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We hypothesize that the selective accumulation of systemically administered cytokines at tumor sites can alter tumor microenvironments to favor the induction of anti-tumor immune responses. We further hypothesize that this can be accomplished by pre-targeting tumors with antibody-streptavidin immunoconjugates and then administering biotinylated cytokines. The purpose of this research program is to identify antibody-pretargeted cytokine therapy strategies that lead to tumor-selective cytokine accumulation, the development of host inflammatory cell infiltrates in tumor, and the induction of tumor-specific immunity. The ultimate goal of this research is to identify candidate strategies for clinical development, alone or in combination with tumor vaccines. We have made significant progress toward achieving these goals. Because biodistribution results did not suggest that the previously used streptavidin-biotin system would yield therapeutic results we have focused efforts in the past year on developing new systems employing bispecific minibodies that contain anti-tumor binding domains and domains that bind to a metal chelate that can serve as a universal acceptor for metal-coniugated cvtokines.

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## INTRODUCTION

We hypothesize that the selective accumulation of systemically administered cytokines at tumor sites can alter tumor microenvironments to favor the induction of anti-tumor immune responses. We further hypothesize that this can be accomplished by pre-targeting tumors with antibody-streptavidin immunoconjugates and then administering biotinylated cytokines. The purpose of this research program is to identify antibody-pretargeted cytokine therapy strategies that lead to tumor-selective cytokine accumulation, the development of host inflammatory cell infiltrates in tumor, and the induction of tumor-specific immunity. The ultimate goal of this research is to identify candidate strategies for clinical development, alone or in combination with tumor vaccines.

## **BODY**

### **Technical Objectives**

1. To determine the conditions required to selectively pretarget streptavidin to human tumor xenografts growing in immunodeficient scid mice.
2. To determine conditions required for the selective accumulation of intravenously-administered biotinylated proteins and peptides to antibody-streptavidin pretargeted human tumor xenografts growing in immunodeficient scid mice.
3. To examine the host cellular infiltrate at tumor sites in mice following therapy with cytokines pretargeted to tumors by streptavidin-conjugated antibodies.
4. To examine the growth properties of tumors in mice treated with antibody-pretargeted cytokines.

### **Work Accomplished**

We previously achieved objectives 1 and 2. Careful analysis of biodistribution results using antibody-streptavidin tumor targeting and biotin-IL-2 delivery to the pretargeted tumors (Specific Aim 2) did not predict for therapeutic success due to innate deficiencies in the tumor targeting properties of the antibody-streptavidin conjugates. Accordingly, we devised an alternate strategy that utilizes a related pretargeting concept that addresses many of the deficiencies of the previously-employed approach.

NR-LU-10 – streptavidin immunoconjugate was obtained from NEORx Corporation, and was shown to bind by flow cytometry to HT-29 cells that overexpress Ep-CAM antigen (not shown). IL-2 was labeled through its carboxy-terminal cysteine according to manufacturer's instructions (Pierce). The biotinylated IL-2 was purified and removed over an avidin column (Pierce) and eluted with 100 mM glycine, pH3.0.

### **Characterization of Biotinylated IL-2(Bt-IL-2)**

The binding of IL-2 species to the IL-2 dependent NK92 cell line known to express the high affinity IL-2 receptor was measured by flow cytometric analysis. The results are depicted in the table below. The biotinylated species exhibited significant, but reduced binding to the IL-2 receptor. Bt-IL-2 bound to tumor cells coated with NRLU-10 – streptavidin. Half-maximal stimulation of T cell proliferation occurred at 0.005 – 0.01 nM IL-2, and at 0.1 – 0.5 nM biotinylated IL-2, respectively. Biotinylation reduced the T-cell proliferative effects of IL-2, but had relatively little impact on the ability of this cytokine to activate lymphocytes for tumor lysis. However, the immunoconjugate did not promote significant ADCC, presumably because the bulky streptavidin conjugation sites interfere with Fc domain interactions with lymphocyte Fc receptors.

Initial in vivo studies showed that best tumor to organ ratios were achieved at 30 h following injection with NRLU1—streptavidin. NRLU10-streptavidin then was injected into cohorts of scid mice bearing HT-29 subcutaneous xenografts (250 mg) followed by



Thus far we have created an initial template containing antibody domains that we have extensively worked with in the past. One antibody contains anti-HER2/*neu* binding specificity and the other antibody has specificity for a different ligand, FcγRIII (which is the Fcγ receptor, CD16, with specificity for Fc domains of immunoglobulin G (Figure 2).

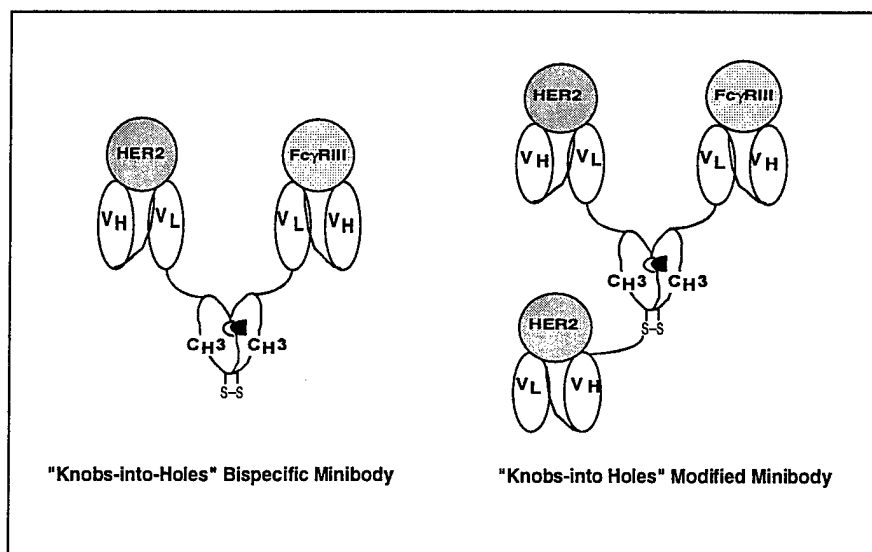
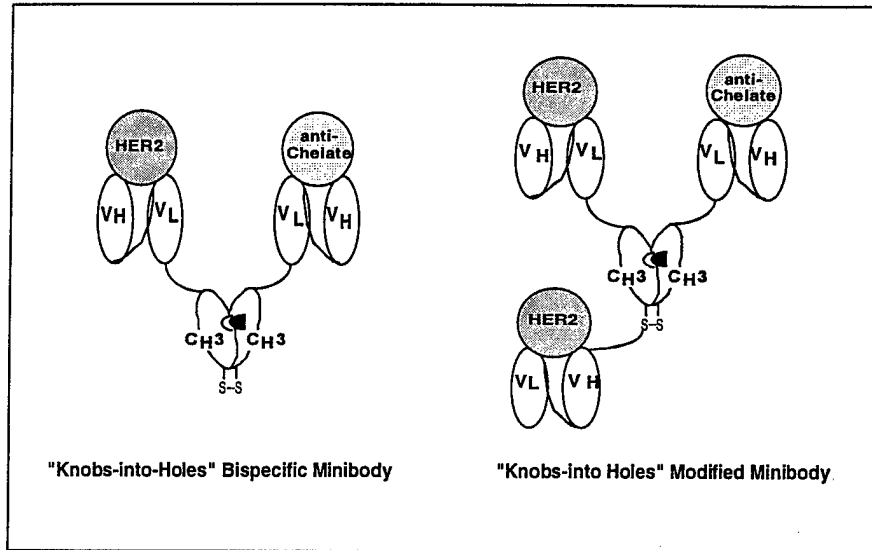


Figure 2. Schematic depicting the Structure of the two distinct minibody structures

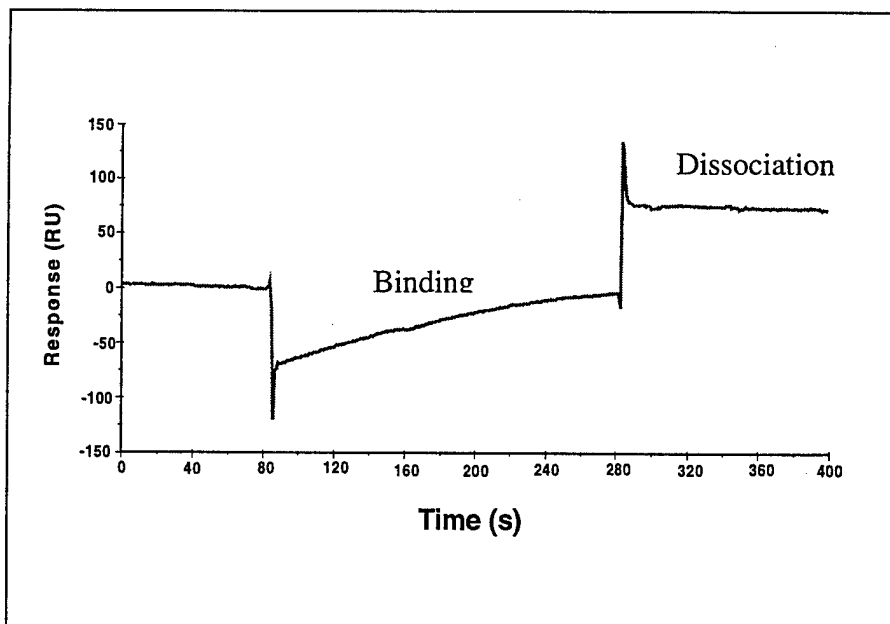
As is evident in this figure, both structures employ the "knobs-into-holes" concept, whereby heterodimerization based on affinities of CH3 domains is favored. Two distinct bispecific minibodies have been produced. The first is a dimeric molecule (left figure), while the other is trimeric, as depicted on the right side of the figure. Tetramers can be produced as well (not shown). Since the genes for these molecules are in hand, it is relatively straightforward to excise the anti-FcγRIII variable heavy and light chain genes and replace them with V<sub>H</sub> and V<sub>L</sub> genes encoding protein domains that bind to the metal chelate, CHX-A". To that end, we are obtaining single-chain Fv molecules that bind to this chelate from a long-time collaborator, Dr. James Marks and intend to create bispecific minibodies that bind to HER2/*neu* and to CHX-A" (see Figure 3 below).

Figure 3. Bispecific Antibodies targeting HER2/*neu* and CHX-A”



We anticipate success in producing the bispecific minibodies schematized above. The anti-HER2/*neu* x anti-FcγRIII minibodies exhibit the desired binding properties in a plasmon sensor resonance assay (BIAcore, Pharmacia). Shown below (Figure 4) is a representative tracing employing the dimeric bispecific minibody (Figure 2, left).

Figure 4. Anti-HER2/*neu* binding and slow dissociation of a dimeric, bispecific minibody



Similar results were demonstrated for binding to Fc $\gamma$ RIII (not shown).

A novel "sandwich" based BIAcore assay was developed to investigate the capacity of the minibody to mediate bispecific binding. Figure 5 and Table 1 below show that the minibody, but not a monospecific antibody, was able to simultaneously bind to HER2/*neu* and to Fc $\gamma$ RIII. Thus, these data support the idea that bispecific proteins can be produced and shown to mediate the desired binding properties. When the anti-CHX-A" variable heavy and light chain genes have been introduced into these structures, we intend to employ these molecules in tumors that overexpress HER2/*neu*; we have extensive experience with SK-OV-3 and with the breast cancer line MDA-MB-231-DYT2; both lines grow well in immunodeficient mice and express abundant HER2/*neu* on the cell surface.

Figure 5. Sandwich BIAcore assay demonstrates binding properties of a bispecific minibody

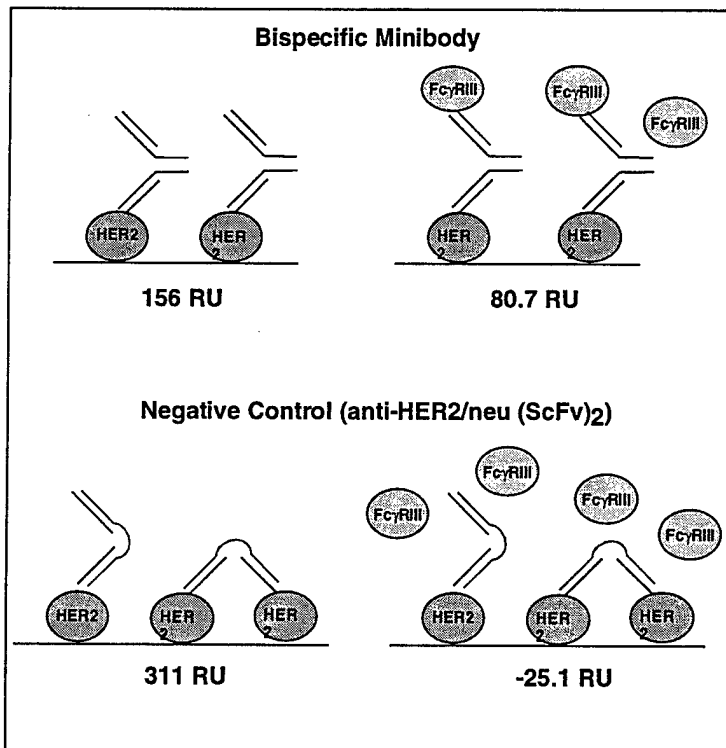


Table 1. Sandwich assay demonstrates bispecific binding properties of anti-HER2/*neu* x anti-FcγRIII minibody

Structure	HER2 (RU)	CD16 (RU)	Bispecific Binding
YCMC	+ (75)	+ (98)	+
TriBi	+ (154)	+ (310)	+
KHYCMC	+ (635)	+ (162)	+
KHTriBi	+ (440)	+ (242)	+
Neg. Control	-	-	-

We intend to modify IL-2 by covalently adding a labeled metal species (probably Indium-111) to its carboxyl terminus using standard technology extensively employed in our laboratory. The ability of the labeled IL-2 to be selectively retained at antibody-pretargeted tumor sites then will be determined.

### KEY RESEARCH ACCOMPLISHMENTS

1. Biotinylation of interleukin-2 (IL-2).
2. Characterization of biotinylated IL-2 binding properties, T-cell activation properties and capacity to promote lymphocyte-mediated cytotoxicity of tumor cells.
3. Demonstration that admixture of streptavidin-conjugated antibody with biotinylated IL-2 is associated with retention of antibody binding and IL-2 binding properties.
4. Proof of concept that antibody pretargeting can promote some selective tumor retention of IL-2.
5. Development of new vehicles for antibody-directed pretargeted cytokine therapy.

**REPORTABLE OUTCOMES**

L. Shahied, R.K. Alpaugh, G.P. Adams, H.H. Simmons, E.M. Horak, C.C. Shaller, D.B. Axworthy, A.R. Amoroso, and L.M. Weiner (2000) Pretargeting Mechanism for the Delivery of Interleukin-2 to Tumor Site. The 11<sup>th</sup> Annual International Conference on Antibody Engineering (Poster Presentation).

**CONCLUSIONS**

The results to date warrant continuation of this line of research. The new direction of the research results from a thorough analysis that showed the particular pretargeting strategy employed to date was not likely to yield successful IL-2 targeting and therapy. The new strategy builds directly upon other work in our laboratory and is felt to be a more promising avenue of research. Preliminary data demonstrate the feasibility of the proposed work.

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