Etoposide Induced Blood-Brain Barrier Disruption in Rats: Duration of Opening and Histological Sequelae

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ABSTRACT

The intracarotid infusion of the antineoplastic compound etoposide enhances blood-brain barrier (BBB) permeability. In a rat model system, the functional reversibility and anatomic sequelae of etoposide induced BBB disruption were investigated. Etoposide, in a dose range from 3.0 to 22.5 mg/kg, was infused into the left internal carotid artery of Sprague-Dawley rats. BBB disruption was evaluated qualitatively by the appearance in the infused hemisphere of systemically administered Evans blue dye and quantitatively by the ratio of counts of the technetium labeled chelate of diethylenetriaminepentaacetic acid in the infused to the noninfused hemisphere. Functional reversibility of altered BBB permeability was investigated at three dose levels of etoposide (3.0, 15.0, and 22.5 mg/kg) by the administration of Evans blue dye at the time of etoposide infusion and the administration of the technetium labeled chelate of diethylenetriaminepentaacetic acid at varying time intervals after etoposide infusion. Fourteen groups of 12 rats each were studied to define the time course of altered BBB permeability at these three doses.

The anatomic sequelae of etoposide induced BBB disruption were investigated at varying time intervals (up to 3 weeks) after intracarotid etoposide infusion. Nineteen rats were examined after sacrifice by intracardiac fixation perfusion with 10% formalin. Each brain was sectioned coronally and examined under light microscopy after hematoxylin and eosin staining.

Evidence of BBB disruption was seen at all dose levels of etoposide. The degree of BBB disruption increased with increasing doses of etoposide. The duration of altered BBB permeability increased from less than 1 day at 3.0 mg/kg to between 3 and 4 days at 22.5 mg/kg. Histological studies revealed no evidence of parenchymal damage, although at 4 days postdisruption, a mild perivascular lymphocytic infiltration was noted in the infused hemisphere.

Etoposide infusion and subsequent BBB disruption were well tolerated by all test animals. In a rat model system the intracarotid infusion of etoposide is capable of producing prolonged reversible BBB disruption.

INTRODUCTION

There is evidence from both human and animal studies that the BBB plays a limiting role in the delivery of chemotherapeutic agents to central nervous system cancers (1–4). In various tumor models, the BBB has been found to be intact in small central nervous system neoplasms and usually at least partially intact in larger tumors (5, 6). Clinical attempts to overcome the resultant limitation in drug delivery have included the intracarotid perfusion of hyperosmotic mannitol (3, 4, 7) or the i.v. administration of dimethyl sulfoxide (8) to disrupt the BBB transiently and the administration of chemotherapeutic agents directly into the arterial supply of the tumor bed (9, 10).

In a rat model system, we have investigated the effects of a variety of compounds on BBB permeability and found that the intracarotid infusion of the antineoplastic drug etoposide produces a dose dependent disruption of the BBB (11). We now report the functional reversibility of etoposide induced BBB disruption and the anatomic effects of intracarotid etoposide infusion. Altered BBB permeability was documented qualitatively by the appearance in the brain parenchyma of the systemically administered dye, Evans blue. This dye is normally excluded from brain parenchyma (12). A quantitative measure of BBB disruption was obtained using 99mTc-DTPA. This γ emitting radiopharmaceutical compound is commonly used in humans to assess BBB integrity (13).

MATERIALS AND METHODS

Experiments were performed on female Sprague-Dawley rats (250–350 g) that had free access to pellet food and tap water until surgery. Anesthesia was induced in all animals as described previously (11).

BBB Disruption (Acute Experiments). Four groups of 12 rats each were studied. After induction of anesthesia, catheters were inserted into the left femoral vein and left external carotid artery as described previously (11). Depending on the preassigned group to which the rat belonged, 5 ml of normal saline containing etoposide (VePesid; Bristol Laboratories, Syracuse, NY) at doses of 0.0, 3.0, 15.0, or 22.5 mg/kg were given by constant infusion into the left internal carotid artery via the catheter in the external carotid artery (11). Immediately after the carotid infusion, 0.5 ml of a 2% solution of Evans blue dye and 150 μCi of 99mTc-DTPA in 0.1 ml of normal saline were injected into the femoral vein catheter. Rats were sacrificed 2 h after the end of the intracarotid infusion by intracardiac perfusion with normal saline. This was done through a midline thoracotomy followed by perfusion of normal saline at 120 mm Hg through a left ventricular catheter along with blood drainage through a right atrial incision. After removal of the brains, staining of the cerebral hemispheres by Evans blue was evaluated by direct visual inspection without knowledge of etoposide dosage. Evans blue staining was graded as follows: grade 0, no staining; grade 1+, just noticeable staining; grade 2+, moderate staining; grade 3+, dark staining (14). The brains were sagittally hemisected and the cerebrum, brain stem, pitui-

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3 The abbreviations used are: BBB, blood-brain barrier; 99mTc-DTPA, 99mTc-labeled chelate of diethylenetriaminepentaacetic acid.
femoral vein catheter. Two hours after the Tc-DTPA injection, rats were allowed to awake from the anesthesia. Carotid artery catheter, the neck incision was sutured and the rats were anesthetized (with xylazine and ketamine as described above) and sacrificed to one of 10 groups. These groupings were based on the concentration of etoposide (either 3.0, 15.0, or 22.5 mg/kg) and on the time interval from carotid infusion to sacrifice of the animal. The carotid infusion procedure was then performed as described above. One group was infused with etoposide, 3.0 mg/kg, four groups with etoposide, 15.0 mg/kg, and five groups with etoposide, 22.5 mg/kg. Immediately after the etoposide infusion, 0.5 ml of a 2% solution of Evans blue dye was injected into the femoral vein catheter. After removal of the external carotid artery catheter, the neck incision was sutured and the rats were allowed to awake from the anesthesia.

At varying time intervals after the initial intracarotid infusion, each rat received 150 µCi of 99mTc-DTPA in 0.1 ml of normal saline through the femoral vein catheter. Two hours after the 99mTc-DTPA injection, rats were anesthetized (with xylazine and ketamine as described above) and sacrificed, and their brains were inspected and counted as described in the acute experiments. On the basis of prior data, the group that had been infused with etoposide, 3.0 mg/kg, was sacrificed 1 day after the etoposide infusion; the four groups infused with etoposide, 15.0 mg/kg, were sacrificed at 1, 2, 3, and 4 days after intracarotid infusion; and the five groups that received 22.5 mg/kg were sacrificed at 1, 2, 3, 4, and 5 days, respectively, after etoposide infusion.

Histopathological Studies. Nineteen animals were evaluated for possible histopathological effects of intracarotid etoposide infusion. All rats were anesthetized as described above. Five rats were studied for acute sequelae of etoposide infusion and 14 for possible longer term effects.

After induction of anesthesia in five animals, polyethylene catheters (PE-50) were inserted into the left femoral vein and left external carotid artery as described in "BBB Disruption (Acute Experiments)." Through the external carotid artery catheter, two of the rats were infused with 5 ml of normal saline alone and three received 5 ml of normal saline containing etoposide, 22.5 mg/kg. Infusions were given over 25 min, with the pterygopalatine branch of the internal carotid artery temporarily occluded. Immediately after the carotid infusion, 0.5 ml of a 2% solution of Evans blue dye was injected into the femoral vein catheter. Rats were sacrificed 2 hours after the end of the intracarotid infusion by intracardiac perfusion with 35 ml of normal saline followed by 10% formalin. Brains were removed and graded for the presence of Evans blue staining of the cerebral cortex as in the above experiments. The left hemisphere was marked by notching the cortex with a scalpel blade. Coronal sections for histological examination were taken from frontal, parietotemporal, and occipital lobes. Sections were fixed in 10% neutral buffered formalin and processed routinely for paraffin embedding and hematoxylin and eosin staining.

After induction of anesthesia, the remaining 14 animals also underwent catheter placement in the left femoral vein and external carotid artery. Four of these rats received intracarotid infusions of normal saline only, while 10 received etoposide, 22.5 mg/kg. Each rat was also given Evans blue dye i.v. The femoral and carotid catheters were then removed, skin incisions were sutured, and animals were allowed to awaken. At predetermined time intervals after carotid infusion (from 1 day to 3 weeks), rats were sacrificed and underwent the same procedures as the above 5 animals. Of the 4 rats infused with saline alone, 2 were sacrificed at 4 days postinfusion and the remaining 2 were sacrificed at 3 weeks. Of the 10 rats infused with etoposide, 2 were sacrificed at 1 day, 2 days, 3 days, 4 days, and 3 weeks, respectively, post-etoposide infusion.

Statistical Methods. Radioisotopic counts of the cerebral hemispheres were analyzed as the ratio of counts per unit weight in left to right hemispheres. There was a wide range among the variances of the ratios in the five groups. Even after transformation of the ratios to the natural logarithms, the variances were significantly different. Therefore, the distributions of the ratios among the groups were compared using nonparametric methods (Kruskal-Wallis analysis of variance on the ranks). Comparisons of the control group with groups of rats infused at each dose level were done using a treatment versus control multiple comparison procedure based on Kruskal-Wallis rank sums (15). An overall level of α = 0.05 was used to determine which individual groups were significantly different from the control.

RESULTS

BBB Disruption. The acute effect of the intracarotid infusion of etoposide on the BBB as measured by the presence of Evans blue dye in the ipsilateral cerebral hemisphere is shown in Table 1. None of the animals infused with saline alone had evidence of Evans blue staining. However, even at the lowest dose of etoposide (3.0 mg/kg), 11 of the 12 animals had some evidence of BBB disruption. At the higher doses (15.0 and 22.5 mg/kg), all rats had evidence of BBB disruption with a progressive shift to higher grades with increasing doses of etoposide.

The distribution of staining within the left hemisphere was variable. Brains which were graded as 1+ usually had most prominent staining in the subtemporal area. Brains graded as 2+ or 3+ were usually stained diffusely, although the most prominent areas of staining varied. When there was 2+ or 3+ staining noted in the left hemisphere, there was frequently some disruption to Evans blue dye noted in the right hemisphere also. Evans blue staining of the right hemisphere was usually seen near the midline in the distribution of the anterior cerebral artery. The number of rats in each group with evidence of any Evans blue staining in the contralateral (right) cerebral hemisphere increased with increasing doses of etoposide. None of the saline controls had evidence of right hemispheric staining, whereas 3 of 12 rats infused with etoposide, 3.0 mg/kg, 6 of 12 with etoposide, 15.0 mg/kg, and 10 of 12 rats infused with etoposide, 22.5 mg/kg, had some evidence of Evans blue staining in the right hemisphere.

The median ratios of the concentrations of 99mTc-DTPA between left and right hemispheres for all groups of rats are shown in Table 2. Comparison of the saline control group with the groups infused with etoposide, 3.0 mg/kg (groups 2 and 3), reveals a significant difference (Kruskal-Wallis analysis of variance, χ² = 24.53; P = 0.0001). Treatment versus control comparisons reveal that the median ratio for group 2 is significantly greater than that for the control group (P < 0.05). Comparison of the control group with all groups infused with etoposide, 15.0 mg/kg (groups 4, 5, 6, 7, and 8), also reveals significant differ-

<table>
<thead>
<tr>
<th>Etoposide (mg/kg)</th>
<th>No. of rats</th>
<th>0</th>
<th>1+</th>
<th>2+</th>
<th>3+</th>
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<td>12</td>
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<td>12</td>
<td>12</td>
<td>1</td>
<td>8</td>
<td>3</td>
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<tr>
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<td>0</td>
<td>2</td>
<td>9</td>
<td>1</td>
</tr>
<tr>
<td>22.5</td>
<td>12</td>
<td>0</td>
<td>1</td>
<td>7</td>
<td>4</td>
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</table>

* All doses were diluted in normal saline to a total volume of 5.0 ml and infused over 25 min.
* Staining grades: 0, none; 1+, just noticeable; 2+, moderate; 3+, dark.
Table 2

<table>
<thead>
<tr>
<th>Group</th>
<th>Etoposide (mg/kg)</th>
<th>Time from end of infusion to injection of radionuclide (h)</th>
<th>Median ratio</th>
<th>Range</th>
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<td>24</td>
<td>1.17</td>
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<tr>
<td>4</td>
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<td>0</td>
<td>3.50</td>
<td>2.72–4.65</td>
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<tr>
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<td>15.0</td>
<td>24</td>
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<td>6</td>
<td>15.0</td>
<td>48</td>
<td>1.91</td>
<td>1.33–3.61</td>
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<tr>
<td>7</td>
<td>15.0</td>
<td>72</td>
<td>1.35</td>
<td>1.06–2.13</td>
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<tr>
<td>8</td>
<td>15.0</td>
<td>96</td>
<td>1.11</td>
<td>1.00–1.53</td>
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<td>22.5</td>
<td>120</td>
<td>1.12</td>
<td>0.93–1.38</td>
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* Etoposide was diluted in normal saline to a total volume of 5.0 ml and infused over 25 min. There were 12 rats in each group.

ences ($\chi^2 = 56.26, P = 0.0001$). Multiple comparisons show that at an overall level of $\alpha = 0.05$, the groups studied at 2 h and at 1 and 2 days after etoposide infusion (groups 4, 5, and 6) are significantly different from the saline infused rats (group 1). In comparing all groups infused with etoposide, 22.5 mg/kg, and the saline control (groups 1, 9, 10, 11, 12, 13, and 14), there is again a significant difference between the groups ($\chi^2 = 68.24; P = 0.0001$). Comparisons of these etoposide infused groups with the control group reveals all etoposide infused groups except those sacrificed at 4 and 5 days after disruption (groups 13 and 14) are statistically different from group 1 ($P < 0.05$). A separate analysis examining all 14 groups together yields identical results.

Visual inspection of the brains in the chronic experiments revealed that 8 of the 120 animals (groups 3, 5–8, and 10–14) had no evidence of Evans blue staining. These included 2 animals in groups 3, 8, and 13 and 1 rat in groups 7 and 12.

**Histopathology.** Of the 5 rats studied 2 h after etoposide or saline infusion, the 3 infused with etoposide all had Evans blue staining of the left hemisphere (2 were graded 2+ and one as 3+). Both of the saline infused animals had no evidence of Evans blue staining. The four chronic saline animals (2 sacrificed at 4 days postinfusion and 2 sacrificed 3 weeks postinfusion) had no evidence of Evans blue staining. Of the 14 chronic etoposide infused rats, all but 2 (one studied at 4 days and one at 3 weeks) had evidence of Evans blue staining.

No pathological changes were seen under light microscopy in the brains of any animals sacrificed up to 4 days after intracarotid infusion of either saline or etoposide. Specifically there was no evidence of edema, inflammation, hemorrhage, or neuronal degeneration. However, at 4 days postinfusion, a mild perivascular lymphocytic infiltration was noted in the left hemisphere only of the two etoposide treated animals (Fig. 1). No other lesions were found. Animals examined 3 weeks after disruption showed no pathological lesions at all (Fig. 2).

**Toxicity.** Intracarotid saline or etoposide infusion was well tolerated by all test animals. Animals receiving higher doses of etoposide were somewhat more lethargic than those rats receiving less drug or none at all. Grossly normal behavior was regained by all animals allowed to live more than 2 h after intracarotid infusion.

**DISCUSSION**

Because of the potentially vital role of the BBB as a limiting factor in drug delivery to central nervous system neoplasms, a variety of techniques for enhancing BBB permeability have been studied (8, 16, 17). Of those which are reported to cause...
reversible BBB disruption, only one has thus far been shown to increase permeability for more than 2 to 4 h (17). Chemotherapeutic drugs other than etoposide have also been reported to cause BBB disruption. MacDonnell et al. (18) initially reported that the i.v. administration of 5-fluorouracil in cats caused BBB disruption. Neuwelt et al., however, could not reproduce those findings in a rat model system (19) but subsequently reported that the intracarotid infusion of cis-platinum in the dog caused BBB disruption that was associated with parenchymal damage (20). Feun et al. (21) have presented data in abstract form that the intracarotid infusion of hydroxyurea also causes enhanced BBB permeability.

Evans blue staining of the contralateral hemisphere is common to techniques of BBB disruption which rely on intracarotid infusions (17, 22). Since the contralateral staining is almost always within the distribution of the anterior cerebral artery, this finding is probably due to bilateral drug delivery through the anterior communicating artery or because rats frequently have a common anterior cerebral artery (23). As seen by the presence of some contralateral hemispheric staining with Evans blue, the use of hemispheric ratios as a measure of BBB disruption probably results in an underestimation of the amount of disruption. It is of interest that similar to the findings in this study, data from hyperosmotic, hypertensive, and sodium dehydrocholate induced BBB disruption also reveal peak hemispheric ratios which range from 2 to 5 even though the tracers used ([3H]norepinephrine, [14C]inulin, [125I]albumin, and [99mTc-DTPA) and methods of disruption have varied considerably (17, 22, 24).

The mechanism of etoposide induced enhanced permeability is not yet known. Etoposide is relatively water insoluble and therefore is formulated by Bristol Laboratories in a complex solvent solution containing 400 mg Tween 80, 3.25 polyethylene glycol 300, 10 mg anhydrous citric acid, 150 mg benzyl alcohol, and absolute alcohol to 5 ml for each 100 mg of etoposide (25). As we have shown previously, the effect of this solvent solution alone on BBB permeability is small compared to the effect of solvents plus etoposide (26). The osmolarity of the etoposide containing solutions used in this study ranged from 0.36 to 0.91, values below that reported by Rapoport et al. (27) as necessary for hyperosmotic disruption. In fact, because of the relatively slow infusion rate and therefore marked dilution of infusion with serum proteins across the vascular basement membrane. We are unaware, however, of this finding having been reported with any other method of BBB disruption. This appears to be a transient phenomenon which was not seen in those animals sacrificed 3 weeks postdisruption. The potential for brain injury which may occur after 3 weeks or which may occur with repeated treatments was not investigated.

Recent clinical phase I studies on the i.v. administration of high doses of etoposide have recommended a dose of approximately 2.5 g/m² (approximately 65 mg/kg) for phase II studies. It is of interest that this is the recommended dose both with or without autologous bone marrow transplantation (28, 29). This dose is much higher than the maximum dose used in this study implying that from the point of systemic toxicity use of intracavitary etoposide in humans may be feasible even at doses that produce marked degrees of BBB disruption in this study.

The finding of BBB opening from the intracarotid infusion of etoposide for up to 4 days stands in marked contrast to the maximum durations of BBB opening of 1 to 4 h which can be achieved with hyperosmotic or hypertensive BBB disruption (22, 27). The ability to alter both the degree and duration of BBB opening without damage to brain parenchyma has potential value for basic studies of the role of the BBB in normal and pathological states and for investigations on the potential therapeutic role of BBB disruption.

REFERENCES


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