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14. ABSTRACT

The project is based on the generation of metastatic brain tumors using a brain selective cell line, 231-BR, derived from human breast cancer. Therefore, the experimental model to be used must be immune compromised. The other requirement we have for this project is that the experimental animals express human HLA-A2 complexes (major histocompatibility complex class I). The antibody we want to evaluate as a potential therapeutic agent is a T-cell receptor mimic restricted to human HLA-A2. Therefore, we must use HLA-A2 transgenic mice. In experiments conducted to date the mouse strain, which fulfills both requirements, JAX 9617, and the corresponding non-transgenic control strain, 5557, were receptive for tumor growth not only in brain, but in a number of peripheral organs (e.g. lung, liver, spleen). To avoid the confounding influence of peripheral metastases, we currently explore alternative methods to generate brain tumors (intracarotid injections, stereotaxic brain implantation. Regarding the analytical side, we have established an immunoradiometric assay for the RL6A antibody with high sensitivity. In saturation and in competition experiments, radioiodinated RL6A and unlabeled antibody showed a Kd value of 1.16 nM and a Ki of 1.26 nM, respectively.

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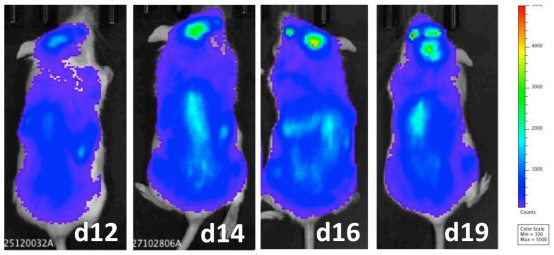
Introduction

The increased occurrence of brain metastases in breast cancer patients is a major clinical problem that becomes more relevant with the longer survival times secondary to better therapeutic options now available to treat the primary tumor and metastatic disease in peripheral organs. Unfortunately, current therapeutic options for brain metastases are dismal. The most significant obstacle to progress in the treatment of brain metastasis is the limited penetration of anticancer drugs (small molecule based agents as well as macromolecules like antibody-based drugs) into the CNS tissue. Although tumor vasculature may be leakier than the healthy blood-brain barrier (BBB), drug levels achievable in the vast majority of brain metastases are below drug concentrations in peripheral tumor metastases by almost a log order or more (1). The purpose of the present project is to evaluate a novel approach, in which we exploit the unique property of a particular antibody, dubbed RL6A, to both undergo active transport across the BBB (2) and to have significant antitumor effects (3). RL6A belongs to a novel class of antibodies called T-cell receptor mimics (TCRm), because these antibodies have binding specificities analogous to TCR. In case of RL6A, this antibody recognizes the YLLPAIVHI peptide, derived from p68 RNA helicase (a tumor antigen), only when presented in the binding groove of human HLA-A2 (4). A murine model of metastatic brain tumor is utilized and our research plan has two specific aims. Studies under *specific aim #1* are designed to determine the uptake of the RL6A antibody into brain and brain tumors after systemic administration. Experiments under *specific aim #2* are designed to determine the therapeutic effect of RL6A in the brain metastasis model.

Body



HLA-A2 transgenic (stock 9617) mouse #4

Figure 1. Representative series of bioluminescent images of a mouse injected with 231-BR cells (175,000 into the left cardiac ventricle). Imaging was performed on the days indicated 15 min after i.p. injection of luciferin.

In the first year of this grant we have performed the following work (with reference to tasks as described in the Statement of Work).

We have established cell culture propagation of the brain-seeking subclone of the MDA-MB-231 cell line (in the following abbreviated BR-231) in the lab of the PI (*task 1*). Hybridomas generating RL6A and a control TCRm, R21A, have been grown in the lab of Dr. Weidanz (Co-Investigator) and endotoxin free, affinity purified antibodies in sufficient quantities for ongoing work are available (*task 2*). Similarly, the corresponding antigens (peptide-HLA-A2 complexes in tetramer format) have been provided by the Weidanz lab.

Local IACUC approval at Texas Tech HSC, and approval by ACURO, has been obtained for 2 animal protocols: one experimental protocol (#11037) covering all planned experiments, and more recently a breeding protocol (*task 3a*). The latter has been initiated to permit the establishment of a breeding colony of the HLA-A2 transgenic mouse strain (Jackson Labs stock 9617) in our local animal facility. The strain is kept at JAX as "live repository", i.e. at relatively low supply levels. While delivery rates provided by JAX appear to be at sufficient level for the pharmacokinetic studies under aim #1 (*task 3b*), we feel it will be advantageous to have additional in-house supply for the required higher animal numbers planned under specific aim #2. We have already seen breeding success in the last 2 months.

The intracardiac injection of 231-BR cells and subsequent live imaging using the IVIS system (Caliper Lumina XR) has been adopted in the PI's lab with support from Dr. Lockman's lab (Co-Investigator). The 231-BR subclone of the widely used breast cancer cell line, MDA-MB-231, is known to metastasize selectively to brain (5), in particular when inoculation is performed by the intracardiac injection approach, as utilized by Dr. Steeg's lab at NCI (6). To date we have performed tumor cell injections in 3 separate groups of mice of the HLA-A2 transgenic strain (9617) and one round in the control strain (JAX 5557). While we encountered no major technical difficulties with the intracardiac injection method (although localizing anatomical landmarks like the xiphoid process is somewhat more difficult compared to nude mice due to presence of fur in these scid strains), there appears to be a difference in susceptibility to metastasis pattern and rate of tumor growth. Starting with a number of 175,000 cells per animal (as in most of the published papers by the Steeg group and as used by Dr. Lockman's lab), we noticed rapid tumor growth in the CNS, but also significant tumor load in the periphery of most animals (see a representative series of live bioluminescent images in Fig. 1). Most animals injected with this number of cells developed brain and peripheral tumors between 8 and 12 days after injection

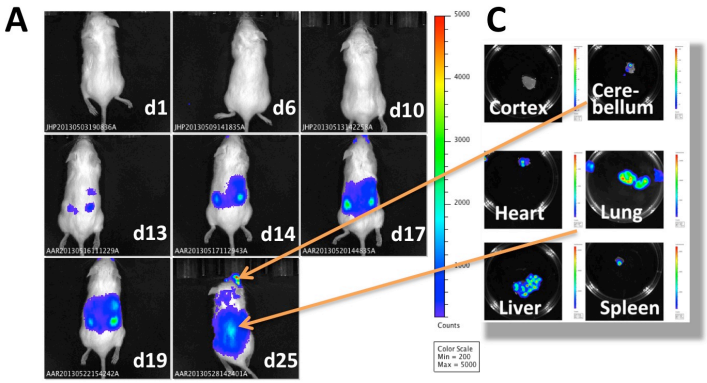


Figure 2. Representative imaging series of a control (A) and a HLA-A2 transgenic mouse (B) after intracardiac injection of 50,000 231-BR cells on day 1. Panel C shows bioluminescent images of the organs sampled form the mouse after euthanasia on day 25, confirming the presence of tumors in cerebellum, heart, lung, liver and spleen.

and all succumbed to tumor or had to be euthanized by day 19. In contrast, inoculated Nu/Nu mice typically start to develop brain tumors as detectable by IVIS around 3 weeks after inoculation and live > 4 weeks (w/o drug treatment). Importantly, with the 231-BR cell line few Nu/Nu mice (<10%) develop metastases in peripheral organs. For the next group of animals we then reduced the number of injected cells to 50,000. As expected, this reduced the rate of tumor growth and extended survival. However, both control and transgenic NSG mice still developed peripheral tumors (including in lung, liver and spleen) as shown in Fig 2.

Because we want to avoid peripheral metastases, which would confound the outcome of any treatment studies targeted to brain metastases (planned under task 6), we will next evaluate two alternative approaches: (a) Injection of a low amount of cells (~5-10,000) directly into a carotid artery (unilaterally), or (b) stereotaxic implantation of the cells into brain. Approach (a) would be preferable, as it will maintain the more physiological model of metastasis induction, approach (b) is a fallback option with predictable success (the standard method of inducing experimental brain tumors in the literature).

Towards accomplishing *task 4*, we have set up a highly sensitive immunoradiometric assay (IRMA) for RL6A by plating the antigen (tetramer of YLL-HLA-A2 complex) to the bottom of 96-wells. The RL6A antibody was labeled to a specific activity of 2,600 mCi/µmol by a modified Cloramine-T method, and saturation binding assays with increasing tracer concentrations, as well as competition binding assays with fixed amounts of tracer and increasing concentrations of unlabeled RL6A were performed. Fig. 3 shows as saturation curve and Fig. 4 depicts a competition experiment. Using the tracer saturation curve as a standard, the detection limit of the assay should be as low as 10 pM of tracer. Nonspecific binding (to wells plated with an irrelevant peptide-HLA-A2 complex was negligible (<1%).

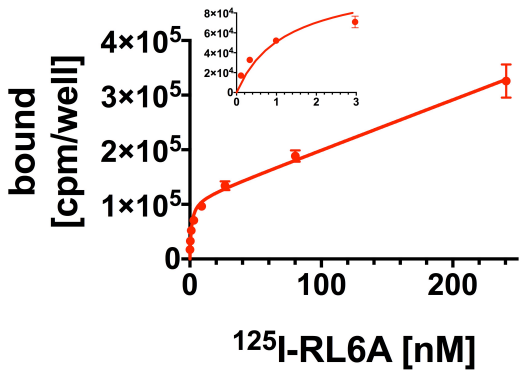


Figure 3. IRMA saturation curve. Total binding, one specific binding site model, curve fitting by nonlinear regression (GraphPad Prism 6). Kd of tracer binding estimated as 1.165 nM (CI = 0.454 – 1.876)

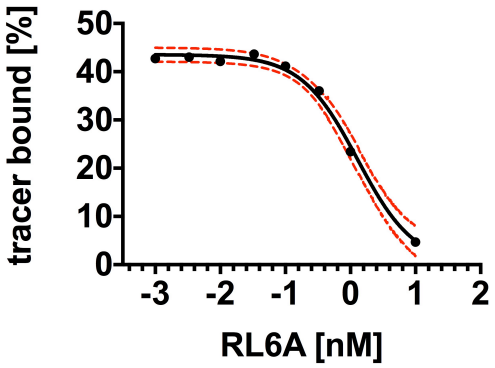


Figure 4. Competition curve with ^{125}I-RL6A and unlabeled antibody. Curve fitting to a one site binding model by nonlinear regression (dashed red curves = 95% confidence interval). The Ki is estimated as 1.26 nM (CI = 0.958- 1.66).

Key Research Accomplishments

- The 231-BR cell line generates reliably brain metastases
- It is however apparently less brain selective in the *scid* mouse models used in this project, compared to the nude mice mostly used in published literature.
- Radiolabeling of the RL6A antibody with ^{125}I does not appreciably compromise binding affinity (the Kd of the native antibody was previously estimated at 0.4 nM in Biacore experiments).
- We expect to have sufficient sensitivity to detect antibody binding in brain homogenate following systemic administration of tracer to mice.

Reportable Outcomes

To date we have not published the new data.

Conclusion

We are currently in the middle of the experimental plan. The strong growth of 231-BR metastases in peripheral organs was not anticipated, but is not surprising either. 231-BR is a brain selective, not brain specific, cell line, and the near complete absence of the adaptive and innate immune system in the NOD-scid gamma (NSG) mice may offer better conditions for tumor growth throughout the body than just the T-cell deficiency in the athymic nude mice. As outlined above, we have a plan to address this problem and it does not require a significant change in the overall plans. We will then proceed with the pharmacokinetic and therapeutic studies, according to the statement of work. Using the IRMA, we are confident that we will be able to show the brain uptake of intact antibody.

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