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FOREWORD

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N/A In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

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## INTRODUCTION

The objective of this research project is to test the hypothesis that immune responses to a cancer antigen that is commonly overexpressed in breast cancer can be selectively enhanced and lead to protection against such tumors. This hypothesis of Fc $\gamma$  receptor-targeted immunization is being tested through the construction of conjugates or fusion proteins containing the HER2/*neu* antigen and an antibody that targets HER2/*neu* to antigen-presenting cells via Fc $\gamma$  receptor IIIA. Appropriate immunoconjugates have been prepared and are undergoing testing in a murine model transgenic for human Fc $\gamma$ RIII. If the hypothesis is validated in the initial experiments, we will construct recombinant fusion proteins with similar specificities and will determine if the sera of immunized mice possess anti-tumor effects.

## BODY

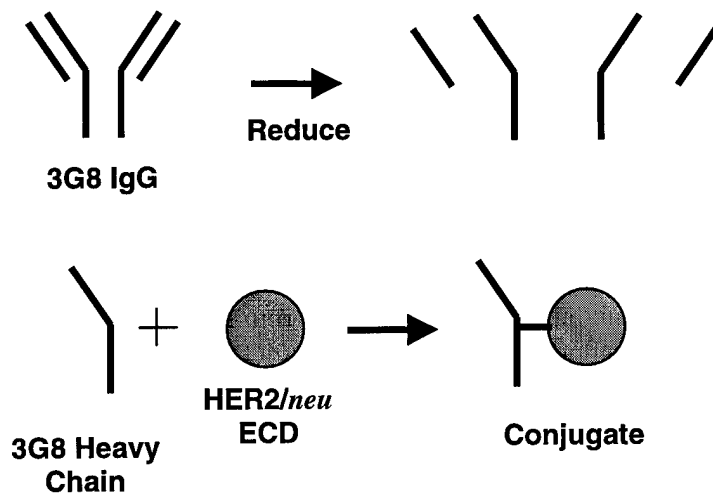
Significant progress has been made in achieving the specific objectives of this research project in the past year. This progress is summarized for each of the major areas of research encompassed by this project.

Creation of a Relevant Animal Model: We now have a fully syngeneic murine model for evaluating human Fc $\gamma$ RIII targeted immunization. Mice transgenic for human Fc $\gamma$ RIIIA (e.g., CD16A) have been backcrossed into mice of the C57Bl/6 strain, and mice in the F11 generation are being used for the immunization studies described below. These mice express human CD16A on natural killer cells and differentiated mononuclear phagocytes.

Production of 3G8 Monoclonal Antibody: The 3G8 hybridoma was obtained from the American Type Culture Collection and cells were cultured in appropriate media. Culture supernatants were collected and the 3G8 antibody was purified using Protein G columns. The purified antibody was found to bind to human neutrophils by flow cytometry similar to previous lots of 3G8 tested in our laboratory (not shown).

Production of Recombinant HER2/neu Extracellular Domain: Plasmids containing the HER2/neu extracellular domain cDNA and a hexahistidine tag to facilitate purification were transfected into the HEK 293 mammalian expression system and cells incorporating the transfected DNA were isolated by standard antibiotic selection procedures. These cells were then cultured to confluence and supernatants were collected. HER2/neu ECD was purified from cultural supernatants by affinity chromatography on an immobilized metal affinity column. The purified material is monomeric, highly purified (based on SDS-PAGE) and exhibits excellent binding to anti-HER2/neu antibodies by surface plasmon resonance imaging (BIAcore; data not shown). This method yields an excess of 1 mg of purified ECD per week.

Preparation of HER2/neu ECD – anti-Fc $\gamma$ RIII Conjugates: Chemical conjugation was chosen as an initial strategy to create reagents to test proof of concept for this



project. Using standard conjugation kits (Pierce), several methods were explored. The currently employed methodology employs reduction of the IgG, followed by blockage conjugation to ECD and subsequent blockage of available sulfhydryl groups using glutathione. The resulting conjugates have molecular weights of 120 kDa (not shown) and possess both anti-Fc $\gamma$ RIII and HER2/neu binding activities. The proposed structure of the 120kDa conjugate is shown in the preceding figure.

**Animal Experimentation:** The initial immunization experiments have commenced. Cohorts of 5 C57Bl/6 mice that are transgenic for human Fc $\gamma$ RIIIA are being immunized by four every other weekly subcutaneous injections. Retro-orbital eye bleeds are taken weekly, and on week 10 the animals will undergo terminal bleeds with concomitant isolation of splenocytes. The cohorts are as follows:

1. HER2/neu ECD (10  $\mu$ g per dose)
2. 3G8 Heavy Chain – HER2/neu ECD (10  $\mu$ g ECD per dose)
3. 3G8 Heavy Chain + HER2/neu ECD (10  $\mu$ g ECD per dose)
4. Phosphate-buffered Saline

The objective of this experiment is to obtain proof-of-concept that targeting the HER2/neu ECD to antigen presenting cells via human Fc $\gamma$ RIII induces more brisk immunization than does immunization with HER2/neu ECD alone. Murine immune responses to HER2/neu ECD will be monitored by ELISA assays. HER2/neu ECD will be adhered to 96 well plates and wells will be incubated with various dilutions of mouse sera obtained at different time points. After washing, the plates will be developed with anti-mouse antibody conjugates suitable for colorimetric assays. Positive results with anti-mouse antibody reagents will stimulate the analysis of isotypic responses. Splenocytes from mice with detectable immune responses will be used in intracytoplasmic cytokine assays using flow cytometry to examine Th1 and Th2 immune responses.

If the above studies demonstrate the promise of Fc $\gamma$  receptor-targeted immunization, we will proceed along several fronts. Firstly, the ability of the elicited antisera to inhibit the growth of HER2/neu overexpressing cancer cells will be studied in vitro and in vivo. We will prospectively compare the efficacy of immunization of the conjugate with that of the HER2/neu ECD admixed with conventional adjuvants. This will allow us to determine if the benefits of Fc receptor targeting are of sufficient magnitude to warrant continued clinical development. Also, we will proceed with existing plans to construct recombinant fusion proteins containing the 3G8 heavy chain fused to the HER2/neu ECD, using the mammalian expression system described above. Finally, we will employ similar strategies employing the 2.4G2 antibody targeting murine Fc $\gamma$ RII/III.

## KEY RESEARCH ACCOMPLISHMENTS

- Expression of HER2/*neu* Extracellular Domain in a Robust Mammalian Expression System
- Development of a strategy to produce conjugates containing equimolar concentrations of HER2/*neu* ECD and the heavy chain of the 3G8 anti-Fcγ receptor III monoclonal antibody. This conjugate possesses intact binding properties of its constituent components.
- Development and characterization of a mouse strain that is syngeneic with C57Bl/6 and transgenic for human FcγRIIIA (CD16A)



## REPORTABLE OUTCOMES

Amoroso AR, Alpaugh RK, Barth MW, McCall AM, Weiner LM. Production and characterization of mice transgenic for the A and B isoforms of human Fc $\gamma$ RIII. *Cancer Immunol Immunother* 48:443-455, 1999.

Weiner LM. Fc $\gamma$  Receptor-targeted immunization against HER2/neu. Department of Defense Breast Cancer Research Program Meeting: Era of Hope. June, 2000 (Abstract)

## CONCLUSIONS

Conclusions regarding this project must be viewed as premature, as definitive experimentation is under way at this time. It has proven difficult to assemble the necessary reagents to conduct such experimentation, but we have succeeded in producing HER2/neu extracellular domain and in developing reactive and reproducible immunoconjugates with minimal aggregation properties. Finally, a mouse model transgenic for human Fc $\gamma$ RIII has been developed and fully characterized.

**REFERENCES**

Not applicable

**APPENDICES**

None

**BINDING**

Not applicable

**FINAL REPORT**

Not applicable