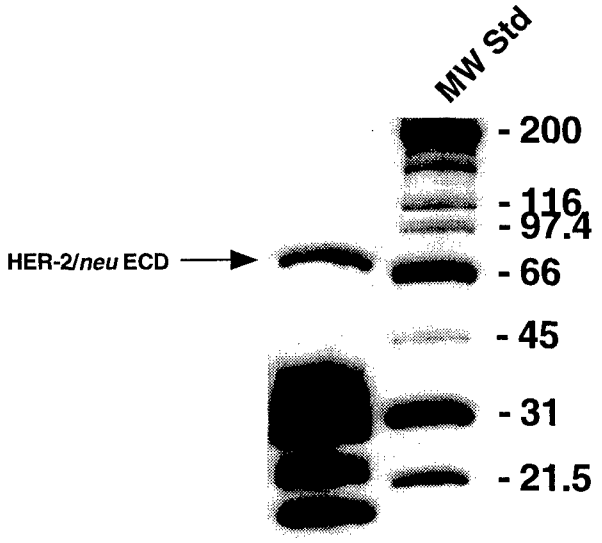

MW Std
- 200
- 116
- 97.4
HER-2/neu ECD
- 66
- 45
- 31
- 21.5

Figure 2.

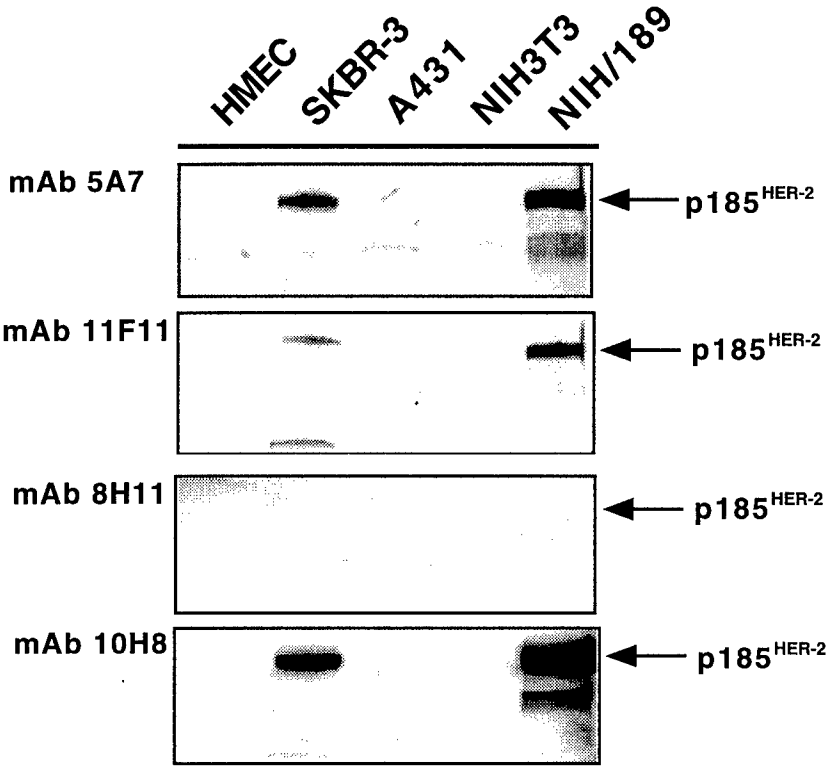

HMEC
SKBR-3
A431
NIH3T3
NIH/189
mAb 5A7
p185HER-2
mAb 11F11
p185HER-2
mAb 8H11
p185HER-2
mAb 10H8
p185HER-2

Figure 3.

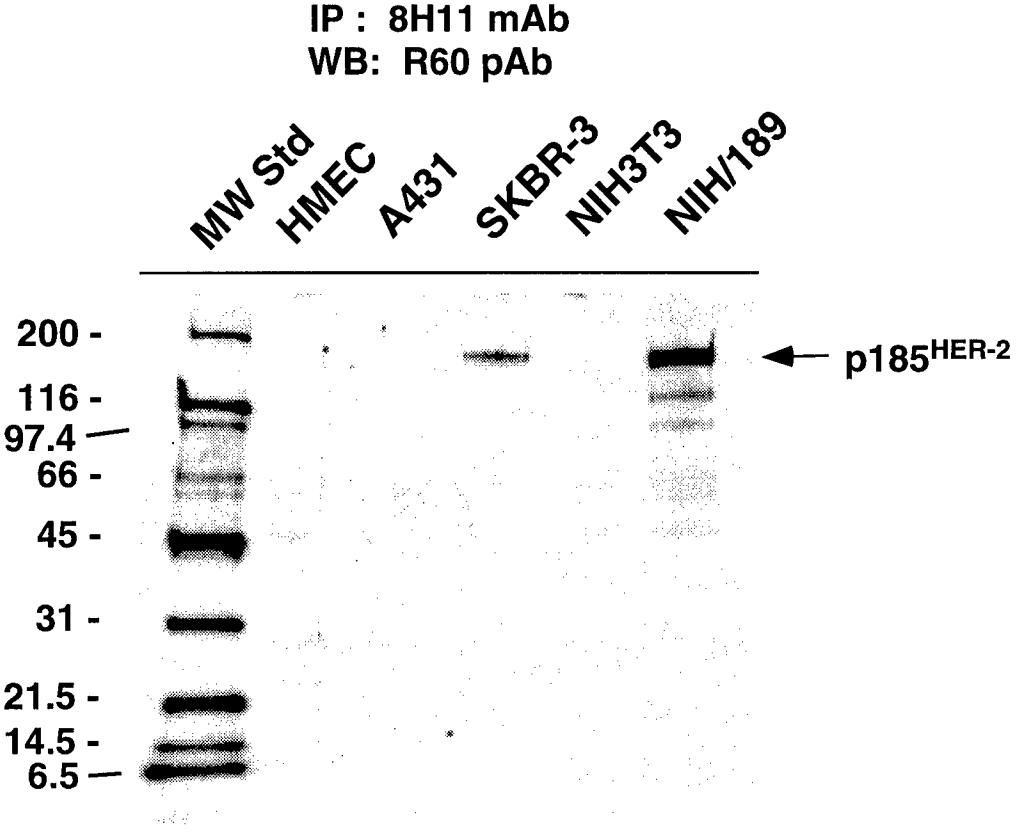
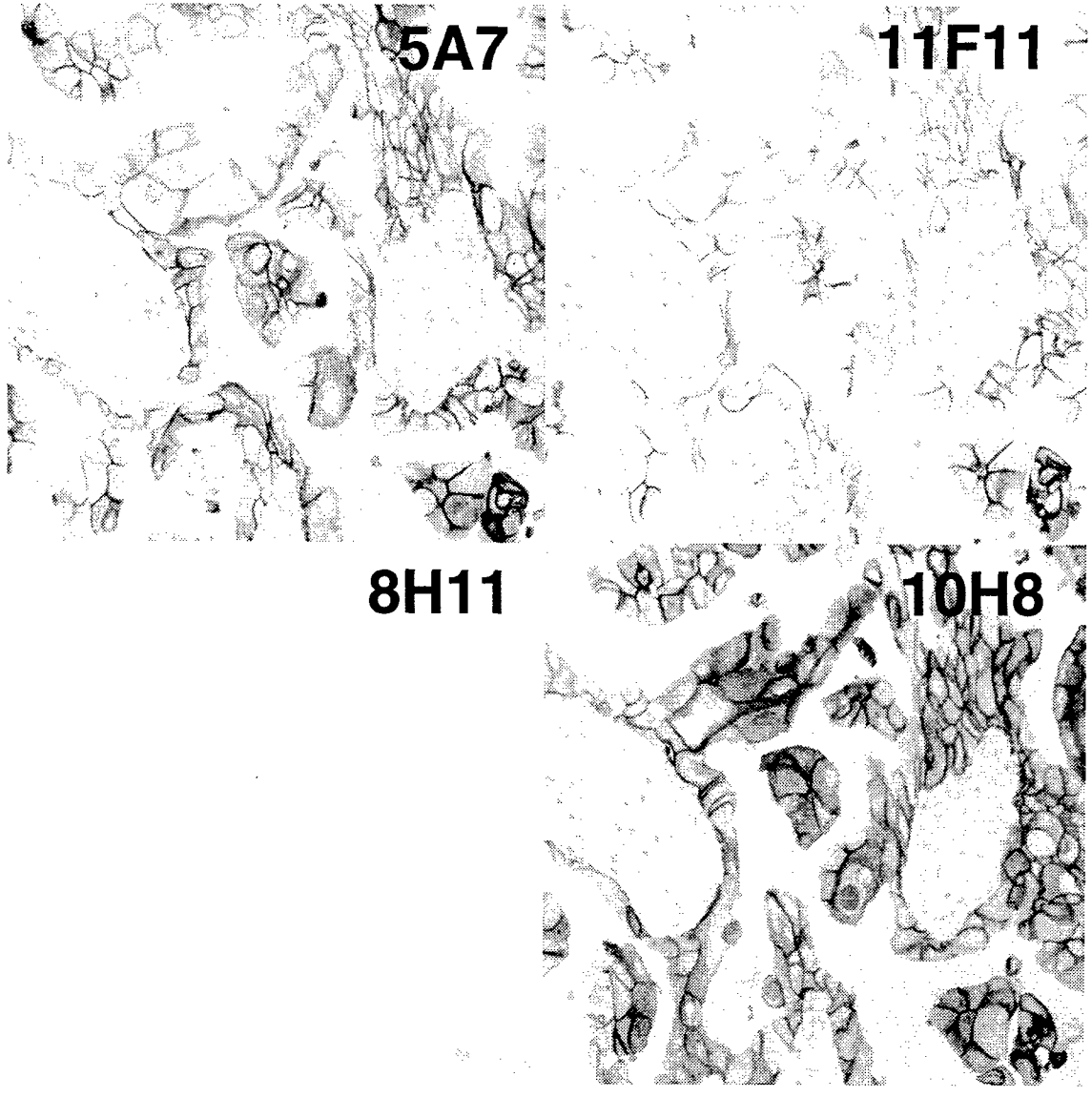

IP : 8H11 mAb
WB: R60 pAb
MW Std
HMEC
A431
SKBR-3
NIH3T3
NIH/189
200 -
116 -
97.4 -
66 -
45 -
31 -
21.5 -
14.5 -
6.5 -
p185HER-2

Figure 4.



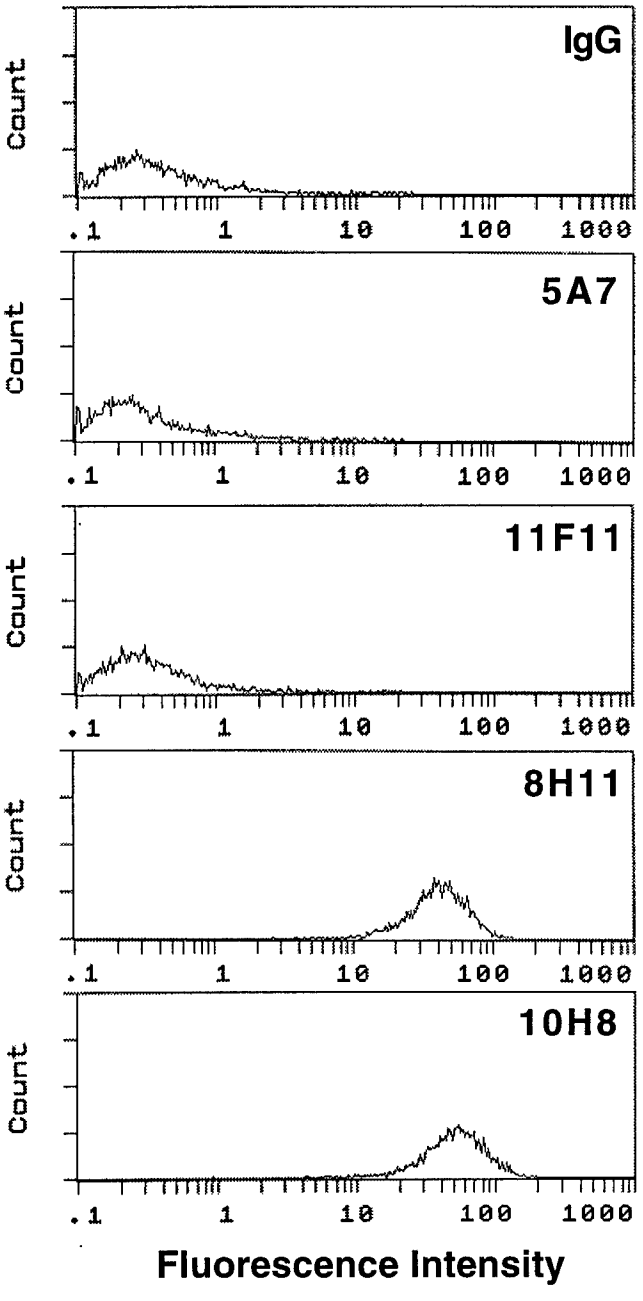
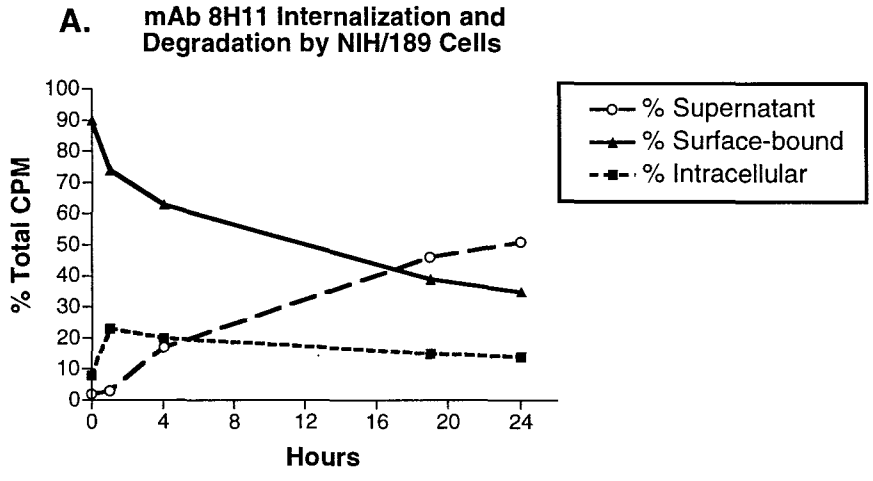
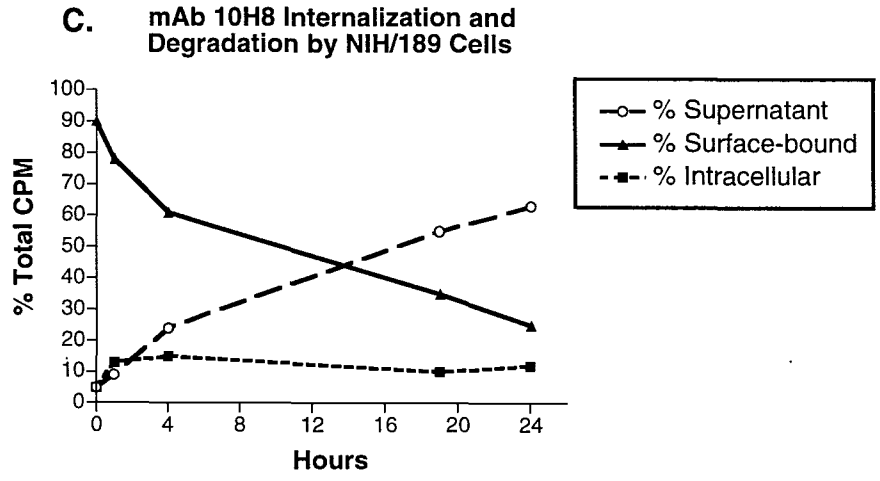
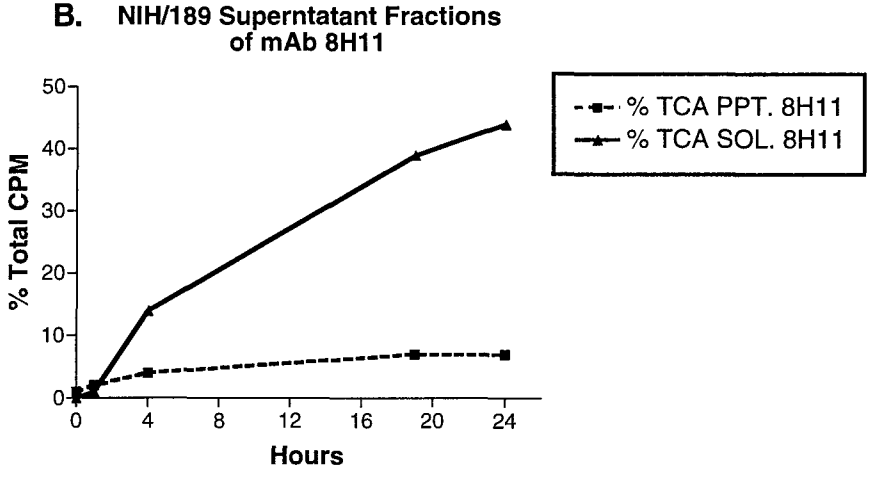

IgG
5A7
11F11
8H11
10H8
Count
.1
1
10
100
1000
Fluorescence Intensity

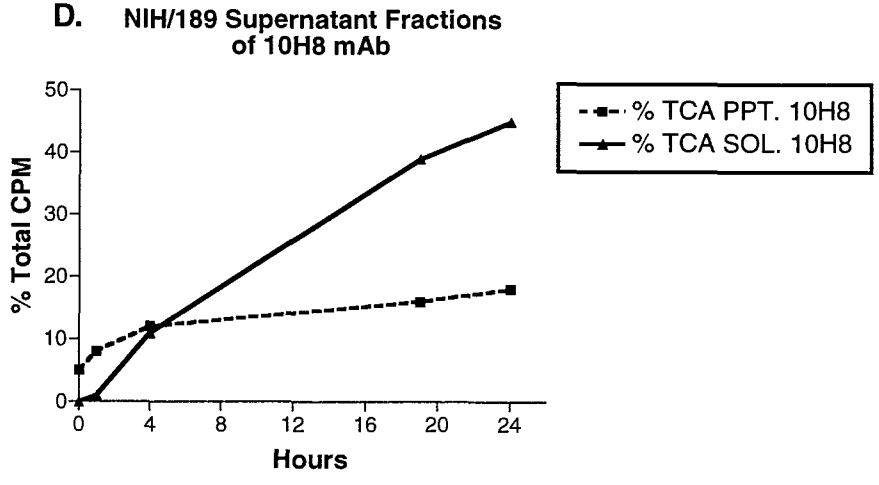
Figure 5.

Figure 6.


A.
mAb 8H11 Internalization and Degradation by NIH/189 Cells
% Total CPM
Hours
% Supernatant
% Surface-bound
% Intracellular


C.
mAb 10H8 Internalization and Degradation by NIH/189 Cells
% Total CPM
Hours
% Supernatant
% Surface-bound
% Intracellular


B.
NIH/189 Superntatant Fractions of mAb 8H11
% Total CPM
Hours
% TCA PPT. 8H11
% TCA SOL. 8H11


D.
NIH/189 Supernatant Fractions of 10H8 mAb
% Total CPM
Hours
% TCA PPT. 10H8
% TCA SOL. 10H8

>1240%, 5/8 LTS; (2 μg) >1240%, 5/9 LTS; (3 μg) 420%, 3/8 LTS. The treatment effect of MR1 is specific and highly effective in vivo for the treatment of EGFRvIII expressing tumors. Clinical trials are in development.

#2986 Ricin-A/anti-HER-2-immunoconjugate-induced p53-independent programmed cell death is associated with selective depletion of intracellular bcl-2 protein in ovarian cancer cells. Pusztai, L., Bozorgi, K., Xu, F.J., Bast, Jr., R.C. *University of Texas, M.D. Anderson Cancer Center, Houston, TX 77030.*

Our laboratory has recently demonstrated that the anti-HER-2/ricin-A-chain immunotoxin (TA1-RTA) induces p53-independent apoptosis in ovarian cancer cell line SKOV-3 clone 9002-18, that overexpresses HER-2. The TA-1 monoclonal antibody alone does not interfere with the ligand binding ability of HER-2 receptor or affect the growth or survival of these cells. We hypothesized that the antibody-ricin conjugate leads to apoptosis through inhibition of protein synthesis, induced by ricin, which leads to an imbalance between the intracellular quantities of pro-apoptotic (*bcl-xs, bax*) and anti-apoptotic (*bcl-xl, bcl-2*) molecules due to different half-life of these proteins. Changes in *bcl-2, bcl-xl, bcl-xs* and *bax* protein expression were detected by Western blotting at 0, 6, 12 and 36 hours after 3-hour incubation with TA1-RTA. Protein expression was quantified by densitometric analysis of Western blots. *Bcl-xl* and *bcl-xs* levels have not changed over a 36 hour follow-up period. *Bax* protein level also remained stable until about 36 hours after exposure. In contrast, *bcl-2* protein level has dropped to 50% by 12 hour after exposure and remained low throughout the 36 hour follow-up period. The results suggest that *bcl-2* has the shortest half-life among the proteins assessed in this study when protein synthesis is inhibited in ovarian cancer cells. These findings raise the possibility that ricin-immunoconjugate induces p53-independent programmed cell death through depletion of intracellular bcl-2 protein.

#2987 Monoclonal Antibody FC-2.15 specifically recognizes Lewis X antigen and lyses Lewis X (+) colon carcinoma cells. Capurro, M., Bover, L., Portela, P. and Mordoh, J. *Instituto de Investigaciones Bioquímicas "Fundación Campomar", Buenos Aires, Argentina.*

Monoclonal Antibody (MAb) FC-2.15 is a murine IgM that lyses antigen (Ag) 2.15 (+) cells in the presence of human complement (C') and that has shown activity in a Phase I clinical trial. Ag-2.15 has been now identified as Lewis X (LeX) which is found in normal peripheral granulocytes (PMN), chronic myeloid leukemia (CML) and several carcinomas. In different cells, LeX may be found in N-linked glycosidic moieties of glycoproteins (PMN, CML) in O-linked glycosidic moieties of mucins (MCF-7 cells) or in both (colon carcinoma). Since LeX is abundantly expressed in human colon carcinomas, the lytic effect of FC-2.15 was tested in several colon carcinoma cell lines. The C' mediated lysis measured by the MTT assay was for the different cell lines: DLD-1 35.1 ± 4.8%; T84: 27.2 ± 10.2%; CACO-2: 3.9 ± 3.9%; HT-29: 0 ± 2.7%; HCT 116: 0 ± 6.8%. Previous treatment of colon carcinoma cells with neuraminidase invariably increased the lytic ability of FC-2.15 to: 48.6 ± 5.1%; 37.8 ± 7.6%; 14.1 ± 4.8%; 8.4 ± 3.2% and 6.5 ± 10.2% respectively. Cell lysis is proportional to the expression of LeX at the cell membrane, which is increased after neuraminidase treatment, suggesting that a combination of MAbs directed against LeX and Sialyl LeX may have additive cytotoxicity in colon carcinoma.

#2988 Radioimmunotherapy of established human colon carcinoma xenografts using CC49 diabody. Pavlinkova, G., Beresford, G., Booth, B.J.M., Batra, S.K., and Colcher, D. *University of Nebraska Medical Center, Departments of Pathology/Microbiology and Biochemistry/Molecular Biology, Omaha, NE 68198-6495.*

Murine monoclonal antibodies (B72.3 and CC49), reactive with the tumor-associated antigen TAG-72, are among the most extensively studied for cancer therapy. However, progress in the treatment of solid tumors has been limited by a number of factors including poor penetration of the labeled IgG into the tumors, their inability to reach the tumor in sufficient quantities without significant normal tissue toxicity, as well as the development of a HAMA response to the injected MAb. One method to alter the pharmacology of antibodies has been to clone the MAb variable regions and develop a single chain Fv (scFv). A new CC49/205C diabody, composed of two noncovalently associated scFvs which are capable of binding two antigen molecules, were generated and shown to bind TAG-72 epitopes with a similar binding affinity as the murine IgG. The therapeutic potential of this construct was examined in a radioimmunotherapy studies performed in athymic mice bearing human colon carcinoma (LS-174T) xenografts. Treatment groups (n=10) received a single dose of ^{131}I-labeled CC49 diabody (500 to 2000 μCi) or ^{131}I-labeled CC49 IgG (250 to 500 μCi). The mice treated with radiolabeled MAbs showed a significant reduction in the rate of tumor growth and prolongation of survival as compared to the control group. At 20 days post administration, the tumors were averaging 25% the size of control tumors in mice given 500–750 μCi of labeled diabody and 15% the size of control tumors in animals given 1000–2000 μCi of labeled diabody or 250–500 μCi of labeled IgG. In conclusion, the CC49 diabody demonstrates *in vitro* and *in vivo* targeting specificity and provides a promising delivery vehicle for therapeutic applications.

#2989 Radioimmunotherapy of pancreatic cancer by ^{131}I-labeled monoclonal antibody Nd2 in orthotopic transplanted model. Yamamoto A, Sawada T, Nishihara T, Yamashita Y, Hirakawa K, Sowa M, Ho J.J.L, Kim Y. S. *Osaka City University Medical School, Osaka 545, Japan*

Radioimmunotherapy of malignant tumors by monoclonal antibody (MoAb) have been investigated mostly in nude mice xenografted subcutaneously, however concerning the blood perfusion and/or microenvironment of clinical primary tumors, orthothopic transplanted tumors are more relevant to investigate in vitro experimental effect. We have previously demonstrated the therapeutic potential of ^{131}I-labeled MoAb Nd2 in nude mice xenografted pancreatic cancer SW1990. In this study, we evaluated the tumor accumulation and inhibitory effect of ^{131}I-labeled Nd2 in orthotopic transplanted pancreatic cancer tumor. A piece of 30 mg of SW1990 tumors was transplanted on the bodies of pancreas in 4 week-old female BALB/c nude mice, and the mice whose tumor reached to approximately 0.7 cm in diameter were selected in this study. Twenty μCi of ^{131}I-Nd2 was injected via the tail vein for radioimmunoscintigraphy and biodistribution study. Distinct tumor image was detected in scintigraphy and specific strong distribution in tumor of Nd2 was observed, tumor to blood ratio reached to 9.4 4 days after administration. About therapy, orthotopic transplanted mice injected 200 μCi of ^{131}I-Nd2 revealed significant inhibition of tumor growth and long-term survival compared with control mice injected PBS. These result suggested that ^{131}I-labeled Nd2 may have high possibilities for clinical application in the treatment of pancreatic cancer.

#2990 Clinical treatment of stomach adenocarcinoma with the apoptosis-inducing human monoclonal antibody SC-1. Vollmers, H.P.(a), Krenn, V.(a), Zimmermann, U.(b), Timmermann, W.(c), Illert, B.(c), Wilhelm, M.(d), Thiede, A.(b), and Mueller-Hermelink, H.K.(a). *Inst.f.Pathologie (a), Inst.f.Biotechnologie (b), Chirurgische Klinik (c), Medizinische Poliklinik (d), Universität Würzburg, Würzburg, Germany.*

The human monoclonal antibody SC-1(IgM), isolated from a patient with a diffuse type adenocarcinoma of the stomach, inhibits stomach cancer growth in vitro and in vivo by inducing tumor-cell specific apoptosis. For a clinical phase I/II study eight patients with poorly differentiated adenocarcinoma received purified antibody intravenously prior to gastrectomy. Neither during nor after infusion the patients develop serious complications or toxic crossreactivity symptoms. Liver enzymes showed no significant changes induced by antibody infusion and all patients are still alive five months after the start of the study. Tumors and lymphnodes were investigated immunohistochemically for apoptosis-induction and morphologically for antibody-induced reactivity. All primary tumors showed a significant increase of apoptotic tumor cells, and histologically, tumor regression was observed characterized by a decreased cellular density of carcinoma cells, an inflammatory infiltration of granulocytes and macrophages and the occurrence of pycnotic nuclei.

#2991 Internalization of monoclonal antibodies by HER-2/*neu* overexpressing cells. Park, J.M., Yang, X., Press, O.W., Press, M.F. *University of Southern California, Los Angeles, CA 90033, University of Washington, Seattle, WA 98195.*

Amplification of the HER-2/*neu* oncogene is a genetic alteration which is correlated with poor prognosis in breast cancer. The HER-2/*neu* protein is a cell-surface receptor over-expressed in 25–30% of breast cancers. We have generated four novel monoclonal antibodies, 5A7, 8H11, 11F11, and 10H8, against the extracellular domain of this receptor. All four have been screened from hybridoma supernatants against an NIH3T3 cell line over-expressing the HER-2/*neu* receptor and counter-screened against a wild-type NIH3T3 cell line. Immunohistochemical analysis shows binding to HER-2/*neu* in a frozen breast cancer biopsy by all four mAbs. Only three monoclonal antibodies identify the receptor by Western Blot suggesting that abrogation of a native conformational epitope prevents binding by the 8H11 antibody. Two mAbs, 8H11 and 10H8 can bind to the membranes of live SKBR-3 human breast cancer cells by indirect immunofluorescence. A cellular radioimmunoassay shows that two of the mAbs, 8H11 and 10H8, are bound to HER-2/*neu* over-expressing cells and internalized by receptor-mediated endocytosis. Monoclonal antibodies with the potential to specifically target human breast cancer cells have been generated and characterized for future use as a therapeutic reagent.

#2992 The role of affinity in the bispecific scFv$_2$-mediated killing of HER2+ tumor cells by FcγRIII+ peripheral blood lymphocytes. McCall, A.M., Amoroso, A.R., Zhang, L., Schier, R., Marks, J.D. and Weiner, L.M. *Fox Chase Cancer Center, Philadelphia, PA, 19111; University of California, San Francisco, CA, 94110*

Bispecific monoclonal antibodies (BsAb) which recognize tumor antigens and Fc receptors are effective in promoting relevant tumor lysis by peripheral blood lymphocytes (PBL). However, the role of affinity in regulating the effector functions of these BsAb has not been investigated. Two bispecific scFv$_2$ were constructed using the C6.5 and B1D2 (anti-HER2) scFv in association with the NM3E2 (anti-human FcγRIII) scFv. The K_D values of the C6.5 and B1D2 scFv were 1.6×10^{-8} M and 1.5×10^{-11} M, respectively, while the K_D value of the NM3E2 scFv was 5.8×10^{-8} M. PBL were treated with 1,000 U/ml of IL-2 for 18 hrs and used with ^{51}Cr-labeled SKOV3 cells. At an E:T ratio of 50:1, there was 15.5% cytotoxicity. 100ng/ml of C6.5 × NM3E2 scFv$_2$ and B1D2 × NM3E2 scFv$_2$