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Most of the recent progress in tumor vaccines has resulted from the identification of new tumor antigens and their epitopes, from advances in the understanding of the biology of antigen presentation, and delineation of the cell types and functions responsible for the induction of protective immunity. Recent studies have shown that proteins can be presented for Class II MHC processing via Fcγ receptors expressed by leukocytes, with sufficient "leakiness" in the process for proteins to exit endosomes and be processed for Class I MHC presentation as well. To translationally exploit such knowledge, we are testing the novel hypothesis that fusion of the extracellular domain (ECD) of the breast cancer antigen HER-2/neu to antibodies reactive with human Fcγ receptor III (FcγRIII) will promote specific immunization that confers resistance to the growth of tumors expressing HER-2/*neu*. The long-term goals of this project are to develop such fusion proteins for clinical use. We will further refine this immunization strategy by introducing KDEL targeting signals and ubiquitin proteosome targeting sequences into the fusion proteins to direct the internalized antigen into both Class I and II processing pathways.

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FOREWORD

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Principal Investigator's Signature

5/1/99

Date

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Progress Report: DAMD 17-98-1-8084. Fcγ Receptor-targeted Immunization

Introduction

We have previously shown that treatment employing bispecific antibodies targeting the HER2/*neu* human tumor antigen and human Fcγ receptor III leads to the induction of antibody responses against HER2/*neu*. The objective of this research program is to investigate the value of inducing targeted immune responses through this mechanism.

Body of Report

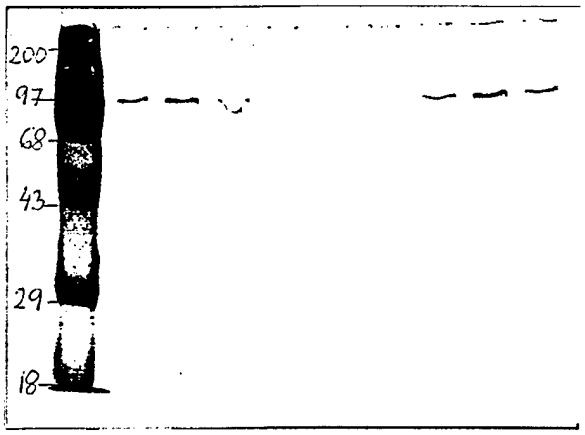
In the past year, our efforts have focused on several key areas:

- Production of HER2/*neu* extracellular domain
- Characterization of anti-Fcγ receptor targeting antibodies
- Production of mice transgenic for a mutated, kinase-deficient form of human HER2/*neu*

Progress in each of these areas is summarized below.

- **Production of HER2/*neu* extracellular domain (ECD)**
  We have produced trace amounts of ECD in baculovirus (**Figure 1**). However, the yield has been insufficient to permit the production of chemical conjugates or fusion proteins. Accordingly, we have turned to the expression of the ECD in CHO cells. The plasmid employed for production in CHO cells are indicated in **Figure 2.**

Figure 1.



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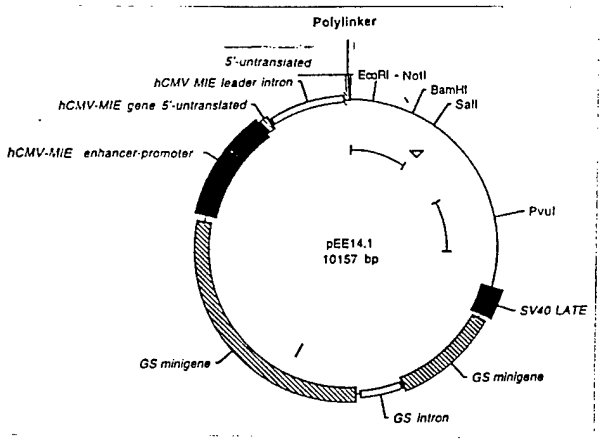

A leader sequence has been introduced into the pEE14-1 vector to permit the expression of the protein in the CHO cells. We anticipate the successful production of HER2/*neu* ECD within a few months.

- **Characterization of anti-Fcγ receptor targeting antibodies**

We have completed the characterization of the NM3E2 (also referred to as Y3) scFv, and have shown that it possesses moderate affinity for FcγRIII, the low affinity Fcγ receptor for immunoglobulin complexes. **Table 1** summarizes the affinity properties of NM3E2.

Table 1. Affinity Properties of NM3E2

Dissociation Constant (Kd)	On Rate (kon)	Off Rate (koff)
3.2×10^{-8}	5.5×10^{5}	1.8×10^{-2}

We have elected to modify our core strategy by developing chemical conjugates and fusion proteins that broadly target antigen-presenting cells. The underlying core principles are that the HER2/*neu* ECD should be targeted most efficiently to Fcγ receptor bearing cells that most actively participate in antigen presentation. Since B cells and dendritic cells do not express FcγR III, we have chosen to conjugate ECD to the murine antibody 2.4G2, which binds to murine FcγR II/III. These conjugates will be tested to provide proof of concept that targeting antigens via Fcγ receptors can induce augmented immune responses against the antigens. the hybridoma clone for 2.4G2 is in our laboratory, and antibody is being produced and purified for these experiments. Once proof of concept has been obtained, we then will turn our attention to the design and production of additional chemical conjugates and fusion proteins targeting human or murine FcγR I, II and III, respectively.

- **Production of mice transgenic for human HER2/*neu***

Mice transgenic for human HER2/*neu* have been produced as well. Since mice transgenic for HER2/*neu* develop fatal developmental abnormalities, C57Bl/6 mouse embryos were injected with a HER2/*neu* construct with a mutation of an invariant lysine at position 752 to methionine, thus eradicating the molecule's tyrosine kinase activity. Mutation was accomplished using overlapping 5' and 3' PCR primers encoding the mutated sequence. The mutated cDNA was cloned into a plasmid incorporating the MMTV LTR promoter as described. Microinjections were performed by the Fox Chase Cancer Center Transgenic Mouse Facility. Tail vein DNA from 30 pups was screened by PCR using primers specific for the mutated sequence. The DNA from fourteen pups had evidence of the mutated HER2/*neu* transgene. Southern blot confirmation of transgene copy number is under way.

Plans for Coming Year

- **Production of HER2/*neu* extracellular domain**

Leader sequences have been identified and are being cloned into a modified pEE14 vector for expression in CHO cells. We anticipate the production of sufficient HER2/*neu* ECD for purification, analysis for epitope preservation and use in conjugation strategies by June, 1999.

- **Production of anti-Fcγ receptor targeting antibodies**

The 2.4G2 hybridoma is being cultured and monoclonal antibody will be purified for use in conjugates with HER2/*neu* ECD. The ECD and antibody will be conjugated using standard

- **Characterization of anti-Fcγ receptor targeting antibodies**

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techniques and then tested for binding to murine peritoneal macrophages by flow cytometry. Once this has been demonstrated, cohorts of C57Bl/6 mice will be injected sc and ip with the conjugate and each of its components. We will determine if antibody and cellular immune responses directed against HER2/*neu* ECD can be demonstrated. MVM2 cells (C57 background) that have been stably transfected with human HER2/*neu* will be used as target cells for cellular responses.

If we demonstrate that these conjugates promote enhanced immunization, the conjugates will be tested in mice transgenic for HER2/*neu* (see below) bearing syngeneic tumors that overexpress HER2/*neu*. Protection against tumor growth will be an indication that the concept of Fcγ receptor-targeted immunization is valid, and will stimulate the formal testing of conjugates and fusion proteins composed of HER2/*neu* and multivalent antibody structures targeting FcγR I, II, or III.

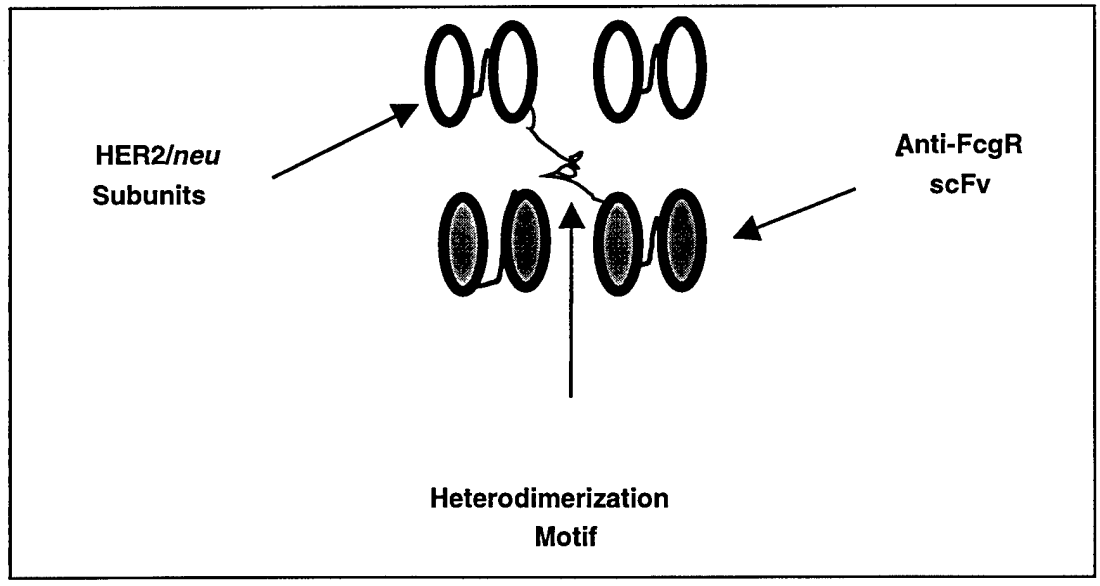
- **Production of mice transgenic for human HER2/*neu***

Pups from two different matings with evidence for the mutated HER2/*neu* sequence by PCR are being screened by Southern analysis to identify founders with introduction of the mutated gene in genomic DNA. These mice will be selectively backcrossed in a C57Bl/6 strain background to produce specific transgenic lines. These mice will be used in tumor protection studies as noted above.

- **Design and production of fusion proteins**

The demonstration of increased immunity resulting from immunization with HER2/neu ECD conjugated to 2.4G2 antibody will indicate the validity of the underlying hypothesis of this research. This will lead to the production of defined fusion proteins containing the HER2/neu ECD and multivalent antibodies targeting human FcγR I, II or III. We have produced NM3E2 (see above) and will produce a fusion protein containing NM3E2 and HER2/*neu* ECD as indicated in **Figure 3**. If this proves successful, we will construct fusion proteins containing the scFv isolated from the IV.3 (anti-human FcγRII) and 22 (anti-human FcγRI) hybridomas as well.

Figure 3. Production of HER2/neu ECD – anti-FcγR Fusion Proteins



Conclusions

The past year has focused on the development of reagents to test the hypothesis that tumor vaccines targeted to antigen-presenting cells via cellular Fcγ receptors can enhance immune responses against defined tumor antigens. In the coming year we will use these reagents to test the fundamental hypothesis and commence the production of reagents that may have applications in the human setting.

References

Not applicable