Blood-Brain Barrier Dysfunction in Cats following Recombinant Interleukin-2 Infusion

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ABSTRACT

The effects of systemic human recombinant interleukin-2 (rIL-2) infusion upon blood-brain barrier status and cerebral vascular ultrastructure were examined in cats. Each of eight animals received a single bolus i.v. infusion of rIL-2 (100,000 units/kg). Six control animals were infused with rIL-2 excipient only. Following a 1-h postinfusion survival time, the brain tissue of five rIL-2 infused and three excipient infused animals was processed and examined by light microscopy and electron microscopy for evidence of altered cerebral permeability to systemically circulating horseradish peroxidase. The brain tissue of three additional rIL-2 infused animals and three excipient infused animals, sacrificed 4 h postinfusion, was examined at the light microscopic and electron microscopic levels for the presence of extravasated endogenous IgG.

All animals infused with rIL-2 and four of six excipient infused animals showed increased cerebrovascular permeability to the probe used. Altered blood-brain barrier permeability, when present, was recognized in multiple loci throughout the brain, being most prominent within white matter regions. Horseradish peroxidase and IgG were observed within perivascular basal laminae and within the interstices of the brain parenchyma. Numerous endothelial lesions were observed as was flooding of endothelial cytoplasm by horseradish peroxidase or IgG. Every animal studied, regardless of permeability status, showed, within the perivascular brain parenchyma, numerous disrupted neuronal and glial processes as well as expanded intercellular spaces. This study suggests that a single systemic infusion of rIL-2 profoundly alters blood-brain barrier integrity and cerebrovascular morphological integrity. The data also suggest that some of the observed cerebrovascular effects of systemic rIL-2 infusion are due to components of the vehicle for rIL-2.

INTRODUCTION

Clinical trials involving the adoptive transfer of systemically administered autologous LAK cells together with rIL-2 are currently under way in a number of centers. These treatment regimens were implemented in the clinical setting following laboratory studies which showed that infusion of LAK cells with rIL-2 mediated the regression of metastatic tumors in experimental animals (1–3). Clinical trials have also produced encouraging results. Rosenberg and coworkers (4–6) have administered rIL-2 therapy, alone and in conjunction with LAK cells, to 157 patients with metastatic cancer in whom standard therapies have been ineffective. Twenty-nine of these patients showed objective regression of cancer (more than 50% of tumor volume). Pulmonary, hepatic, and subcutaneous metastases from melanoma, colorectal cancer, renal cell cancer, and lung adenocarcinoma regressed with therapy in these patients.

Thus, preliminary studies offer hope that rIL-2 administration, alone or in conjunction with LAK cells, represents a treatment regimen which may be effective in combatting certain human cancers. Various treatment protocols are presently under clinical investigation in an effort to achieve the most effective therapy.

Subsequent to preliminary clinical and animal trials it has been observed that rIL-2, at effective therapeutic dosage levels, produces toxic side effects. Among these are weight gain due to massive fluid retention, hypotension due to reduced blood volume, and pulmonary interstitial edema, often accompanied by dyspnea and occasionally by severe respiratory distress (4–6). Such clinical reports and additional testing of rIL-2 in animal models have linked its toxicity in large part to increases in systemic capillary permeability (4–8). In animal studies, vascular permeability to circulating radiolabeled serum albumin was significantly increased, following rIL-2 administration, within the splenic, hepatic, pulmonary, and renal vascular beds (8).

Those conducting clinical trials with rIL-2 have noted that patients receiving rIL-2 exhibit not only pulmonary and hemodynamic abnormalities but also changes in neurological function. CNS symptoms include confusion, depression, dementia, paranoia, somnolence, and coma (5, 6). The causative mechanisms underlying these symptoms have not been investigated; however, it can be suggested that rIL-2 is acting upon the cerebral vasculature and, in turn, elicits change within the brain parenchyma.

The present study was undertaken in an effort to determine whether cerebrovascular permeability to circulating proteins is altered following systemic rIL-2 administration and to identify the affected brain regions. Additionally, electron microscopy was used to examine the ultrastructural correlates of any altered cerebrovascular permeability and to determine if other abnormalities occurred in vessels not demonstrating altered permeability.

MATERIALS AND METHODS

Animals. Fourteen adult cats weighing 2.5 to 4 kg were used.

rIL-2. Recombinant human interleukin-2 was supplied in sterile vials by the Cetus Corporation (Emeryville, CA). Each vial contained 1.2 mg (3 × 10⁴ units) rIL-2, lyophilized, 141 mg mannitol, plus a stabilizer, SDS (150 µg/mg rIL-2). The dosage used in each animal was 100,000 units/kg diluted in 2 ml sterile saline delivered by infusion into the femoral vein over 15 min. Excipient, in sterile vials containing 135 mg mannitol and SDS (54 µg/vial), was also supplied by the Cetus Corporation. Diluted with sterile saline, the excipient was administered as described above, with a mannitol concentration (7–11 mg/ml) approximately equivalent to that in the rIL-2 infusate and with a SDS concentration proportionately reduced.

Permeability Probes. To assess cerebrovascular permeability, two circulating large molecular weight proteins were used. Both proteins, exogenous HRP (M, 40,000) and endogenous blood-borne IgG (M, 160,000), are normally excluded from brain parenchyma by the BBB. Horseradish peroxidase, visualized through the use of various chromagens, is readily seen in fixed tissue by both light and electron microscopy. As such, peroxidase can be injected into the systemic circulation and can serve as an excellent probe for identifying anatomical sites of altered cerebral vascular permeability and for examining ultrastructural correlates accompanying permeability change (9–11). IgG, an endogenous immunoglobulin, is also normally excluded from both brain tissue and cerebrovascular endothelium (12–15). With the
aid of immunocytochemistry, IgG, like HRP, can be visualized within brain tissue at both the light and electron microscopic levels, allowing anatomical localization of vessels with altered permeability. Moreover, previous studies have shown that endothelial cytoplasmic flooding by IgG, when observed, is indicative of irreversible endothelial cell damage (12-14). Thus, IgG can be used not only as a marker of barrier compromise, but also as an indicator of profound endothelial cell damage.

Eight animals were given injections of HRP (Sigma type VI, 50 mg/kg in 2 ml sterile saline) via the femoral vein prior to rIL-2 or excipient infusion. In an additional 6 animals, infused with either rIL-2 or excipient, endogenous circulating IgG served as a marker of barrier alteration. These animals did not receive peroxidase.

Experimental Design. All animals were anesthetized with sodium pentobarbital (30 mg/kg) and equipped with femoral arterial and venous cannulae for physiological monitoring (arterial blood pressure, arterial blood gases) and drug administration. Animals receiving peroxidase were then given injections of HRP as described above and were infused with either rIL-2 or excipient as described previously. Peroxidase-injected animals were sacrificed at 1 h postinfusion in order to assess initial vascular change. Animals studied for IgG extravasation were sacrificed 4 h following infusion. The 4-h survival period was chosen for the IgG group to allow not only for the identification of any altered blood-to-brain IgG passage but also for the consideration of any potential endothelial cell flooding with the immunoglobulin, a condition linked with overt damage and impending cell death. With the longer survival period, it was felt that the potential for identifying any such IgG endothelial flooding would be enhanced. At the designated time, postinfusion, each animal was administered an overdose of sodium pentobarbital and then was perfused transcardially with 0.9% sodium chloride followed by fixative. The fixative used for peroxidase-injected animals consisted of 2.5% glutaraldehyde:2% paraformaldehyde in 0.1 M phosphate buffer. Fixative for brains to be processed by immunocytochemistry consisted of 4% paraformaldehyde, 0.1% glutaraldehyde in 0.1 M phosphate buffer.

Histological Preparation. Brains were serially sectioned on a vibrotome at 40 µm. Brain tissue from the HRP-injected animals was processed according to the cobalt-glucose oxidase method (16) for visualization of HRP reaction product. Brain sections processed for the visualization of IgG were immersed in 10% NGS with 0.1% Triton X for 1 h followed by a 1-h wash in 1% NGS in phosphate-buffered saline. The tissue was then incubated in biotinylated goat anti-cat IgG (1:200; Vector) in phosphate-buffered saline with 1% NGS for 30 min at room temperature. As a control, some tissue was incubated in the same manner without the addition of anti-cat IgG. All tissue was then washed in phosphate-buffered saline and immersed in an avidin:biotinylated horseradish peroxidase complex (ABC; Vector; 1:100) for 30 min. Following several washes in phosphate-buffered saline, followed by Tris-HCl buffer, the tissue was reacted for the visualization of the HRP reaction product through the use of diaminobenzidine.

Following histochemical and immunohistochemical processing, brain sections were prepared for examination by either light or electron microscopy. Sections for LM examination were mounted on glass slides, cleared, and coverslipped. Based upon the LM observation of altered barrier status, adjacent brain sections were osmicated, dehydrated, and flat embedded in Medcast resin. Thin sections were cut on a diamond knife, viewed, and photographed on a transmission electron microscope.

Data Analysis. All brain sections prepared for light microscopy were examined for evidence of tracer extravasation. Brain regions demonstrating HRP/IgG passage at the LM level were carefully delineated and subsequently identified in adjacent sections prepared for electron microscopy. These regions were dissected free from the flat plastic embedded brain sections, were sectioned at 70 nm, and were examined with a transmission electron microscope. Some brain regions not exhibiting tracer extravasation were also sampled and examined ultrastructurally.

RESULTS

rIL-2 Infusion. Each of the 8 rIL-2-infused animals, whether sacrificed at 1 or 4 h postinfusion, demonstrated increased cerebral vascular permeability with extravasation of the tracer into the substance of the brain parenchyma (Figs. 1 and 2). Altered permeability to the tracer used, whether HRP or IgG, was recognized in multiple loci throughout the brain and was particularly prominent in subcortical white matter including the internal capsule, corona radiata, and corpus callosum. Vessels demonstrating increased permeability were also observed consistently within the thalamus and, to varying degrees, within cortical gray matter.

Ultrastructurally, both HRP and IgG were observed within perivascular basal laminae of many intraparenchymal arterioles, venules, and capillaries (Figs. 3 and 4). Such vessels were often observed to be surrounded by enlarged perivascular spaces flooded with tracer (Fig. 3A). Although the route of blood-to-brain tracer passage could not be identified with certainty, many endothelial cells were inundated with the tracer, either HRP or IgG, suggesting direct transendothelial passage (Figs. 3C, 5, and 6). The cytoplasm of some endothelia appeared entirely flooded by IgG. Although occasional endothelia showed evi-
Fig. 3. Transmission electron micrographs from rIL-2-infused animal showing extravasated peroxidase reaction product within perivascular basal laminae. a, intraparenchymal arteriole with subendothelial and perimucosal basal laminae flooded with reaction product (small arrows). Enlarged perivascular spaces are filled with tracer (large arrows) which can also be observed diffusing into the surrounding parenchyma (arrowheads). Swollen perivascular astrocytic processes are visible (A) as is a vacuolated perivascular phagocyte (P). Inset, portion of vessel wall (b). Bar, 1 μm. b, endothelium containing peroxidase-filled vesicles (arrows) which may participate in increased blood-to-brain protein passage. Bar, 1 μm. c, additional vascular segment from same animal showing, in addition to protein-laden vesicles, profound endothelial cytoplasmic flooding with protein (arrows) which may serve as a conduit for blood-to-brain protein passage. Swollen astrocytic processes are also indicated (A). Bar, 1 μm.
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Fig. 4. Transmission electron micrograph from rIL-2-infused animal showing extravasated immunoglobulin within perivascular basal lamina (arrows) of intraparenchymal microvessel. IgG is also visible within surrounding brain parenchyma (arrowheads), within perivascular phagocyte (P), and within cytoplasm of fibrous astrocyte (A). Asterisks indicate enlarged perivascular spaces. Unstained tissue. Bar, 2.5 μm.

Fig. 5. Transmission electron micrograph of arteriole from rIL-2-infused animal showing endothelial cytoplasmic flooding with peroxidase (arrows). Expanded extracellular spaces are notable (asterisks) as is the presence of a peroxidase-laden perivascular phagocyte (P). Bar, 1 μm.

Fig. 6. Transmission electron micrograph of venule from rIL-2-infused animal showing endothelium (END) to be abnormally electron lucent and completely inundated by IgG. Unstained tissue. Bar, 1 μm.

Fig. 7. Transmission electron micrograph of arteriole from rIL-2-infused animal showing vacuolated perivascular phagocyte (P) with extensive cytoplasmic processes (arrows) and peroxidase-filled cytoplasm. Although protein permeability appears normal, numerous swollen perivascular astrocytes are visible (A). Bar, 2.5 μm.

dence of increased pinocytotic activity (Fig. 3B), no visible evidence of interendothelial tight junctional cleaving was observed. Arteriolar vascular smooth muscle cells appeared normal. Within the interstices of the related brain parenchyma, both tracers were observed to be confined primarily to the extracellular compartment. However, occasional neuronal and glial somata were noted to contain IgG (Fig. 4).

Interspersed among those vessels showing increased permeability to protein were other vessels which did not demonstrate altered permeability. Although a majority of these appeared morphologically normal, some manifested severe endothelial blebbing, reminiscent of luminal endothelial surface alterations observed following free radial damage (17, 18).

In addition to these changes, further study revealed that a majority of vessels, regardless of permeability status, were surrounded by enlarged extracellular spaces and swollen neuronal and glial processes (Figs. 3 and 7). The cell membranes of such processes were occasionally disrupted. In association with many vessels, demonstrating either normal or altered permeability to protein, were highly vacuolated phagocytic cells localized along abluminal arteriolar walls (Figs. 3A, 4, 5, 7). Such cells displayed extensive cytoplasmic processes and large vacuoles containing peroxidase (in HRP-injected animals).

Excipient Infusions. Among the six excipient-infused animals, four showed increased cerebrovascular permeability. All three of the animals examined at 4 h postinfusion showed abundant immunoglobulin within endothelial cytoplasm, perivascular basal laminae, and the brain parenchyma. Only one of the animals examined at 1 h postinfusion showed increased permeability to peroxidase. Increased cerebrovascular permeability predominated within the same brain regions demonstrating increased permeability following rIL-2 infusion. However, overall, both the magnitude of altered permeability and the number of vessels involved appeared reduced in the vehicle-infused group. Altered barrier function was, again, identified by the
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presence of reaction product within perivascular basal laminae, the surrounding brain interstices, and within endothelial cells themselves, where the cytoplasm frequently showed partial flooding by HRP or IgG. Some endothelia were observed to be entirely flooded by IgG.

Ultrastructural examination of the tissue also revealed other findings similar to those observed in rIL-2-infused animals. Such findings included endothelial blebs, expanded extracellular spaces, swollen perivascular glial processes, and perivascular phagocytic cells, all of which were also observed in the two excipient-infused animals which did not show increased permeability to protein. Except for the widespread distribution of phagocytic cells, consistent among IL-2 and excipient infused animals, other cerebrovascular and brain parenchymal alterations appeared less dramatic in excipient infused animals, regardless of postinfusion survival time. In the excipient group, perivascular spaces appeared less markedly expanded, and neuronal/glial membrane damage was more rarely observed.

DISCUSSION

Adoptive immunotherapy involving rIL-2 administration, although sometimes effective, is accompanied by some serious, occasionally lethal, systemic side effects, many of which appear directly related to increased systemic capillary permeability. The present study demonstrates that brain vascular permeability, following rIL-2 infusion, is also increased and accompanied by profound morphological vascular and parenchymal change.

The finding that a single rIL-2 infusion results in increased cerebrovascular permeability is of more than mere academic interest. The brain neuronal/glial microenvironment is carefully regulated by the BBB which, by virtue of certain features of cerebral endothelial cells, limits the blood-to-brain passage of many substances. Not only does the BBB exclude certain blood-borne solutes (e.g., neurotransmitters, plasma proteins, certain drugs) from the brain parenchyma, but it also serves to regulate blood/brain electrolyte gradients and the delivery to brain tissue of essential metabolic substrates (amino acids, glucose, amines, etc.). This transport of nutrients through the BBB is now being recognized as a potential site of cerebral metabolic regulation (19). When cerebral endothelial integrity is pathologically altered such that BBB permeability increases, the consequences for cerebral function may be significant, especially when barrier alterations are of a chronic nature, as may be the case during rIL-2 therapy. The present study has demonstrated increased cerebrovascular permeability to macromolecules following a single rIL-2 or excipient infusion. In general, patients undergoing rIL-2 therapy receive 3 such infusions per day over 5 consecutive days. It is feasible that rIL-2-excipient-induced barrier alterations persist throughout the days of rIL-2 therapy, leaving the brain indiscriminately exposed to circulating solutes, including drugs such as indomethacin, normally excluded from the brain and routinely given in the course of IL-2 therapy (7, 20). Thus, the neurological abnormalities reported in rIL-2-treated patients may reflect altered cerebral metabolism secondary to chronic barrier alterations. Although, to date, no long-term neurological deficits have been attributed to transient BBB opening, such as is commonly observed following head injury, acute hypertension, and hyperosmotic infusion, the consequences of long-term barrier compromise, over a period of days, are unknown.

Although the cause underlying IL-2-excipient permeability changes is as yet undetermined, the present study demonstrates that the vehicle for rIL-2 contributes to many of the observed morphological and barrier changes. As the low concentration of mannitol used (0.04–0.06 M) would not be anticipated to have any biological effect and as previous studies have linked SDS to rIL-2 vehicle-related toxicity (7), it appears reasonable that SDS is a causative agent. In preliminary studies, one cat was given an injection i.v. of SDS (EM Industries; electrophoresis grade, 20 μg in 2 ml saline). This SDS concentration is equivalent to that contained in a rIL-2 infusion. The brain showed increased cerebrovascular permeability to endogenous IgG when examined at 4 h postinfusion. Altered vessels were observed almost exclusively within the corona radiata, internal capsule, and thalamus, as observed in animals infused with rIL-2 or excipient alone. Ultrastructural observations were also reminiscent of those reported in the present study. That excipient-infused animals showed less dramatic, although consistently observed, cerebrovascular and brain parenchymal alterations is consistent with the lower SDS content of the excipient and supports the suggestion that SDS contributes to some or all of the observed changes.

It may be argued that some other technical aspect of the experimental protocol (i.e., rIL-2-excipient infusion technique, use of HRP, method of anesthesia, saline/aldehyde perfusion technique, physiological animal management) was responsible for the observed pathology. In our laboratory, we have extensively studied, in animal models, BBB alterations and their morphological correlates following multiple forms of CNS insult (11, 21–23) using the same techniques, tracers, solutions, and equipment. In controls for these studies, we have not observed any of the presently described vascular alterations. Moreover, many of the presently described alterations appear to differ markedly from vascular changes we have observed heretofore. Barrier alterations observed in our laboratory following head injury and acute hypertension are most commonly observed within the cerebral cortices rather than white matter. Additionally, these previous studies have suggested that many alterations in barrier status are mediated by increased endothelial pinocytosis transferring solutes from blood to brain front (11, 21, 23). The presently observed profound flooding of endothelial cytoplasm by both tracers was an unanticipated finding and a clear departure from a majority of the literature. Studies by both other investigators have linked endothelial inundation by IgG, such as that observed in the present study, with endothelial cell injury and impending endothelial cell death (12–15). Thus, based upon this finding, one can infer that rIL-2 and/or its vehicle exerts a lethal influence on at least some of the cerebrovascular endothelia.

Furthermore, in no other experimental model of vascular injury studied in our laboratory have we observed the presently described perivascular vacuolated phagocytic cells visualized following IL-2(excipient) infusion. Nor have we encountered the profound perivascular neuronal/glial membrane damage or cerebral extracellular spaces swollen to the degree observed in the present study.

In conclusion, we have demonstrated that rIL-2 and/or its excipient, administered in dosages similar to or identical to those being widely used clinically (6), has profound effects on cerebrovascular permeability to proteins in cats, accompanied by endothelial, neuronal, and glial cell membrane damage. The duration of such changes following a single infusion is unknown, and whether multiple infusions exacerbate the alterations is also as yet undetermined.

As patients receiving rIL-2 therapy show signs of altered

Unpublished observations.
neurological function, it seems reasonable to speculate that the presently reported rIL-2/exciptent induced cerebrovascular effects underlie the genesis of those clinical findings. In view of the apparent effects of one of the vehicle's constituents, SDS, on the cerebral vasculature and associated parenchyma, it is reasonable to conclude that some of the serious systemic side effects, linked to rIL-2 administration, are vehicle related. It is hoped that the presently reported study and future studies will accelerate the development of an effective rIL-2 therapeutic treatment, fully characterized and free of long-term adverse effects.

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REFERENCES

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