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TITLE: Viral Immunotherapy to Eradicate Subclinical Brain Metastases

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**Purpose**: To develop an immunologically based strategy for the treatment of breast cancer metastases to the brain.

**Scope**: To establish an animal model of breast cancer metastases to the brain and to use this model to demonstrate that anti-tumor host memory T-cells can be re-activated to enter and destroy early BM by viral infection of Her2-positive breast BM by a recombinant vesicular stomatitis virus (VSV), which liberates tumor antigens by viral oncolysis and induces acute inflammation in BM and CSF via the cytokine transgenes it carries and/or by adoptive transfer of cytotoxic, cytokine-producing natural killer (NK) cells capable of accumulating in smaller metastases of the brain.

**Major Findings**:
- Breast cancer cell line (D2F2/E2) and model established, which easily produce animals with brain/meningeal breast cancer metastases expressing Her2/neu and allows CSF sampling.
- VSV and natural killer (NK) cells containing cytokine transgenes successfully produced.
- Demonstrated that animals with extra-cranial D2F2/E2 tumors can be cured by treatment with VSV, cyclophosphamide, and anti-CTLA-4 and that cured animals are protected against intracranial challenge with D2F2/E2.
- Donor T cells and A-NK cells traffic to brain tumors but less well than previously seen in pulmonary and hepatic tumors.

**Subject Terms**: Breast cancer metastases to brain; Her2/neu pseudotyped VSV; anti-tumor memory T cells; NK cells; cytokines and cytokine transgenes; effector cell traffic.
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Viral immunotherapy to eradicate subclinical brain metastases

INTRODUCTION

Subject: Breast cancer metastases to the brain.

Purpose: To develop an immunologically based strategy for the treatment of breast cancer metastases to the brain.

Scope: To establish an animal model of breast cancer metastases to the brain and to use this model to demonstrate that anti-tumor host memory T-cells can be re-activated to enter and destroy early BM by viral infection of Her2-positive breast BM by a recombinant vesicular stomatitis virus (VSV), which liberates tumor antigens by viral oncolysis and induces acute inflammation in BM and CSF via the cytokine transgenes it carries and/or by adoptive transfer of cytotoxic, cytokine-producing natural killer (NK) cells capable of accumulating in smaller metastases of the brain.

BODY OF REPORT

Grey text: Original SOW text

Black text: Work performed mainly in the Bergman laboratory.

Blue text: Work performed mainly in the Basse laboratory.

Tasks

1. Demonstrate that anti-tumor memory T-cells are activated when tumor antigens and inflammatory mediators are delivered to CSF macrophages (Months 0-9)

   a. Generate donor survivor animals by treatment with replicating recombinant Vesicular Stomatitis Virus (rrVSV)
      i. Months 0 to 24.
      ii. Bergman
      iii. These mice are generated weekly
      iv. 300 mice/year
      v. All donor mice are Balb/c
      vi. Tumor implants use the D2F2/E2 mammary cancer cell line

   This task has been accomplished and works consistently. Tumors develop in 100% of animals implanted in the peritoneum with the D2F2/E2 mammary cancer cell line (Fig.1-Bergman). Therapy with rrVSV achieves > 90% cure rate. We are currently generating 5-10 cured mice per week and have available abundant donor animals required for our experiments. The availability of unlimited cured donor animals means that we can test different components of donor cells (CD4 T-cells, CD8 T-cells, B-cells or total spleen cells) in transfer experiments and use a large number of hosts each with a single experimental variable.

   b. Production of rrVSV and non-replicating VSV expressing various cytokines (IL-12, IL-1α, IL-6, TNFα, IL-4, IL-7, IL-15, IFNγ, IL-23, TGFβ and CD40L)
      i. Months 0-24
This task was technically challenging but has been accomplished for every cytokine. We have an extraordinary set of recombinant VSV expressing a wide variety of cytokines. These cytokines have a very broad range of inflammatory and immunologic effects that can now be manipulated in a controlled fashion in the local setting of the tumor.

c. Animal studies: Inject non-replicating VSV-infected tumor cells via cisterna magna (CM) into hosts with donor anti-tumor CD4 cells
   i. Months 1-9
   ii. Bergman
   iii. 100 mice

We performed the following experiment which provided a clear answer to the question posed by this task. Seven animals received IP D2F2/E2 tumor implants and were then cured of tumor by standard therapy with rVSV. These cured animals were then challenged with D2F2/E2 tumor implants in the cisterna magna (CM). None of these experimental animals developed neurological disease. Control naïve animals implanted with CM tumor all developed neurological disease and died within 2 weeks. It is therefore clear that cured animals not only develop immunity to the original tumor cells but that this immunity easily crosses the blood-brain-barrier and prevents growth of tumor in the meninges. This result is consistent with the clinical hypothesis of this project. We suggested a two-phased strategy to treat breast cancer metastases in the brain before they produce debilitating symptoms. The first phase was to generate anti-tumor memory T-cells when the patients

Fig. 1 - Bergman. D2F2/E2-J9 brain tumors: D2F2/E2-J9 cells were implanted into the cisterna magna. The mouse was sacrificed 10 days later when it first manifested lethargy and weakness. The brain was fixed overnight in buffered formalin, cut in the sagittal plane and embedded in paraffin. Slides were cut at 5 µM and stained with hematoxylin and eosin.
   A. A large nodular tumor is seen (left side of picture) in the meningeal space anterior to the olfactory bulb (right side of picture). Scale bar is 100 µM.
   B. A nodule of tumor (left side of picture) is seen in the 4th ventricle adjacent to the cerebellar vermis (right side of picture). Scale bar is 100 µM.
first presented by treating the primary breast tumors with rrVSV. The second phase was to activate the patient’s anti-tumor memory T-cells to enter the brain and find and destroy early metastases. In this experiment, implantation of the tumor cells may have provided sufficient inflammatory signals to bring memory T-cells to the tumor. In further experiments, we will explore methods to generate these beneficial signals when the tumors themselves are growing silently.

a. Analysis of CSF: Number of cells and types of cytokines that mediate inflammation in the central nervous system (CNS)
iv. Months 1-9
v. Bergman

This task has been delayed by technical difficulties that have been substantially resolved. Initially, the methods used to implant tumor cells into the cisterna magna did not produce consistent leptomeningeal (LM) tumors. We tried several techniques reported in the literature and generated LM tumors some of the time but felt that consistent implantation was required to compare experimental to control groups. In response, we improved both the method of implantation and the cells implanted. D2F2/E2 cells were implanted into the CM and new primary cultures were made from successful tumor implants. This was done several time and we now have a subclone, D2F2/E2-J9 that consistently implants in the LM. In addition, we developed our own very simple method for CM puncture that in combination with the adapted cell line currently yields 100% implantation (Fig. 1- Bergman). We plan to confirm the presence of LM implants in some animals using MRI prior to therapy. Use of this imaging technique has recently been approved by the institutional animal committee and is being submitted to ACURO.

To determine if the D2F2/E2 brain tumor cells expressed Her-2 also in vivo, fresh frozen sections were stained with antibody against human Her-2. We found a strong and almost homogenous expression of human Her-2 in all tumors analyzed (Fig. 2-Basse).

![Fig. 2-Basse. Expression of human Her-2 by D2F2/E2 brain tumors. Sections of brain tumors were stained with humanized mouse anti-huHer-2 followed by FITC-labeled rabbit anti-human Ig.](image-url)
We have also adapted our simple method for CM puncture to be able to sample CSF percutaneously on living, lightly anesthetized animals. Surgery is not required and sample can be obtained on multiple different days. Only 2-6 ul of CSF can be obtained so analysis on any one sample is limited. The CSF can undergo cytospin for examination of cells or can be diluted for multiplex analysis of cytokines. Our major finding to date using this technique is that spleen cells from cured donor animals transferred intravenously to host animals bearing LM tumor can traffic to the CSF as illustrated in the figure below (Fig. 3- Bergman). The next step is to define the conditions that allow and increase this trafficking and these studies are underway. These further studies will also analyze CSF cytokines as outlined in the grant.

One technical difficulty that has only partially been resolved is implantation of tumor by carotid artery injection. The surgical technique of isolating and injecting cells into the carotid artery of the mouse turned out to be very challenging. We can now accomplish this task consistently with the help of 34 gauge needles that we special order from

Fig. 4-Bergman. D2F2/E2 tumors induced by carotid injection. D2F2/E2 cells were implanted into the right carotid artery. The mouse was sacrificed 20 days later when it first manifested lethargy and weakness. The brain was fixed overnight in buffered formalin, cut in the coronal plane and embedded in paraffin. Slides were cut at 5 µM and stained with hematoxylin and eosin. Extensive tumor deposits are seen in the right hemisphere (top picture) in the meningeal and perivascular spaces and infiltrating the brain. Tumor is not visible in the left hemisphere (bottom picture).
Japan. The remaining problem is that the tumor cells form growing tumors only about 30% of the time (Fig. 4-Bergman). Once more, we are serially making primary cultures from tumors that have grown in the brain to develop a sub-clone of D2F2/E2 that consistently implants and grows following carotid injection.

From our previous work with a lung metastasis model, we learned that i.v. injection of a single tumor spheroid (composed of less than 50 tumor cells) consistently gave rise to one tumor metastasis, whereas we needed to inject at least 10,000 tumor cells to induce 1-10 metastases. Based on this observation, the Basse lab is generating D2F2/E2 tumor spheroids (diameter ~50 microns) of which we will inject 1-2 per mouse via the internal carotid artery.

In order to be sure that our surgical methods are appropriate, we also plan to test a mouse colon cancer cell line transfected to express human Her2/neu called MC38/E2. This cell line was shown by others to implant in the brain following carotid injection. Use of this cell line has recently been approved by the institutional animal committee and is being submitted to ACURO. The carotid artery model will provide useful information but is not essential to this grant which can be accomplished with the CM model that is fully effective.

d. Analysis of tissues: Determine the density and state of activation of donor (especially CD4 T cells) and host immune cells in metastases and normal tissues
   i. Months 1-9
   ii. Basse

As indicated in the grant, the first task was to determine optimal methods for labeling donor cells. A basic aim is to determine how anti-tumor memory T-cells from donor animals traffic to brain tumors and different methods of labeling the donor cells may be appropriate for different experiments. We have tested 3 methods of labeling and detecting donor cells: label with CFSE, label with Hoechst 33342 or use Thy 1.2 donors in Thy 1.1 recipients. We determined that Hoechst and CFSE were best visualized in brain tissue following fixation in 2-4% paraformaldehyde and dehydrating in sucrose. Thy 1.2 is best stained in flash frozen brain tissue. Hoechst and CFSE labeled cells are readily visualized in fresh CSF. The brightest staining is achieved with Hoechst and is detectable in pathological specimens for more than 3 days following cell transfer. Hoechst binds to DNA preventing cell replication and transcription. Trafficking of these transferred cells is therefore limited to properties they already possess at the time of labeling and not properties that they acquire in the host animal. CFSE labeled cells can replicate and transcribe new message but we found that label could only be clearly visualized within donor cells in the brain for 24 hours following cell transfer. Transfer of Thy 1.2 donor T-cells into Thy 1.1 hosts allows full replication, transcription and activation of the donor cells. Being a genetic marker, Thy1 is not diluted by replication. We have therefore decided to use congenic Thy 1 as our primary method of visualizing transferred donor cells while still using Hoechst and CFSE for special purposes.

The need to use Thy 1.1 Balb/c mice created an additional Task. We had to establish a breeding colony for these animals because they are not available commercially. The
first breeding pair did not produce a litter and consequently these experiments were slowed but we are proud to report that we now have a fecund breeding colony with an ample supply of Thy 1.1 mice.

Initial experiments showed that transfer of donor cells required intravenous administration and that intraperitoneal delivery did not yield satisfactory results. We attempted IP delivery because our previous work showed that IP delivery of anti-tumor T-cells from donor animals cured peritoneal tumors in host animals. Current work using flow cytometry quantified that the IV route delivered 5 to 18-fold more donor cells to the host spleen than the IP route (Fig. 5-Bergman).

![Fig. 5-Bergman.](image)

Hoechst-labeled spleen cells (5 x 10^7 cells) were transferred IV (top figure) or IP (bottom figure) to donor mice. Mice were sacrificed 24 hours later and spleen harvested, converted to single cell suspensions and analyzed by flow cytometry. There were 3% donor cells following IV transfer (top figure) and 0.2% following IP transfer (bottom figure).

Histo-pathological analysis of liver and spleen tissue (Fig. 6-Basse) similarly showed that cured spleen cells transferred i.p. to host animals inadequately reached the systemic circulation. We now transfer cells exclusively using the IV route.

Early experiments with Hoechst-labeled donor cells showed that neither cells from naïve animals nor donor cells from animals that had been cured of peritoneal tumor with rrVSV therapy were seen in CSF or meninges of host animals bearing CM tumor. These findings are consistent with our initial hypothesis that anti-tumor memory T-cells would not find tumor in the brain unless there was an inflammatory reaction to bring them to the
In a handful of animals, we have not seen Hoechst-labeled donor cells in meningeal tumors following infection of the tumor with pseudotype VSV. However, these Hoechst-labeled cells may not traffic to the brain because they cannot be stimulated in local lymph nodes to become activated and divide. Much deeper explorations of these results are now being carried forward using congenic Thy 1.1/1.2 mice. Using the congenic system, we have been able to demonstrate homing of CD8+ donor T cells to brain tumors (as shown in Fig. 6E-Basse). However, the number is low, and homing of effector cells to these intracranial tumors must be improved. As outlined in the SOW’s Tasks 3+4, we believe this can be achieved by injection of cytokine transgene-carrying virus and/or NK cells. In addition, we plan to try replicating virus as
well as pseudovirus in future work. Use of replicating virus has recently been approved by the institutional animal committee and is being submitted to ACURO.

Experiments using Hoechst and CFSE label were performed in the Bergman lab. All other immunohistochemical stainings and analyses needed to accomplish this and subsequent tasks were performed in the Basse lab.

2. Establish the ability of A-NK cells to infiltrate brain metastases of Her2/neu positive mammary cancer.
   a. Generate animals with brain and leptomeningeal (LM) metastases using the D2F2/E2 mammary cancer cell line
      i. Months 1-24
      ii. Bergman
      iii. 60 mice
      iv. All host mice are Balb/c
   b. Generate A-NK cells: Adenovirally transduced to express rmIL-2 and rmIL-12
      i. Months 1-6
      ii. Basse
      iii. 60 mice (same as 2. a. iii)

As noted above, by improving the method of CM implantation and developing a sub-clone of D2F2/E2 that grows easily in the meninges, we are able to consistently produce an unlimited number of mice with LM metastatic implants.

   To date, we have successfully transduced A-NK cells with two adenoviral vectors simultaneously (IL-2 and IL-12). Preliminary data suggest that even triple-transduction is possible, which would allow us to produce A-NK cells secreting, e.g., CTL-attracting chemokines or mediators of acute inflammation, in addition to IL-2 and IL-12 (Fig. 7-Basse).

![Fig. 7-Basse. Adenoviral transduction of IL-2 activated NK (A-NK) cells with adenoviral vectors containing the genes for IL-2 and IL-12. A-NK cells prepared from Balb/c splenocytes were incubated for 2 hours with adenoviral vectors – at different MOIs - containing the genes for IL-2 (AdIL-2) and IL-12 (AdIL-12). While AdIL-2 transduction at MOIs up to 20 provided growth/survival advantage, higher MOIs were inhibitory/toxic. While AdIL-12 at MOIs above 50 were clearly inhibitory, lower MOIs of AdIL-12 provided a synergistic effect when combined with AdIL-2. The optimal growth/survival of the A-NK cells were seen when an MOI of 5 of both vectors were used.](image-url)
c. Animal studies: Inject A-NK cells intravenously (IV) and via cisterna magna (CM)
   i. Months 1-6
   ii. Basse

A-NK cells have been produced from Thy1.2+ Balb/c splenocytes and we have confirmed that they are cytotoxic against tumor cells, which are resistant to non-activated NK cells. We have injected A-NK cells by the i.v. route into congenic, Thy1.1+ Balb/c mice with tumors in the brain and in the peritoneal cavity (Task 2a). So far, all the A-NK cells used for transfer have been non-transfected and have therefore been supported by intraperitoneal (IP) injections of Peg-IL-2. To avoid development of severe toxicity (vascular leakage and hypotension), the IL-2 treatment was limited to two injections per day for 3 days.

d. Analysis of tissue: Distribution of A-NK cells
   i. Months 1-9
   ii. Basse

We have determined the tissue distribution of intravenous (IV) injected A-NK cells in

![Fig. 8-Basse: Adoptively transferred and host NK cells in D2F2/E2 brain tumors.](image)

Animals with 9 day old D2F2/E2 brain tumors received 5 million Thy1.2+ A-NK cells via the IV route. Three days later, brains were removed and processed for immune-histology. A) Sections were stained with Alexa-488 (greenish) anti-NKp46 antibody to reveal NK cells and with PE (red/purple)-anti-Thy1.1 to reveal endogenous lymphocytes of T and NK cell origin. Several NKp46+Thy1.1neg A-NK cells are seen (at purple arrows), but also a substantial number of activated NKp46+Thy1.1+ NK cells of host origin (at white arrows). Red-only cells represent host T cells. Original magnification; 200X. B) Brain sections from animals, which did not receive exogenous A-NK cells, revealed that endogenous NK cells also infiltrate the brain tumors (cells stained green). However, almost all of the tumor-infiltrating host NKp46+ cells were negative or only very weakly positive for the Thy1.1 marker, indicating that they are of a non-activated phenotype. The red/purple-only cells are host T-cells. Original magnification; 400X.
tumor-bearing animals using the Thy1.1/1.2 system. Thus, organs were removed at 3 and at 5 days after injection of the A-NK cells. Representative tissue sections or various organs were stained with antibodies against the congenic markers (host=Thy1.1 and donor cells = Thy1.2) and against NKp46 (a marker of NK cells). Thy1 is a marker of all T cells as well as activated NK cells. Very few A-NK cells were found in any tissues at 5 days after injection. This was expected, since the A-NK cells in these animals were without IL-2 support from day 3 to day 5 after injection, resulting in their death by apoptosis. However, at 3 days after injection, NKp46+/Thy1.2+ (not shown)/Thy1.1 negative cells – i.e., NK cells of donor origin, were found in tumor (Fig. 8A-Basse), liver and spleen (not shown). Somewhat surprisingly, a substantial number of NK cells of host origin were found in tumors from non-treated control mice (Figure 8B-Basse). However, in contrast to the host NK cells found in tumors of mice receiving A-NK cells (Figure 8A-Basse), the NK cells in the non-treated animals did not co-express NKp46 and Thy1.1, i.e., they did not appear to have an activated phenotype. Whether the host NK cells found in tumors from the A-NK cell treated animals are activated by IFNγ secreted by the donor A-NK cells or by the exogenous IL-2 or both, is being investigated.

We are now repeating these experiments with AdIL-2+Ad IL-12 transduced A-NK cells which are injected without support by exogenous IL-2. We expect that these A-NK cells can support themselves with sufficient IL-2/IL-12 to survive in the tumors and we predict, according to our hypothesis, that they will secrete sufficient amounts of these cytokines to also support infiltrating anti-tumor CTLs and possibly infiltrating host NK cells.

3. Determine that pseudotype VSV infection and A-NK cell infiltration of small brain and LM tumor nodules delivers tumor antigens to CSF macrophages and activates anti-tumor memory T-cells (months 6-21)

These experiments are just beginning. Personnel and technical difficulties have been overcome. The work will be completed between months 12-24 instead of 6-21 as planned.

a. Generate donor survivor animals by treatment with rrVSV (same as 1.a)
b. Generate animals with brain and leptomeningeal (LM) metastases using the D2F2/E2 mammary cancer cell line. Transfer T-cells from donor mice (from 3.a)
   i. Months 6-21
   ii. Bergman
   iii. 200 mice
c. Animal studies: Non-replicating VSV injected into tumor bearing animals (from 3b)
   i. Months 6-21
   ii. Bergman
   iii. 100 mice
d. Animal studies: A-NK cells injected into tumor bearing animals (from 3b)
   i. Months 6-21
   ii. Basse
   iii. 100 mice
e. Analysis of CSF: Number of cells and types of cytokines that mediate inflammation in the central nervous system (CNS)
   i. Months 6-21
   ii. Bergman

f. Analysis of tissues: Determine the density and state of activation of donor T-cells and host innate and adaptive immune cells in metastases and normal tissues
   i. Months 6-21
   ii. Basse

4. Establish that activated memory anti-tumor CD4 T-cells in the CSF target an immune response to tumor nodules (months 12-24)

These experiments are also just beginning. All procedures are in place and preliminary work performed or underway. The work will be completed between months 12-24 as planned.

a. Generate donor survivor animals by treatment with rrVSV (same as 1.a)

b. Generate animals with brain and leptomeningeal (LM) metastases using the D2F2/E2 mammary cancer cell line. Transfer T-cells from donor mice (from 4.a)
   i. Months 12-24
   ii. Bergman
   iii. 300 mice

c. Animal studies: Non-replicating VSV injected into tumor bearing animals (from 4b)
   i. Months 12-24
   ii. Bergman
   iii. 100 mice

d. Animal studies: A-NK cells injected into tumor bearing animals (from 4b)
   i. Months 12-24
   ii. Basse
   iii. 100 mice

e. Animal studies: Non-replicating VSV injection followed by A-NK cells injection into tumor bearing animals (from 4b)
   i. Months 12-24
   ii. Basse and Bergman
   iii. 100 mice

f. Analysis of tissues: Determine the density of donor and host immune cells in metastases compared with normal brain
   i. Months 12-24
   ii. Basse

g. Analysis of treatment effects: Survival curves
   i. Months 12-24
   ii. Basse and Bergman
KEY RESEARCH ACCOMPLISHMENTS

- Breast cancer cell line (D2F2/E2) and model established, which easily produce animals with brain/meningeal breast cancer metastases expressing Her2/neu and allows CSF sampling.
- VSV and nat ural killer (NK) cells containing cytokine transgenes successfully produced.
- Demonstrated that animals with extra-cranial D2F2/E2 tumors can be cured by treatment with VSV, cyclophosphamide, and anti-CTLA-4 and that cured animals are protected against intracranial challenge with D2F2/E2.
- Demonstrated, using various cell labels and routes of administration, that adoptively transferred memory T cells and IL-2-activated NK (A-NK) cells, although in low numbers, traffic to D2F2/E2 brain tumors.

REPORTABLE OUTCOMES

1 Development of D2F2/E2-J9, a subclone of D2F2/E2 that consistently implants in the leptomeninges.

CONCLUSIONS

- We believe we have produced a very robust experimental model system for preclinical studies of breast cancer metastases to the brain and the ability of immune effector cells to reach these metastases.
- The fact that animals cured of their primary extra-cranial tumor reject a subsequent intracranial challenge with the same tumor cells, as shown here, indicate that immune effector cells can be active against intracranial breast metastases.
- Although adoptively transferred T effector cells and A-NK cells traffic to intracranial breast metastases, the number of cells reaching these tumors is, as expected, low.
- “So what now”: We are excited about the application of this model to study the mechanisms of effector cell trafficking to breast cancer brain metastases and, very importantly, to test our strategies of augmenting it, as described in the SOW’s Tasks 3+4, to produce a therapeutic response.

REFERENCES

NA

APPENDICIES

NA

NB: Unfortunately, an unexpected protracted illness led to the retirement of the very skilled and experienced technician who was responsible for all of the animal surgery and animal processing for this study. While this initially slowed progress of the project, the PI and another member of the lab trained and assumed control of all of the animal procedures and work resumed at a rapid pace with outstanding quality.