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Department of the Army position, policy or decision unless so
designated by other documentation.
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. In the first year of this award 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. Animal experiments to characterize the biodistribution properties of the antibody – streptavidin conjugate and of the conjugate admixed with biotinylated IL-2 are underway, and pretargeting experiments are about to commence.
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In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

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

[Signature]

Date
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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 in the past year, and anticipate completing these and 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

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.

<table>
<thead>
<tr>
<th>IL-2 (nM)</th>
<th>MFI</th>
<th>Bt-IL-2 (nM)</th>
<th>MFI</th>
<th>% of native IL-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>21.6</td>
<td>1</td>
<td>16.5</td>
<td>76</td>
</tr>
<tr>
<td>5</td>
<td>22.6</td>
<td>5</td>
<td>16.4</td>
<td>73</td>
</tr>
<tr>
<td>20</td>
<td>23.3</td>
<td>20</td>
<td>16.8</td>
<td>72</td>
</tr>
</tbody>
</table>
These results indicate that the biotinylated species exhibits significant, but reduced binding to the IL-2 receptor.

**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. $^3$H 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 $^{51}$Cr 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 immunooconjugate retains an ability to activate lymphocytes to promote tumor lysis. In one experiment employing the HT-29 tumor cell line overexpressing Ep-CAM, the immunooconjugate promoted tumor lysis by human lymphocytes at 25:1 effector: target ratios (not shown). However, the immunooconjugate 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. These animal experiments have commenced. A biodistribution study is under way. In this experiment, cohorts of scid mice bearing HT-29 subcutaneous xenografts (250 mg) are treated with $^{125}$Iodine-labeled antibodies. Two cohorts were treated with NR-LU-10 – streptavidin and were sacrificed at 24 and 48 hours, respectively. 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 will be assayed for radioactive content and results used to calculate % injected dose per gram of tumor or organ, and to calculate tumor: normal organ ratios. These results will be used to validate prior results employing the NR-LU-10 – streptavidin immunooconjugate and to determine if its conjugation to IL-2 affects its biodistribution properties in scid mice. Most importantly, the results will form the basis for the next experiment, in which the NR-LU-10 – streptavidin immunooconjugate will be used to pretarget tumors, followed by administration of a clearing agent and then by radiolabeled biotinylated IL-2. Separate cohorts of mice will be treated with biotinylated IL-2 alone. Mice have been inoculated with tumors for this next set of experiments.

The results of these studies will form the basis for further investigations into the potential value of antibody-pretargeted cytokine therapy.
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.
REPORTABLE OUTCOMES

None to date (still in first year of support).
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.
Not applicable
None.

APPENDICES