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PRINCIPAL INVESTIGATOR: Louis M. Weiner, M.D.

CONTRACTING ORGANIZATION: Fox Chase Cancer Center
Philadelphia, Pennsylvania 19111

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13. ABSTRACT (Maximum 200 Words) <p>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. Interleukin-2 (IL-2) has been biotinylated, and its biological properties have been thoroughly characterized. We have obtained a streptavidin-conjugated monoclonal antibody that recognized the Ep-CAM tumor antigen that is frequently overexpressed in breast cancer specimens. The biodistribution properties of the antibody - streptavidin conjugate and of the conjugate admixed with biotinylated IL-2 have been characterized, and pretargeting therapy studies are about to commence.</p>				
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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 have made significant progress in achieving objectives 1 and 2, and anticipate commencing work on objectives 3 and 4 in the coming year.

NR-LU-10 – Sterptavidin Immunoconjugate

This 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).

Biotinylation of Interleukin-2 (IL-2)

IL-2 was labeled through its carboxy-terminal cysteine according to manufacturer's instructions (Pierce). Excess biotin was removed by dialysis. The biotinylated IL-2 was purified and removed over an avidin column (Pierce) and eluted with 100 mM glycine, pH3.0. The final product was dialyzed against PBS with a final recovery of 25%, and was frozen into 200 µl aliquots at concentration of .228 mg/ml. A HABA assay was used to determine the molar biotin: IL-2 ratio.

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

IL-2 Receptor Binding. 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.

IL-2 (nM)	MFI	Bt-IL-2 (nM)	MFI	% of native IL-2
1	21.6	1	16.5	76
5	22.6	5	16.4	73
20	23.3	20	16.8	72

These results indicate that the biotinylated species exhibits significant, but reduced binding to the IL-2 receptor. Furthermore, in order to determine if the Bt-IL-2 could

bind to NRLU-10 – streptavidin on cells a second flow cytometric analysis was performed in which the antibody was incubated with HT-29 cells followed by the addition of the Bt-IL-2. The interaction was detected using an anti-human IL2-FITC-labeled antibody. Results from this experiment showed that indeed the Bt-IL-2 could bind to the NRLU-10 – streptavidin which was bound to cells.

T cell proliferation assays. Unmodified and biotinylated IL-2 were added at various concentrations to 200,000 human peripheral blood lymphocytes and incubated for 72 hours. ³H thymidine was added to the cell cultures and incorporation was measured and extrapolated for proliferation. Half-maximal stimulation occurred at 0.005 – 0.01 nM IL-2, and at 0.1 – 0.5 nM biotinylated IL-2, respectively.

Cytotoxicity assays. In another set of experiments, human carcinoma cell line SK-OV-3 was labeled with ⁵¹Cr and added to human lymphocytes that had been incubated in varying concentrations of unmodified or biotinylated IL-2. At 25: 1 effector: target ratios, 14% lysis of tumor was mediated by lymphocytes activated in 2000 IU unmodified IL-2, while equivalent levels of tumor lysis were seen using lymphocytes activated in 5000 IU biotinylated IL-2. Thus, biotinylation reduces the T-cell proliferative effects of IL-2, but has relatively little impact on the ability of this cytokine to activate lymphocytes for tumor lysis. When biotinylated IL-2 is admixed with NR-LU-10 – streptavidin, the resulting immunoconjugate retains an ability to activate lymphocytes to promote tumor lysis. In one experiment employing the HT-29 tumor cell line overexpressing Ep-CAM, the immunoconjugate promoted tumor lysis by human lymphocytes at 25:1 effector: target ratios (not shown). However, the immunoconjugate does not promote significant ADCC, presumably because the bulky streptavidin conjugation sites interfere with Fc domain interactions with lymphocyte Fc receptors.

In vivo experiments. Based upon the above studies we concluded that the biotinylated IL-2 possessed adequate properties for in vivo studies with NR-LU-10 – streptavidin. In the first set of mouse experiments ¹²⁵I-labeled NRLU-10-streptavidin antibody was evaluated to determine its optimal tumor targeting timepoint. These studies showed that best tumor to organ ratios were achieved between the 24 and 48 h timepoints. This finding confirmed prior observations that 30 h is the optimal timepoint to inject Bt-IL-2. In the following experiments, NRLU10-streptavidin was again injected into cohorts of scid mice bearing HT-29 subcutaneous xenografts (250 mg) followed by the injection of a clearing agent 24 h later. This clearing agent was administered in order to remove any unbound NRLU10-streptavidin from the bloodstream. Thirty hours post-antibody injection, ¹²⁵I-labeled Bt-IL-2 was injected into the mice. Two additional cohorts(24 h and 48 h timepoints) were treated in the same manner, with the exception that the IL-2 that was administered was not biotinylated, this provided a negative control. Further controls included injecting both the IL-2 and Bt-IL-2 without antibody to compare its targeting alone. Other cohorts were treated with NR-LU-10 – streptavidin admixed ex vivo with equimolar concentrations of biotinylated IL-2 and sacrificed at the same time points. Tumors and normal organs were assayed for radioactive content and results used to calculate % injected dose per gram of tumor or organ, and to calculate tumor: normal organ ratios. Results showed that under these

pretargeting conditions the ^{125}I -Bt-IL-2 was successfully targeted to tumor sites as compared to pretargeted ^{125}I -IL-2 or either of these molecules administered alone (see attached figure). Although these results were encouraging the amount of tumor to normal organ ratios were not high. However, it is possible that they amount of Bt-IL-2 delivered to tumor site is sufficient to induce tumor regression. Future therapy studies in mice will allow us to elucidate this answer. Furthermore, we are currently investigating other moieties (eg. barnase – barstar) besides the biotin-streptavidin interaction for use in this pretargeting strategy. These initial results demonstrate that the concept of antibody-pretargeted cytokine therapy continues to have merit and is worth pursuing.

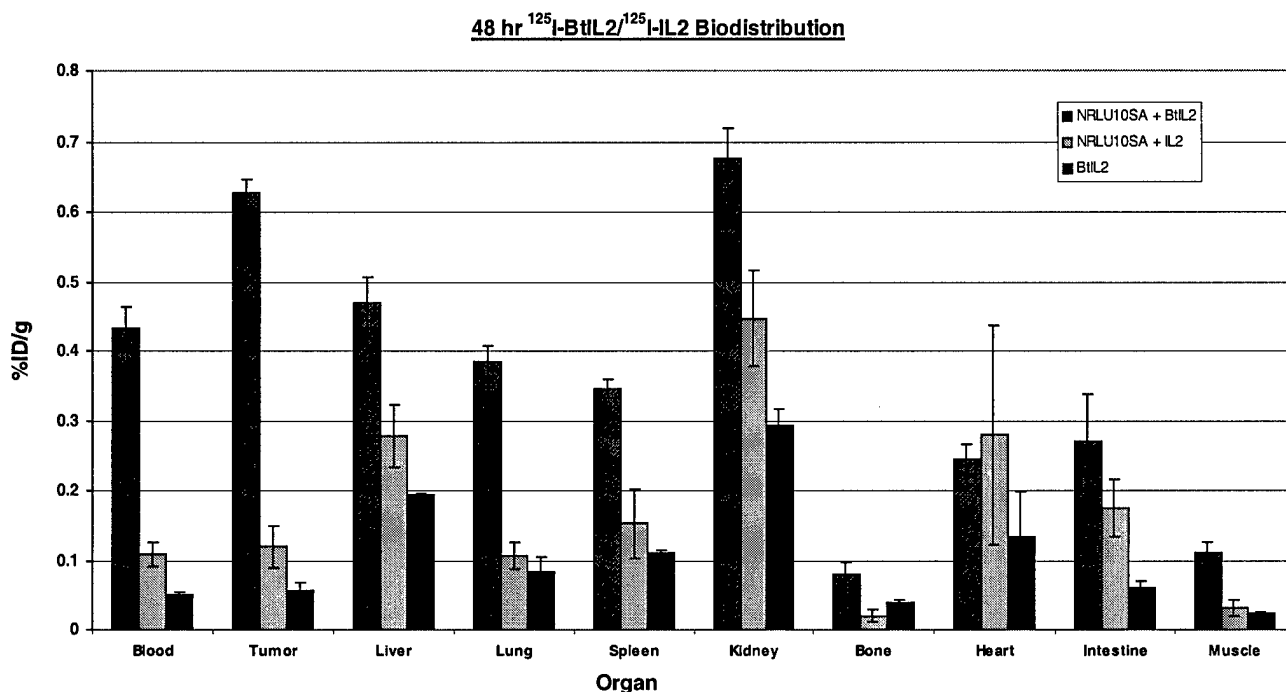


Figure legend:

^{125}I -BtIL2 Biodistribution

In this experiment 400 μg of the NRLU-10SA antibody was first administered to HT29 tumor bearing SCID mice followed by injection with 100 μg of galactosylated-biotinyl-HSA, a clearing agent used to remove excess streptavidin-labeled antibody from circulation. The mice were then injected with 5 μg of either ^{125}I -BtIL2 or ^{125}I -IL2. One cohort received ^{125}I -BtIL2 alone as a second negative control. Shown is the average percent injected dose per gram of radiolabeled material in several organs and the blood.

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 the selective tumor retention of IL-2.

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 11th Annual International Conference on Antibody Engineering (Poster Presentation).

CONCLUSIONS

The results to date warrant continuation of this line of research. The biotinylation of IL-2 does lead to a loss of IL-2 biological activity, but we hypothesize that this loss will be overcome by a decrement in host toxicity coupled with the concentration of the cytokine at tumor sites by antibody pretargeting. Therapy studies employing this system are commencing and will be adapted to a *HER2/neu* transgenic mouse model in the coming year.

REFERENCES

Not applicable

APPENDICES

None.