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Award Number: DAMD17-00-1-0167

TITLE: Enhanced T Cell Attack of Brain Micro-Metastases

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REPORT DATE: July 2003

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	July 2003	Annual (1 Jul 02-30 Jun 03)

4. TITLE AND SUBTITLE
Enhanced T Cell Attack of Brain Micro-Metastases

5. FUNDING NUMBERS
DAMD17-00-1-0167

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

Brain-metastasizing breast cancer is a major clinical problem. Cell-mediated immunotherapy is well-suited to attack it, but the efficacy must be increased. Our work addresses two basic research problems: ***A)*** The need for appropriate small animal models for blood-borne brain metastases, and ***B)*** the need for greater basic understanding of immune modulation in the brain. In **year 1**, we reported initial characterization of a novel rat model. In **year 2**, we reported identification, analysis, and solutions for a series of technical problems. Now, in **year 3**, we report : ***A)*** Further work in the tumor model, including: *1)* Methods for quantitative analysis and depiction of micro-tumor distribution, *2)* the distribution of blood-borne micro-metastases in untreated control rats, *3)* provocative evidence that a simple needle wound can change the distribution; ***B)*** Further work on our proposed immunotherapy (injection of gamma interferon (IFN-g), including: *1)* New basic findings on immune modulation and delivery routes, *2)* our approach to further probing the functional consequences of attempted immunotherapy; ***C)*** Related efforts, including a comprehensive special issue of the *Journal of Neuro-Oncology* on Brain Tumor Immunotherapy, co-edited and with an introductory overview by the PI.

14. SUBJECT TERMS
Rat mammary carcinoma, metastatic breast cancer, brain metastases

15. NUMBER OF PAGES
30

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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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. Our work addresses two basic research problems: ***A)*** The need for appropriate small animal models for blood-borne brain metastases, and ***B)*** the need for greater basic understanding of immune modulation in the brain. In **year 1**, we reported initial characterization of a novel rat model. In **year 2**, we reported identification, analysis, and solutions for a series of technical problems. Now, in **year 3**, we report : ***A)*** Further work in the tumor model, including: ***1)*** Methods for quantitative analysis and depiction of micro-tumor distribution, ***2)*** the distribution of blood-borne micro-metastases in untreated control rats, ***3)*** provocative evidence that a simple needle wound can change the distribution; ***B)*** Further work on our proposed immunotherapy (injection of gamma interferon (IFN-g), including: ***1)*** New basic findings on immune modulation and delivery routes, ***2)*** our approach to further probing the functional consequences of attempted immunotherapy; ***C)*** Related efforts, including a comprehensive special issue of the *Journal of Neuro-Oncology* on Brain Tumor Immunotherapy, co-edited and with an introductory overview by the PI.

BODY OF REPORT. In previous years, we established an appropriate tumor model (**Tasks 1 and 2**), and, during the course of **Task 3**, identified and addressed major technical problems. In the period of the current report, we completed validation of the technical improvements, then resumed our remaining tasks, **Tasks 3-5.**

Please note: A no-cost extension has been granted; this is *not* the final report.

Tasks 3-5 (continuing).

In our tumor model, a rat mammary carcinoma cell line (highly metastatic *13762 MAT BIII*), which we had transduced with the lacZ reporter gene (*MATB/lacZ*), is injected stereotactically into the carotid artery of syngeneic CD Fischer (CDF) rats. Blood-borne tumor metastasizes to the brain, by the route that is appropriate for human tumors. Our focus is on earliest, smallest tumor (micro-metastases).

Our original *hypothesis* was that injection of an immune activating drug (*gamma interferon, IFN-g*) into the brain can enhance immune activity against micro-metastases.

The current tasks are:
Task 3. Define short-term effects on immune parameters.
Task 4. In longer term studies, define therapy.
Task 5. In parallel with task 4, study prophylaxis.

Performing these tasks requires quantitative analysis and depiction of the distribution of blood-borne micro-metastases in the brain (**A**. below), and of the functional consequences of IFN-g injection into the brain during ongoing CNS disease (**B**., below). The studies below are thus fundamental to each task.

A. QUANTITATIVE ANALYSIS OF TUMOR DISTRIBUTION.

Overview. Tasks 3-5 define the effect of different manipulations on blood-borne micro-metastases to the brain. Extending our original characterization, we made a more detailed analysis of the tumor distribution, especially of parenchymal micro-metastases. Technically, methods for quantitative analysis, and for depiction of the tumor patterns, were selected and adapted, and lab members became experienced with each method. Conceptually, we found that a simple needle wound could change the pattern. This finding, the work leading up to it, and the implications, are described below.

1. Quantitative analysis of time 0 distribution, after intra-carotid injection.

a. Time 0 distribution of inert microspheres. To eliminate biological variables (cell death, adhesion, etc.), inert polystyrene microspheres were used to define the initial distribution after intracarotid injection.

Methods. Each rat received a bolus 100 ul injection of 100,000 polystyrene microspheres in PBS, either 10u diameter or 50u diameter in different rats, to the carotid artery. The brain was harvested immediately, cryoprotected, and snap frozen, according to our usual methods (Lampson 92, 93).

To define the distribution of the microspheres, 20u cryostat sections, taken at regular intervals through the brain, were lightly stained with hematoxylin. The smooth, dark spheres are clearly visible, without special staining (fig. 1, APPENDIX).

The sites in which microspheres were seen were scored as to whether they were in the brain proper (parenchyma), ventricles, or meninges. Adjacent spheres within a single vessel were scored as one "site". (For illustrations, please see fig. 1, APPENDIX).

Results.

1) General pattern. In each rat, microspheres were concentrated on the side of the intra-carotid injection, where they were widely distributed.

At higher magnification, the microspheres were confirmed to be within vessels. They appeared in parenchymal and meningeal vessels and, in the ventricles, they were associated with the vessels of the choroid plexus.

The distribution between parenchyma, ventricles, and meninges was generally in proportion to the number of vessels at each site, with most microspheres seen in the parenchyma (Table 1, APPENDIX).

The distribution of the microspheres was consistent with the known blood flow pattern of the rat.

2) Details for individual rats. Rats #1-3 (Table 1, APPENDIX) received 10u diameter microspheres, chosen because they are similar in size to individual MATB/lacZ tumor cells (8 u diameter). Rat #4 received one million 10u microspheres (instead of 100,000). This rat showed a similar distribution, although there were many more sites in total.

Larger microspheres, 50u diameter, which would correspond to a small tumor embolus, were tested in two rats (#5 and 6). In the first rat (#5), most microspheres were again trapped in the cerebral vessels (71%), and relatively few were again seen in the choroid plexus (3%), but a relatively higher proportion was trapped in the meninges (26%) than for the smaller diameter spheres (rats #1-3). The second rat (#6) showed a comparable pattern, although counts were not made.

Conclusions.

- Taken together, this data confirmed that our intracarotid injection method did deliver tumor-sized objects to the brain parenchyma, which is the focus of our project.
- We note that material is also delivered to the meninges, and this is equally important as a site of brain metastasis for human breast cancer patients. Therefore, In future studies, we can evaluate effects on both parenchymal and meningeal tumor.

b. Time 0 distribution of tumor.

Methods / results. To compare the initial distribution of tumors cells to what had been defined for the microspheres, rats received a bolus intracarotid injection of 10 million MATB/lacZ tumor cells in PBS, using the same methods as for the microspheres.

The rats survived the procedure (which had not been possible when microspheres were injected), and were sacrificed at different times within the first 20 hours. This interval was chosen to allow time for adhesion of the cells to cerebral vessels, according to the literature.

To reveal b-gal+ tumor cells, the optimal procedure is to perfuse the rats with fixative (2% glutaraldehyde) at the time of sacrifice. However, to avoid washing cells out of vessels, this step was omitted. Instead, the brains were frozen immediately, as described above for the microspheres.

When sections were stained histochemically for b-gal+ tumor cells, staining was relatively weak, and the cells did not appear well-preserved, consistent with the fact that this was not the optimal fixation method (as explained above). In later studies, tumor cells from perfused tissue appeared healthy and were darkly stained (below).

The original 13762 tumor cell line is keratin+, as we had confirmed in preliminary studies. Therefore, as a complementary way of identifying the tumor cells, sections from some brains were also stained for keratin, and this method did give clearer staining in the non-perfused tissue.

In all, 7 rats were evaluated for b-gal distribution and 5 of those were also stained for keratin. The distribution of keratin staining was consistent with what had been established for the microspheres (above). The distribution of b-gal staining was also consistent, although less staining overall was seen.

Conclusions.

- Following intracarotid injection, the initial distribution of tumor was similar to that seen with inert microspheres.
- The distribution we observed is consistent with the known blood-flow of the rat.
- Taken together, this data confirms that we are performing the intra-carotid injections correctly, and sets the baseline for the analysis of later times, as described below.

2. Quantitative analysis of tumor distribution 9-12 days after injection.

Methods. To define the pattern of tumor distribution at later times, graded numbers of MATB/lacZ cells (0.5, 1, or 2 x 10^6) were injected into the carotid artery of female CDF rats, following the methods described above.

Rats were checked daily, and sacrificed when they displayed the first signs of illness, which were usually failure to groom and/or aberrant movement. In the dose range used, the rats show these signs of illness after 9-12 days, with a trend towards shorter survival as the cell dose increased, as reported previously.

In this study, our focus was on tumor within the brain itself. Because we were less concerned with tumor that still remained in the vessels, we were able to use optimal fixation conditions. Accordingly, rats were sacrificed by intracardiac perfusion with fixative (2% paraformaldehyde), after which the brains were removed and cryoprotected, according to our usual methods (Lampson 92, 93).

For each brain, 6u cryostat sections taken at regular intervals through the brain were stained histochemically to reveal b-gal+ tumor cells, according to our published methods (Lampson 92, 93). (During the past year, we found it important to re-check the pH of all assay buffers immediately before each assay.)

The distribution of tumor between parenchyma, ventricle, and meninges was defined as described above, and compared to what had been seen at time 0 (**1.**, above).

Results.

By 9-12 days, tumor had often grown within the ventricles and meninges. Often, the lateral ventricle on the injection side was filled with tumor and enlarged; in many cases, the basal cistern was also tumor-filled and enlarged (fig. 2, APPENDIX).

Parenchymal tumor foci, in contrast, were relatively small and did not distort the surrounding brain (fig. 2, APPENDIX). The individual tumor cells could usually be seen to be associated with small vessels.

Conclusions.

- At 9-12 days after injection of MATB/lacZ cells into the carotid artery, tumor was seen in the parenchyma, ventricles and meninges.
- In the brain parenchyma, only small micro-tumor foci were seen. Thus, we are able to focus our study on parenchymal micro-tumor, which is the main focus of our project.
- Larger masses of tumor were seen at other sites (ventricles and meninges). This is most likely to reflect regulatory differences in the different environments.
- A more subtle example of local regulatory differences became apparent in our further analysis, as described below.

3. Further quantitative analysis of parenchymal tumor at days 9-12.

Methods. The analysis described in **2.**, above, was continued as described below.

a) Defining the region of interest. The 4 rats that had received the highest cell dose (2 x 10^6 cells) were tested first. Sections at regular intervals through the brain were stained to reveal b-gal+ tumor, according to our usual methods, and the slides were examined by light microscopy.

We noted that the distribution of parenchymal b-gal+ tumor cells was more restricted than had been seen immediately after injection (**1.**, above). Rather than being spread throughout the hemisphere, parenchymal tumor was now concentrated in the thalamus and surrounding grey matter. In the remaining rats, therefore, analysis focused on slides from this region, and a more detailed analysis of the distribution within this region was made:

b) More detailed analysis of the region of interest. For the remaining rats to be analyzed (listed in Table 2, APPENDIX), sections at regular intervals through the region of interest (encompassing thalamus and surrounding grey matter) were stained for b-gal+ tumor, as above.

For this analysis, only tumor cells in the brain parenchyma proper were counted. Cells in the meninges or ventricles and cells at the edge of the ventricle were excluded. Small clusters of closely adjacent cells were counted as one "site".

We first identified the slide with the most parenchymal tumor from each rat. To do this, the slides were scanned at low power (40x) and the number of sites of parenchymal tumor were counted as necessary.

For each rat, the location of the slide with the most parenchymal tumor was then defined with reference to the rat brain atlas of Paxinos and Watson (86).

Results.

For all 12 rats analyzed, the slide with the most tumor was always within a particular region of the brain, corresponding to plates 32-35 in the atlas, with tumor most concentrated in the thalamus. The data is shown in Table 2, APPENDIX, and depicted visually in fig. 3, APPENDIX.

Conclusions. This quantitative analysis confirmed what had been seen by inspection in *a)*, above: 9-12 days after intracarotid injection, parenchymal tumor was most concentrated in the thalamus and surrounding grey matter.

There is high blood flow to this gray matter. This might contribute to the distribution in two ways (which are not mutually exclusive):

1) The *initial distribution* of tumor may be greater in the thalamus than in surrounding regions.

Our analysis of the time 0 distribution had not suggested that the thalamus was favored (**1.**,above). However, at the time of the time 0 analysis, we did not know that this region was of special interest. We must now re-analyze that particular region, to confirm that it is not, in fact, favored at time 0.

2) Alternatively (or in addition), the high blood flow and/or other features of the thalamus and surrounding grey matter may offer a more favorable environment for *survival* of the tumor cell line we are using, as compared to other brain regions.

This explanation is consistent with much other work showing the importance of the tumor micro-environment.

Our own studies of site-specific immune regulation provide an intriguing context for pursuing this explanation. Briefly, we showed that the local neurochemical environment influences local immune control (McCluskey 00, 01).

As we continue Tasks 3-5, we will continue to define how the manipulations we make (a needle wound, injection of IFN-g) may change the favored region of tumor growth and/or immune attack. In fact, our most recent data suggests that a simple needle wound can change the pattern, as described below.

4. Effect of a needle wound on the tumor pattern. The data above is taken from rats that had received an intra-carotid injection of tumor cells with no further manipulation. The effect of a needle wound was studied in parallel, as defined below.

Methods.

a) As part of each experiment, other rats were included that received an intracerebral needle wound. Within each experiment, rats that received the needle wound and control rats (with no wound) received aliquots of the same preparation of tumor cells, and were handled in the same way.

In different experiments, the wound was made at different times before or after the intracarotid injection of tumor cells (day -5, +5, or +7, respectively, where the tumor was given on day 0).

The other methods were as described in **3.**, above. Briefly:

Rats were followed daily and sacrificed when they displayed failure to groom and/or aberrant movement, as described above. Rats receiving an intracerebral needle wound showed these signs at the same time, or at most one day earlier, than the

corresponding controls (without a needle wound). The slight difference may reflect the trauma of the surgery.

To compare parenchymal distributions, sections encompassing the region of interest were stained to reveal b-gal+ tumor, as described above.

For each rat, we identified the slide with the most parenchymal tumor, and identified the corresponding plate in the rat brain atlas, as described above.

b) To continue the analysis, we selected the four rats with the most parenchymal tumor, and counted sites of parenchymal tumor in a set of four slides for each rat. The set included the slide with the most parenchymal tumor (which was re-counted) and slides rostral and caudal to it. For each rat, all sections had been handled in the same way, and stained for b-gal in the same assay.

To plot the data, the location of each slide counted was defined with reference to the rat brain atlas of Paxinos and Watson, as described above. The cell counts were then plotted against the position, where the position is given as the plate number from the atlas (figure 4C, APPENDIX). For comparison, the distribution of parenchymal tumor in three control rats (with no wound), was plotted in the same way (figure 4B). The initial distribution of inert beads was also plotted (figure 4A).

Results.

- The 4 rats that received a wound 5 days *before* tumor (day -5) were similar to the 12 rats that had received no wound (as described above): The slide with the most tumor was in a characteristic position, corresponding to plates 32-35 in the atlas (table 2 and fig. 3, APPENDIX), with tumor most concentrated in the thalamus.
- In contrast, in the 12 rats that received a needle wound 5 or 7 days *after* tumor injection (day +5 or +7), the slide with the most tumor was shifted in the direction of the needle wound, corresponding to plates 22-25 in the atlas. Now, the tumor was most concentrated in the striatum (where the wound had been made), rather than the thalamus (table 2, fig. 3, APPENDIX).
- Among the rats receiving a needle wound on day +5 or +7, one rat did not fit the pattern (marked ^ in Table 2). It is possible that the injection site was missed in this rat.
- Including data from *all* rats (including the one that did not fit the pattern), the difference between experimental groups was highly significant: For the location of the slide with the most parenchymal tumor, the mean value for rats that received no wound (n=12) differed significantly from that seen with rats that received a needle wound on day +5 or +7 (n=12), with $p < 0.0001$ (2-tailed t test).
- In rats receiving the needle wound on days +5 or +7, the distribution of tumor also appeared to be more sharply focused around the wound, as compared to controls (fig. 4, APPENDIX).
- The total amount of tumor was similar for the rats in fig. 4B and 4C. Thus, it appeared that a needle on days +5 or +7 had not increased the amount of parenchymal tumor, but, rather, had changed its distribution.

Conclusions and follow-up.

- The data above suggests that a simple needle wound had changed the distribution of blood-borne parenchymal metastases. Further studies must determine if this reflects a change in the initial site of entry and/or the favored site of growth.
- Our findings are consistent with previous work suggesting that a wound can change the distribution of blood-borne cells in the brain. This has been reported for both metastases and inflammatory cells. However, in most cases, the wound was more damaging than what we have done. Our finding that a simple needle wound (insertion of the needle of a Hamilton syringe) can have this effect is thus of interest.
- Our finding with tumor metastases complements our previous finding that the same kind of simple needle wound can also change the pattern of entry for inflammatory cells (Sloan 92).
- These follow-up questions will now be asked:
- Can a simple needle wound be used to localize blood-borne metastases to *any* pre-selected region of the brain? In particular, is it possible to divert metastases to a site that is relatively accessible to treatment?
- Can blood-borne metastases be localized to a pre-selected region *outside* the brain? In particular, is it possible to divert the metastases away from the brain, to a site that is more accessible to treatment?

Reportable outcome. The work above was presented as an abstract at the most recent annual meeting of the American Association of Neuropathology (AANP) (Please see section on **Reportable Outcomes**.) A full-length manuscript is in preparation. Rupa Kapoor, who participated in this project as a Harvard undergraduate, graduated *summa cum laude*, based on her work.

5. Problems and responses: **A complementary tumor candidate**. In the course of the above studies, inherent limitations in the intra-carotid injection method became important: The time required for each rat limits the size of experimental groups. Also, the method is sufficiently difficult so that more time is required for training than for our other neurosurgical methods, and not all lab members are sufficiently adept.

The PI took advantage of the recent "Era of Hope" meeting (Orlando 2002) to seek a complementary small animal model, one that might simplify study of blood-borne brain metastases.

The most promising lead came from a poster about the mouse mammary carcinoma cell line, 4T1 (Luznik et al. 02). Although this was not the main point of the poster, the poster contained the information that, after a simple subcutaneous injection, tumor could metastasize to the brain. After the meeting, review of the relevant literature supported our interest in this cell line.

The PI requested an aliquot of the cells from Dr. Fred Miller (Wayne State), and is pleased to report that he provided a vial of the cells immediately, with "no strings" attached.

The PI then established a collaboration for the purpose of introducing the lacZ gene into the cells. (Although we have done this ourselves for our other cell lines, we thought

that collaborating with a large biotechnology company would expedite the process. In fact, this has not been the case; it seems we will have to do it ourselves after all.)

Under current regulatory requirements, the necessary steps are to introduce the lacZ gene, establish a stable subclone, send the cells out for contaminant testing (before we can inject them into animals), and amend our animal protocol for use of the new cell line.

Conclusions.

The advantages of our *current* model cell line, MATB/lacZ, are:

- The species is rat, which is more suitable for intra-carotid injection and stereotactic neurosurgery (to inject drugs into different brain sites).
- Blood-borne micro-metastases are seen in the brain parenchyma (our original focus) and also the meninges (also an important site for brain metastasizing breast cancer in human patients).
- We have accumulated considerable data and experience with this model.
- We have much background information about basic immune manipulation in the rat.

The complementary potential advantages of the 4T1 mouse model would be:

- In the MATB/lacZ rat model, the intracarotid injections are difficult and time-consuming, which limits the size of experimental groups. If, in the 4T1 mouse model, blood-borne metastases can indeed be studied after subcutaneous implantation of tumor (as has been reported), this would be much simpler.
- It is desirable to have more than one model, to show the generality of important findings.
- A mouse model would have greater potential for using genetically-manipulated animals in future studies, since there are many more kinds of genetically-modified mice than rats available.
- This mouse model would also complement our rat model in terms of the biology of invasion and immune modulation. In the rat model, where we inject tumor directly into the blood, we can focus on the later steps in invasion and immune modulation. In contrast, in the mouse model, earlier steps in the invasion process (from the primary site to the blood) would come into play. Moreover, there would an opportunity for tumor at the primary site to affect immune parameters.

Taking these considerations together, we will continue to work in the existing rat model (improving efficiency and simplicity as possible), and also move towards development of the complementary mouse model, as described in the text above.

B. IMMUNE MODULATION IN THE BRAIN. Our proposed therapy includes intracerebral injection of IFN-g, in order to enhance the effector phase of the T cell-mediated anti-tumor response. The rationale is based on current understanding of the T cell-mediated response, as reviewed in Lampson 03a, b, 04):

Briefly, T cells must recognize antigen *twice*:

- The response is *initiated* when tumor antigen is presented to naive T cells. This happens most efficiently in organized lymphoid tissue. This step is the goal of current efforts in many laboratories to develop anti-tumor vaccines.
- In the *effector* phase of the response, activated T cells must *re-recognize* their antigen at the tumor site. We are one of few labs to focus on this phase of the response for brain tumor immunotherapy (Dutta 03, Lampson 03a).

We had shown that intracerebral injection of IFN-g can affect two steps that would be important for recall recognition of antigen at a tumor site (Dutta 03, Phillips 99a, b):

- It can enhance the entry of activated, tumor-specific T cells from the blood.
- It can activate mononuclear phagocytes (microglia, monocytes, macrophages), which can serve two functions: They can serve as antigen presenting cells (APC), presenting ingested antigen to the newly entered T cells, and can also attack tumor directly.

At the same time, the functional consequences of "activation" of T cells or phagocytes are now being re-evaluated by many investigators, as described below.

Current ideas. We -- and others -- have become increasingly interested in the possibility that both T cells and phagocytes can be "activated" into different pathways (reviewed in Dutta 03; Lampson 03a, b, 04).

- T cells can indeed be "activated" to attack tumor. Alternatively, they can be "activated" into regulatory pathways which would suppress the anti-tumor response or into ineffective states (anergy, ignorance, lysis).
- Similarly, phagocytes can be "activated" to present antigen and/or to attack tumor directly. Alternatively, they can be "activated" to suppress anti-tumor activity.

Our most recent findings focus our attention on these possible alternatives, as described below.

1. Our most recent findings. Our planned therapy includes intracerebral injection of IFN-g, in order to enhance the effector phase of the T cell-mediated anti-tumor response. Our working model was that IFN-g would increase anti-tumor activity (as described above), and that the delivery route would be an important factor.

The literature about the effect of IFN-g on immune activity in the brain is controversial. Based on our original review of the literature, we formed the hypothesis that the delivery route is a major factor in determining the functional consequences. Our *specific hypothesis* was that direct, intracerebral injection of IFN-g and more generalized delivery (such as into the *cerebrospinal fluid, CSF*) would have opposite effects.

However, our recent work has led us to change our ideas. This work was done in the well-characterized model of CNS autoimmunity, *EAE* (*experimental allergic*

encephalitis). We had introduced this model into the lab during the previous year, when it served as a positive control for our basic injection and assay methods.

The studies done in the past year involved *substance P* (*SP*), which we had shown can enhance the "immune-activating" activity of IFN-g. In particular, SP can enhance the ability of IFN-g to increase T cell entry, and to activate phagocytes (McCluskey 00, 01).

IFN-g is already present in the brain during EAE. We predicted that SP would enhance it's immune-activating effects, and so increase disease severity. However, our findings were quite different. The work, stressing implications for micro-tumor therapy, is described below.

a) Effect of SP.

Methods. EAE was induced by a single injection of myelin basic protein (MBP) in Complete Freund's adjuvant (CFA) according to the methods of Swanborg (88, 01), as reported previously. 8-10 days later (and just before the onset of clinical signs, as determined previously), rats received injection of SP to the CNS. (Each rat received 1 injection.)

Two injection sites were used:

Local injection. In this case, SP was injected stereotactically to the brainstem (nucleus of the solitary tract, NTS), which is involved in the disease, as confirmed in our previous work. Based on our work in disease-free rats, the doses tested were 0.01 and 1 ng, delivered in 2 ul.

CSF injection. In this case, SP was injected into the cerebrospinal fluid (CSF) via the cisterna magna. CSF injection was chosen because it gives a more widespread distribution than local injection. To partially compensate for the dilution effect, a higher dose was used (10 ng).

Results and discussion.

- Contrary to our prediction, injection of SP during ongoing EAE did *not* increase disease severity. In fact, it was *protective* (fig 5, APPENDIX).

  This finding directly connects our work to a topic of growing current interest, relevant to all forms of immunotherapy: The different functional consequences of immune "activation" in vivo. Specific implications for immunotherapy of brain micro-metastases, and how we will follow up, are discussed below.

- SP was protective whether given locally (into the brain parenchyma) or more generally (into the CSF) (fig. 5A and 5B, respectively).

  In response to this finding, we are now re-examining the literature to form an alternative hypothesis to fit both the published data about alternative delivery routes and our own results; appropriate follow-up studies will then be planned.

  In fact, delivery of an immune modulating drug into the CSF, rather than the brain parenchyma proper, has several advantages. The drug can spread more readily through the brain, and the procedure can be less invasive. Therefore, we will compare direct and CSF delivery in our upcoming studies.

b) Effect of IFN-g. Although IFN-g is already present in the brain in EAE, we also asked how additional IFN-g would affect disease severity.

Methods. EAE was induced and IFN-g was injected using the methods described above. IFN-g (20,000 U) was injected into the striatum 8 days after MBP injection (fig. 6, APPENDIX). Our previous work had shown that, in disease-free rats, this dose has a widespread effect on phagocyte activation, and can also affect T cell entry.

Results and discussion.

Under the conditions tested, injection of IFN-g to the striatum did *not* increase disease severity, and there was a suggestion of protection (fig. 6, APPENDIX)

It is possible that injection of IFN-g directly into an involved site (brainstem rather than striatum) or into CSF would show a more pronounced effect.

The important point here is that these findings were contrary to our original working model (in which we expected IFN-g to *exacerbate* EAE), but consistent with what we found with SP (above).

Follow-up.

- These results prepare us for the possibility that -- whether given directly or into the CSF -- injection of IFN-g may not enhance anti-tumor attack in our tumor model, and may even reduce it.
- There is a general functional reciprocity between some cytokines, such as between IFN-g and IL-4 or IL-10. Therefore, if INF-g in fact *reduces* anti-tumor immune activity, it would be logical to ask if a complementary cytokine, such as IL-4 or IL-10, can increase it.
- In any case, it is important to have appropriate tools and controls for analyzing functional consequences of immuno-active drugs in vivo. Our approach is described in **2.**, below.

Implications for brain tumor therapy.

- If, in fact, IFN-g and agents (such as SP) that enhance IFN-g action can *reduce* anti-tumor activity in the brain, this has important implications.

  It may help to explain some clinical disappointments, since it is generally assumed that certain agents, such as IFN-g, will be immune-activating.

  It would stimulate fresh thinking about new trials, for the same reasons.
- In fact, most investigators have focused their attention on the initiation of the anti-tumor response (tumor vaccines), rather than enhancing the effector phase. Our focus on the effect of immune-"activating" agents in the effector phase enables us to make an important contribution.
- There is little background information available about the effect of IFN-g on metastatic tumor in the brain; indeed, there is littler background literature about even baseline immune parameters. Our results illustrate how the well-characterized EAE model can provide a helpful framework for testing basic hypotheses. It also provides useful positive control for methods and reagents, which we exploited in the previous grant year, and will continue to exploit as described in **2.**, below.

2. Tools for further analysis.

Selection of markers and reagents. An on-going aim for our laboratory has been to identify reagents can be used *in tissue sections* to mark the different activation pathways of T cells and phagocytes. This is important because much of the existing work has been done in vitro, which does not take into account the anatomic and pharmacologic complexity of immune regulation in the brain (Lampson 95; McCluskey 00, 01).

We had previously identified monoclonal antibody OX40 as an attractive marker for recently re-activated T cells in the rat.

During the past year, we identified commercially-available reagents that can be used to discriminate, in tissue sections, between the two main regulatory subpopulations of T cells (Th1 and Th2) in the rat. Monoclonal antibodies for characteristic cytokines, IFN-g and IL-4 or IL-10, respectively, were selected to be tested first. (Although these subpopulations and markers are very widely studied, relatively few studies have used perfusion-fixed rat brain tissue sections; this is why our own preliminary analysis is important.) Selection of appropriate reagents for different functional pathways of mononuclear phagocytes is in progress.

For each selected reagent, the steps are to verify the specificity, in our hands, using well-defined reagent controls, and also tissue controls in which the expression is already well-defined. The development of appropriate tissue controls is described below.

Appropriate positive controls. To provide ourselves with appropriate control tissue, we took advantage of the well-characterized EAE model described in **1.**, above.

In the EAE model we are using, the rats follow a stereotyped clinical course. Over a timeframe of several days, inflammation and clinical signs rise from baseline, peak, then return to normal. Thus, tissue at different times can serve as controls for T cells and phagocytes in different regulatory states. Our first goal is to confirm that, in our hands, we are able to detect these in well-preserved tissue sections.

Methods / results (ongoing). EAE was induced according to the methods we established in the previous year. The time course in our hands was defined and found to correspond with the literature. At selected times during the course, the rats were sacrificed, and, brain, brainstem, and different levels of the spinal cord were prepared for preliminary analysis.

We found that the method we were using to take out the spinal cord did not give reproducible segments. We adapted our methods, using better landmarks when we remove and section the cord.

Having prepared the way, EAE was then induced in a larger set of rats, so that rats can be sacrificed in duplicate at relevant times along the clinical course. The tissue will be used as positive controls for commercial reagents chosen to define alternative differentiation pathways for T cells and phagocytes, as explained above.

Importance for brain tumor therapy. Our goal is to evaluate immune parameters at sites of blood-borne metastatic mammary carcinoma. Because there is little information already available, a complementary positive control for the reagents and methods is needed. We are preparing the well-characterized EAE model to serve us in this way.

C. Other relevant activities.

1. The 2002 Era of Hope meeting was very helpful to us, particularly in helping us to identify the complementary 4T1 mouse model (as described in **A.5.**, above).

2. As a reviewer of grants, the PI has been active in encouraging more fair and constructive reviews. Partly in acknowledgment, she was appointed as chair of her NIH study section (NSD-B), and then re-appointed for an extra term.

3. The PI co-edited a comprehensive special issue of the *Journal of Neuro-Oncology* on ***Brain Tumor Immunotherapy*** (Glick RP and Lampson LA, editors, *Journal of Neuro-Oncology*, 2003, in press), editing each manuscript and also providing an overview of the field, ***An Immunologist's Perspective*** (Lampson, *Journal of Neuro-Oncology*, 2003 in press).

4. The PI recently attended and presented work at a small meeting in New York, sponsored by the Cancer Research Institute (CRI) on *Cancer Vaccines 2003 - Cancer & HIV Vaccines: Shared Lessons*.

This meeting brought together some of the most prominent tumor immunologists and viral immunologists, together with basic scientists. Points that became clear are:

- Clinical immunotherapy is not yet successful.
- Ongoing work in basic science can help to explain past disappointments and suggest more successful alternatives.
- The meeting thus validated our own basic science approach, which extends and complements others' in several ways, as described in the text above. Specific examples include:
- Our emphasis on micro-tumor (rather than large tumor masses) and our development of appropriate tumor models.
- Our emphasis on enhancing the effector phase of the anti-tumor response (rather than vaccination).
- Our emphasis on the subtleties of immune regulation within the brain itself (rather than at other sites or in vitro).

RESPONSE TO REVIEWER COMMENTS ON PREVIOUS REPORTS.

The PI is proud to have received the reviewer's commendation on the previous progress report (year 2), as described below.

From the review of previous report, page 3:

Format / editorial issues: "All issues raised by the previous reviewer were thoroughly addressed by the PI in this report."

Specific discrepancies and recommendations: "The PI is commended for presenting such a complex situation in an organized and detailed format."

We have tried to retain these strengths in the present report.

We have also continued to adhere to the suggestions made after the first progress report. As requested: Care was taken to identify all journals. We have reported problems and surprises as fully as favorable data. Several tables and figures are provided.

KEY RESEARCH ACCOMPLISHMENTS

- We continued to develop methods for quantitative analysis and depiction of the distribution of blood-borne metastasis to the brain.
- We showed that, although blood-borne material (tumor or inert beads) has a broad initial distribution, within 9-12 days, parenchymal tumor is confined to a particular region of the brain, the thalamus and surrounding grey matter.
- Taking all our data together, we suggest that the limited distribution of parenchymal tumor is most likely to reflect local regulatory differences. This complements our previous finding of local regulatory differences in immune regulation.
- We showed that a simple needle wound can shift the distribution of parenchymal tumor, so that tumor is now concentrated around the wound. This has important clinical implications.
- Taking advantage of the 2002 Era of Hope meeting, we identified a murine tumor model that may have complementary advantages to our current rat model.
- We continued to exploit the EAE model we had developed in the previous year, as a well-characterized positive control for methods and reagents, and a well-studied framework for testing hypotheses. In particular:
- It has become increasingly important to distinguish between different pathways that immunologically relevant cells (T cells, mononuclear phagocytes) can take after they have become "activated" during immunotherapy. In our own work, agents that were expected to *exacerbate* EAE were, in fact, *protective*. This has important implications for understanding past disappointments in tumor immunotherapy, and for planning new strategies.
- The EAE model is serving as an important positive control for testing methods and reagents, to further probe this complexity in vivo.
- We have also used the EAE model to refine ideas about drug delivery. Based on the literature, we had predicted that local delivery and delivery to the CSF would have opposite functional outcomes. However, we have found that local and CSF delivery need *not* have opposite effects. This is important for tumor immunotherapy because CSF delivery has many practical advantages.
- The PI co-edited a comprehensive special issue of the *Journal of Neuro-Oncology* on ***Brain Tumor Immunotherapy*** (Glick RP and Lampson LA, editors, *Journal of Neuro-Oncology*, 2003, in press), editing each manuscript and also providing an overview of the field, *An Immunologists Perspective* (Lampson, *Journal of Neuro-Oncology*, 2003 in press).

REMAINING TASKS. Please note: We have received a no-cost extension for this project. Therefore, this is *not* the final report.

Tasks 3-5 are **in progress**. The work in the past year is fundamental to each.

Our original *hypothesis* was that injection of an immune activating drug (*gamma interferon, IFN-g*) into the brain can enhance immune activity against micro-metastases. As we continue our Tasks, we will take the past year's results into account, as described below.

Task 3. Define short-term effects on immune parameters.

This remains essential. However, we are now more aware of the possibility that, even if we do see increased entry of "activated" T cells and increased "activation" of mononuclear phagocytes, this will not necessarily increase anti-tumor efficacy; it may even reduce it. Expanding our ability to evaluate the differentiation state of T cells and phagocytes in well-fixed tissue sections (**B.2**, above) will allow a more subtle analysis of the functional potential of these "activated" cells.

Task 4. In longer term studies, define therapy.

This remains essential, but, again, we are now more aware of the possibility that IFN-g treatment may *reduce* rather than *enhance* anti-tumor efficacy. If this is found, an appropriate response would be to ask if agents that generally have the opposite effect (such as IL-4 or IL-10) would be more beneficial.

In addition, because of our results in the past year (**B.1.**, above), we will evaluate CSF delivery as well as local delivery of IFN-g or other agents.

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

Similarly to task 4, if IFN-g is not found to be efficacious, an appropriate response would be to ask if agents such as IL-4 or IL-10 are more beneficial; and we will try CSF delivery as well as local delivery of the agents to be tested.

REPORTABLE OUTCOMES not previously reported (published or in press)

Glick RP and Lampson LA, eds. Brain Tumor Immunotherapy. [Special issue of the journal of Neuro-Oncology]. *J. Neuro-Oncol.* 2003, in press.

Dutta T, Spence A, Lampson LA: Robust ability of IFN-g to upregulate class II MHC antigen expression in tumor-bearing rat brains. *J. Neuro-Oncol.* 2003, in press.

Lampson LA: Brain tumor immunotherapy: An immunologist's view. *J. Neuro-Oncol.* 2003, in press.

Recent abstracts

Lampson LA, Kapoor R, Kondo Y, Durham J, Laying the foundation for T cell-mediated immunotherapy in a rat model of brain micro-metastases. Era of Hope 2002, DOD-BCRP meeting, Orlando.

Kapoor R, Lampson LA, A model for blood-borne parenchymal metastasis of mammary carcinoma: Basic pattern and effect of a needle wound. AANP 2003 Ann. Mtg, *JNEN* 2003, in press.

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Kapoor, R., Blood-borne metastases in a breast cancer model, senior thesis, Department of Biology, Harvard University, 2003. (*Summa cum laude*)

CONCLUSIONS

The past year provided new findings for two key topics.

Regarding metastasis itself, we found that blood-borne metastases in the brain proper (parenchyma) were concentrated in a particular region of the brain. The localization appeared to reflect regulatory differences in tumor growth or survival, rather than a limited initial distribution. This complements our previous finding that immune regulation in the brain is also under local regulatory control.

The pattern of tumor localization could be shifted by a simple needle wound. This complements previous findings that a needle wound can affect the entry of blood-borne inflammatory cells.

These findings raise important questions: Will the factors that underlie the localization of tumor growth and immune function work together, to favor immune attack? Or, rather, will they work in opposition, to hamper immune attack? More speculatively, can the factors that underlie the localization be exploited therapeutically, for example, to divert metastatic tumor to pre-selected sites?

Regarding immunotherapy, in a well-characterized model of CNS immunity (EAE), we found that injection of drugs that were expected to *enhance* immune attack were, in fact, *protective*. Most of the work was done with substance P (SP), which we had shown enhances the immune "activating" effects of IFN-g; and some work was done with IFN-g itself.

This finding ties our work to a growing awareness that immunologically-relevant cells (T cells, mononuclear phagocytes) can be "activated" into different pathways, with different functional outcomes. It may help explain why some immunotherapy trials have been disappointing, and it suggests alternative approaches for the future.

Our tumor model, plus the complementary EAE model we are exploiting to test ideas and reagents, create an ideal environment for pursuing these ideas and their implications for immunotherapy against blood-borne brain metastases.

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APPENDIX

Lois A. Lampson PhD

DAMD17-00-1-0167

Enhanced T cell attack of brain micro-metastases

2 Tables

6 figures

INERT MICROSPHERES

Fig. 1, below, illustrates appearance of microspheres; counts are given in **Table 1**, next page.

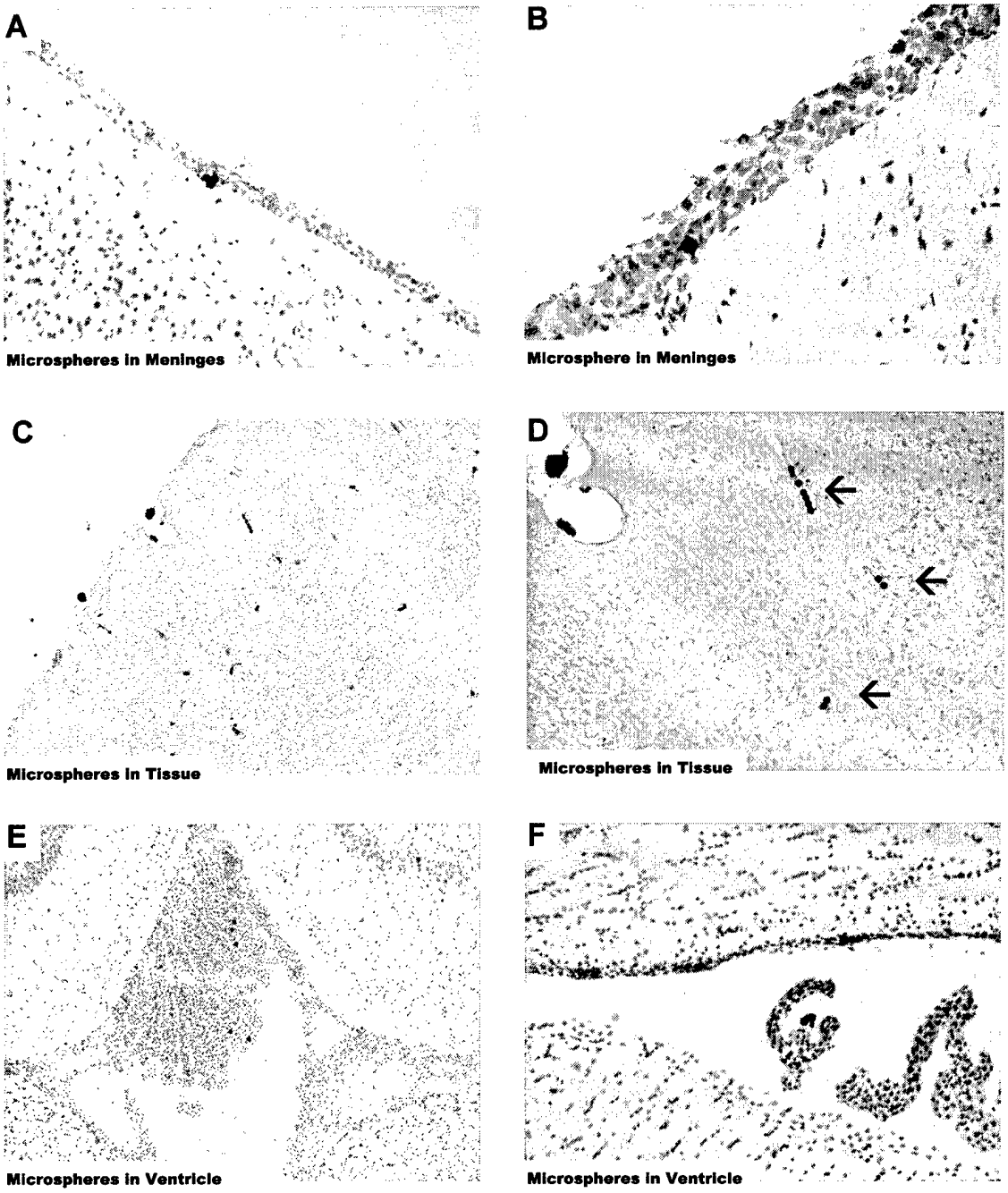


Fig. 1. Inert microspheres, appearance and location. The microspheres appear dark, without further staining. ***A***, low power view of spheres in meninges (center of panel); ***B***, higher power view of sphere in meninges. ***C***, low power view of spheres in meningeal vessels (at edge) and cerebral vessels; ***D***, higher power view. *Arrows* show spheres in cerebral vessels;. each arrow marks one "site." ***E***, low power view of spheres in choroid plexus, within ventricle (center of panel); ***F***, higher power view of spheres in choroid plexus (center of panel).

Table 1. Distribution of Microspheres within the Brain.

Rat	Size of micro-sphere[a]	Total sites counted	% of total sites in: Parenchyma	Ventricle	Meninges
1	10 u	175	88	9	3
2	10 u	390	91	3	6
3	10 u	429	89	8	4
4	10 u	n/a[b]	similar to 1-3, but not counted[b]		
5	50 u	262	71	3	26
6	50 u	n/a[b]	similar to 5, but not counted[b]		

[a] Rats #1-4 received 10u spheres, similar in size to individual tumor cells. Rats #5-6 received larger microspheres (50u), corresponding to a small tumor embolus. Note that the % of meningeal tumor is increased for the larger spheres.

[b] The pattern was confirmed by inspection, but counts were not made.

TUMOR

Fig. 2, below, illustrates appearance and location of tumor 9-12 days after intracarotid injection. Results of quantitative analysis are shown in **Table 2** and **fig. 3**, next page.

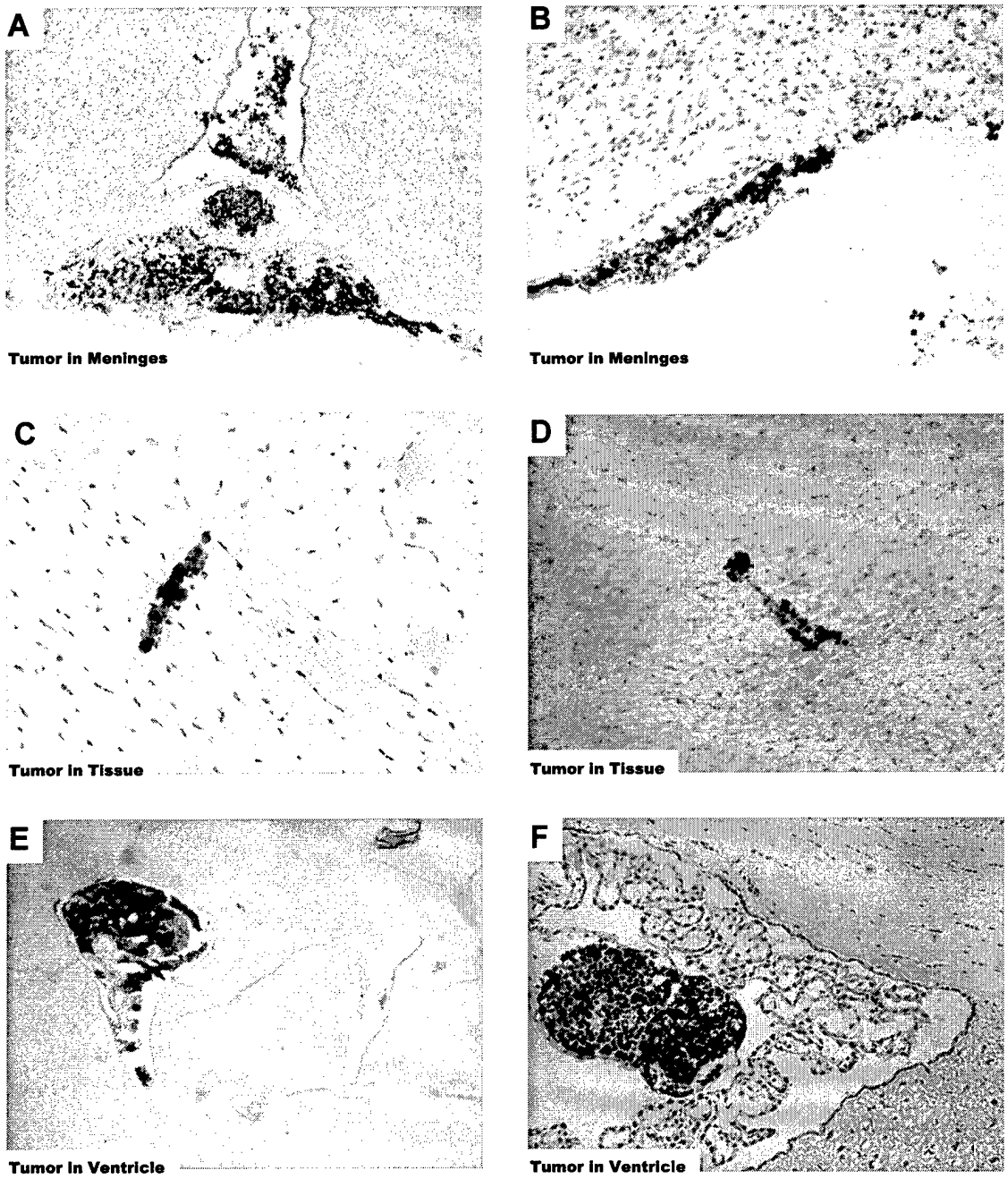


Fig. 2. Tumor. MATB/lacZ mammary carcinoma cells appear dark after staining for the lacZ reporter gene product, b-gal. ***A, B***, tumor growing in ventricle (at edge of panel) and, in ***A***, extending into brain. ***C, D***, Higher power view of tumor growing around vessels in parenchyma (tumor-surrounded vessel in the center of each panel). ***E***, low power overview of tumor growing in the lateral ventricle. Ventricle is enlarged and distorted, as compared to ventricle (clear space) on opposite side. ***F***, Higher power view of tumor growing in lateral ventricle.

DISTRIBUTION OF PARENCHYMAL TUMOR

Table 2. Plate numbers.*

Day of wound		
None	***-5***	***+5 or 7***
n=12	*n=4*	*n=12*
34	34	23
33**	32	22**
32	32	22
35	34	22
34		25
33**		23
33**		23
32		22**
32		32^
33		23**
32		22
32		23**

Fig. 3. Graphic depiction.*

*Table and figure show location of slide with the most parenchymal tumor. For each rat, the slide with the most tumor was identified, and matched to the corresponding plate in the rat brain atlas. The plate number from the atlas is listed in the table, and depicted visually in the figure.

Comparing rats that received *no wound* ("*none*", n = 12) vs rats that received a wound on day +5 or +7 (n = 12), $p < 0.0001$ (2-tailed t test).

** Tumor distribution for these rats is graphed in **fig. 4**, next page.

^ This was the only rat that did not follow the pattern. (For discussion, see text.)

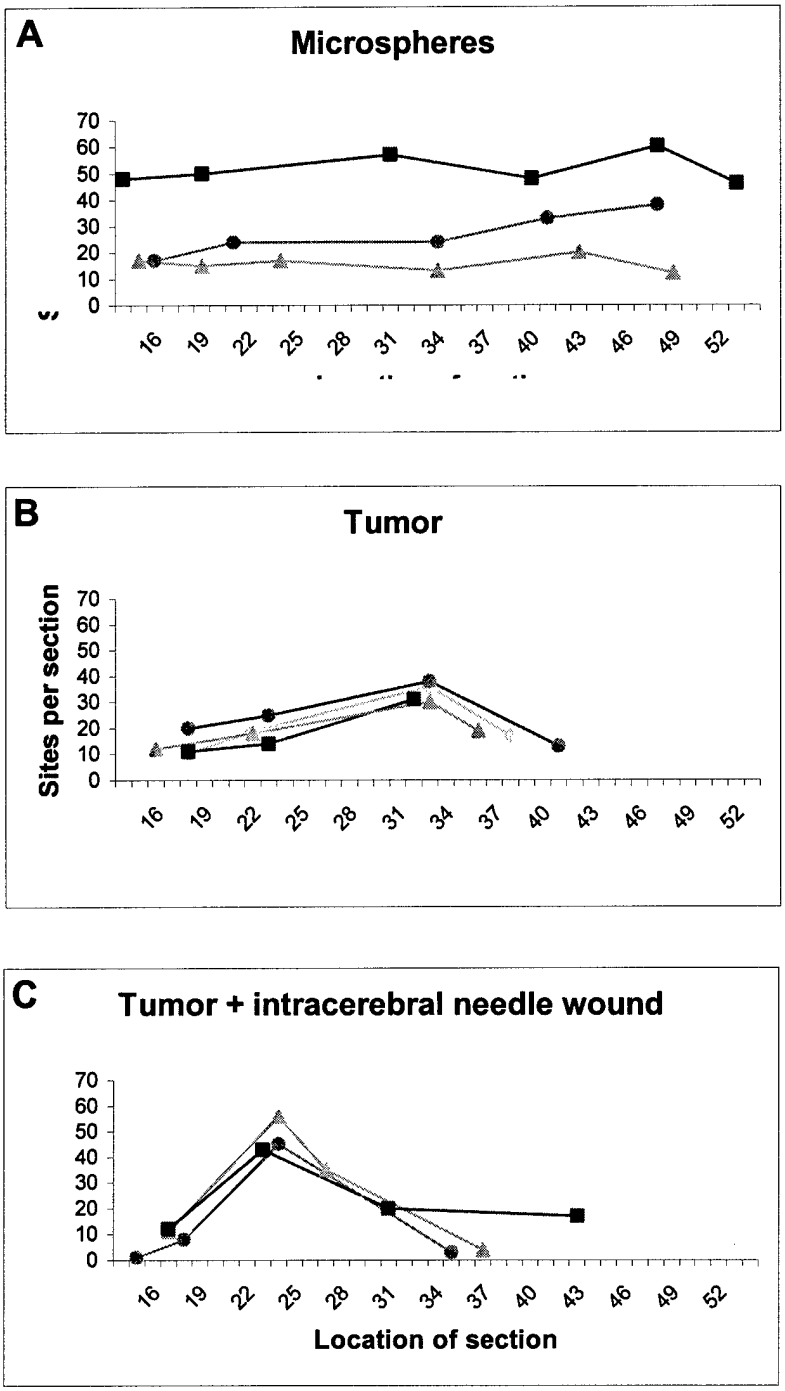
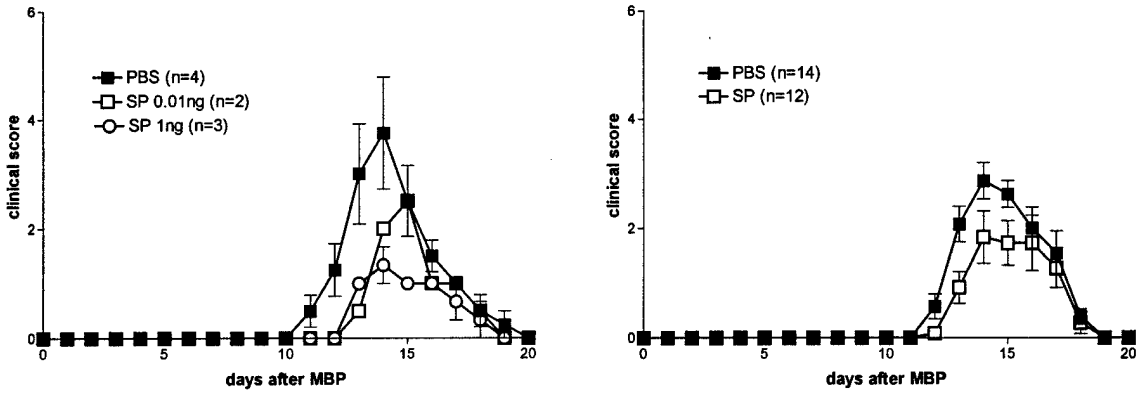


Fig. 4. Tumor distributions. Sites of parenchymal blood-borne metastases were counted in the slide with the most parenchymal tumor, and in slides rostral and caudal to it, as explained in the text. The location of the slide, according to the corresponding plate number in the rat brain atlas, is shown on the X axis. The number of sites counted is shown on the Y axis. Note that in ***C***, there is a sharper peak around the needle wound than in unwounded control rats (***B***). In ***A***, the distribution of inert microspheres at time 0 is shown for comparison.

IMMUNE MODULATION

Work in the complementary EAE model that led us to re-evaluate our ideas about IFN-g action in the brain are shown. Results with SP, which is expected to enhance IFN-g activity (**fig. 5**), and with IFN-g itself (**fig. 6**), are shown.



A. Local delivery of SP is *protective*. **B. Delivery of SP to CSF is also *protective*.**

Fig. 5. Effect of substance P (SP). SP, which is expected to enhance IFN-g activity (as explained in the text), was delivered to the CNS just before the onset of clinical signs in EAE. In each case, the SP was delivered in 0.2 ul 8- 10 days after sensitization with MBP. (Each rat received only 1 injection.)

A. Local delivery. 0.01 or 1 ng of SP was injected stereotactically into the brainstem (nucleus of the solitary tract, NTS). The curve for each dose differed from the curve for the PBS control rats, $p < 0.05$, $p < 0.001$, respectively.

B. CSF delivery. SP was injected stereotactically into the cisterna magna. To partially account for the dilution effect, a higher dose, 10 ng, was given. The curve for the SP-treated rats differed from that for the PBS-treated control rats, $p < 0.001$.

Note that SP was *protective*. Moreover, it was protective whether given to the brainstem (**A**) or to the CSF (**B**).

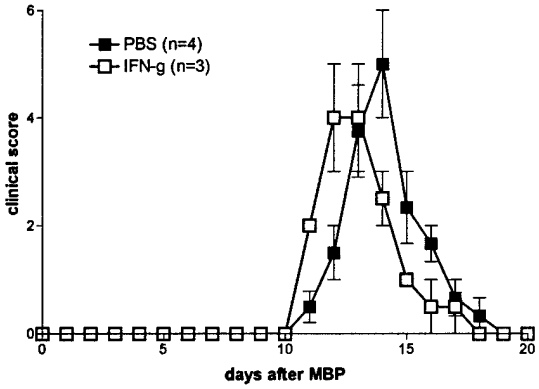


Fig. 6. Effect of IFN-g. On day 8 after MBP, rats received injection of 20,000 U IFN-g (or PBS control) to the striatum. Under the conditions tested, local injection of IFN-g did not increase disease severity, and may have been slightly protective. Injection into alternative sites may show a greater effect, as explained in the text.
