Journal of Neuro-Oncology **20**: 17–25, 1994.

Laboratory investigation

Blood-brain barrier changes following intracerebral injection of human recombinant tumor necrosis factor-α in the rat

James L. Wright[1] and Randall E. Merchant[1,2]
Department of[1] Anatomy, and Department of[2] Surgery, Division of Neurosurgery, Viginia Commonwealth University, Medical College of Virginia, Richmond, VA, USA

Key words: rTNF-α, blood-brain barrier, toxicity, histopathology

Summary

Human recombinant tumor necrosis factor-α (rTNF-α) was administered to normal Fischer 344 rats by stereotaxic intracerebral (IC) injection. Animals receiving a single injection of either 6×10^4 U rTNF-α or an equivalent volume of excipient (vehicle) in their right parietal lobe. In order to demonstrate any effects rTNF-α might have on the blood-brain barrier (BBB), two studies were conducted, one employing exogenous horseradish peroxidase (HRP, 44 kD) as a tracer of BBB permeability and the other using endogenous IgG (150 kD). Rats given rTNF-α showed transitory BBB permeability to HRP by 24 hours post-injection; this BBB compromise was determined to be no longer than 60 hours. In the other study, IgG was seen to cross the BBB by 48 hours post-rTNF-α injection. Alternatively, rats injected IC with excipient showed only limited BBB opening as a result of injection-induced trauma. We conclude that human rTNF-α, injected IC into normal rats triggers a temporary breakdown in BBB integrity which begins sometimes between 12 and 24 hours post-injection, is large enough to permit macromolecules of at least 150 kD to pass, and resolves by 72 hours post-injection.

Introduction

Immunotherapy has gained widespread attention in the past decade as an increasingly powerful oncologic tool. Our laboratory has availed itself of the newfound ability to modulate the immune response to tumors using recombinant cytokines and found them to be particularly applicable in immunotherapeutic approaches to the treatment of malignant brain tumor. Using an animal model, we have examined the effects that local or systemic injection(s) of cytokines have on the growth and histopathology of malignant glioma including human recombinant interleukin-2 (rIL-2), human recombinant tumor necrosis factor-α (rTNF-α) and murine recombinant interleukin-1β (rIL-1β) [1–6].

An effect which is naturally of great concern when assessing the potential use of any cytokine to treat an intracerebral (IC) neoplasm is its ability to influence BBB permeability in the tumor and in the surrounding normal tissue. In animal studies, we have found that systemic and IC injections of human rIL-2 compromised the integrity of the BBB [1, 8, 9, 6] and that IC injections of murine rIL-1β also temporarily increased BBB permeability in the normal rat brain [1]. In a clinical trial of human rIL-2 in patients with recurrent glioma, we observed an increase in peritumoral edema following intralesional injection(s) of the cytokine [9, 10].

Our lab has examined the effects of one or more intravenous (IV) injections of rTNF-α on the cerebrovasculature of normal and glioma-bearing rats [2]. We confirmed prior reports that IV rTNF-α has no effect on BBB permeability in normal rodents

[11], but in rats with large intracerebrally implanted gliomas, a single IV injection of rTNF-α caused increased extravasation of a horseradish peroxidase (HRP) tracer. Multiple injections caused widespread hemorrhagic necrosis, neutrophil adherence to tumor vasculature and subsequent infiltration into the tumor.

Other *in vitro* studies have suggested that TNF-α plays an indirect role in increasing vascular permeability by inducing endothelial cells to produce platelet activating factor and IL-1β, two cytokines responsible for vascular leak and leukocytic influx into inflammatory foci [12, 13]. Studies have shown a vascular permeability effect within the CNS as well. At the site of the blood-cerebrospinal fluid (CSF) barrier in mice, Saukkonen and colleagues detected serum proteins in the CSF six hours following an injection of 10^7 U rTNF-α into the cisterna magna [14]. In a similar study, intracisternal challenge with 10^6 U rTNF-α was seen to induce inflammatory infiltrate into the subarachnoid space and a breakdown in the BBB in rats, although the potency was less than that of IL-1β [15]. In the clinical setting, however, serum TNF-α levels (and not IL-1 levels) are seen to correlate with BBB disruption, disease severity and indices of meningeal inflammation [16]. *In vitro*, effects on endothelial monolayers ascribed directly to rTNF-α include decreased inter-endothelial tight junction resistance, suppression of DNA synthesis and, in conjunction with interferon, tumor cytotoxicity [17–19].

Recently, we reported on the histopathological changes affected by one or more intracerebral injection(s) of human rTNF-α in rats [6]. In this study which focused on the ability of rTNF-α to induce leukocytic infiltration into normal brain, we found that a single dose of 6×10^4 U human rTNF-α induced an infiltration of monocytes and neutrophils which peaked at 48 hours and resolved three to five days following injection. The inflammatory reaction was localized at the injection site and had no apparent effect on behavior, body weight or core body temperature. These results are in direct contradiction with a similar study by Andersson *et al.* which showed no leukocytic infiltration into the parenchyma of the mouse hippocampus following intracerebral injection of 10^4 U murine rTNF-α [20]. Reasons for this discrepancy may lie in the different doses and volumes used; in our study, 6×10^4 U rTNF-α was injected in 5 μl while Andersson used 1 μl to inject 10^4 U rTNF-α. Any extra edema resulting from our methods could only serve to facilitate neutrophil diapedesis through cerebral endothelium.

In the present study, we further define the nature of TNF-α's actions on normal brain cerebrovasculature. Horseradish peroxidase (44 kD) and endogenous IgG (150 kD) were used as tracers to detect any compromise in the BBB. An exogenous tracer like HRP allowed us to delineate the duration of any breach in the BBB, while an endogenous tracer indicated the accumulative effects of BBB breakdown in response to rTNF-α.

Materials and methods

Animals

Female Fischer 344 rats (Harlan Sprague Dawley, Inc.) weighing 140 g to 160 g were used. Animals were housed in individual cages and maintained on a 12 hour light/dark cycle with food and water supplied *ad lib*. Weights and body temperatures were monitored throughout the study.

Cytokine

Lyophilized human rTNF-α was provided by the Cetus Corporation (Emeryville, CA). Purity was > 99% as determined by SDS-PAGE, with a specific activity of 24×10^6 U/mg protein and an endotoxin content of 0.02 ng/ml (as determined by the Limulus amoebocyte lysate assay). Human rTNF-α in a bulking agent of 2.6% mannitol and 0.9% sucrose was reconstituted with sterile, endotoxin-free water. Excipient for injection into all control animals was composed of 2.6% mannitol (Sigma) and 0.9% sucrose (Sigma) in sterile, endotoxin-free water.

Exogenous tracer studies

Animals were anesthetized by intraperitoneal (IP) injection of sodium pentobarbital (40 mg/kg). Single stereotaxic injections were made 1 mm posteri-

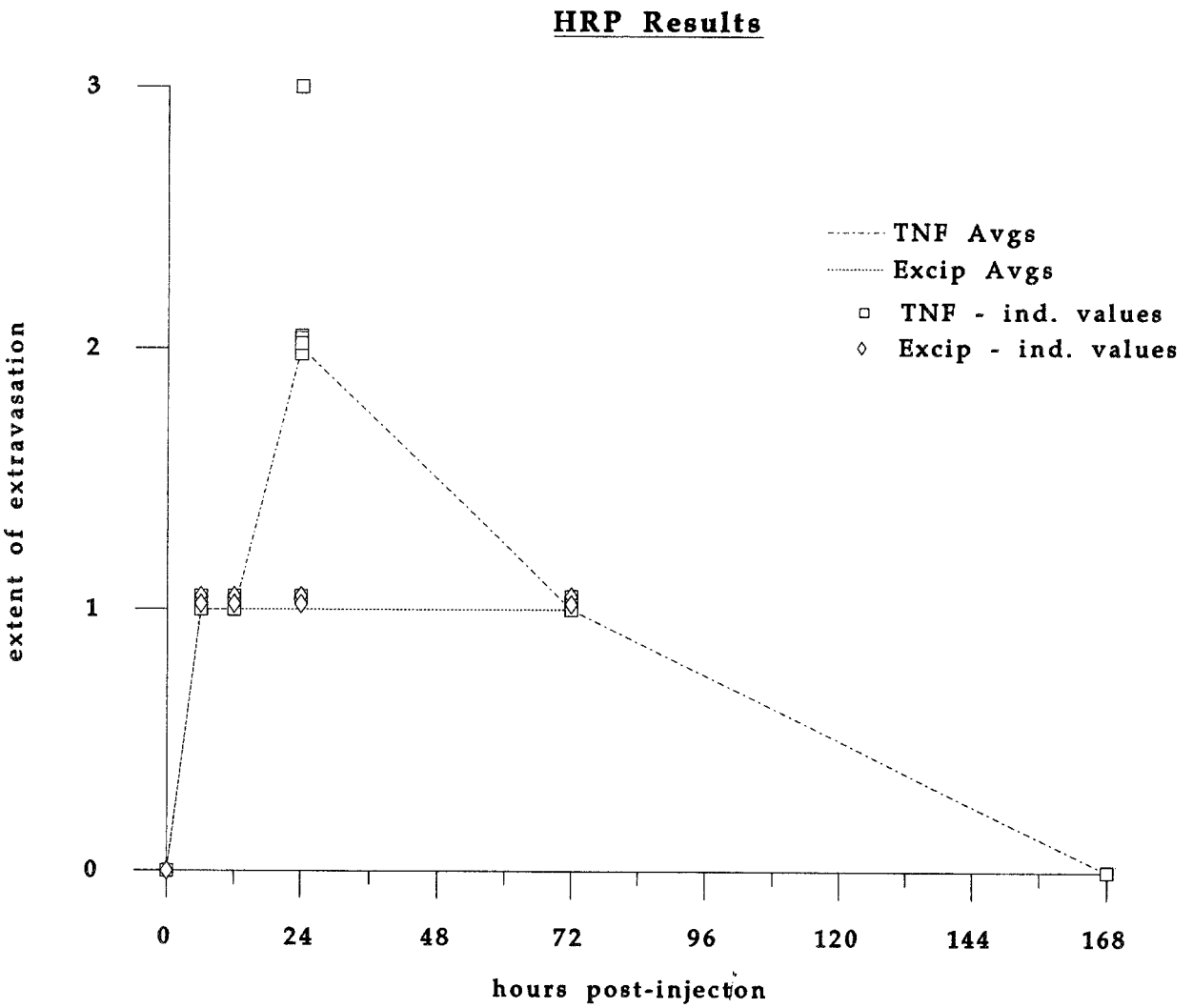

Fig. 1. Graph of individual and averaged results of histological grading of HRP samples. rTNF-α recipients display HRP extravasation throughout entire right hemisphere (grade 2) at 24 hours post-injection. Excipient animals show HRP extravasation limited to the injection site (grade 1) at all time points considered.

or and 4 mm to the right of bregma at a depth of 1.5 mm into the parietal cortex using a Hamilton syringe fitted with a 28 G needle. Animals received either 6×10^4 U rTNF-α or excipient in 5 µl delivered over a 10 minute period. Rats were later sacrificed after 6 (n = 4) and 12 (n = 4) hours and after 1 (n = 8), 3 (n = 4) and 7 (n = 2) days. At each time point, rats were divided evenly between those receiving rTNF-α and excipient (24 hour study rats were divided so that 6 received rTNF-α and 2 received excipient; rats sacrificed 7 days post-injection were injected with rTNF-α only). Horseradish peroxidase (Type VI, Sigma Chemical Co., St. Louis, MO) was suspended in 200 µl sterile saline and given to anesthetized rats at a concentration of 100 mg HRP/kg body weight through a cannula in the femoral vein. Infusions were made over a period of 5 minutes. One hour later, animals were transcardially perfused first with physiologic saline followed by a 0.1 M phosphate-buffered fixative containing 2.5% paraformaldehyde and 2% glutaraldehyde. Brains were removed and held in buffer until processed further. Vibratome sections were made up to the mid-injection site for HRP staining and histologic evaluation. Sections were reacted with 0.005% tetramethylbenzidine (TMB, Sigma)

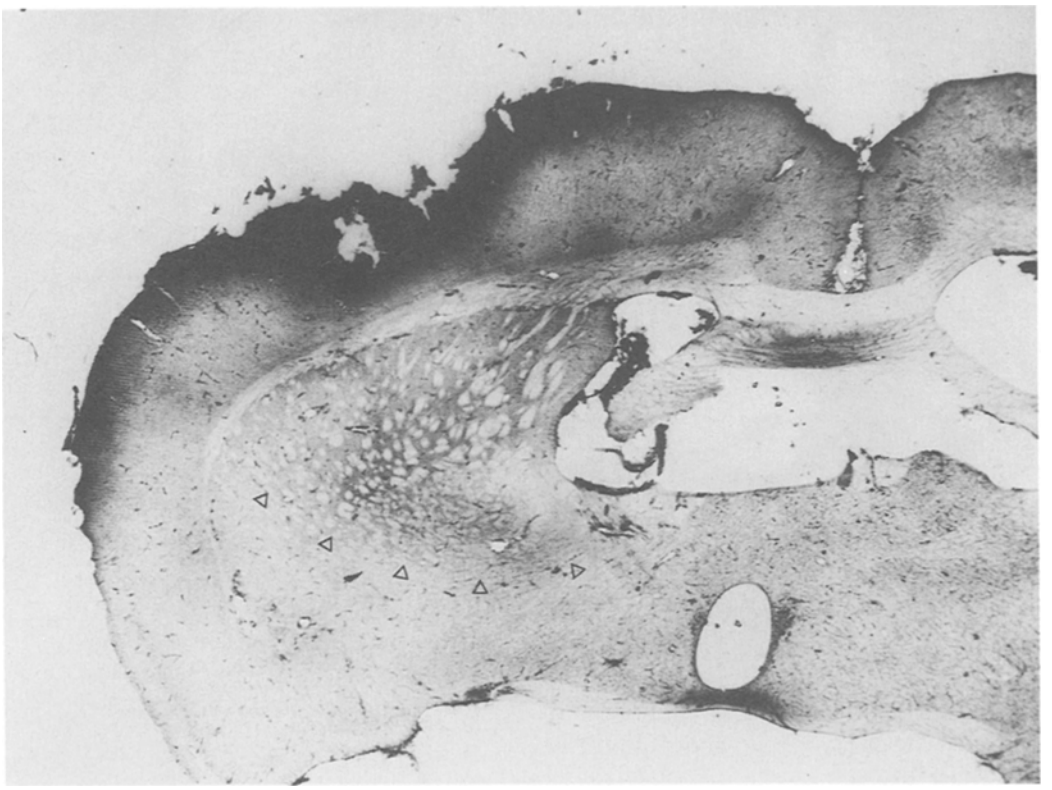

Fig. 2. Twenty-four hours following an injection of rTNF-α. HRP reaction product is seen throughout the right parietal cortex and basal ganglia (arrows). × 14.

and then 0.3% hydrogen peroxide solution for 20 minutes and counterstained with 1% neural red (Sigma) as described previously [4].

Endogenous tracer studies

Rats were implanted with cannulas as described in our earlier study [6]. Briefly, cannulas were implanted in the right parietal bone 1 mm posterior and 4 mm lateral to bregma. Three days later, a 28 G blunt-tip injection cannula was inserted into the parietal lobe at a depth of 3.5 mm. After 3 minutes accommodation, a 5 µl volume of either rTNF-α (6×10^4 U) or excipient was injected as before. Following a subsequent 5 minute accommodation period, the injection cannula was slowly withdrawn.

In order to examine the effects of cannula implantation on BBB integrity and leukocytic infiltration, sham operated animals (n = 3) were implanted with a guide cannula and sacrificed 3 days later. Tissue for all animals was processed for endogenous IgG visualization and for routine hematoxylin and eosin (H&E) staining as decribed below. Rats were sacrificed 4 (n = 4), 6 (n = 6) and 12 (n = 5) hours and 1 (n =4), 2 (n = 6), 3 (n = 7), 5 (n = 6), and 7 (n = 8) days following injection. Each group of animals was divided evenly between those receiving rTNF-α and excipient (12 hour study rats were divided so that 2 received rTNF-α and 3 received excipient; rats sacrificed after 3 days were divided so that 4 were injected with rTNF-α and 3 with excipient).

Anesthetized rats were transcardially perfused first with physiologic saline followed by a 0.1 M phosphate-buffered fixative containing 4% paraformaldehyde and 0.1% glutaraldehyde. Brains were removed and held in buffer until processed further. Vibratome sections of 50 µm thickness were made up to the mid-injection side. To stain for extravasated IgG, sections were first immersed 1 h in phosphate buffered saline (PBS) containing 10%

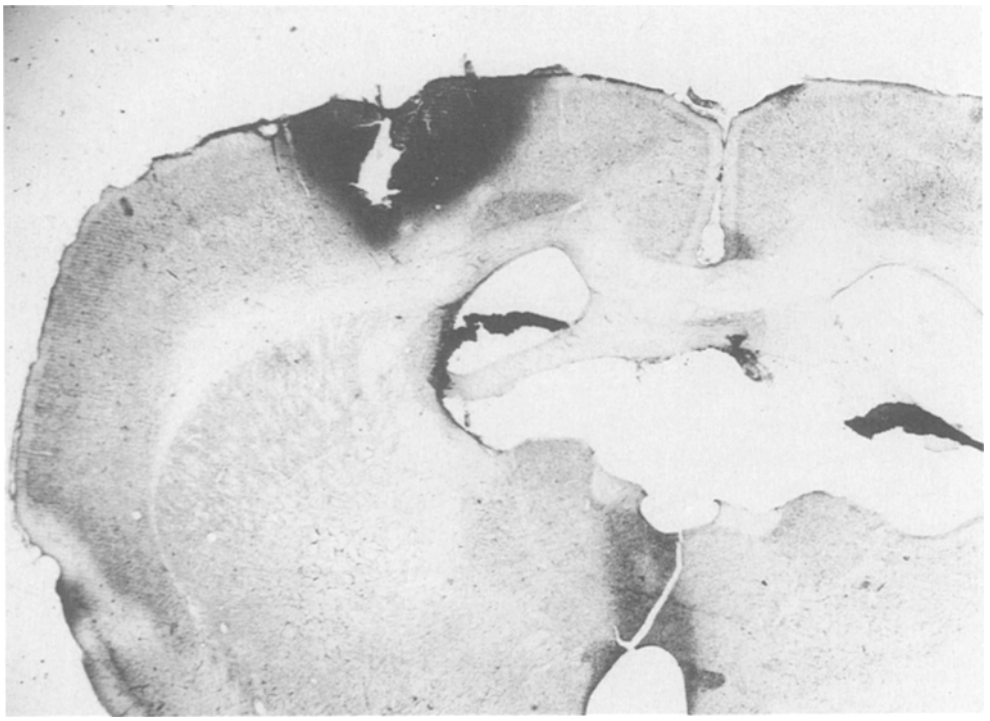

Fig. 3. Twenty-four hours following an injection of excipient. The pattern of HRP staining typical of all excipient-injected rats is presented. Reaction product is concentrated around the injection site only. × 14.

normal rabbit serum (NRS, Vector Labs) and 0.1% Triton X (Sigma) followed by a 1 h wash in 1% NRS in PBS. Sections were then incubated for 45 min in 1% NRS containing 0.5% biotinylated rabbit anti-rat IgG (Vector Labs). Sections were then washed with PBS and incubated for an additional 30 min in PBS containing 1% avidin-biotinylated HRP complex (Vector Labs). Sections were washed in three changes of PBS followed by a Tris-CHl buffer wash (pH 7.6) and reacted first with a 1% diaminobenzidine solution (DAB, Aldrich, Milwaukee, WI) for 10 minutes and then a 0.05% DAB/0.01% hydrogen peroxide solution (Sigma) for 4–5 minutes. Sections were then mounted on gelatin-coated slides and counterstained with 1% neutral red (Sigma).

Histological grading

A semiquantitative system of histological grading was devised to assess the extent of IgG an HRP extravasation. Four histological patterns were evident and were assigned numerical values as follows: 0 – none detectable, 1 – located within the edematous tissue surrounding the injection site only (IgG: average diameter of 1.4 mm; HRP: average diameter of 2.0 mm), 2 – observed throughout the injection hemisphere, and 3 – observed also within contralateral white matter (corpus callosum and basal ganglia). Rats reveiving rTNF-α were compared to those receiving excipient by averaging results from combined observations. Averages for rTNF-α and excipient groups are presented in Figs 1 and 4.

Results

As in our earlier study, a single IC injection of 6×10^4 U human rTNF-α had no adverse effect on behavior, weight, or body temperature of rats. Figure 1 shows results from histological survey of brain tissue in which HRP was used to mark areas of BBB breakdown. At 6 and 12 hours following injection, HRP reaction product was apparent only in the immediate area of the injection site (grade 1). This pattern of BBB disruption most likely resulted from trauma caused to the cerebrovasculature during drilling and needle placement as it was identical to

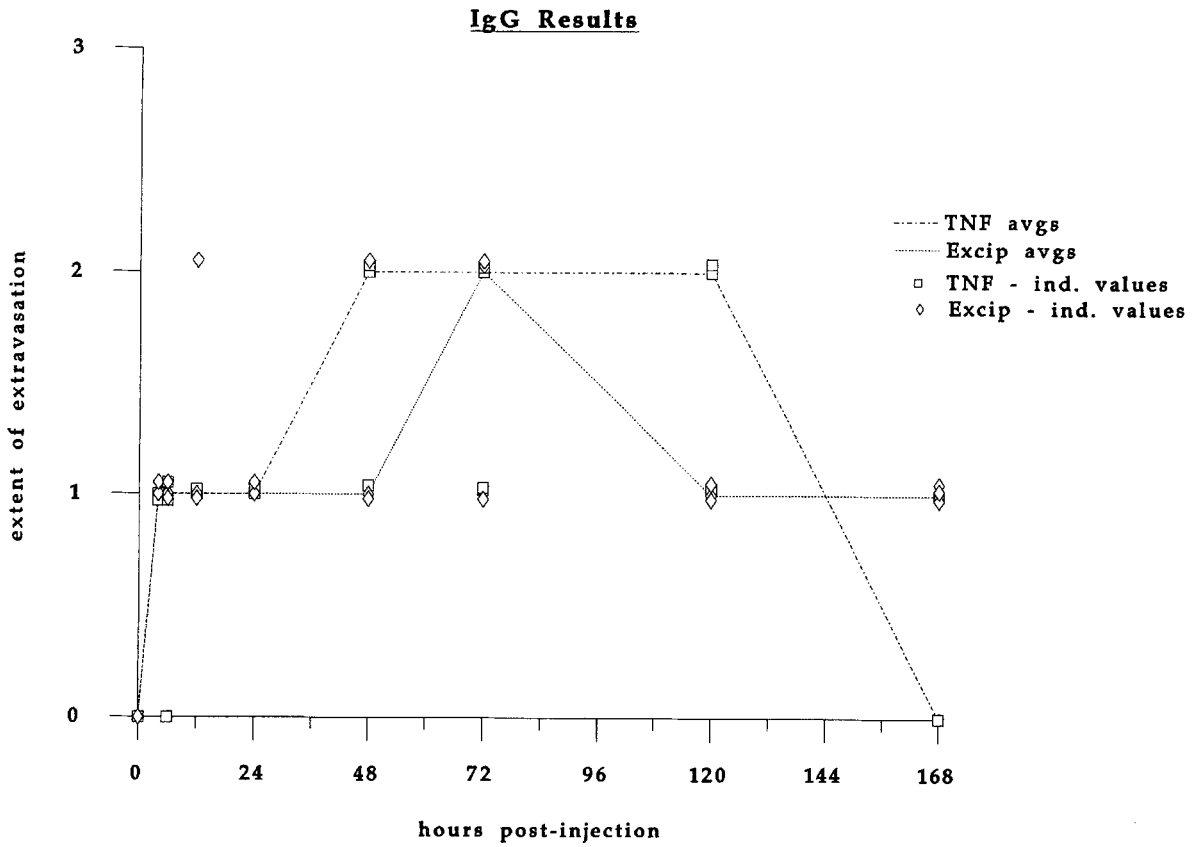

Fig. 4. Graph of individual and averaged results of histological grading of animals in IgG studies. rTNF-α recipients display endogenous IgG staining throughout the injection hemisphere (grade 2) from 48 hours to 5 days post-injection. Animals receiving excipient mainly show IgG staining limited to the injection site (grade 1) except for a peak of grade 2 at 3 days post-injection.

results observed in excipient-injected rats. Twenty-four hours following an injection of rTNF-α, HRP reaction product could be detected throughout the right parietal cortex and basal ganglia (Fig. 2). This widespread disruption of the BBB, however, was not observed at 3 days post rTNF-α injection, which showed HRP reaction product at the injection site, or at 7 days, where no HRP was seen. Figure 1 also demonstrates that in rats receiving excipient, the typical pattern of HRP distribution remained focused around the injection site at all time points examined (Fig. 3).

In animals where IgG was used as a tracer, Fig. 4 shows that the BBB in these rats remains impermeable to IgG until a time point between 24 and 48 hours. Forty-eight hours post-rTNF-α injection, IgG was seen throughout the injection hemisphere (grade 2) where it remained until 5 days post-injection (Fig. 5). Seven days following rTNF-α injection, IgG could not be detected in samples. In contrast to those receiving rTNF-α, rats receiving excipient typically showed IgG concentrated in a well-defined pattern around the injection site (Fig. 6) except for 3 days post-injection when IgG could be detected throughout the injection hemisphere.

Discussion

Tumor necrosis factor-α's effects on the integrity of the BBB to exogenous and endogenous proteins of different sizes was assessed in the rat. A single IC injection of 6×10^4 U rTNF-α was found to compromise BBB resistance to the 44 kD HRP tracer 24 hours after injection; this effect was resolved by 3 days following injection.

This paper surveys two independent BBB tracer studies, one with HRP and one with IgG. In the ini-

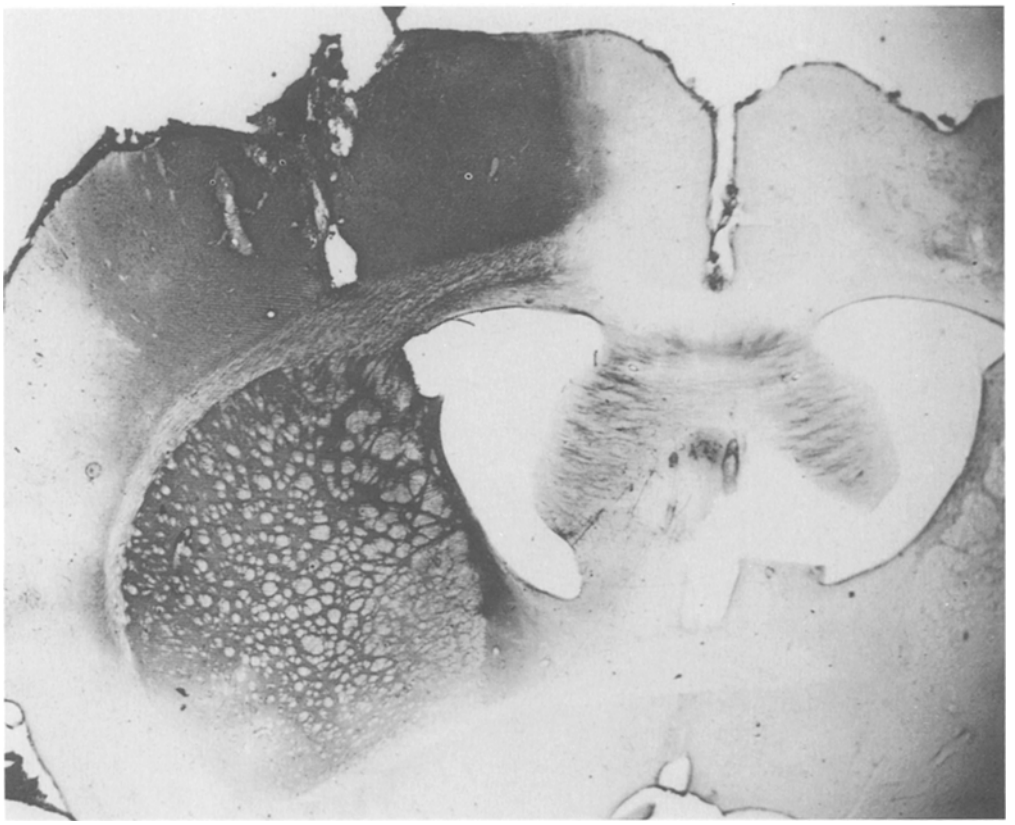

Fig. 5. Forty-eight hours post rTNF-α injection. Immunohistochemical demonstration for endogenous IgG reveals heavy staining throughout most of the injection hemisphere. × 14.

tial experiment employing HRP as a tracer, IC injections of rTNF-α were made in the parietal cortex at a depth of 1.5 mm. Problems were encountered, however, in differentiating between method-derived trauma and those phenomena attributable to the actions of rTNF-α. In the more recent studies employing IgG, a deeper injection site of 3.5 mm and an implanted cannula was used to produce an area within the brain which was separated from trauma induced by drilling and needle entry to such an extent that subtle differences between excipient and rTNF-α-injected animals could be examined at time points as early as 4 hours post-injection.

Since our results with HRP indicate an intact BBB by 3 days post-injection, IgG observed in brain tissue at 3 and 5 days post-rTNF-α injection most likely represents residual tracer which accumulated during BBB breakdown.

One of the conceptual problems in the use of rTNF-α as part of an immunotherapeutic regimen to treat glioma is its actions on vascular permeability, and its obvious potential for aggravation of pre-existing cerebral edema caused by the tumor. The present study shows that a single injection of 6×10^4 U rTNF-α into the normal rat brain has a transitory effect on BBB permeability which is limited to the injection hemisphere and which resolves spontaneously after a few days. Furthermore, at this dose, rTNF-α has no effect on temperature, feeding, or normal activity of the animal. Future studies will examine rTNF-α's effects on malignant brain tissue in a rat glioma model.

Acknowledgements

This investigation was supported in part from the A.D. Williams Research Fund and by gifts from the Lind Lawrence Fund, Davenport Research Fund, and the family and friends of Christine Armstrong,

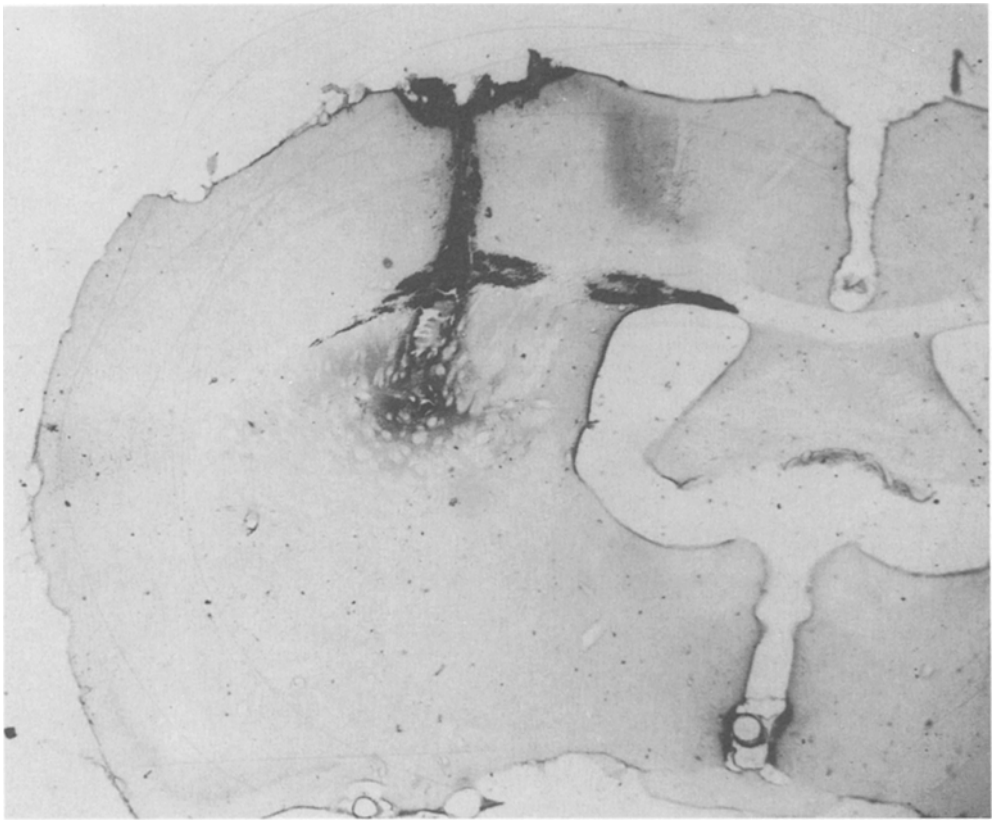

Fig. 6. Forty-eight hours following excipient injection. Endogenous IgG staining is seen only under the burr hole and immediately adjacent to the needle extract. × 14.

Gilles Fiechter, Jack Harvey, Reginald Morris, and Christopher Wemple.

The authors wish to thank Charles D. Rice, Ph.D. and Goro Kido, M.D. for their valuable technical assistance.

References

1. Gordon CR, Merchant RE, Marmarou A, Rice CD, Marsh JT, Young HF: Effect of murine recombinant interleukin-1 on brain edema in the rat. Acta Neurochir Suppl 51: 268–270, 1990
2. Kido G, Wright JL, Merchant RE: Acute effects of human recombinant tumor necrosis factor-α on the cerebral vasculature of the rat in both normal brain and in an experimental glioma model. J Neuro-Oncol 10: 95–109, 1991
3. Rice CD, Merchant RE: Systemic treatment with murine recombinant interleukin-1β inhibits the growth and progression of malignant glioma in the rat. J Neuro-Oncol 13: 43–55, 1992
4. Watts RG, Wright JL, Atkinson LL, Merchant RE: Histopathological and blood-brain barrier changes in rats induced by an intracerebral injection of human recombinant interleukin 2. Neurosurgery 25: 202–208, 1989
5. Watts RG, Merchant RE: Cerebrovascular effects and tumor kinetics after a single intratumoral injection of human recombinant interleukin-2 alone or in combination with intravenous chemotherapy in a rat model of glioma. Neurosurgery 31: 89–99, 1992
6. Wright JL, Merchant RE: Histopathological effects of intracerebral injections of human recombinant tumor necrosis factor-α in the rat. Acta Neuropathol 85: 93–100, 1992
7. Ellison MD, Povlishock JT, Merchant RE: Blood-brain barrier dysfunction in cats following recombinant interleukin-2 infusion. Cancer Res 47: 5765–5770, 1987
8. Ellison MD, Krieg RJ, Merchant RE: Cerebral vasomotor responses after recombinant interleukin-2 infusion. Cancer Res 50: 4377–4381, 1990
9. Merchant RE, Grant AJ, Merchant LH, Young HF: Adoptive immunotherapy for recurrent glioblastoma multiforme using lymphokine activated killer (LAK) cells and recombinant interleukin-2. Cancer 62: 665–671, 1988
10. Merchant RE, Merchant LH, Cook SH, McVicar DW, Young HF: Intralesional infusion of lymphokine-activated killer (LAK) cells and recombinant interleukin-2 (IL-2) for

the treatment of patients with malignant brain tumor. Neurosurgery 23: 725–732, 1988
11. Clark I, Ilschner S, MacMicking J, Cowden W: TNF and *Plasmodium berghei* ANKA-induced cerebral malaria. Immuno Lett 25: 195–198, 1990
12. Braquet P, Hosford D, Braquet M, Bourgain R, Bussolino F: Role of cytokines and platelet-activating factor in microvascular immune injury. Int Arch Allergy Appl Immunol 88: 88–100, 1989
13. Movat HZ: Tumor necrosis factor and interleukin-1: role in acute inflammation and microvascular injury. J Lab Clin Med 110: 668–681, 1987
14. Saukkonen K, Sande S, Cioffe C, Wolpe S, Sherry B, Cerami A, Tuomanen E: The role of cytokines in the generation of inflammation and tissue damage in experimental gram-positive meningitis. J Exp Med 171: 439–448, 1990
15. Quagliarello V, Wispelwey B, Long W, Scheld W: Recombinant interleukin-1 induces meningitis and blood-brain barrier injury in the rat. Characterization and comparison with tumor necrosis factor. J Clin Invest 87: 1360–1366, 1991
16. Sharief M, Ciardi M, Thompson E: Blood-brain barrier damage in patients with bacterial meningitis: association with tumor necrosis factor-alpha but not interleukin-1 beta. J Infect Dis 166: 350–358, 1992
17. Mullin JM, Snock KV: Effects of tumor necrosis factor on epithelial tight junctions and transepithelial permeability. Cancer Res 50: 2172–2176, 1990
18. Saegusa Y, Ziff M, Weikovich L, Cavender D: Effect of inflammatory cytokines on human endothelial cell proliferation. J Cell Physiol 142: 488–495, 1990
19. Yoshida S, Minakawa T, Takai N, Tanaka R: Effects of cytokines on cultured microvascular endothelial cells derived from gerbil brain. Neurosurgery 25: 373–377, 1989
20. Andersson P-B, Perry V, Gordon S: Intracerebral injection of proinflammatory cytokines or leukocyte chemotaxins induces minimal myelomonocytic cell recruitment to the parenchyma of the central nervous system. J Exp Med 176: 255–259, 1992
21. Kuroiwa T, Cahn R, Juhler M, Goping G, Campbell G, Klatzo I: Role of extracellular proteins in the dynamics of vasogenic brain edema. Acta Neuropathol 66: 3–11, 1985

Address for offprints: R.E. Merchant, Virginia Commonwealth University, Medical College of Virginia, Department of Anatomy, MCV Station, Box 709, Richmond, Virginia, 23298-0709, USA