Imaging and therapy with rituximab anti-CD20 immunotherapy in an animal model of central nervous system lymphoma

Leslie L. Muldoon1,2, Seth J. Lewin1, Edit Dósa1, Dale F. Kraemer4,6, Michael A. Pagel6, Nancy D. Doolittle1, and Edward A. Neuwelt1,3,6

Departments of 1Neurology, 2Cell and Developmental Biology, 3Neurosurgery, 4Public Health and Preventive Medicine, and 4Medical Informatics, and Clinical Epidemiology, Oregon Health & Science University, Portland OR 97237. 5Department of Pharmacy Practice, Oregon State University, Portland, OR 97239. 6Portland Veterans Administration Medical Center, Portland OR 97239.

Corresponding author: Edward A. Neuwelt, MD; Oregon Health & Science University, 3181 S.W. Sam Jackson Park Road, L603; Portland, OR 97239-3098. Phone: (503) 494-5626, Fax: (503) 494-5627, Email: neuwelte@ohsu.edu

Running title: Rituximab in a rat model of CNS lymphoma

Abbreviations: blood-brain barrier (BBB); blood-brain barrier disruption (BBBD); central nervous system (CNS); magnetic resonance imaging (MRI); monoclonal antibody (mAb); primary central nervous system lymphoma (PCNSL); whole-brain radiotherapy (WBRT)
Statement of Translational Relevance. The standard of care for primary central nervous system lymphoma (PCNSL) is currently undefined, with clinicians moving away from radiotherapy options that cause cognitive loss. Two options are the addition of rituximab anti-CD20 monoclonal antibody to target B-lymphoma cells, or the use of osmotic blood-brain barrier disruption (BBBD) to enhance the delivery of methotrexate chemotherapy to infiltrating tumor cells throughout the brain. We assessed the effects of rituximab in a rat model of human CNS lymphoma, and tested the hypothesis that enhanced delivery with BBBD and/or combination with high dose methotrexate would improve anti-tumor efficacy. We also measured early changes in tumor volume on MRI to determine if this outcome correlated with survival. We found that rituximab was effective as a single agent in extending survival in the rat model of CNS lymphoma. We suggest rituximab warrants additional clinical trials in PCNSL.
ABSTRACT

Purpose: To evaluate the effect of rituximab monoclonal antibody (mAb) on magnetic resonance imaging (MRI) tumor volumetrics and efficacy in a rat model of central nervous system (CNS) lymphoma when delivery to the brain was optimized with osmotic blood-brain barrier disruption (BBBD).

Experimental Design: Female nude rats with intracerebral human MC116 B-cell lymphoma xenografts underwent baseline MRI and were randomized into five groups (n = 6/group): 1) BBBD saline control; 2) methotrexate with BBBD; 3) rituximab with BBBD; 4) rituximab and methotrexate with BBBD; and 5) intravenous rituximab. Tumor volumes were assessed by MRI at 1 week and rats were followed for survival.

Results: BBBD increased delivery of $^{90}$Y-radiolabeled mAb in the CNS lymphoma model. Control rats showed 201 ± 102% increase in tumor volume on MRI 1 week after entering the study and median 14 day survival (range 6-33). Tumor growth on MRI was slowed in the methotrexate treatment group, but survival time (median 7 days, range 5-12) was not different from controls. Among 17 evaluable rats treated with rituximab, 10 showed decreased tumor volume on MRI. All rituximab groups had increased survival compared to control, with a combined median of 43 days (range 20-60, P<0.001). There were no differences by route of delivery or combination with methotrexate.

Conclusions: Rituximab was effective at decreasing tumor volume and improving survival in a CNS lymphoma model, and was not affected by combination with methotrexate or by BBBD. We suggest that rituximab warrants further study in human primary CNS lymphoma.
INTRODUCTION

Primary central nervous system lymphoma (PCNSL) is an aggressive malignancy of the brain, spinal cord and/or eye that accounts for up to 4% of primary brain tumors (1-3). The prognosis for patients with PCNSL remains poor, with median overall survival of only 16 to 40 months (4-9). Treatment of PCNSL usually consists of high dose methotrexate-based chemotherapy, with or without the addition of whole-brain radiotherapy (WBRT) or bone marrow transplantation (4, 7, 10, 11). However, at least 50% of patients relapse, usually within 2 years of initial diagnosis, and combined methotrexate and WBRT is associated with high rates of delayed neurotoxicity, particularly in long-term survivors over 60 years of age (4, 6, 8, 11). A treatment regimen providing long-term effectiveness and minimal toxicity is necessary in PCNSL.

Approximately 95% of PCNSL are diffuse large B-cell lymphomas that express the membrane-associated phosphoprotein CD20 (12). The chimeric anti-CD20 monoclonal antibody (mAb) rituximab is approved for the treatment of B-cell lymphomas and has improved the prognosis of diffuse large B-cell lymphomas lacking central nervous system (CNS) involvement (13, 14). Case studies and small trials suggest that intraventricular and/or intravenous rituximab has potential anti-tumor activity in PCNSL (15-20). Additionally, targeting CD20 with the radioimmunoconjugate $^{90}$Y-labeled ibritumomab tiuxetan (Zevalin) has also demonstrated efficacy in PCNSL (15, 21). We have hypothesized that the high molecular weight of rituximab likely limits penetration and, therefore, efficacy in PCNSL, particularly in the tumor-infiltrated brain around the main tumor mass.

Limited leakage of chemotherapy and mAbs across the blood-brain barrier (BBB) can be overcome using the osmotic BBB disruption (BBBD) technique to increase transvascular delivery to brain tumors. BBBD improves delivery and efficacy of chemotherapy and
immunoconjugate therapy in rat brain tumor models (22-24), and is safe and effective for enhanced delivery in humans (5, 25). We recently reported improved survival without the cognitive loss due to WBRT in a multi-institutional trial with 149 newly-diagnosed PCNSL patients treated with osmotic BBBD and intra-arterial methotrexate-based chemotherapy (5).

We have previously reported a rat model of human B-cell CNS lymphoma that closely mimics the clinical situation (26, 27) and provides a useful tool for evaluating therapies for human PCNSL. Rituximab and methotrexate showed anti-tumor efficacy as determined by early changes on magnetic resonance imaging (MRI) in the rat model (26). The purpose of the current study was to determine if BBBD-enhanced delivery of mAb increased efficacy in the rat CNS lymphoma model. Additionally, we evaluated whether the early MRI changes correspond to improved survival in this model.

MATERIALS and METHODS

Cell culture. The MC116 human B-cell lymphoma cell line was obtained from the American Type Culture Collection (ATCC, Manassas, VA), and was characterized by cytogenetic analysis and antigen expression (28). It forms an infiltrative CNS lymphoma when injected in nude rat brain (27). Cells were cultured in suspension in RPMI1640 medium supplemented with 20% fetal bovine serum, 2 mM L-glutamine, and antibiotics. Cells were harvested immediately prior to intracerebral implantation and were used only if viability exceeded 80%. New vials of MC116 cells were obtained from ATCC every 1-2 months throughout this 10-month study.

Animal use and tumor inoculation. The care and use of animals was approved by the Institutional Animal Care and Use Committee and were supervised by the Oregon Health &
Science University (OHSU) Department of Comparative Medicine. Female athymic nude rats (rnu/rnu, 180-240 g, from the OHSU Blood-Brain Barrier Program in-house colony) were used for all studies. Intracerebral tumor inoculation was performed as previously described (26, 27). Rats were anesthetized with intraperitoneal ketamine (60 mg/kg) and intraperitoneal diazepam (7.5 mg/kg). Animals received 1.2 to 1.5 x 10⁶ of >80% viable MC116 cells in a volume of 15 µl, stereotactically injected in the right caudate putamen (vertical, bregma 5 mm; lateral, bregma 3 mm). The needle was initially advanced to a depth of 6.5 mm and then withdrawn to a depth of 6 mm to limit reflux up the needle track. Our previous studies have demonstrated that intracerebral growth of the MC116 model is very inconsistent (26, 27); therefore, all rats were treated with intraperitoneal cyclophosphamide (100 mg/kg) 24 hours prior to tumor implantation and two weeks after tumor implantation to decrease innate immunity as a mechanism to improve the consistency of tumor growth (27, 29).

Pharmacology study design. Ibritumomab tiuxetan (Zevalin, Biogen Idec Inc.) was conjugated with the high energy β-emitting radioisotope Yttrium-90 (⁹⁰Y) using a kit supplied by the manufacturer. Rats with MRI-confirmed tumor received 0.2 mCi/kg ⁹⁰Y-Zevalin given intravenously with or without BBBD. At 10 min, 24 h, or 3 days after mAb administration, rats (n = 3 per group per time point) were perfused with saline to clear the vasculature. The brains were harvested and radioactivity was measured in tumor, 1-2 mm brain around tumor, 1-2 mm ipsilateral brain distant to tumor, and contralateral (left hemisphere) normal brain.

Treatment study design. Rats with MRI-confirmed tumor were randomized into five groups: 1) Control with BBBD and intra-arterial saline (n=6); 2) methotrexate 1 g/m² intra-arterially with BBBD (n=6); 3) rituximab 375 mg/m² intravenously with BBBD (n=6); 4) rituximab 375 mg/m² intravenously + methotrexate 1 g/m² intra-arterially with BBBD (n=5); and 5) intravenous rituximab 375 mg/m² (n=7). Saline or methotrexate were injected into the internal carotid artery...
immediately after BBBD, and rituximab was given into the femoral vein just prior to BBBD. In the methotrexate groups (groups 2 and 4), a rescue regimen of folinic acid (Leucovorin 40 mg/kg intraperitoneal), sodium bicarbonate (334 mg/kg intraperitoneal), and subcutaneous saline was administered twice daily for three consecutive days starting 24 h after methotrexate treatment. The rats were scanned again seven days after treatment, or earlier (day 5-6 after treatment) if clinical symptoms necessitated early sacrifice. Some animals received a third MRI if they survived more than 2 weeks after treatment. The animals were examined and weighed at least weekly, and were sacrificed using intracardiac thiopental injection (0.5 ml) if they showed severe clinical symptoms or >20% weight loss. The predetermined end time for the study was 60 days (approximately 4X control survival); rats that survived to 60 days were killed.

The MC116 CNS lymphoma model shows inconsistent tumor take and growth, as demonstrated in our previous studies (26, 27). Therefore, rats were entered into the treatment study only if intracerebral tumor volume between 4 mm$^3$ and 40 mm$^3$ was confirmed on T2-weighted MRI. Rats underwent MRI beginning approximately 16 days after cell implantation; rats lacking tumors on MRI were scanned again at weekly intervals, and were excluded from the study if they did not meet the inclusion criterion by 4-6 weeks after tumor implantation. In rats lacking tumor, little or no signal change was detected along the needle track on T2-weighted MRI (data not shown). A total of 92 rats were used in this study; 87 rats survived the tumor inoculation, and 56 showed tumors on MRI (64% tumor take). Among these, 5 showed tiny tumors that did not grow to meet the inclusion criterion, 4 had tumors that were too large for inclusion, and 6 died due to anesthesia or technical issues during MRI. Thus 41 rats entered the survival study. Eleven rats died during or within 24 h of therapy, due to technical error or treatment-related toxicity. Treatment deaths were in the following groups: group 1, n=1; group 2, n=2; group 3, n=2; group 4, n=5; and group 5, n=1. The high number of deaths in group 4 receiving methotrexate and rituximab with BBBD reached the pre-determined stopping rule of three consecutive deaths or
50% of planned inclusion, so only 5 rats completed the study in this group. Methotrexate-treated rats showed no obvious signs of drug toxicity, such as diarrhea or precipitous weight loss. In total, 30 rats were evaluated for survival; 29 of these rats had both pretreatment and 1 week post-treatment MRI.

**MRI.** Rats were anesthetized with ketamine (60 mg/kg intraperitoneal) and dexmedetomidine (Dexdomitor™, Pfizer Animal Health, Exton, PA, USA; 0.5 mg/kg intraperitoneal), with Antesedan 1.2 mg/kg intraperitoneal following the procedure for reversal. Rats were imaged on a 3 Tesla MRI scanner (Siemens Magnetom Trio, Erlangen, Germany) using a custom rat head transmitter-receiver coil. The imaging sequences were: T1 spin echo (SE) with relaxation time (TR) = 750 ms and echo time (TE) = 12 ms; and T2 turbo spin echo (TSE; TR = 5430 ms, TE = 78 ms, turbo factor = 13). The voxel size was 0.26 x 0.26 x 2 mm for coronal scans. Horizontal and coronal T1 scans were done before and after intravenous gadolinium (Omniscan, Amersham Health AS, Oslo, Norway) at a dose of 0.1-0.3 mmol/kg. Pre- and post gadolinium T1-weighted MRI scans and T2-weighted images were evaluated for tumor response and changes in tumor characteristics by a neuroradiologist (ED) who was blinded to treatments. Images were uploaded in MIPAV (Medical Image Processing, Analysis, and Visualization, BIRSS; NIH, Bethesda, MD, USA). The lesions were outlined on each MR image and the software calculated the tumor volume. Volumetric analysis was expressed in units of cubic millimeter. T1-weighted volume measurements were generally smaller and more variable than the T2 data, consistent with our previous findings (26); therefore we present only the T2 results.

**Osmotic BBB**. Rats were anesthetized with isoflurane (5% induction, 2% maintenance AEErrane; Anaquest, Inc., Madison WI), then switched to 1.5 L/min of 50% N₂O and intravenous propofol anesthesia (800 µg/kg/min, Zeneca Pharmaceuticals, Wilmington DE). A catheter filled with heparinized saline was tied into the right external carotid artery for retrograde infusion (22,
Mannitol (25%) warmed to 37°C was infused into the right internal carotid artery via the right external carotid artery catheter at a rate of 0.09 ml/sec for 25 sec using a Harvard Instruments model 11-Plus constant flow pump (Harvard Apparatus Inc., Holliston MA). Rituximab was administered intravenously in a volume of 1-1.5 ml prior to BBBD. Methotrexate in 1-1.5 ml saline, or saline alone, was delivered via the carotid cannula over 5 min immediately after infusion of mannitol for BBBD. The external carotid artery was ligated after drug infusion, and the skin was sutured closed.

**Histology.** Brains were excised and fixed in 10% buffered formalin for vibratome sectioning at 100 µm in the coronal plane. For tumor volumetrics, every 6th brain section was stained with hematoxylin and then imaged at high resolution (35 µm pixel diameter) on an Epson 1640XL flatbed scanner using Adobe Photoshop software. Tumor volume was assessed using NIH ImageJ software by a biologist blinded to treatments (LLM) as previously described (26, 27). Histological volume included the caudate inoculation site and infiltrating tumor in the cortex, subdural space, and in the ventricles. Ventricle volume was measured in the brain section just anterior of the fornix, using NIH ImageJ software.

**Statistical considerations.** No power calculations were made *a priori* or *post hoc*. ⁹⁰Y-Zevalin delivery to tumor and brain regions was compared using a mixed model, repeated measures analysis of variance (ANOVA) with a first-order auto-regressive covariance structure. MRI tumor volume data obtained from T2-weighted scans were compared using the generalized Wilcoxon test. Differences from baseline (seven-day volume minus baseline volume) were compared using an ANOVA to compare each active arm to the control after adjusting for the baseline volume. A mixed model, repeated measures ANOVA model was also fit to T2 volumes using an unstructured covariance structure. T1 scans had smaller values but greater variations and were not fit. Covariance structures were selected from among multiple candidate structures using the
Akaike Information Criterion. Changes from baseline volume, with each animal serving as its own pre-treatment control, were also compared using Student’s t-test. Overall survival, calculated from the day of treatment until death, was estimated using the Kaplan-Meier product limit method. A Cox proportional hazards model was fit to compare groups (each active group is compared to control) and control for the percent change in T2-weighted tumor volume from day 0 to day 7. The significance level was 0.05 (two-sided) for all statistical tests. All analyses were performed with Microsoft Excel or version 9.2 of SAS® (SAS Institute, 2002-2008).

RESULTS

Pharmacology study. To evaluate the effect of BBBD on delivery of mAb to the CNS lymphoma tumor mass and tumor-infiltrated rat brain, we used $^{90}$Y-Zevalin, which targets CD20 expressed on B-cells, similar to rituximab (15, 21, 31). BBBD improved Zevalin delivery throughout the disrupted hemisphere compared to intravenous mAb administration (Figure 1A). At 10 minutes, there was a significant interaction ($p=0.0291$) between site in the brain and delivery method (BBBD vs. IV) in the repeated measures ANOVA model. Site ($p=0.0152$) and delivery method ($p=0.0361$) were also significant. Comparisons between the delivery methods were statistically significant only for brain distant to tumor ($p=0.0271$) and not for the other three sites ($p>0.25$). Levels of mAb in tumor-infiltrated brain around tumor and brain distant to tumor were elevated at 10 min and 24 h, but not 3 days (Figure 1B).

Treatment study. Early changes in tumor volumes were evaluated by MRI in 29 rats in the imaging and therapy study. Three response patterns were found: tumor growth on MRI with short survival (Figure 2A), tumor growth with long survival (Figure 2B), and decreased tumor volume (tumor response) with long survival (Figure 2C). No rats showed a decrease in tumor volume on MRI accompanied by short survival. In the control rat shown in Figure 2A, tumor...
volume increased from 10.3 mm$^3$ at baseline to 48.9 mm$^3$ at 6 days, and the animal died from tumor burden. Hematoxylin histochemistry demonstrated tumor in the caudate inoculation site, but also spreading to the subdural space and to the contralateral ventricle, which was not detected by MRI. Figure 2B shows a tumor that initially increased in volume on MRI after treatment with rituximab administered with BBBD, from a baseline of 7.4 mm$^3$ to 11.1 mm$^3$ at 1 week, and then subsequently regressed. Histochemistry after death at day 44 shows trauma and macrophage infiltration in the inoculation area, without tumor, while tumor growth is evident in the cortex, base of the brain, and contralateral ventricle. The tumor in Figure 2C showed a rapid response to the combination of rituximab and methotrexate administered with BBBD, from a baseline of 19.6 mm$^3$ to 4 mm$^3$ at 1 week, which was maintained for over 7 weeks. After sacrifice at the predetermined endpoint (60 days), histology demonstrated trauma and macrophage infiltration in the inoculation area, without tumor, with tumor growth detected in the cortex.

Baseline T2-weighted tumor volumes were not significantly different among all groups (Table 1, P=0.77). All control rats (saline with BBBD) showed more than a doubling of tumor volume at one week (Figure 3 and Table 1). Mean volume increased from 10.6 ± 3.9 mm$^3$ to 30.2 ± 11.3 mm$^3$ (mean 201 ± 102% increase from baseline). All tumors in Group 2 (methotrexate with BBBD) showed increased tumor volumes on T2-weighted MRI one week after treatment; however the mean increase of 70 ± 42% was reduced compared to controls (P=0.016). Among rats in all treatment groups receiving rituximab, over half (10 of 17) showed a decrease in tumor volume on MRI one week after therapy (Figure 3), but several animals showed markedly increased tumor volume. In the ANCOVA model, there is a significant difference between group 4 and control (p=0.0044) and between group 5 and control (p=0.0010). Group 5 (intravenous rituximab) demonstrated a significant decrease in tumor growth compared to control, with an
actual reduction of tumor volume from 18.8 ± 15.1 mm$^3$ at baseline to 10.4 ±10.1 mm$^3$ after 1 week (mean 39 ± 36% decrease in tumor volume).

Individual and mean survival times for each treatment group and Kaplan-Meier survival estimates are shown in Figure 4A and 4B respectively. Median survival in the saline control was 14 days (range 6-33). Survival time after methotrexate (median 7 days, range 5-19) was not significantly different from controls (P=0.18). All rituximab groups had increased survival compared to control: Group 3 (rituximab BBBD) median 37.5 days, range 20-60 days, P=0.013; Group 4 (rituximab and methotrexate BBBD) median 42 days, range 27-60, P=0.0042; and Group 5 (intravenous rituximab) median undefined, range 26-60, P=0.0049. There were no obvious differences among the rituximab groups, but the study lacks adequate power to determine if there were minor differences.

We evaluated differences among the groups and whether or not the percent change in T2-weighted tumor volume on MRI was associated with survival. There were significant differences between the control group and group 2 (hazard ratio=9.11; p=0.0081) and between the control group and group 5 (hazard ratio=0.14; p=0.0450). There were no significant differences between the control group and groups 3 (hazard ratio=0.30; p=0.092) and 4 (hazard ratio=0.29; p=0.091). The association between the change in T2 volume and survival was significant (p=0.017) with a hazard ratio of 1.85 (for each 100% increase in T2 volume).

Six rats, all from the rituximab treatment groups, survived until the predetermined endpoint of 60 days. Three of these rats showed complete tumor response on histology. In rats that died from tumor burden, histological tumor volume was similar between groups, although variability was increased in the treatment groups: control mean tumor volume at death was 182 ± 21 mm$^3$ (range 158-212 mm$^3$, n=6); methotrexate mean 213 ± 156 mm$^3$ (range 16-413 mm$^3$, n=6); and
combined rituximab treatment groups mean 232 ± 134 mm$^3$ (range 11-397 mm$^3$, n=9). In the controls, tumor was largely localized to the caudate nucleus inoculation site, although infiltration into the cortex, subdural space, and contralateral ventricle was common (Figure 2A). Many of the rituximab-treated tumors showed decreased tumor localization in the injection site and increased localization elsewhere (Figures 2B, 2C), although this was highly variable. Animals in the rituximab treatment groups that died from tumor burden showed increased cerebral ventricular volume (6.2 ± 4.0 mm$^3$, P=0.039; see Figure 2C) compared to the control (2.3 ± 0.9 mm$^3$) and methotrexate (2.1 ± 1.5 mm$^3$) treatment groups, which may represent hydrocephalus ex vacuo.

DISCUSSION

The investigation of CNS lymphoma biology, pathology, imaging characteristics, and novel treatment approaches, has been difficult in humans, mainly due to the rarity of the disease. Animal models of human CNS lymphoma are relatively rare (26, 27, 32). Intracerebral implantation of MC116 human B-cell lymphoma cells in nude rats provides an animal model of CNS lymphoma that closely mimics human PCNSL (26, 27). We have previously reported our experience with intravenous methotrexate- and rituximab-based treatment regimens in the rat model using changes in tumor volume on MRI imaging modalities as end points (26). The current study confirms our previous results and extends the findings to the effects of these agents on survival when delivery to brain tumor is enhanced with BBBD. The major finding is that rituximab was highly effective at extending survival in the rat model. Although BBBD enhanced the delivery of radiolabeled mAb to the CNS lymphoma model, a single treatment with rituximab BBBD did not improve survival compared to intravenous rituximab. Additionally, the efficacy of a single dose of rituximab was not improved by combination with methotrexate.
The imaging results in this study indicate that the snapshot provided by a single MRI scan is not sufficient to discern efficacy. Several of the tumors in the rituximab treatment groups initially increased in volume on the 1 week scan but then showed subsequent decreased tumor volume and long survival (Figure 2B), indicating that serial imaging may be more appropriate to monitor efficacy. Decreased tumor volume on MRI correlated with increased survival, but a partial response, as indicated by reduced rate of growth, did not. This mimics the human situation, in which PCNSL patients with partial responses to methotrexate chemotherapy have the same overall survival as those with progressive disease (33). The imaging did not show the subdural or intraventricular growth of tumor that was evident on histology (Figure 2). A contrast agent that can delineate inflammatory cell infiltration may be better than either T2-weighted images or standard T1-weighted MRI with gadolinium-based contrast for detecting infiltrative tumor. In this context, iron oxide nanoparticle MRI contrast agents have shown utility for imaging tumor-associated inflammation (34).

Previously we reported that MC116 cells are highly sensitive to methotrexate in vitro, but efficacy in vivo was modest, as determined by changes in tumor volume on MRI (26, 27). High dose methotrexate-based chemotherapy, with or without WBRT is commonly used to treat PCNSL, but responses are often transitory (4, 5, 7, 35). We have hypothesized that enhancing delivery of chemotherapy to the brain tumor with osmotic BBBD would improve the anti-tumor efficacy of methotrexate. Osmotic BBBD and intra-arterial methotrexate-based chemotherapy, without WBRT, is safe and effective in patients with newly-diagnosed PCNSL (5). In our recent report of 149 subjects, the overall response rate was 81.9% and median overall survival was 3.1 years. Low-risk patients (age less than 60 y with Karnofsky performance score greater than or equal to 70), survived a median of 14 years, with a plateau after about 8 years (5). These
results are superior to other clinical studies and were obtained without the cognitive loss due to WBRT.

We tested the hypothesis that enhanced delivery with BBBD would improve the efficacy of a single dose of methotrexate in the rat CNS lymphoma model. MRI one week after treatment confirmed that BBBD delivery of methotrexate slowed tumor growth but the magnitude of responses was not elevated compared to our previous report (26). Survival in the MC116 CNS lymphoma model was not enhanced by a single dose of methotrexate even when delivery was optimized with BBBD. A number of issues may explain the discordance between the in vitro sensitivity and positive MRI effects versus the lack of improvement in survival. This experiment used a single treatment with a relatively low dose of methotrexate (1 g/m²) compared to the clinical BBBD regimen of 2 consecutive days every 4 weeks for a year (5, 25). A higher dose (3 g/m²) was evaluated in several animals; however, this dose induced dehydration and diarrhea even with three days of hydration and folinic acid rescue. Rats showed no obvious toxicities at the 1 g/m² dose. Methotrexate has a 3-10 h half life, so the effective dose is low in the rat. BBBD can only be performed once in the rat, due to surgical cannulation of the external carotid artery (23, 24). Without extensive tumor kill with the single treatment, there may be accelerated repopulation after the initial slowed tumor growth, as demonstrated by increased tumor volumes at death in many animals in the methotrexate group. Overall, the transient response to a single low dose of methotrexate without increased survival shows that the rat model of CNS lymphoma is not appropriate to assess multiple courses with high dose chemotherapy.

Current clinical PCNSL treatment regimens with high dose methotrexate and WBRT are not optimal and are often neurotoxic, so it is critical to evaluate new approaches (4, 6, 8, 11). Rituximab seems an obvious choice for treatment of human PCNSL. Alone or in combination with chemotherapy, the anti-CD20 mAb is effective against non-Hodgkin’s B-cell lymphoma.
lacking CNS or ocular involvement (13, 14). The use of rituximab in PCNSL is supported by a number of preclinical studies (26, 36) and small clinical studies (3, 16, 17, 19, 20, 35, 37). In mouse models of CNS and intra-ocular lymphoma, intracerebral and intravitreal injection of rituximab, respectively, induced tumor regression (36). However, mice were treated only 1 d after cell injection, before the development of tumor, and the authors did not evaluate intravenous rituximab. In patients with newly diagnosed PCNSL, addition of rituximab nearly doubled the rate of complete response to an aggressive chemotherapy (methotrexate, procarbazine, and vincristine) and WBRT regimen (35). In recurrent PCNSL, rituximab has shown potential for disease control in combination with methotrexate (15), temozolomide (16), and multiple chemotherapy regimens (17).

Prior to this study, we hypothesized that intravenous rituximab would show minimal or transient efficacy in PCNSL, because the high molecular weight of rituximab and the low permeability of the BBB would limit the delivery of the mAb to the brain tumor. Others have argued that the BBB does not play a role in PCNSL, at least in the main tumor mass with its relatively permeable vasculature (2, 3, 19, 37). Data from anti-CD20 radioimmunotherapy supports the idea that the BBB limits efficacy. Both Doolittle et al. and Maza et al. have demonstrated responses targeting CD20 with intravenous $^{90}$Y-Zevalin radioimmunoconjugate (15, 21). Sufficient mAb crossed the leaky BBB within the PCNSL mass to provide an effective dose of radiotherapy to the tumor cells as demonstrated by regression of the original enhancing mass. However, response to Zevalin was transient, with recurrence in multiple additional locations distant to the original lesion (15). Nevertheless, the survival results indicate that the rat model may be effective for evaluating the potential efficacy of intravenous therapies.

In the CNS lymphoma model in immunocompromised athymic rats, single dose, single-agent rituximab was effective at increasing survival in the immunologically privileged site of the brain.
This result suggests that rituximab may be appropriate for immunocompromise patients who retain some immune function, as can occur with AIDS (38). In contrast to our original hypothesis, the efficacy of a single dose of rituximab was not enhanced by BBBD or by combination with methotrexate. The lack of additional effect from methotrexate is likely due to the single low dose and short half life, as discussed above. In contrast, the single dose of intravenous rituximab was effective alone. BBBD significantly enhanced delivery of $^{90}$Y-Zevalin in the MC116 CNS lymphoma model, but by 3 days there was no difference in mAb localization between the intravenous and BBBD groups. The half life of rituximab is 21 days (range 14 to 62 days) (39). We suggest that a slow leak of the long half life mAb into the main tumor mass, even in the absence of barrier opening, was then trapped by binding to CD20 on the tumor cells, attaining sufficient concentration for anti-tumor efficacy. In support of this hypothesis, in many rats the tumor mass in the caudate nucleus injection site, where permeability is highest, was eliminated by rituximab; however, the effect was transient. Tumor recurred in the subdural space, contralateral ventricle, and cortex distant to the original tumor mass (Figures 2B, 2C), similar to the findings with $^{90}$Y-Zevalin therapy in human PCNSL (15). Thereby, we hypothesize that in order to obtain a complete and durable response, multiple courses of enhanced delivery will be necessary to treat micrometastases in tumor-infiltrated brain far from the main enhancing tumor mass.

In conclusion, our results show good evidence of rituximab efficacy in a CNS lymphoma model and suggest that this mAb should be investigated as a single agent in the clinic. Rituximab may improve outcomes for patients with newly diagnosed PCNSL, either alone or in conjunction with high dose methotrexate, and allow patients to avoid or delay WBRT. We hypothesize that in PCNSL with areas of low BBB permeability in disseminated tumor, BBBD will enhance the efficacy of rituximab. Unpublished pilot data from this group in patients with newly diagnosed PCNSL treated with BBBD-enhanced delivery of rituximab in combination with high dose
methotrexate, without WBRT, suggests that rituximab increases progression-free survival to 3.5 years compared to previously reported progression-free survival of 1.8 years without rituximab (5). A multi-center clinical trial of rituximab with high dose methotrexate-based BBBD in patients with newly-diagnosed PCNSL is currently underway.

ACKNOWLEDGEMENTS: This study was supported by a Veterans Administration Merit Review Grant and by NIH grants NS053468, CA137488, and NS44687 to EAN.

Disclosure of Conflicts of Interest: No author has a financial conflict of interest.
References:


Table 1. Early anti-tumor efficacy determined by T2-weighted MRI tumor volumetrics

<table>
<thead>
<tr>
<th>Group</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>3, 4, 5</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Treatment</td>
<td>Control</td>
<td>MTX</td>
<td>RTN</td>
<td>RTN+MTX</td>
<td>RTN IV</td>
</tr>
<tr>
<td>Volume day 0 (mm³)</td>
<td>10.6 ± 3.9</td>
<td>12.5 ± 9.9</td>
<td>10.1 ± 4.7</td>
<td>14.4 ± 9.8</td>
<td>18.8 ± 15.1</td>
<td>14.4 ± 10.8</td>
</tr>
<tr>
<td>Volume day 7 (mm³)</td>
<td>30.2 ± 11.3*</td>
<td>19.5 ± 14.3*</td>
<td>22.4 ± 22.5</td>
<td>10.6 ± 4.9**</td>
<td>10.4 ± 10.1**</td>
<td>14.7 ± 15.2*</td>
</tr>
<tr>
<td>Volume change (mm³)</td>
<td>19.5 ± 9.8</td>
<td>7.0 ± 5.3*</td>
<td>12.3 ± 18.1</td>
<td>-3.8 ± 10.6**</td>
<td>-8.5 ± 10.5***</td>
<td>0.3 ± 15.9*</td>
</tr>
<tr>
<td>% change</td>
<td>201 ± 102</td>
<td>70 ± 42*</td>
<td>88 ± 105</td>
<td>51 ± 176</td>
<td>-39 ± 36***</td>
<td>32 ± 122**</td>
</tr>
</tbody>
</table>

Mean ± s.d. are indicated for tumor volume in mm³ on day 0 before treatment and day 5-7 after treatment, the absolute change in volume (in mm³), and the paired % change comparing treatment to baseline. MTX = methotrexate; RTN = rituximab; all RTN = combined groups 3, 4, and 5. Significant differences are indicated as * P<0.05, ** P<0.01, *** P<0.001 compared to control; ^ P<0.05 compared to day 0.
FIGURE LEGENDS

Figure 1. **BBBD increases antibody delivery in the rat CNS lymphoma model.** Rats with intracerebral MC116 xenografts received $^{90}$Y-Zevalin (0.2 mCi/kg) intravenously with or without BBBD (n = 3 per group per time point). (A) Radiolabel in tumor, brain around tumor (BAT), ipsilateral brain distant to tumor (BDT), and contralateral left hemisphere (LH) was determined 10 min after antibody administration. There was a significant effect of BBBD in the repeated measures ANOVA model (P=0.0361). (B) Radiolabel localized in BDT is shown at 10 min, 24 h, and 3 days after antibody administration. Data are indicated as mean ± standard deviation of % delivered dose.

Figure 2. **MRI and histology of response patterns in the CNS lymphoma model.** Rats with intracerebrally implanted MC116 CNS lymphoma underwent T2-weighted MRI before and approximately 1 week after treatment, with additional follow up MRI if applicable (day of MRI indicated on each scan). At death or sacrifice, hematoxylin histochemistry (htx) was performed to demonstrate pathology and tumor location and size.

**A. Tumor growth in a control rat.** This rat received IA administration of saline with BBBD. The second MRI (middle) was performed at 6 days due to clinical symptoms of tumor burden, and shows an example of tumor growth on MRI (arrow). Hematoxylin histochemistry immediately after the 6 day MRI (right) shows tumor in the inoculation area (arrow), extending into the cortex in brain around tumor, as well as tumor in the subdural space and contralateral ventricle (arrow heads).

**B. Late response to rituximab.** The 1 week MRI shows tumor growth (arrow), while the follow up MRI shows near complete regression 14 days after administration of rituximab intravenously with BBBD. Hematoxylin histochemistry (right) after death at day 44 shows macrophage infiltration in the inoculation area (arrow), without tumor, with tumor growth in the cortex, base of the brain, and both ventricles (arrow heads).
C. Early response to rituximab. MRI shows decreased tumor volume (arrow) 7 days after treatment with rituximab and methotrexate intra-arterially with BBBD, with maintained near complete regression 48 days after treatment. Hematoxylin histochemistry (right) after death at day 60 shows macrophage infiltration in the inoculation area (arrow), without tumor, with tumor growth in the cortex (arrow head), and enlarged ventricles.

Figure 3. Changes in tumor volume on MRI show early responses to therapy. Rats with intracerebrally implanted MC116 CNS lymphoma underwent T2-weighted MRI on day 16-26 prior to entering the treatment groups, and approximately 1 week after treatment. The change in absolute tumor volume from baseline is indicated for each rat, and the mean is shown by the line. In the ANCOVA model, there is a significant difference between group 4 and control (p=0.0044) and between group 5 and control (p=0.0010). MTX, methotrexate; RTN, rituximab.

Figure 4. Rituximab increases survival in the rat CNS lymphoma model. Rats with MRI-confirmed MC116 CNS lymphoma were randomized to treatment groups and followed for survival. (A) Survival time is indicated for each rat, and the mean is shown by the line. (B) The Kaplan-Meier survival curves demonstrate that MTX was ineffective in this model (P=0.18), while all rituximab (RTN) groups improved survival compared to control (RTN BBBD P=0.013; RTN+MTX BBBD P=0.0042; RTN IV P=0.0049). MTX, methotrexate; RTN, rituximab.
Survival time (days)

Control  MTX  RTN  RTN + MTX  RTN IV
BBBD  BBBD
Imaging and therapy with rituximab anti-CD20 immunotherapy in an animal model of central nervous system lymphoma


Clin Cancer Res  Published OnlineFirst March 8, 2011.

Updated version
Access the most recent version of this article at:
doi:10.1158/1078-0432.CCR-10-2923

Author Manuscript
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, use this link http://clincancerres.aacrjournals.org/content/early/2011/03/05/1078-0432.CCR-10-2923. Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.