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Award Number: DAMD17-98-1-8036

TITLE: Anti-HER2/Toxin Expressing Lymphocytes for Breast Cancer Therapy

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REPORT DATE: May 2001

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

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20010731 076

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)

2. REPORT DATE
May 2001

3. REPORT TYPE AND DATES COVERED
Annual Summary (1 May 98 - 30 Apr 01)

4. TITLE AND SUBTITLE
ANTI-HER2/TOXIN EXPRESSING LYMPHOCYTES FOR BREAST CANCER THERAPY

5. FUNDING NUMBER
DAMD17-98-1-8036

6. AUTHOR(S)
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
Report contains color graphics

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 Words)*

Cyclin E is a key regulator of the mammalian cell cycle, as it is rate limiting for progression from G1 to S phase. Deranged cyclin E expression has been found both quantitatively (overexpression) and qualitatively (multiple isoforms) in almost all breast cancer cell lines and patient tissue samples. In spite of the apparent significance of altered cyclin E expression, the role of cyclin E overexpression in the transformation and proliferation of breast cancer cells has not been established. Using an intrabody approach, we are developing a model to phenotypically knock out cyclin E in breast cancer cells to investigate the role cyclin E plays in the tumorigenicity of these cells. We have constructed two anti-cyclin E single-chain antibodies (sFv) and have displayed their ability to bind cyclin E by ELISA. We have successfully targeted expression of these intrabodies to the cytosol and nucleus of breast cancer cell lines (SKBR3 and MCF-7) as conjugates with the human IgG constant region fragment(sFv-Fc). We are currently investigating the effect of anti-cyclin E intrabodies on the growth and tumorigenicity of clonal SKBR3 and MCF-7 cell lines either stably or inducibly expressing the intrabodies.

14. SUBJECT TERMS
Breast Cancer, Cyclin E, Intrabody, SFV, Cell Cycle

15. NUMBER OF PAGES
8

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18
298-102

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Introduction

Cyclin E is an important regulator of the coordinated cell cycle, as it is rate limiting for the G1/S phase transition. Deranged cyclin E expression has been found both quantitatively (overexpression) and qualitatively (multiple isoforms) in almost all breast cancer cell lines and patient tumor tissue examined to date. Disruption of such an important cell cycle protein could potentially contribute to transformation and/or tumorigenicity in breast cancer, but the direct involvement of cyclin E in breast cancer has yet to be elucidated. Intracellular antibodies, or intrabodies, are a powerful tool for evaluating the *in vivo* function of a given protein. Single-chain antibodies (sFvs), consisting of the heavy and light chain variable regions of an IgG linked by a flexible peptide spacer, are able to fold and retain the antigen binding specificity of the parental antibody. SFvs can be modified for targeted expression to the cytosol, nucleus, or endoplasmic reticulum (ER), where they can bind to and inactivate their target antigen, thus generating a "phenotypic knockout" of the protein of interest. In this study we have constructed two anti-cyclin E sFvs and generated constructs that successfully target their expression as intrabodies to the cytosol or nucleus of breast cancer cells. We propose to use breast cancer cell lines stably or inducibly expressing these anti-cyclin E intrabodies to evaluate their effect on the nuclear trafficking and associated kinase activity of cyclin E. We will then evaluate the biological effects (i.e. doubling time, cell cycle distribution, growth in soft agar, tumorigenicity in nude mice) of anti-cyclin E intrabody expression in these cell lines. This study should provide direct evidence to determine the functional significance of cyclin E in the abnormal growth and transformation of breast cancer, as well as determine if there is merit in cyclin E targeted strategies for the treatment of breast cancer.

Annual Summary

The first aim of this proposal was to generate anti-cyclin E single chain antibodies (sFvs) based on the parental anti-cyclin E monoclonal antibody producing hybridoma cell lines HE-12 and HE-172. These hybridomas were generated and characterized in the lab of Dr. Edward Harlow at Harvard University. We obtained these hybridomas and grew them in culture for the purpose of isolating poly-adenylated messenger RNA (mRNA) from log phase cells using the Micro-FastTrack mRNA Isolation Kit (Invitrogen). Using this mRNA as template, we then performed RT-PCR to amplify the approximately 350 base pair cDNA fragments which encode the heavy (V_H) and light (V_K) chain variable regions of the anti-cyclin E antibodies (Fig 1). RT-PCR was performed using degenerate primers designed against the Framework 1 (5') and Framework 4 (3') regions of the IgG heavy and light chain genes. These primers incorporated HindIII (5') and XbaI (3') restriction sites to facilitate cloning into a sequencing vector. Several clones were sequenced and the resulting nucleotide and deduced amino acid sequences were subjected to GeneBank query to ensure they encoded unique peptide sequences. Unique heavy and light chain cDNAs were PCR amplified to add overlapping oligonucleotides encoding a $(Gly_4Ser)_3$ linker, and the two fragments were then linked by an additional overlap extension PCR into sFvs in the order of V_H-Linker-V_K.

Using this procedure, we have successfully cloned the heavy and light chain variable region genes of both the HE-12 and HE-172 hybridomas, and linked them into SFvs (Figure 1). The nucleotide and deduced amino acid sequences for these sFv constructs were reported last year.

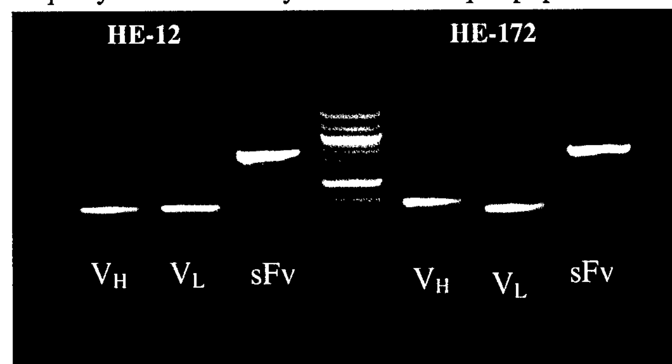
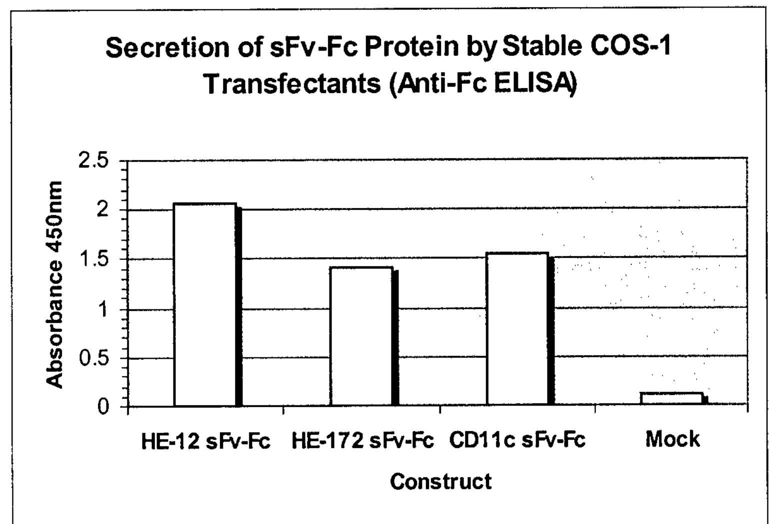


Figure 1

In order to test the binding activity of the anti-cyclin E intrabodies, we cloned the sFvs into a mammalian expression vector (pRc/CMV) for expression and purification of soluble sFv-Fc immunoconjugates from COS-1 supernatants. Addition of the Fc fragment has been shown to increase protein stability, enhances sFv secretion, and facilitates purification of the sFv-Fc fusion proteins by Protein A affinity chromatography. The IgG heavy chain signal sequence was added 3' to the sFv-Fc constructs to target them to the secretory pathway (Appendix A). For production of recombinant immunoconjugate protein, COS-1 cells were transfected with the anti-cyclin E immunoconjugate vectors using Gene Porter reagent (Gene Therapy Systems). Two days after

transfection, cells were trypsinized and re-plated in 6-well plates at a dilution of 1:50 in selective medium containing 500ug/ml G418 (Invitrogen). Cells were grown in selective medium until visible colonies formed, at which time the cells were trypsinized, pooled, and expanded in selective medium containing 250ug/ml G418. Supernatants from stably transfected cells were assayed by an anti-human Fc Enzyme Linked Immunosorbent Assay (ELISA) to confirm secretion of immunoconjugate protein into the supernatant (Figure 2).

Figure 2. To confirm secretion of sFv-Fc constructs from COS-1 cell transfections, 96 well plates were coated with 50 ng/well of goat anti-human IgG (Fc specific) antibody (Jackson). Wells were blocked with blocking buffer (PBS, .1%BSA, 0.005%Tween 20, 5% heat-inactivated goat serum), and incubated for 2 hours with supernatants. Plates were washed and incubated with peroxidase-conjugated anti-human IgG (Fc Specific) antibody (Jackson) diluted 1:30,000 in PBS + 5% heat inactivated goat serum, 0.01% Tween 20. The reaction was developed with 1 Component Peroxidase Substrate (KPL) and stopped with 4N Sulfuric Acid. Absorbance was read at 450 nm on a Perkin Elmer HTS 7000 Plus BioAssay Plate Reader. CD11c-sFv-Fc protein (anti-mouse CD11c) was purified for use as a non-cyclin E binding sFv-Fc control in an anti-cyclin E ELISA.



For harvesting protein, stably transfected COS-1 cells were grown to near confluence in 150 cm^2 flasks, then grown in serum free DMEM for 48 hours. The supernatants were clarified by centrifugation and incubated at 4°C overnight with 250ul of protein A Agarose (Sigma). The protein A was then applied to a disposable chromatography column and rinsed sequentially with 100 mM Tris pH 8.0 and 10 mM Tris pH 8.0, then eluted in 500ul fractions with 100mM Glycine pH 2.7. The fractions were immediately neutralized by addition of 50ul of 1.0 M Tris pH 9.0 to each fraction. Protein containing fractions were identified using a Bradford-based protein assay (Bio-Rad) and analyzed by SDS-PAGE and Western Blot (Figure 3).

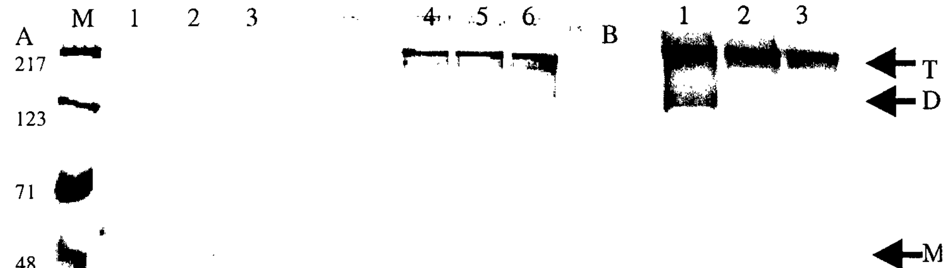
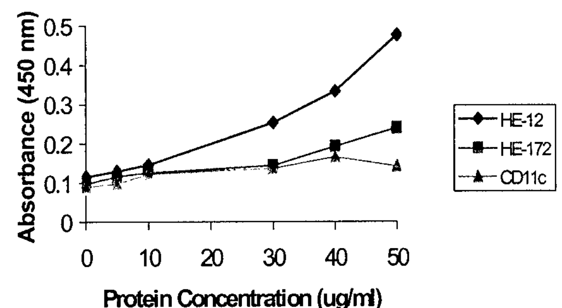


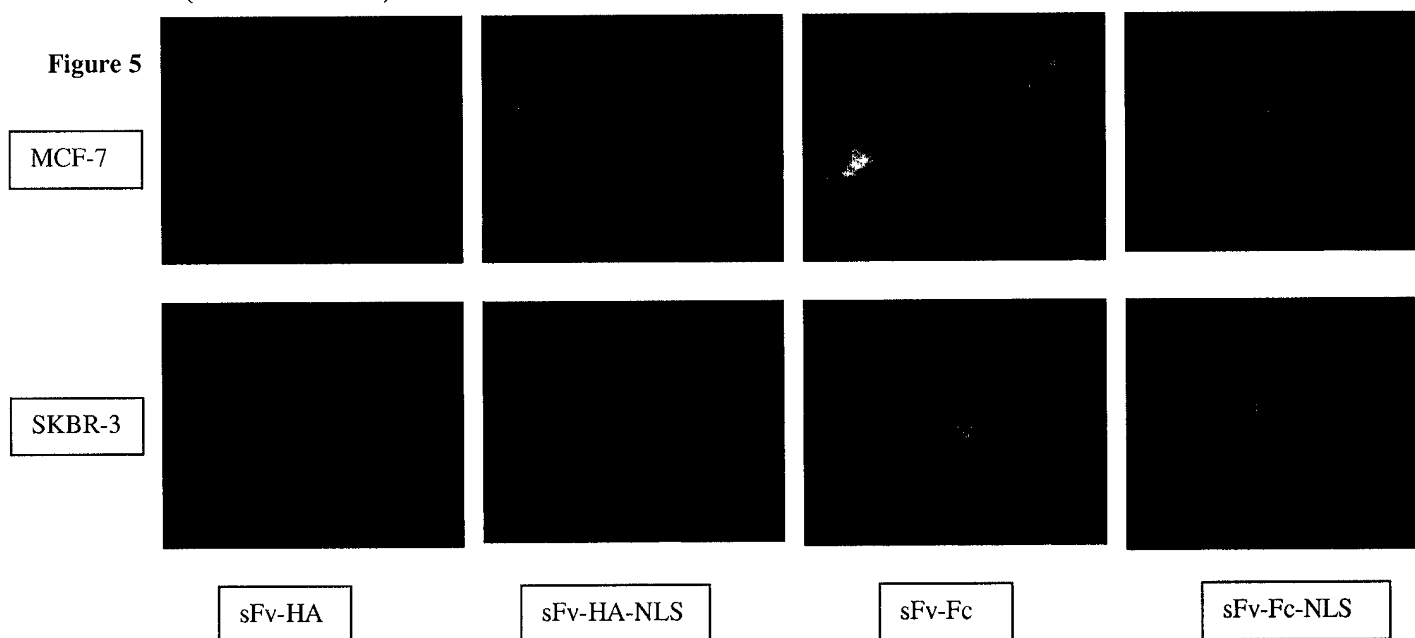
Figure 3. SDS-PAGE analysis of recombinant sFv-Fc immunoconjugates. *A)* SDS-PAGE and Coomassie stain of HE-12 (lanes 1,4), HE-172 (2,5), and CD11c (3,6) protein under reducing (lanes 1-3) and non-reducing (4-6) conditions. Molecular weight markers (M) in kD. *B)* Western blot of non-reduced HE-12 (lane 1), HE-172 (2), and CD11c (3) immunoconjugates. Protein was separated by SDS-PAGE, transferred onto Hybond P membrane (Amersham), and blocked. The membrane was then blotted with peroxidase-conjugated anti-human Fc specific antibody (Jackson) at a dilution of 1:20,000. The membrane was washed, developed with ECL-Plus reagent (Amersham), and exposed to film. Tetramers(T), dimers(D), and monomers(M) are indicated by arrows.

Finally, to confirm the ability of sFvs to bind cyclin E, we performed an anti-cyclin E ELISA using recombinant immunoconjugate protein (Figure 4.) Briefly, 96 well plates were coated overnight with recombinant human cyclin E-GST fusion protein (50ng/well) purified from insect cell lysates. The wells were then blocked and incubated with varying concentrations of HE-12, HE-172, or CD11c (control) recombinant protein for 2 hours at room temperature. Wells were then washed and assayed for the presence of bound immunoconjugate as described above using peroxidase-conjugated anti-human Fc-specific antibody. We found that both the HE-12 and HE-172 sFv-Fc proteins displayed cyclin E binding activity, whereas the CD11c control did not bind cyclin E as expected (Figure 4).



The second aim of this proposal was to generate mammalian expression vectors for use in the targeted expression of anti-cyclin E intrabodies to the cytosol or nucleus of the breast cancer cell lines MCF-7 and SKBR-3. A schematic representation of the constructs for cytosolic and nuclear expression of sFv and sFv-Fc intrabodies is shown in Appendix A. All of the constructs depicted have been completed for the HE-12, HE-172, and CD11c sFvs, and were used in transient transfection assays using MCF-7 and SKBR-3 cells. The sFv was modified by addition of the HA epitope tag for use in immunodetection. Additional sFv-Fc constructs were made to assess the effect of the Fc fragment on expression, stability, and targeting of sFvs in breast cancer cells. Antibodies specific for the human Fc fragment were used for immunodetection of sFv-Fc constructs. Addition of the SV40 nuclear localization signal (NLS) to the 3' end of the sFv or sFv-Fc construct was used to target expression of the protein to the nuclear compartment. Figure 5 presents immunofluorescence results from transient transfection of MCF-7 and SKBR-3 cells. Briefly, MCF-7 or SKBR-3 cells were grown in 6-well plates and transfected with the indicated constructs using GenePorter transfection reagent (Gene Therapy Systems). 48 hours post-transfection, cells were fixed and stained with either anti-HA-FITC or anti-human Fc-FITC antibody. These results indicated the ability to successfully target expression of sFv and sFv-Fc intrabodies to the cytosol or nucleus of both MCF-7 and SKBR-3 cells. In addition, the fluorescence intensity seen in these experiments supported the notion that intrabody stability was increased by either 1) targeting expression of the sFv to the nucleus, thereby removing the protein from the harsh cytosolic environment (sFv vs sFv-NLS), or 2) addition of the Fc fragment, thereby increasing cytosolic (sFv vs sFv-Fc) as well as nuclear compartment (sFv-NLS vs sFv-Fc-NLS) stability. Similar results were shown for the HE-12 and CD11c constructs (data not shown).

Figure 5



The third aim of this proposal was to establish breast cancer cell lines expressing anti-cyclin E intrabodies either stably or under the influence of an inducible promoter. We initially established pools of SKBR3 cells stably transfected with HE-12, HE-172, and CD11c (control) sFv-Fc and sFv-Fc-NLS constructs due to the apparent increase in intrabody stability gained by addition of the Fc fragment. Immunoprecipitation and Western blot analysis of cell lysates from these stable transfectants confirmed intrabody expression in all cell lines (Figure 6). An initial growth assay using the stably transfected SKBR3 cell lines indicated expression of the nuclear-targeted HE-172 intrabody resulted in inhibition of cell growth, whereas the HE-172 intrabody targeted to the cytosol, and the HE-12 and CD11c nuclear-targeted constructs had no effect on cell growth (Figure 7). We have recently repeated the generation of the pooled SKBR3 stable transfectants, and have been unsuccessful In attempts to repeat this growth inhibition. We are currently investigating the effects of anti-cyclin E intrabody expression in clonal populations of SKBR3 cells. We are also establishing SKBR3 and MCF-7 cell lines expressing the anti-cyclin E intrabodies under the control of a tetracycline inducible promoter using the Tet-On system (Clontech).

HE-12 HE-172 CD11c
V sFv-Fc Fc-N sFv-Fc Fc-N sFv-Fc Fc-N

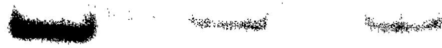


Figure 6. Equal amounts of cell lysates from stably transfected SKBR3 cells were immunoprecipitated overnight using 1ug/ml anti-human Fc antibody and protein A agarose. Immunoprecipitates were washed, separated by SDS-PAGE, and transferred to Hybond-P membrane. The membrane was then blocked, blotted with peroxidase-conjugated anti-human Fc-specific antibody, and developed using ECL-Plus. Vector control cells (V) were transfected with the empty pRc/CMV vector and subjected to stable selection, lysed, and processed for IP/Western.

Asynchronous Growth Assay

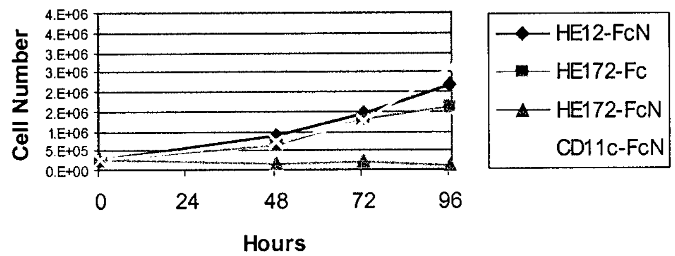


Figure 7. 100mm dishes were seeded with 250,000 SKBR3 cells stably transfected with the indicated constructs. Cells were plated in duplicate and counted 48, 72, and 96 hours after seeding. For counting, cells were trypsinized, pelleted by centrifugation, and viable cells counted by trypan blue exclusion.

Key Research Accomplishments

- Completion of HE-12 and HE-172 single-chain antibody cloning
- Completion of HE-12 and HE-172 sFv-Fc mammalian expression vectors for use in expression, purification, and binding analysis of sFv-Fc proteins manufactured in a mammalian cell line
- Successful expression, purification, and characterization of HE-12 and HE-172 immunoconjugates produced in COS-1 cells
- Display of anti-cyclin E binding activity by HE-12 and HE-172 intrabody constructs.
- Completion of HE-172 mammalian expression vectors for targeted cytosolic and nuclear targeting of sFv and sFv-Fc constructs
- We have shown sFvs can be expressed and successfully targeted to the cytosol and nucleus of the breast cancer cell lines MCF-7 and SKBR-3
- Establishment of SKBR3 cell lines stably expressing cytosolic and nuclear targeted anti-cyclin E intrabodies

Reportable Outcomes

No puplications have been or are currently submitted concerning this work to date. Abstracts have been published and poster presentations have been made at the following meetings:
1998- Cold Spring Harbor Gene Therapy Meeting (Cold Spring Harbor, NY)
2000- DAMD Era of Hope Meeting (Atlanta, GA)
2000- First China International Symposium on Antibody Engineering: Technologies and Applications (Tianjin, China)

Appendix A. Schematic Representation of Anti-cyclin E sFv, sFv-Fc, and Immunoconjugate Constructs.