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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*
Brain-metastasizing breast cancer is a major clinical problem. Cell-mediated immuno-therapy is well-suited to attack it, but the efficacy must be increased. We developed a rat model and began to test our proposed therapy. A key step is to inject *gamma interferon* (*IFN-g*) into the brains of rats with metastatic mammary carcinoma. During the period of this report, technical and theoretical problems were identified and addressed. ***A)*** Technical: the positive controls for the IFN-g injections did not give the expected results. This was puzzling and unprecedented. We systematically re-evaluated each step in the injection and assay methods, and the training of all lab members. Problems with the assay, anesthesia apparatus, and micro-injection pump were identified and corrected. ***B)*** Theoretical: We reviewed the conflicting literature on effects of IFN-g in *central nervous system* (*CNS*) immunity, as defined in other (non-tumor) contexts. Our analysis supports our plan to inject IFN-g directly into the brain, rather than deliver it by other routes. ***C)*** We reviewed recent advances and remaining concerns in small animal models. In all, this establishes a more secure technical and theoretical foundation for the final year.

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Table of Contents

INTRODUCTION

Brain-metastasizing breast cancer is a major clinical problem. Cell-mediated immunotherapy is well-suited to attack it, but the efficacy must be increased. We developed a rat model and began to test our proposed therapy. A key step is to inject *gamma interferon* (*IFN-g*) into the brains of rats with metastatic mammary carcinoma. During the period of this report, technical and theoretical problems were identified and addressed. ***A)*** Technical: the positive controls for the IFN-g injections did not give the expected results. This was puzzling and unprecedented. We systematically re-evaluated each step in the injection and assay methods, and the training of all lab members. Problems with the assay, anesthesia apparatus, and micro-injection pump were identified and corrected. ***B)*** Theoretical: We reviewed the conflicting literature on effects of IFN-g in *central nervous system* (*CNS*) immunity, as defined in other (non-tumor) contexts. Our analysis supports our plan to inject IFN-g directly into the brain, rather than deliver it by other routes. ***C)*** We reviewed recent advances and remaining concerns in small animal models. In all, this establishes a more secure technical and theoretical foundation for the final year.

BODY OF REPORT.

This report covers the period from the last report (November 2001) until August 2002. **Tasks 1, 2, 3** were carried out as described in the first progress report.

The work in this report begins with our analysis of the controls from an experiment of **Task 3**. The relevant text is summarized from the last report:

*"**Task 3. Use the model to study T cell surveillance.***

***Exp. 2** Methods. Rats received 0.5 x 10^6 MATB/lacZ cells in the carotid artery, using the methods of Task 1.*

The rats were divided into 3 groups. Group 1 received 1000 U IFN-g, injected stereotactically into the striatum, 5 day after tumor injection. Group 2 received vehicle instead of IFN-g. Group 3 received no treatment.

Rats were sacrificed at the first signs of illness, as described above. Sites of metastatic tumor away from the ventricle were measured as described above.

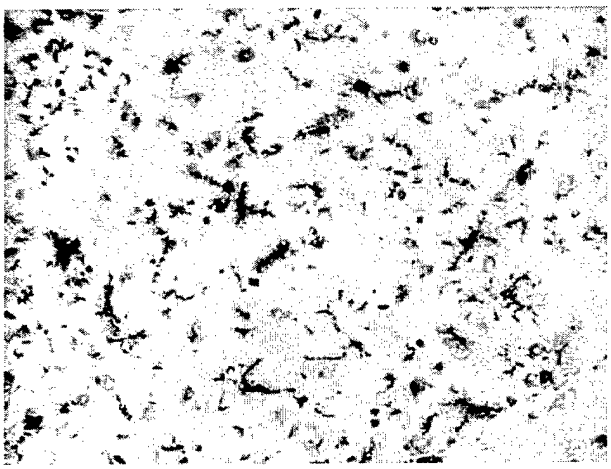
... We are now continuing the analysis: ... Taken together, this will define how a needle wound alone, and how injection of IFN-g alone, affect the pattern of metastases, when the IFN-g is injected 5 days after tumor has been placed in the blood."

As indicated above, our first analysis of these experiments focused on the behavior of the tumor. An unexpected and puzzling finding was obtained when we did assays that were intended to confirm the activity of the IFN-g.

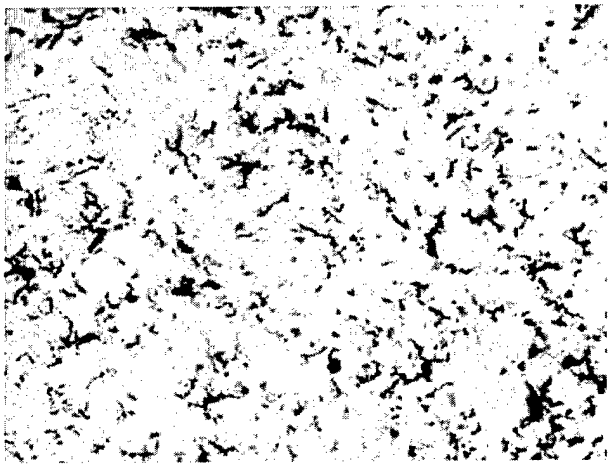
Following our usual methods, slides were stained with monoclonal antibody OX6 to reveal activated parenchymal microglia with strong class II *major histocompatibility complex* (*MHC*) upregulation. (Little OX6 staining is seen in microglia of control rats.) To our great surprise, little evidence of class II MHC upregulation was seen (fig. 1). The reasons for our surprise, and the way we approached the problem, are given below.

A. A technical problem and how we addressed it. Our proposed therapy involves intracerebral injection of IFN-g to activate microglia in the brain. This is a common, widely-accepted way of activating microglia. It has worked consistently in our laboratory since 1990 (Sethna 91, Phillips 99, McCluskey 01). It has worked in the presence of many different CNS tumors (Wen 92, Dutta 98). Indeed, the PI is internationally acknowledged for her expertise in the effects of intracerebral IFN-g in normal and tumor-bearing brains (Lampson 95, 02, 03).

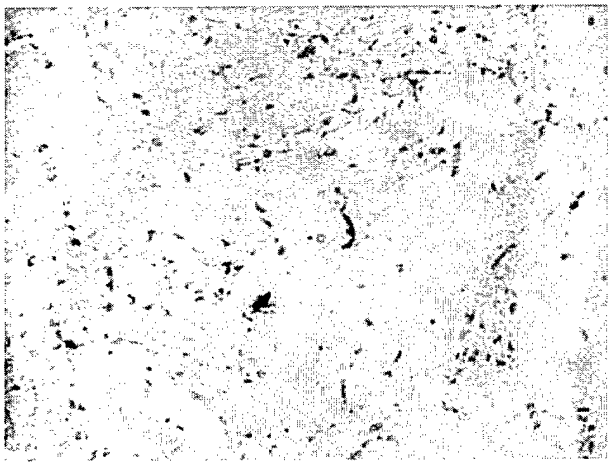
- Normally, few class II MHC+ activated microglia are seen in control buffer-injected brains away from the needle wound (Lampson 95, 02, 03, Sethna 91, Phillips 99, McCluskey 01).
- Normally, 2-5 days after intracerebral injection of a sufficient dose of IFN-g, activated parenchymal microglia (with a characteristic morphology) with strong class II MHC expression are seen over a wide area (Lampson 95, 02, 03, Sethna 91, Phillips 99, McCluskey 01).



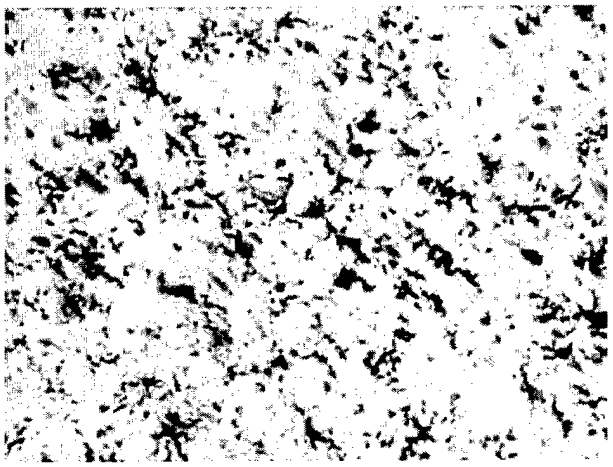
A. Positive control. Assay of tissue from a previous experiment in which 800 U IFN-g had been injected stereotactically into the rat striatum. Class II MHC+ activated microglia appear black after staining with monoclonal antibody OX6 in the ABC assay with DAB substrate. *Photographed at 200 X.*



B. Microglial activation by an independent method. EAE was induced in LEW rats and brain sections were stained to reveal activated microglia as in **A**. This confirms we can obtain and detect microglial activation by an alternative method. *Antibody staining and magnification same as in A.*



C. The problem. In recent experiments, few class II MHC+ activated microglia were detected after intracerebral injection of ≥10,000 U IFN-g. *Antibody staining and magnification same as in A.*



D. Identifying the variables. After intracerebral injection of 5,000 U IFN-g into 4 rats, eliminating use of oil (see text), the first rat showed class II+ activated microglia, as shown. The other 3 rats had minimal responses. This focused our attention on finding additional variables in the injection procedure.
Antibody staining and magnification same as in A.

Figure 1. Class II+ activated microglia under different conditions.

Table 1. Identifying reasons that class II+ microglia were not seen after IFN-g injection.

Exp.	IFN-g (n) (or alternative)	IFN active?	After re-assay?	Relevant variable(s)	Conclusion
YK 19	Original IFN-g dose-response.	yes	yes	Earlier exp.	Widespread class II+ microglia after **≥ 100U**.
GP2	300 U IFN-g	yes	yes	Earlier exp.	*Do* see class II+ microglia.
GP3	100 U IFN-g (2)	no	no	Repeat low dose IFN.	Contradicts YK 19 - do *not* detect class II+ microglia.
GP4	(Prelim. EAE; see YZ3 below)	n/a	n/a	(Prelim EAE).	(Prelim for YZ3)
GP5	Dye to ventricle, no pump (4).	n/a	n/a	Confirm co-ords	Did inject into ventricle.
GP6	IFN-g to ventricle, no pump. 10,000 U (2) 5,000 U (1)	no	yes	No pump. No oil.	Sub-optimal assay part of problem. Also suspect oil and/or pump.
GP7	IFN-g to striatum, with pump. 10,000 U, Antigenix (1) 20,000 U, Antigenix (1) 20,000 U, Cell Sciences (1)	no	no	Fresh IFN-g.	Increase suspicion of oil and/or pump.
YZ3	Induce EAE (8)	n/a	n/a	Alternative way to activate microglia.	Successful EAE. Identify slides with cuffs.
YZ3	EAE regions with cuffs.	n/a	n/a	Detect activated microglia ?	Can obtain and detect class II+ activated microglia.
YZ4	IFN-g to striatum. 5,000 U (4).	1st rat – yes. 3 later rats – no.	Same even if longer color dev. (25 min.)	Air bubble instead of oil.	Air bubble may help. Must find sources of variability during injection. Note: Wounds displaced.
YZ5	Inject dye into striatum (3)	n/a	n/a	Air bubble.	Wounds are displaced. Suspect bent needle.
YZ6	Inject buffer into left and right striatum (1).	n/a	n/a	Air bubble. New needle.	Wounds well-localized on each side.
YZ7	IFN-g to left and right striatum 4, 000 U (2)	In progress		Air bubble. New needle.	In progress.

We had shown that the dose-response curve for IFN-g effects can be site-specific (Phillips 99). In preliminary studies for this project, we established dose-response curves for the effect of intra-cerebral IFN-g injected into the site to be used in this project (striatum) in tumor-free brains. We also showed that IFN-g could still be active in the presence of growing tumor. It was totally unexpected that microglia were not activated.

It was possible that, under the particular conditions of the experiment described above, the mammary carcinoma had inhibited the dose of IFN-g injected. Alternatively, it was possible that a one-time technical error had occurred. However, analysis of other rats suggested the problem was more fundamental:

When we injected IFN-g into normal (tumor-free) rats, using a dose (100 U) that had previously been active when we defined the dose-response curve, we again did not see the expected IFN-g activity (Exp. GP 3,Table 1).

Higher doses of IFN-g also failed to show activity, as described below. It became increasingly apparent that were facing a fundamental problem, not limited to the original tumor experiments.

In response, we carried out a systematic, step-by-step evaluation of all of our reagents and procedures. We considered the rats themselves, the source and handling of the IFN-g, the injection methods, and the assay methods.

The activation of microglia by IFN-g is robust (Sethna 91, Phillips 99, McCluskey 01, Lampson 02, 03). It is likely that several problems would have to accumulate before it would fail to be detected. The different potential variables were analyzed in parallel, as described below.

1. Assay methods.

To identify potential problems and personal variations in the assay methods, lab members performed assays in parallel in the presence of the PI. Accumulated mistakes in the final steps were identified:

One key step in the procedure is the timing of the final step, when the color is developing. Our preferred method is to follow the developing stain in parenchymal microglia on control slides under a dissecting microscope, to confirm the correct time for each assay.

It was found that lab members were not performing and interpreting this step correctly and, in fact, the color development was being stopped sooner than necessary. Lab members had settled on a "routine" time of 2 minutes.

When the timing was monitored correctly, it was found that color development can continue for 15 minutes or longer without compromising tissue quality or introducing false positives. Stopping the reaction at 2 min did allow detection of the strongest staining. However, as the staining became weaker, increasing the timing increased the ease and confidence of detection (figs. 2, 3; Table 2).

Compounding the problem, lab members had been using inappropriate tissue as positive controls. These controls were positive even when class II+ activated microglia were not well-stained. This error was explained and corrected.

Brains that had failed to show the expected response to IFN-g were re-assayed, using the corrected assay conditions. Tissue from control brains (from earlier successful experiments) *did* show microglial activation and strong OX6 activity, as expected (fig. 1A).

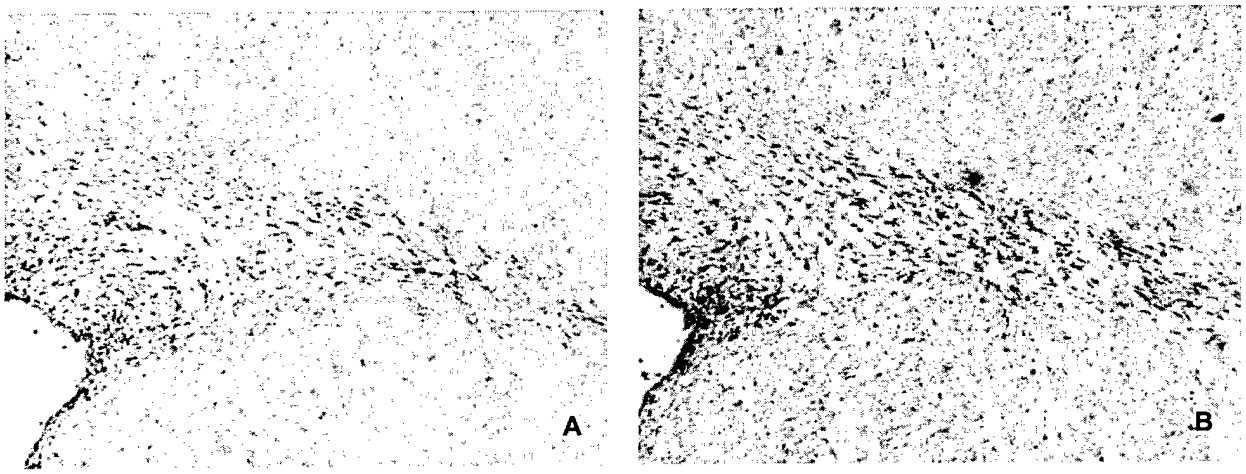
The corrected assay did reveal additional IFN-g activity in one recent experiment (Exp. GP 6,Table 1).

However, the failed positive controls from other recent experiments still did not show the expected activation of parenchymal microglia (Table 1). Thus, other factors (in addition to the assay) had contributed to the problem (below).

Also as part of this process, accumulated modifications and alternative procedures were re-evaluated, in internally controlled assays. No further problems were identified (last rows, Table 2).

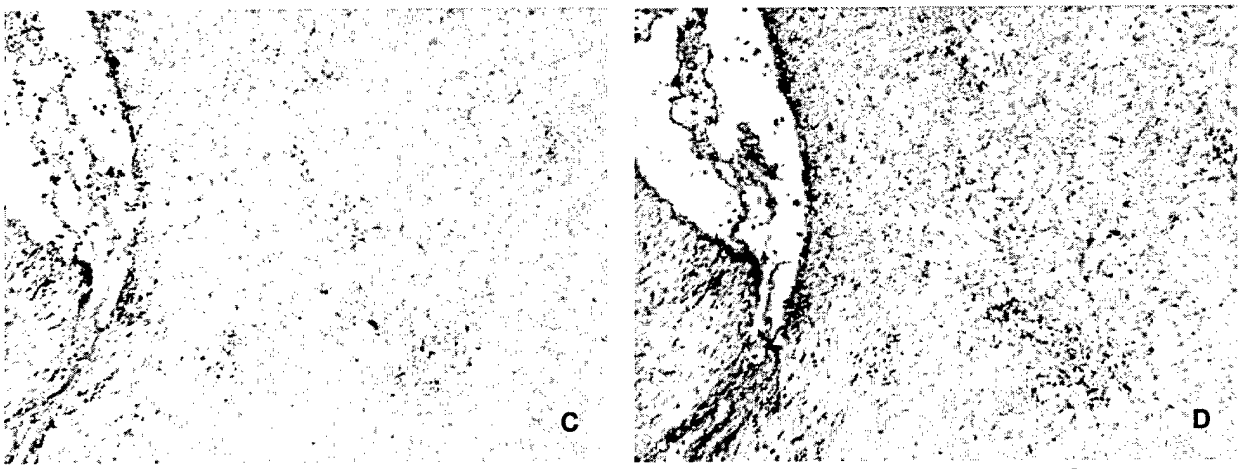
Conclusions. Sub-optimal assay conditions were a factor in our inability to detect microglial activation after IFN-g injection. However, re-assay of recent experiments showed that this was not the only problem.

Color developed for 2 min **Color developed for 15 min**



A. Corpus callosum.

B. Adjacent slide, same location as in **A**.



C. Same slide as **A**, different site (basal ganglia).

D. Same slide as **B**, same site as in **C**.

Figure 2. Effect of incubation time on detection of class II MHC+ activated microglia. Figure illustrates how a sub-optimal incubation time can impede detection of class II MHC+ activated microglia, particularly outside the region of highest activity. All sections taken from a rat that had received 10,000 U IFN-g in the lateral ventricle (*without* use of micro-injection pump) and sacrificed 5 days later. To reveal class II+ activated microglia, 6u frozen sections were stained with monoclonal antibody OX6 in the ABC method with DAB substrate. The timing of the final step, in which the color is allowed to develop, was tested. Within the same assay, sections on the left were incubated for 2 min; sections on the right were incubated for 15 min. Note that the longer incubation time makes staining stand out more clearly, and does not sacrifice tissue quality. In regions of strongest staining (**A**, **B**), class II+ cells are seen even with the short incubation time. The longer time is especially important where the stain is less intense (**C**, **D**). In each panel, the lateral ventricle (where the IFN-g had been injected) is on the left. *All panels photographed at 40x (low power view).* At higher power, stained cells were confirmed to be microglia by their characteristic morphology (please see fig. 3).

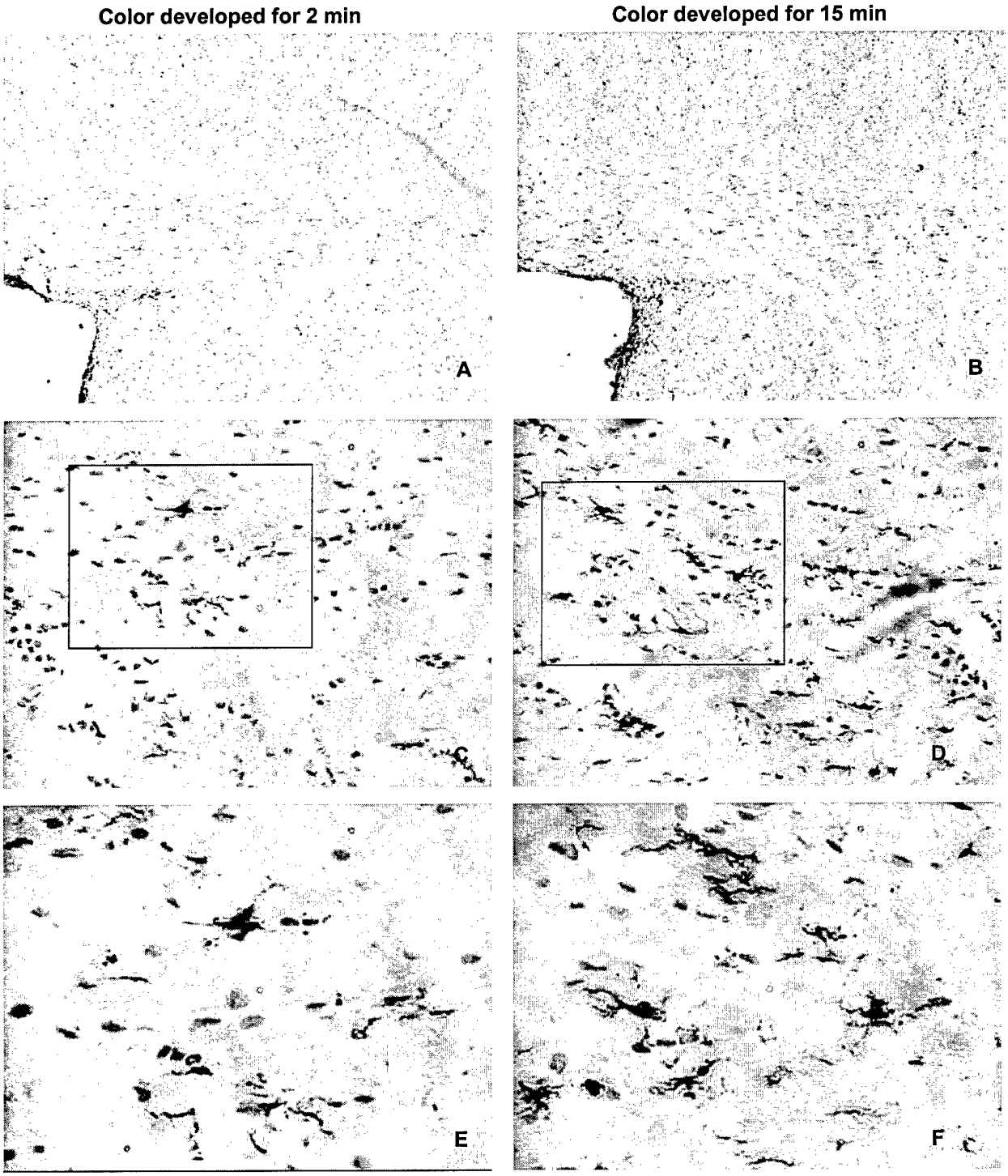


Figure 3. Morphology of class II+ cells. Figure shows staining of class II+ activated microglia in a second rat treated as in fig. 2. Staining of the same areas in adjacent sections, when color was developed for 2 min (*left*) or 15 min (*right*), is shown. **A**, **B**: low power (photographed at 40x). At higher power, stained cells have characteristic morphology of activated microglia. **C**, **D**: 200x. **E**, **F**: 400x.

Table 2. Testing assay conditions.

Assay	Tissue from exp. (see Table 1)	Relevant variable(s)	Conclusion
SK 1	GP 7	Time for color development: 2 - 20 min.	Longer time gives more stain; bk *not* increased (see figs 2-3). IFN-g not active; stained cells mainly at wound.
SK 2	GP 2	Re-test timing.	Confirms results above (figs 2-3).
SK 3	GP 3	Re-stain exp. that did not show IFN activity.	IFN-g still not active.
SK 4	GP 6	Re-stain exp. that did not show IFN activity.	Do detect more class II +activated microglia.
SK 5	YZ 4	Initial assay.	¼ rats has class II+ microglia.
SK 6	YZ 3	Look for cuffs in EAE tissue.	Do detect cuffs, confirming EAE.
SK 7	YZ 4	Look for class II+ microglia near cuffs.	Do detect class II+ microglia. Confirms we can activate microglia and detect them.
SK 8	YZ 4	Re-stain SK 5; develop color even longer, 25 min.	Results of SK 5 not changed.
SK 9	YK 19	Re-stain tissue from earlier exp.	Confirm assay now correct.
SK 10	GP 6	Re-evaluate: buffers, use of imidazole	Confirm assay now correct. Imidazole can intensify stain.
SK 11	YZ 3	Re-evaluate: use of detergent	Confirm detergent makes stain stronger, clearer.
SK 12	YZ 3	Fine-tune time to develop color. Compare 3, 4, 5, 7, 10, 15 min.	7-10 min optimal. Should confirm for each assay.

2. Independent positive control. To determine why we could not detect microglial activation after IFN-g injection, we needed an independent way of activating microglia.

One of the most common ways of activating microglia is to induce *experimental allergic encephalitis*, *EAE*. Among many alternative procedures in use, we sought a simple method with readily available reagents. We reviewed the methods in current use and consulted colleagues. We selected use of commercial guinea pig *myelin basic protein, (MBP)* in *Complete Freund's Adjuvant (CFA)* in Lewis (*LEW*) rats, following the methods of Swanborg (88, 01).

We made the necessary amendment to our animal protocol, and waited until formal permission was received.

We succeeded in inducing EAE in Lewis rats, as shown by the expected clinical signs (limp tail, hindlimb paralysis) (Exp YZ3, Table 1).

During the time they showed clinical signs, rats were sacrificed and frozen sections through the brain and spinal cord were stained with hematoxylin to reveal perivascular cuffs of inflammatory cells, the expected histological change in EAE. Cuffs were seen as expected.

Staining with appropriate monoclonal antibodies confirmed that T cells (R73+) and monocytes (OX6+) were present and could be detected in and near the cuffs, as expected.

In the same tissue, we *did* detect activated microglia with class II MHC upregulation, as revealed by strong OX6 staining and characteristic morphology (fig. 1B).

Conclusion. This confirmed that, in our current animal facility, using our current methods of tissue preparation and our current assay methods, we were able to obtain and detect activation of microglia, including class II MHC upregulation, as revealed by OX6 staining. It also provided fresh tissue for positive controls.

3. Injection methods. In our proposed therapy, IFN-g is injected stereotactically into the brain. Following the general approach above, we reviewed each step in the injection procedure. We paid particular attention to personal variations in performing the methods. The points considered are listed below.

a. Handling the IFN-g. For reproducibility, we keep the IFN-g cold, in an ice-water bath, until the moment of injection. Only one dose is loaded into the injection syringe at a time. The rationale and detailed methods were reviewed. Lab members did understand this step; it was not likely to be the underlying problem.

b. Larger volume of IFN-g.

Because a larger volume can be given into the ventricle than into the brain proper, we decided to inject higher doses of IFN-g into the lateral ventricle. We also decided to simplify the procedure by injecting by hand, rather than using the micro-injection pump. We confirmed we could inject into the ventricle by hand (GP 5, Table 1).

Rats were given IFN-g into the left lateral ventricle (GP 6, Table 1). In the initial assay, the IFN-g showed minimal activity. When we re-analyzed the tissue using the corrected assay conditions (above), class II+ activated microglia were seen in the two rats that received the highest dose (10,000 U) (figs. 2 and 3).

Conclusion. Having seen some activity with higher IFN-g doses in the ventricle, we next wanted to inject higher doses into the striatum, the site to be used for our planned therapy. To do this, we needed to purchase a more concentrated preparation (below).

c. More concentrated IFN-g.

We had been using frozen aliquots of a stock preparation of IFN-g. We wished to test fresh IFN-g, and to obtain a more concentrated preparation.

We reviewed details of the recombinant rat IFN-g currently provided by different vendors and purchased fresh IFN-g from two vendors for comparison (Antigenix and Cell Sciences).

We injected high doses ($\geq$10,000 U) of new IFN-g from each vendor into the caudate of separate rats (GP 7, Table 1), again paying attention to the temperature control (above). Under these conditions, we had consistently activated microglia over a wide area in earlier work (Sethna 91, McCluskey 99).

In the initial assay, we did not detect class II+ microglia. In this case, repeating the assay under optimal conditions did not change the result.

Conclusion. It was unlikely that such high doses of IFN-g, from two different vendors, would be inactive. This study, plus the previous one, focused attention on the injection method. The factors we considered are described below.

d. Use of oil in the micro-pump. We typically inject IFN-g in 2 ul volumes. To achieve the desired slow injection rate (1 ul / min) reproducibly, we use a syringe micro-pump (Stoelting). A piece of plastic tubing is used to connect the syringe on the micro-pump to the injection needle.

It would be too expensive to fill the plastic tubing with IFN-g. Instead, our method has been to fill the tube with buffer, then draw up oil as a separator, then draw up the 2 ul IFN-g to be injected (Phillips 99).

As we made inquiries, many questions about the oil arose. Most troubling, we found that the oil in the animal room had been replaced without appropriate labeling. It became clear that the particular oil being used was different from what had been used originally. When the original oil ran out, it had been replaced – without notification or proper labeling – with an inexpensive mineral oil, not tested or intended for biological use. The timing appeared to be consistent with the changed oil being a component of the problem.

In making further inquiries, further questions about the use of oil arose. For example, there is concern that the oil could form micelles with the aqueous IFN-g solution, and the likelihood of this happening could vary from oil to oil, and also from person to person.

We consulted colleagues and the literature, and decided to use an air bubble as a separator instead of the oil. A new post-doctoral fellow (Yang Zhang, Ph.D.) had extensive experience with this method.

We ordered new syringes, tubing, and needles, to eliminate residual oil as a variable.

Four rats were given injections of the new IFN-g, using an air bubble as a separator (instead of oil) (Exp. YZ 4, Table 1). The first rat injected did show class II MHC+ activated microglia (fig. 1D).

The three rats injected after the first rat did *not* show activated class II+ microglia away from the needle wound. Although we had used the corrected assay conditions, we asked if increasing the time of color development even further would give more staining in the three negative rats. However, the result did not change (Assay SK 8, Table 2).

This focused our attention on identifying additional variables that might be introduced during the injection procedure (see below).

Conclusion. Using an air bubble (instead of oil) in the delivery tube may help to solve the problem, but other problems remained.

e. Control of the micro-pump. We found that some lab members were confused about the correct way of setting the control on the micro-pump. The result of the error would be that the full intended volume would not be injected, and the discrepancy would vary from rat to rat. In a 2 ul injection, this could be an important factor.

Lab members were re-trained as necessary. However, as we reviewed the procedures and estimated the likely % error, we concluded that this was probably not a major factor in our recent experiments.

Conclusion. Incorrect use of the micro-pump was noted and corrected. However, this is probably not the major problem.

f. Injection site. We noted that, in the test rats above (d.), the needle wounds seemed displaced. For further analysis, we injected a marker (carbon black) into the brains of fresh rats (Exp. YZ 5, Table 1). We confirmed that the location of the needle wound was not as predictable or reproducible as in our previous experience. One contributing factor is described below.

g. Anesthesia apparatus. We are currently using gas anesthesia, chosen with the guidance of our veterinary staff.

The gas anesthesia involves use of a nose cone for the rat. We found that, as the apparatus was currently being used, this can cause the following problems: The IFN-g is injected through a needle attached to the arm of the stereotactic apparatus. When the arm is swung into place, it can hit the nose cone. This contact can bend the needle (slightly). Even with a slight bend, the bent needle will not deliver the IFN-g to the intended coordinates. Our needles are custom-made, and re-used. Once the needle is bent, it will affect future experiments.

We ordered new needles, and are re-training lab members to perform this step in a way that avoids contact between the needle and the nose cone.

We also noted that an adjustment should be made in the stereotactic coordinates, to take account of the effect of the nose cone on the position of the head. However, this is probably a minor component of our recent problems.

With a new needle, we injected buffer into the left and right striatum of a normal rat. Sections were cut through the brain, and stained to reveal the needle wound. We confirmed that histological stain (hematoxylin) can reveal the wound well enough; it is not necessary to inject dye. In this case both wounds *were* in the expected location.

Next, IFN-g was injected bi-laterally into the striatum of two (tumor-free) rats, using air as the spacer (instead of oil). The rats have been sacrificed and the tissue is now being prepared for analysis. We will confirm the location of each wound, then use the corrected assay conditions to stain for class II+ activated microglia.

Anticipating that we have probably solved the key technical problems, we are also preparing to grow up our tumor cell line, so that we can resume our tests of tumor therapy.

Conclusion. A bent needle is a likely explanation for the mis-placed injections. This can now be avoided. New needles were purchased. The wound location should be confirmed as soon as the tissue is cut, in every rat. We must also adjust the stereotactic coordinates to take account of the nose cone.

Current status.

1. We have corrected our assay methods, and confirmed that we can now reliably detect activated microglia with upregulated class II MHC expression in the rat brain. Sub-optimal assay conditions had been a component of the problem. However, other factors must also have contributed (below).

2. As an independent way of activating microglia, we adopted a simple method for inducing EAE. We confirmed that we are able to activate microglia in our rats, and able to detect class II MHC+ activated microglia with our current methods for tissue preparation and assay.

3. We have become aware of potential problems in using oil as a spacer for the IFN-g injections, and have stopped using it. We have begun to use an air bubble as an alternative spacer. We have replaced all components that had come into contact with the oil: Hamilton syringes, tubing, and injection needles.

4. We found that our recent stereotactic injections were not delivering material to the expected location, and found one relevant factor: If care is not taken, the nose cone of the gas anesthesia apparatus can come into contact with, and slightly bend, the injection needle. We obtained new needles, adapted our methods to avoid this

problem in the future, and adapted our assay plan to immediately confirm the wound location in each rat.

5. We identified a potential problem in setting the control for the micro-injection pump and re-trained current lab members in the correct procedures.

6. Personnel changes were made. The current team is more dependable, approaches the work more thoughtfully, and has been trained (or re-trained) in the correct procedures.

Current interpretation. The activation of microglia in response to IFN-g is robust. Several problems might have to accumulate before it would fail to be detected. In the PI's judgment, the most likely explanation is that several such factors had accumulated. It is likely that the undocumented change in the oil plus personal differences in handling the oil, a (slightly) bent needle, and sub-optimal staining all contributed to the problem.

Next steps. As stated above, the next step is to repeat injection of IFN-g into (tumor-free) rats, making all of the corrections listed above. This is now in progress.

If, as we anticipate, we can now reliably activate microglia by intracerebral injection of IFN-g (in tumor-free control rats), we will immediately resume our test of tumor therapy.

B. Confirmation of the plan for injecting IFN-g.

Our therapy involves intracerebral injection of IFN-g to enhance immune surveillance. The rationale is based on two well-established effects of IFN-g: ***1)*** It can activate cerebral vessels, making them more permissible to the entry of activated T cells. ***2)*** It can activate microglia and other potential antigen presenting cells (APC) (reviewed in Lampson 02).

We originally chose to inject the IFN-g directly into the brain, rather than into the blood or *cerebrospinal fluid (CSF)*, for two reasons: ***a)*** It minimizes the dose that is needed. ***b)*** It minimizes systemic toxicities.

As we re-evaluated our injection methods (above), in parallel, we re-evaluated the relevant literature. We asked: Is our planned use of IFN-g still justified? Is direct local injection still the method of choice?

We are one of few groups seeking to use local IFN-g to enhance immune activity in the *central nervous system* (*CNS*). However, there is a growing literature on the effects of IFN-g in a complementary context: To *reduce* immune activity in CNS autoimmunity, especially EAE. In a recent invited chapter for a new brain tumor text (Lampson 03), the PI stressed the importance of taking this body work into account.

Provocatively, the effects of IFN-g in autoimmunity have been controversial. Some studies find that IFN-g does enhance immune activity in the CNS. However, other studies find the opposite result.

As we reviewed the literature, it appeared that the route of injection was a key variable. In a variety of laboratories, using a variety of experimental paradigms, a similar conclusion was reached: A local increase of IFN-g, within the CNS parenchyma, *increases* immune activity in the CNS (Table 3). This supports our plan to give local injection of IFN-g into the brain itself.

Table 3. Effect in CNS of local vs more general changes in IFN-g levels.

Location of change in IFN-g level	Effect of increased IFN-g on immune activity in CNS	First author (see below)	Year
local	increases	Simmons	90
local	increases	Corbin	96
local	increases	Horwitz	97
local	increases	Tanuma	97
local	increases	Renno	98
local	increases	Egwuagu	99
i.v.	reduces	Abreu	82
i.v.	reduces	Duong	94
i.p.	reduces	Billiau	88
i.p.	reduces	Voorthuis	90
i.p.	reduces	Willenborg	95
i.p.	reduces	Heremans	96
i.p.	reduces	Espejo	01
CSF	reduces	Voorthuis	90
CSF	reduces	Furlan	01

Table illustrates how effect of altering IFN-g levels can differ, depending on the location in which IFN-g is changed. Table summarizes studies using a variety of experimental models, in which IFN-g levels were changed by injection of IFN-g, antibody to IFN-g, or other agents. *Local* changes in IFN-g levels (within the CNS parenchyma) are compared to changes when IFN-g or other agents are delivered *intravenously* (*i.v.*), *intraperitoneally* (*i.p.*), or into the *cerebrospinal fluid* (*CSF*).

Citations for Table 3.

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In contrast, when IFN-g levels are changed more generally (following *intravenous* (*i.v.*), *intraperitoneal*, (*i.p.*) or *cerebrospinal fluid (CSF*) delivery of IFN-g, anti-IFN-g antibody, or other agents), IFN-g is often found to *reduce* immune activity in the CNS (Table 3). Thus, the literature does not suggest that alternative delivery routes of IFN-g would be preferable for our planned tumor therapy (Table 3).

We suggest that route-dependent differences in IFN-g effects may reflect different functional outcomes of IFN-g exposure for different populations of potential *antigen-presenting cells (*APC), for example, parenchymal CNS microglia vs. blood-borne monocytes. The multiple possible effects of exposing APC to IFN-g are reviewed in Lampson 03. The important point here is that our plan to inject IFN-g directly into the brain, rather than by other routes, is supported by experience in other models.

Conclusion. The literature on IFN-g effects on immune activity in the CNS has been controversial. Our planned therapy for brain metastases of mammary carcinoma is to use IFN-g to enhance immune activity at tumor sites within the brain. Review of the recent (and older) literature supports our plan to inject IFN-g locally, rather than by other routes.

C. Small animal models. We are one of few laboratories to focus on immunotherapy for blood-borne metastases in the CNS. However, immunotherapy for primary brain tumors (glioma, etc.) is a rapidly growing field. The information from the work in primary tumors is relevant to this project in 2 ways: Insights can be obtained about animal models, and about basic principles of tumor immunotherapy.

As a context for reviewing and interpreting this work, the PI has edited a special section of the ***Journal of Neuro-Oncology*** on *Animal models of CNS tumor* (Lampson 01a), and is now co-editing a special issue on *Brain tumor immunotherapy* (in preparation). Relevant points from the PI's introductory essay on tumor models (Lampson 01b) are summarized below.

A general problem in brain tumor therapy has been the failure of clinical trials. One approach has been to develop transgenic models that more accurately mimic known genetic changes in human brain tumors. Several such models are discussed in the special section (Lampson 01a).

Equally important, but given less current emphasis, is to identify problems that can *not* be addressed by genetic manipulation. Specific examples include differences of scale -- both spatial and temporal - between even the most accurate small animal models and

human patients; and species differences – both anatomical and biochemical. In the PI's view, a problem of another kind is the emphasis, by reviewers of grants and papers, on "cures" of small animals, rather than step-by-step understanding.

The PI's essay (Lampson 01b) was intended to stimulate thinking about problems in small animal models that can *not* be solved by genetic manipulation. In the essay, brain tumor therapy was used as the specific context, and implications for brain-metastasizing tumors were noted. As a follow-up, the PI solicited responses from colleagues, to be published together with an introduction by the PI, in an upcoming issue of the same journal (***Journal of Neuro-Oncology***).

Conclusion. It is important to continually ask how even the most favorable results in our rat model might mislead us, and what follow-up questions should be asked. The PI's essay, and the response from colleagues, help identify points that deserve attention.

Schedule for remaining tasks. Our original schedule was conservative, to allow for unanticipated problems, which did in fact occur. The time remaining should be sufficient for us to complete Task 3, and perform Tasks 4 and 5. Progress will be aided by the improved methods, and by the more reliable and better-trained team, as described above.

Task 3. Use the model to study short-term effects ("T cell surveillance").

Task 4. In longer term studies, define therapy.

Task 5. In parallel with task 4, study prophylaxis.

Re: Comments on previous report. The PI wishes to acknowledge the thorough review of the last annual report. The specific suggestions were appreciated and followed:

Formal/editorial issues: As requested, care was taken to identify all journals.

Technical issues: Concern was expressed that we might be presenting only the most favorable data. This report should allay that concern: We report a puzzling and totally unexpected technical problem, and the steps we took to address it. False leads and likely sources of error are both included.

More tables and figures are provided, as requested.

KEY RESEARCH ACCOMPLISHMENTS

- In a routine control assay, we identified what proved to be a fundamental technical problem in the stereotactic injection of IFN-g: Even in (tumor-free) control rats that received high IFN-g doses, activated microglia with strong class II MHC expression could not be detected.
- In response, we reviewed all procedures and reagents and re-trained lab members.
- We identified and corrected problems in the assay methods.
- We confirmed that microglia could be activated and detected by an independent method (induction of EAE).
- We identified and corrected problems in the injection method. The most important changes were using air as a separator (rather than oil), and avoiding and controlling for (slight) bends in the needle.
- In parallel, we reviewed the conflicting reports on IFN-g effects in the CNS. This provided additional support for our plan to inject IFN-g locally, rather than by other routes.
- More generally, we reviewed recent advances in small animal models, stressing remaining problems and concerns.

REPORTABLE OUTCOMES not previously reported (published or in press)

Lampson LA, editor. Animal models of CNS tumor. *J Neuro-Oncol* 2001, 53: 275-318. (A special section of the journal, with papers invited and edited by Dr. Lampson.)

Lampson LA. New animal models to probe brain tumor biology, therapy, and immunotherapy: advantages and remaining concerns. *J Neuro-Oncol* 2001, 53: 275-287.

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CONCLUSIONS

Following up on a failed positive control, we reviewed all methods and reagents associated with stereotactic injection of IFN-g, a key component of our planned tumor therapy. We identified and corrected accumulated problems in current lab methods for stereotactic injection of IFN-g and assay of IFN-g activity. In parallel, a review of the current literature supported our plan to inject IFN-g locally, rather than by other routes. More broadly, a review of current small animal models pointed to areas that deserve attention. This re-evaluation and re-training lays a stronger foundation for the remaining tasks.

Why this is important. Our goal is to exploit the potential of cytokine-enhanced cell-mediated immunotherapy against brain-metastasizing breast cancer, a growing clinical problem for which new approaches are urgently needed. Improved procedures and a better-trained team increase our ability to successfully complete our tasks.

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