Efficacy and MRI of rituximab and methotrexate treatment in a nude rat model of CNS lymphoma

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To determine the efficacy of methotrexate and/or rituximab in a CNS lymphoma model and to evaluate MRI modalities for monitoring efficacy, we inoculated female athymic nude rats (*rnu/rnu*) intracerebrally with human MC116 B-lymphoma cells. Between days 16 and 26, rats were randomized to receive intravenous (IV) treatment with (1) saline (controls, $n = 15$), (2) methotrexate 1,000 mg/m² ($n = 6$), (3) rituximab 375 mg/m² ($n = 6$), or (4) rituximab plus methotrexate ($n = 6$). T2/fluid-attenuated inversion recovery (FLAIR) and gadolinium contrast-enhanced T1 MRI sequences were performed prior to and 1 week after treatment. IV rituximab gave an objective tumor response in four of six animals ($>50\%$ reduction in tumor volume comparing pre- and posttreatment T2/FLAIR MRI) and resulted in stable disease (50%–125% of baseline) in another animal. The percent change in tumor volume on T2/FLAIR images was significantly different in the control versus rituximab group ($p = 0.0051$). IV methotrexate slowed tumor growth, compared to controls, but only one of six ani-

mals had an objective response. In untreated controls, tumor histological volumes correlated well with T2/FLAIR or contrast-enhanced T1 images ($r = 0.877$). In the treatment groups, T2/FLAIR correlation was good, but the gadolinium-enhanced T1 MRI was not significantly correlated with histology ($r = 0.19$). The MC116 CNS lymphoma model seems valuable for preclinical testing of efficacy and toxicity of treatment regimens. IV rituximab was highly effective, but methotrexate was only minimally effective. T2/FLAIR was superior to contrast-enhanced T1 for monitoring efficacy. *Neuro-Oncology* 11, 503–513, 2009 (Posted to *Neuro-Oncology* [serial online], Doc. D08-00178, January 21, 2009. URL <http://neuro-oncology.dukejournals.org>; DOI: 10.1215/15228517-2008-119)

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The incidence of primary CNS lymphoma (PCNSL) has increased almost 3-fold since the 1970s.¹ The optimal treatment for PCNSL is yet to be defined. Postoperative whole-brain radiation therapy (WBRT) improves median survival up to 18 months, but responses are not sustained.² The use of high-dose intravenous (IV) methotrexate alone is associated with survival times of 22.8–25 months.^{3,4} For chemotherapy regimens based

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on methotrexate, a 5-year survival rate of up to 43% has been reported.⁵ Chemoresistance of PCNSL is due at least in part to the blood–brain barrier (BBB) that limits the penetration of chemotherapy into the tumor and the surrounding brain.^{6–8} Combined modality therapy with high-dose methotrexate-based chemotherapy and WBRT improves response and survival rates compared with WBRT alone, and 5-year survival rates range from 9% to 32%.^{9–12} However, this approach is associated with high rates of delayed neurotoxicity, particularly in long-term survivors older than 60 years of age.^{9,10,13,14} Although current therapies have high rates of recurrence and treatment-induced neurotoxicity, high-dose systemic methotrexate, with or without WBRT, remains a standard of care for PCNSL.¹⁵ It is important to develop new therapeutic approaches with increased efficacy that decrease the need for WBRT.

The anti-CD20 monoclonal antibody (mAb) rituximab (Rituxan) and the anti-CD20 mAb radioimmunoconjugate ⁹⁰Y-ibritumomab tiuxetan (Zevalin) have significantly improved outcomes in systemic non-Hodgkin lymphoma.^{16,17} Rituximab has also shown some efficacy in PCNSL,^{18–20} and Doolittle et al.¹⁸ have demonstrated initial responses with the yttrium radioimmunoconjugate. The intact BBB is relatively impermeable to antibodies due to their high molecular weight; therefore, poor CNS delivery must be assumed.^{7,21,22} Conversely, the long plasma half-life of rituximab and the relative high permeability of the blood–tumor barrier may provide adequate delivery of the mAb to achieve antitumor effects. In this report, we tested the efficacy of rituximab and/or methotrexate in an animal model of CNS lymphoma that closely mimics the clinical situation.²³

MRI studies in our previous report suggested that T2-weighted images provided a more accurate representation of tumor size than did T1-weighted sequences with gadolinium contrast.²³ In immunocompetent human subjects, PCNSL is characterized by lesions in contact with the cerebrospinal fluid spaces, which typically show strong enhancement on T1-weighted sequences after IV gadolinium.^{24,25} Brain tumor response criteria are based on reduction in or complete absence of contrast enhancement on MR images,²⁶ although it has been acknowledged that these criteria may not be completely accurate for PCNSL.²⁷ Here, we report our experience using serial MRI to evaluate outcome in the CNS lymphoma model and a comparison of MRI modalities for monitoring efficacy.

Materials and Methods

Tissue Culture

The MC116 human B-cell lymphoma cell line (American Type Culture Collection, Manassas, VA, USA) is Epstein-Barr virus and human immunodeficiency virus negative²⁸ and forms an infiltrative CNS lymphoma when injected in nude rat brain.²³ Cells were cultured in suspension in RPMI 1640 medium supplemented with 20% fetal bovine serum, 2 mM L-glutamine, 10 mM HEPES

buffer, and antibiotics. Cells were harvested immediately prior to intracerebral implantation and were used only if viability exceeded 80%. For in vitro analysis of toxicity, MC116 cells were plated at 1×10^4 to 1×10^5 cells/ml in 12-well or 24-well tissue culture plates and treated for 2–6 days with methotrexate (0.01–1 μ M), with rituximab (20–500 μ g/ml), or with a combination of both. Toxicity was assessed by trypan blue exclusion in three wells per condition.

Animal Use and Tumor Inoculation

The care and use of animals in this study was approved by the Institutional Animal Care and Use Committee and were supervised by the Oregon Health and 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: 81 rats in the efficacy and MRI study, and an additional eight rats for toxicity assessment. All rats were treated with intraperitoneal (IP) cyclophosphamide (300 mg/m²) 24 h prior to tumor implantation to decrease innate immunity,²⁹ as a mechanism to improve the consistency of tumor growth.^{23,30} Intracerebral tumor inoculation was performed as previously described.²³ Rats were anesthetized with IP ketamine (60 mg/kg) and IP diazepam (7.5 mg/kg). Animals received 1.2 to 1.5×10^6 of >80% viable MC116 cells in a volume of 15 μ l, stereotactically injected in the right caudate-putamen (vertical, bregma 5 mm; lateral, bregma 3 mm). The needle was initially advanced to a depth of 5.5 mm and then withdrawn to a depth of 5 mm to limit reflux up the needle track. The animals were examined daily and weighed at least weekly. Animals were sacrificed if they showed severe clinical signs or symptoms or >20% weight loss.

Treatment Study Design

MRI was performed between days 16 and 20 after cell implantation to confirm the presence of intracerebral tumor prior to treatment. Three rats that were negative for tumor on day 19 had tumor on day 26 and received treatment on that same day. The time point of treatment was chosen based on the availability of MRI and our previous experience with the growth and treatment of this model.²³ Rats with no evidence of tumor on both MRI scans, atypical tumor location away from the needle track and the caudate-putamen, or tumor volumes < 4 mm³ were not included in the data analysis. The exclusion of tumors < 4 mm³ was based on our previous experience that below this volume, further tumor growth in control animals is rarely observed.²³ The rats were scanned again 7 days after treatment, or earlier (day 5 or 6 after treatment) if clinical symptoms necessitated early sacrifice.

Rats with MRI-confirmed tumor were randomized into four groups: (1) control group with IV saline ($n = 15$), (2) IV methotrexate 1,000 mg/m² ($n = 6$), (3) IV rituximab 375 mg/m² ($n = 6$), or (4) IV rituximab 375 mg/m² plus IV methotrexate 1,000 mg/m² ($n = 6$).

Drugs were injected into the femoral vein in isoflurane-anesthetized rats. In the methotrexate groups (groups 2 and 4), IP folinic acid (10 mg) was administered twice daily for 3 consecutive days starting 24 h after methotrexate treatment. Immediately after the second MRI, a complete blood count was obtained for assessment of treatment-related hematological toxicity via intracardiac puncture under isoflurane anesthesia, and then the animals were sacrificed using intracardiac thiopental injection (0.5 ml). Eight additional animals were evaluated only for blood/bone marrow toxicity at the posttreatment time point.

MRI

Rats were anesthetized with IP ketamine (60 mg/kg) and IP medetomidine (Domitor; Pfizer Animal Health, Exton, PA, USA; 0.5 mg/kg) and imaged on a 3-T 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, T2 turbo SE (TR, 5,430 ms; TE, 78 ms), and fluid-attenuated inversion recovery (FLAIR; TR, 9,280 ms; TE, 89 ms; inversion time, 2,100 ms). The voxel size was $0.26 \times 0.26 \times 2$ mm for coronal scans. T1 scans were done before and after IP gadolinium (Omniscan, Amersham Health AS, Oslo, Norway) at a dose of 0.5–0.6 mmol/kg. IP gadolinium for MRI studies was administered at a higher dose than IV gadolinium as in our previous study²³ in order to obtain a similar contrast enhancement pattern (Fig. 1A). Pre- and postgadolinium T1-weighted MRI scans (Fig. 1A) and T2/FLAIR images (Fig. 1B) were evaluated for tumor response and changes in tumor characteristics by a neuroradiologist (C.G.V.) who was unaware of treatment status. Tumor volume was determined by measuring the longest axis and width of the tumor on coronal images and multiplying by the height on horizontal scans.

Histology

Brains were excised and fixed in 10% buffered formalin for vibratome sectioning at 100 μ m in the coronal plane. For tumor volumetrics, every sixth brain section was stained with hematoxylin and then imaged at high resolution (30- μ m pixel diameter) on an Epson 1640XL flatbed scanner using Adobe Photoshop software (Photoshop Education version 7.0.1, Adobe Systems Inc.). Tumor volume was assessed using NIH ImageJ software by a biologist unaware of treatment status (L.L.M., ImageJ 1.37v [http://rsb.info.nih.gov/ij]) as previously described.²³ Histological volume included the caudate inoculation site and infiltrating tumor in the cortex and crossing the midline along the corpus callosum, but did not include tumor infiltration in the subdural space, along the base of the brain, or in the ventricles, such as the ventricle indicated by the arrow in Fig. 1C. Immunohistochemistry was performed using the pan-leukocyte marker CD45 (sc-1187), the B-cell marker CD20 (sc-7733; Fig. 1D), and anti-CD31 (sc-46694; Fig.

1E), a marker for neovascularization. All antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Immunoreactivity was visualized by incubation with the appropriate biotinylated secondary antibody followed by avidin and biotinylated peroxidase (Vectastain ABC kit, Vector Labs, Burlingame, CA, USA). Brown reaction products were formed with diaminobenzidine.

Statistical Considerations

The baseline tumor volume was highly variable and not normally distributed; therefore, volume data are presented as median and minimum/maximum values in Table 1. All treatment groups had some very small tumors and some very large tumors, as demonstrated by the range of volumes in Table 1. All efficacy data are presented as changes from baseline, with each animal serving as its own pretreatment control. Two measures of antitumor efficacy were assessed comparing tumor volumes between the pre- and posttreatment MRI, for both the T2/FLAIR and gadolinium-enhanced T1 scans. First, we assessed the percent change in tumor volume on posttreatment versus pretreatment MR images. Second, we assessed whether tumors had an objective response (>50% shrinkage on posttreatment scans). Stable disease (<50% decrease in volume and $\leq$ 25% increase in volume) or progressive disease (>25% increase in volume) were both assessed as no response. For percent changes in volume, Kruskal-Wallis nonparametric analyses were performed separately for T2/FLAIR and gadolinium-

Fig. 1. MRI and histology of the rat CNS lymphoma model. An untreated control rat with intracerebral MC116 B-lymphoma MR image with a 3-T scanner, using T1-weighted sequences with gadolinium enhancement (A) or T2-weighted sequences (B). Immediately after MRI, the rat was sacrificed and brain taken for histology. Vibratome sections were stained for (C) hematoxylin (Htx). The arrow shows spread to the opposite ventricle. Adjacent sections were immunostained for CD20 B-cell marker (D) and CD31 (platelet endothelial cell adhesion molecule), a marker of neovascularization (E). Histology original magnification, $\times 3$.

Table 1. Absolute and relative differences in tumor volumes, comparing pretreatment and posttreatment MR images and posttreatment histology^a

Group (n)	Median (Minimum/Maximum), mm ³		Volume Change from Baseline	
	Initial Volume	Final Volume	Median (Minimum/Maximum), mm ³	Median, %
Control (15)				
T1 + Gd	5.6 (2.2/20.5)	13.7 (2.3/62.0)	8.0 (−2.0/41.5)	187
T2/FLAIR	7.7 (2.6/72.8)	16.0 (5.3/184.3)	7.8 (−2.4/111.5)	196
Histology ^a		17.4 (3.3/90.3)		
Rituximab (6)				
T1 + Gd	11.7 (0/32.6)	4.3 (2.0/82.1)	−3.6 (−14.4/49.4)	41 ^b
T2/FLAIR	16.3 (5.8/113.3)	5.0 (2.0/92.1)	−8.6 (−35.2/48.7)	37
Histology ^a		15.6 (2.8/32.7)		
MTX (6)				
T1 + Gd	20.3 (0/71.8)	17.1 (0/35.7)	0.3 (−36.2/6.1)	103 ^b
T2/FLAIR	26.0 (8.1/128.2)	19.0 (7.8/186.5)	−1.7 (−20.9/58.2)	92
Histology ^a		30.8 (1.1/174.5)		
Rituximab + MTX (6)				
T1 + Gd	13.5 (5.5/111.7)	11.0 (2.1/69.5)	−4.2 (−95.6/59.7)	65
T2/FLAIR	12.0 (5.9/170.8)	35.8 (2.9/227.6)	28.0 (−19.6/56.8)	220
Histology ^a		48.3 (1.0/153.2)		

Abbreviations: Gd, gadolinium; FLAIR, fluid-attenuated inversion recovery; MTX, methotrexate.

^aHistological tumor volume is for parenchymal tumor only, not including tumor detected in the subdural space, ventricles, or base of brain.^bDue to zero volume at baseline, the percentage change is undefined for one rat in each of these two groups.

enhanced T1. The data were skewed within treatment groups, meaning analysis of variance models were not appropriate. If this result was significant at a level of 0.05 (i.e., there was no Bonferroni adjustment of the significance level for testing multiple analyses), pairwise comparisons between groups were performed using the Wilcoxon rank sum test with Bonferroni adjustment for the number of pairwise comparisons, for a comparison-level significance of 0.083. Rates of response were also compared separately for the T2/FLAIR and gadolinium-enhanced T1 images using an extension of Fisher's exact test. If this test was significant at a level of 0.05, each pair of groups was compared using the two-tailed Fisher's exact test with Bonferroni adjustment as described above. The agreement of the response rates between T2/FLAIR and T1 with gadolinium was also compared using the κ statistic. To determine the correlation of imaging modalities with histology, the calculated final MR image tumor volumes for each animal were compared with the actual tumor volumes determined on hematoxylin stain histology using the Pearson correlation. All analyses were performed with Microsoft Excel or version 9.1 of SAS (SAS Institute Inc., Cary, NC, USA).

Results

Characterization of the MC116 CNS Lymphoma Model

A total of 81 rats were inoculated for the efficacy and MRI study. Deviating from our previously used meth-

ods,²³ IP cyclophosphamide (300 mg/m²) was administered 24 h prior to tumor implantation in all rats to decrease innate immunity and to increase the inoculation success rate.^{29–31} On MR images 16–20 days after tumor implantation, 57 of 74 evaluable rats (73%) had evidence of tumor on T2/FLAIR and/or gadolinium-enhanced T1-weighted images. Seven rats died prior to the first MRI. Imaging characteristics of the MC116 CNS lymphoma model confirmed our previously reported findings,²³ with small areas showing contrast enhancement on T1-weighted images with gadolinium (Fig. 1A), and relatively larger areas showing high signal on T2-weighted or FLAIR sequences (Fig. 1B). Tumor sizes in the rat CNS lymphoma model were highly variable on MR images. The median and minimum/maximum of tumor volumes on pretreatment and posttreatment MRI scans are shown in Table 1 for both T2/FLAIR and gadolinium-enhanced T1 sequences.

As on MR images, median tumor volumes on histology were highly variable and did not statistically differ between the groups (Table 1). The MC116 intracerebral tumors showed diffuse hematoxylin staining in the injected cerebral hemisphere as well as leptomeningeal spread (Fig. 1C). The tumors showed cell surface immunostaining for human CD20, a B-cell marker (Fig. 1D), and for CD45, a pan-leukocyte marker. We evaluated tumor vascularity by immunohistochemistry for CD31 (platelet endothelial cell adhesion molecule), a marker of neovascularization. All brains showed CD31 reactivity in and around the area of tumor (Fig. 1E).

Table 2. Toxicity one week after treatments

Group	White Blood Cells, $\times 10^9/\text{liter}$		Platelets, $\times 10^9/\text{liter}$		<i>n</i> ^a
	Median	Minimum/Maximum	Median	Minimum/Maximum	
Control	2.2	0.7/8.8	264	61/1,035	6 (4)
Rituximab	2.6	2.1/7.4	502	116/1,153	4 (2)
Methotrexate	0.7	0.3/1.8	301	60/542	5 (2)
Rituximab + methotrexate	0.4	0.2/3.4	77	43/731	7 (6)

^aNumbers in parentheses indicate animals from the treatment and MRI study included in the blood counts.

Toxicity of Treatments in the CNS Lymphoma Model

Despite folinic acid rescue, clinical toxicity of IV methotrexate treatment was significant and frequently included anorexia, facial or generalized edema, weight loss, and agony. Due to treatment-related toxic effects, several animals could not be included in the study: three animals treated with methotrexate alone had to be sacrificed early, and three rats that received methotrexate in combination with rituximab died less than 1 week after treatment. A subset of animals surviving through the posttreatment MRI were evaluated for hematological toxicity. Additional animals were tested in each study group to assess toxicity (Table 2). Although the rats receiving methotrexate had decreased white blood cell counts at 1 week after treatment, the blood counts did not differ significantly from the control rats due to the very large range of normal values in these animals. Similarly, the animals receiving both rituximab and methotrexate appeared to have low platelet levels (Table 2), but again, this was not significantly different between the study groups.

Efficacy of IV Chemotherapy and mAbs

In vitro, rituximab alone was nontoxic in the MC116 cells after a 3-day treatment at doses up to 500 $\mu\text{g}/\text{ml}$ (data not shown). We have previously reported that MC116 cells are sensitive to methotrexate in vitro.²³ To further assess the sensitivity of these B-lymphoma cells to methotrexate, the dose response and time course for cytotoxicity of low-dose chemotherapy were determined by trypan blue exclusion (Fig. 2A, B). MC116 cells grew exponentially in culture with a doubling time of approximately 24 h. Treatment with 0.1 μM methotrexate decreased cell viability by >80% within 48 h (Fig. 2A). At doses of 0.03 μM and 0.05 μM , methotrexate significantly reduced cell growth over the course of 6 days, while 0.1 μM methotrexate abolished cell growth (Fig. 2B). Rituximab did not alter the dose response to methotrexate in vitro (data not shown).

Treatment efficacy in vivo was established by determining the tumor volumes in the MRI scans 1 week after treatment in comparison to the tumor volumes in the baseline MR image prior to treatment. Separate

Fig. 2. In vitro toxicity of methotrexate (MTX). MC116 human B-lymphoma cells were cultured in the absence or presence of methotrexate at 0.01–1 μM for 2–6 days. (A) Dose response for cell viability after treatment for 2 days (triangles) or 4 days (circles). (B) Time course for the change in cell number as a percentage of the untreated control cultures. Viability and cell number were determined by the trypan blue exclusion assay. Data represent the mean and standard deviation of $n = 3$ wells per point, from two independent experiments.

Fig. 3. Efficacy of tumor treatment. Rats with MRI-confirmed CNS lymphoma were untreated (control) or treated with rituximab (RTX), methotrexate (MTX), or rituximab plus methotrexate (RTX/MTX). The mean $\pm$ SEM change in tumor volume is shown for T1-weighted with gadolinium contrast (T1 + Gd) MRI scans (A) and T2/FLAIR scans (B), comparing pretreatment and posttreatment images.

analyses were performed for the T2/FLAIR and T1 gadolinium-enhanced scans. The median changes in tumor volume between the pre- and posttreatment MRI scans are summarized in Table 1. The mean and standard deviation of the percent change from baseline MR image volumes are shown in Fig. 3. In control (saline IV) animals, median tumor volume 1 week after treatment was 196% of baseline (range, 74%–426%) on T2/FLAIR scans, and 187% of baseline (range, 74%–578%) on T1 gadolinium-enhanced scans. Volumes in animals treated with IV rituximab decreased to a median of 37% of baseline on T2/FLAIR and 41% on T1 plus gadolinium images, and these values were significantly different from the saline controls ($p = 0.0051$ for T2/FLAIR; $p =$

0.0164 for T1 plus gadolinium). Methotrexate treatment reduced tumor growth compared to the control, but this was not significant after the Bonferroni adjustment for multiple comparisons for either T2/FLAIR or T1 plus gadolinium. The combination of rituximab plus methotrexate was not as effective as each agent alone.

We assessed whether tumors had an objective response ($>50\%$ shrinkage on posttreatment scans), stable disease ($<50\%$ decrease in volume and $\leq 25\%$ increase in volume), or progressive disease ($>25\%$ increase in volume). There were significant differences among the groups with respect to both T1 gadolinium-enhanced images ($p = 0.0061$; Fig. 4A) and T2/FLAIR images ($p = 0.0034$; Fig. 4B). For each of these, the differences

Fig. 4. Response rates. Rats with MRI-confirmed CNS lymphoma were untreated (control) or treated with rituximab (RTX), methotrexate (MTX), or rituximab plus methotrexate (RTX/MTX). Tumor objective response ($>50\%$ shrinkage on posttreatment scans, cross-hatched bars) and stable disease ($<50\%$ decrease in volume and $\leq 25\%$ increase in volume; solid bars) were determined on T1-weighted gadolinium-enhanced (T1 + Gd; A) and T2/FLAIR (B) MRI scans.

between control and rituximab were significant ($p = 0.0017$ for T2/FLAIR and $p = 0.0049$ for T1 plus gadolinium) while the other pairs of groups did not differ, with or without Bonferroni adjustment. The agreement between responses as assessed by T2 scans compared to T1 gadolinium-enhanced sequences was moderate to good ($\kappa = 0.688$).

Correlation of MRI and Histology

One of the goals of this study was to determine if serial MRI could be used to evaluate tumor response in the rat CNS lymphoma model and to assess whether T2/FLAIR images or contrast-enhanced T1-weighted images were superior for monitoring this tumor. Tumor growth in the subdural space, in the ventricles, or along the base of the brain was detected by histology in 12 of 43 rats in this study. These areas of tumor growth were difficult

to detect on MR images and were impossible to quantify for technical reasons. Therefore, the analysis of the correlation of histology and MR image volumetrics was limited to tumor within the brain parenchyma, including the caudate inoculation site and infiltration into the cortex and across the midline along the corpus callosum. For example, the tumor located in the left ventricle on histology in Fig. 1C (arrow) was not included in the volume measurement for either the MRI volumes or the histological volumes (Table 1). On the T1-weighted scans, there was subtle gadolinium enhancement in the right cerebral hemisphere before treatment (Fig. 5A, dotted line). In contrast, T1 gadolinium-enhanced images showed small or even nil areas of enhancement in many tumors after treatment (Fig. 5B). In the treated tumors, the T2/FLAIR images matched visually with tumor histology (Fig. 5, C and D vs. E).

Fig. 5. Comparison of MRI and histology of a rat with CNS lymphoma: T1-weighted gadolinium-enhanced (T1 + Gd) (A and B) and T2-weighted (T2) (C, D) sequences before treatment (A, C) and 1 week after treatment with rituximab plus methotrexate (B, D). On the T1-weighted scans, the dotted lines indicate subtle gadolinium enhancement in the right cerebral hemisphere before treatment (A) and increased local enhancement in the cortex after treatment (B). Immediately after the second MRI, the brain was sectioned for histology and stained with hematoxylin (E; original magnification, $\times 3$).

Fig. 6. Correlation of MRI and histology. Tumor volumes were measured on the posttreatment MR images in treated animals ($n = 18$). Volumes on T2/FLAIR images (circles) or T1 gadolinium-enhanced images (triangles) are shown in comparison to histological tumor volumes. Best-fit linear regression and Pearson r -values are shown.

In control tumors, both imaging approaches had good correlations with histology (T2/FLAIR, $r = 0.873$; T1 plus gadolinium, $r = 0.877$). Combining all treatment groups, the T2/FLAIR correlation with histology was $r = 0.89$ (Fig. 6). The T1 gadolinium-enhanced image correlation is $r = 0.19$ (Fig. 6). The slope of the T1 plus gadolinium versus histology line is not significantly different from zero. These results suggest that some of the responses noted on T1 gadolinium-enhanced images shown in Figs. 3A and 4A were false positives.

Discussion

The investigation of CNS lymphoma treatment, biology, pathophysiology, and pathology has been difficult in humans, mainly due to the rarity of the disorder. Intracerebral implantation of MC116 human B-lymphoma cells in nude rats provides an animal model that closely mimics the characteristics of human CNS lymphoma.²³ Here, we report our experience with methotrexate- and rituximab-based treatment regimens in this model and comparison of MRI modalities.

The efficacy measures in this study involved changes in tumor volumes on MR images. Due to the high variability in baseline tumor size, it was not appropriate to use absolute tumor sizes on posttreatment MRI or histology for assessment of treatment response. Instead, we used absolute and relative changes in tumor volumes between pre- and posttreatment MR images, a method also most commonly used in the clinical setting to evaluate CNS tumor response to treatment. The differences between the control arm and the rituximab arm of this study were significant using both T2/FLAIR sequences and T2/FLAIR or T1 gadolinium-enhanced sequences. The statistical analyses used account for the

lack of normally distributed data in these measures and, by use of the Bonferroni adjustment, are also conservative with respect to pairwise comparisons among groups.

There was a significant correlation between tumor volumes within brain parenchyma on MR images and histology in untreated tumors, thus making MRI a valid tool for assessing tumor growth in the MC116 CNS lymphoma model. Median tumor sizes on contrast-enhanced T1 sequences appeared to be smaller than those on T2/FLAIR images in all treatment groups, a finding consistent with the situation in humans.²⁴ The extent of gadolinium enhancement is known to represent the central tumor area with a somewhat leaky BBB, whereas changes on T2/FLAIR are usually less specific for tumor and may as well represent peritumoral edema and brain areas microscopically infiltrated by tumor cells. In untreated animals, the correlation was equally good between the T2/FLAIR and histological volume and between T1 plus gadolinium and histological volume. In treated animals, T2/FLAIR tumor volumetrics correlated closely with histology, as shown in Figs. 1 and 5. In contrast, T1-weighted gadolinium-enhanced MRI did not correlate with histology. Thus, it is clear from the comparisons of MR images and histology that some of the apparent responses on T1 plus gadolinium images were in fact false positives.

Our data confirm observations made in the clinical setting, indicating that both methotrexate and rituximab are effective in PCNSL. However, due to the lack of clinical phase III trials as a result of the rarity of the disease, no definite conclusions can be drawn regarding the superiority of treatment regimens in PCNSL. The standard of care for PCNSL is high-dose systemic methotrexate, with or without WBRT,^{15,32} mainly because there is extensive phase II data on these treatment options. Human PCNSL shows variable sensitivity to methotrexate-based chemotherapies, with complete remission rates ranging from 30% to 50% after multiple courses of chemotherapy.^{3,4,33–35}

Response rates to methotrexate in the MC116 CNS lymphoma model were modest. Possible explanations for the limited efficacy of methotrexate in our study are the comparatively low number of rats, the rats' suppressed innate T-cell immunity with consequent impairment of antitumor activity, the pharmacology of methotrexate or folinic acid rescue,^{21,36} the short time period between treatment and response assessment, and a possible resistance of the MC116 cell line to chemotherapy. Resistance of the MC116 cell line to other cytostatic agents has been reported,³⁷ although our *in vitro* data consistently demonstrate sensitivity of the MC116 line to methotrexate, even at low drug concentrations. However, a difference between *in vitro* and *in vivo* sensitivity of MC116 cells to methotrexate cannot be excluded, and many *in vivo* key determinants of methotrexate toxicity (e.g., drug-evading repair mechanisms and drug penetration into tumoral and peritumoral tissues) remain unknown in this CNS lymphoma model.

Response rates to rituximab monotherapy in our

study were surprisingly high, considering the low permeability of the BBB to antibodies. We hypothesize that a slow leak of the rituximab through the impaired BBB achieved therapeutic concentrations within the tumor. The cyclophosphamide pretreatment in these rats prior to tumor implantation may also have damaged the BBB and enhanced rituximab penetration.³⁸ We did not measure rituximab delivery in the MC116 CNS lymphoma model, although a rat study is in progress (data not shown). However, we have evaluated the delivery of radiolabeled anti-CD20 mAb radioimmunoconjugate ⁹⁰Y ibritumomab tiuxetan, an analog of rituximab, in humans with PCNSL.¹⁸ At 48 h after IV administration of radioimmunoconjugate, tumor received 1,600–3,100 rads, with a delivery of 0.18% injected dose, while normal brain distant to tumor received 720 rads with a delivery of 0.10% injected dose. This degree of mAb delivery was sufficient to achieve a complete response, as determined by disappearance of the enhancing tumor on MR images, but new tumor distant to the existing tumor appeared within 8 weeks.¹⁸ We hypothesize that rituximab efficacy in large baseline tumors should be equal to or even better than in small tumors since the high-molecular-weight agent rituximab is able to penetrate the disrupted BBB in bulky tumors, as we have previously demonstrated in other tumor models.^{39,40} Comparison with human data is difficult due to the lack of prospective data on treatment of PCNSL with single-agent rituximab, but two recent reports indicate excellent efficacy and safety of single-agent rituximab in PCNSL manifesting as intraocular lymphoma.^{41,42} This is further relevant to our work because the eye, like the brain, lacks complement, considered to be an important modulator of systemic rituximab activity.

Rituximab in combination with temozolomide has been used in a low number of patients mainly with relapsed CNS lymphoma, yielding response rates of 53%–100%.^{18–20} Data on PCNSL treatment with a combination of methotrexate and rituximab is not yet available. Due to the different mechanisms of action of methotrexate and rituximab, a synergistic effect of both drugs with improved response rate can be postulated. This study failed to demonstrate a superiority of methotrexate and rituximab combination treatment over methotrexate or rituximab alone. We hypothesize that methotrexate might reduce the permeability of the BBB and blood–tumor barrier to rituximab, thus reducing antitumor efficacy; this hypothesis will be tested in future pharmacology and treatment studies.

This study has several limitations. Generally, animal models of human tumors rarely reflect the clinical situation completely accurately. Specifically, the MC116 CNS lymphoma model demonstrates reproducible tumor inoculation rates and MRI and histology features but fails to yield consistent tumor sizes, representing an important confounding factor for evaluation of tumor response. In an attempt to improve the consistency of tumor growth, the rats were pretreated with cyclophosphamide prior to tumor implantation in order to reduce possible effects of

innate immunity,^{29,31} and because cyclophosphamide has been reported to improve metastatic tumor growth.^{30,31} Although more tumors grew past the micrometastasis volume of <4 mm³ that was noted in the previous study,²³ the inoculation success rate remained low at 73%. A new approach to stabilize the model is to use two or more doses of cyclophosphamide, which may affect vascular endothelial growth factor concentrations and tumor vascularity (data not shown). In a pilot study, eight rats were pretreated with cyclophosphamide 24 h before cell injection and then again at 2 weeks after cell implantation. Our preliminary results show that all rats had small tumors on MR images at 3 weeks (1 week after the second dose of cyclophosphamide), with a median tumor volume of 54.9 mm³ (range, 9.3–115.3 mm³) at 4 weeks. Additionally, in our future studies, inclusion criteria will include baseline tumor volume of approximately 10 mm³ on MR images, rather than aiming for a specific day of treatment as in the present study, thereby reducing potential biases resulting from highly variable tumor sizes at study entry.

In our T1-weighted MR images, IP gadolinium was administered at three times the IV dose to achieve comparable tumor enhancement.²³ This provided adequate T1 images in untreated animals, but the route of administration may have decreased the correlation of T1 MR images with histology in the treated animals. The MC116 CNS lymphoma is an infiltrative model; tumor growth in the meninges and ventricles was noted on histology in 12 rats, which was not quantifiable on MR images. Analysis of the correlation between histology and MRI volumetrics was limited to the parenchymal tumor (caudate inoculation site and infiltration into cortex and across the midline along the corpus callosum), thereby creating a potential bias for assessment of tumor size and response.

Additional limitations of the model include the significant acute toxicity of methotrexate observed in this study. Several rats died due to toxicity, which may have confounded the evaluation of antitumor efficacy. Rats could not be followed for longer than 1 week after treatment, thus preventing evaluation of survival and tumor regrowth. Further modifications of the model to reduce the methotrexate toxicity and the variability in tumor volumes are needed. Due to the presence of the BBB, delivery of chemotherapy/mAb to the brain tumors may not have been optimal. One potential approach is to use the osmotic BBB disruption technique to maximize transvascular delivery.^{8,21,22} In human PCNSL, chemotherapy delivery with BBB disruption improves survival without radiotherapy and without cognitive loss.^{6–8,43}

In summary, the MC116 CNS lymphoma model seems valuable for preclinical testing of efficacy, transvascular delivery, and toxicity of various treatment regimens, especially considering the fact that CNS lymphomas in humans are rare, thus preventing the conduction of phase III trials. Further treatment approaches will be tested, including chemotherapy and mAb- and radioimmunotherapy-based therapies.¹⁷ Different routes of administration (e.g., IV vs. intraarterial plus BBB

disruption), especially for rituximab, could be further studied in this model. We conclude that T2/FLAIR was superior to contrast-enhanced T1 for monitoring the efficacy of treatment regimens in the MC116 CNS lymphoma model, and that IV rituximab was highly effective but methotrexate only minimally effective in this model.

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References

1. Eby NL, Grufferman S, Flannelly CM, Schold SC Jr, Vogel FS, Burger PC. Increasing incidence of primary brain lymphoma in the US. *Cancer*. 1988;62:2461–2465.
2. Nelson DF, Martz KL, Bonner H, Nelson JS, Newall J, Kerman HD. Non-Hodgkin's lymphoma of the brain: can high dose, large volume radiation therapy improve survival? Report on a prospective trial by the Radiation Therapy Oncology Group (RTOG): RTOG 8315. *Int J Radiat Oncol Biol Phys*. 1992;23:9–17.
3. Batchelor T, Carson K, O'Neill A, et al. Treatment of primary CNS lymphoma with methotrexate and deferred radiotherapy: a report of NABTT 96-07. *J Clin Oncol*. 2003;21:1044–1049.
4. Herrlinger U, Kuker W, Uhl M, et al. NOA-03 trial of high-dose methotrexate in primary central nervous system lymphoma: final report. *Ann Neurol*. 2005;57:843–847.
5. Pels H, Schmidt-Wolf IG, Glasmacher A, et al. Primary central nervous system lymphoma: results of a pilot and phase II study of systemic and intraventricular chemotherapy with deferred radiotherapy. *J Clin Oncol*. 2003;21:4489–4495.
6. Kraemer DF, Fortin D, Doolittle ND, Neuwelt EA. Association of total dose intensity of chemotherapy in primary central nervous system lymphoma (human non-acquired immunodeficiency syndrome) and survival. *Neurosurgery*. 2001;48:1033–1040.
7. Neuwelt EA. Mechanisms of disease: the blood-brain barrier. *Neurosurgery*. 2004;54:131–140.
8. Neuwelt EA, Goldman DL, Dahlborg SA, et al. Primary CNS lymphoma treated with osmotic blood-brain barrier disruption: prolonged survival and preservation of cognitive function. *J Clin Oncol*. 1991;9:1580–1590.
9. Abrey LE, DeAngelis LM, Yahalom J. Long-term survival in primary CNS lymphoma. *J Clin Oncol*. 1998;16:859–863.
10. Blay JY, Conroy T, Chevreau C, et al. High-dose methotrexate for the treatment of primary cerebral lymphomas: analysis of survival and late neurologic toxicity in a retrospective series. *J Clin Oncol*. 1998;16:864–871.
11. DeAngelis LM, Seiferheld W, Schold SC, Fisher B, Schultz CJ. Combination chemotherapy and radiotherapy for primary central nervous system lymphoma: Radiation Therapy Oncology Group Study 93-10. *J Clin Oncol*. 2002;20:4643–4648.
12. O'Neill BP, Wang CH, O'Fallon JR, et al. Primary central nervous system non-Hodgkin's lymphoma (PCNSL): survival advantages with combined initial therapy? A final report of the North Central Cancer Treatment Group (NCCTG) Study 86-72-52. *Int J Radiat Oncol Biol Phys*. 1999;43:559–563.
13. Gavrilovic IT, Hormigo A, Yahalom J, DeAngelis LM, Abrey LE. Long-term follow-up of high-dose methotrexate-based therapy with and without whole brain irradiation for newly diagnosed primary CNS lymphoma. *J Clin Oncol*. 2006;24:4570–4574.
14. Poortmans PM, Kluin-Nelemans HC, Haaxma-Reiche H, et al. High-dose methotrexate-based chemotherapy followed by consolidating radiotherapy in non-AIDS-related primary central nervous system lymphoma: European Organisation for Research and Treatment of Cancer Lymphoma Group Phase II Trial 20962. *J Clin Oncol*. 2003;21:4483–4488.
15. Abrey LE, Batchelor TT, Ferreri AJ, et al. Report of an international workshop to standardize baseline evaluation and response criteria for primary CNS lymphoma. *J Clin Oncol*. 2005;23:5034–5043.
16. Feugier P, Van Hoof A, Sebban C, et al. Long-term results of the R-CHOP study in the treatment of elderly patients with diffuse large B-cell lymphoma: a study by the Groupe d'Etude des Lymphomes de l'Adulte. *J Clin Oncol*. 2005;23:4117–4126.
17. Nowakowski GS, Witzig TE. Radioimmunotherapy for B-cell non-Hodgkin lymphoma. *Clin Adv Hematol Oncol*. 2006;4:225–231.
18. Doolittle ND, Jahnke K, Belanger R, et al. Potential of immunochemotherapy and radioimmunotherapy in relapsed primary CNS lymphoma. *Leuk Lymphoma*. 2007;48:1712–1720.
19. Enting RH, Demopoulos A, DeAngelis LM, Abrey LE. Salvage therapy for primary CNS lymphoma with a combination of rituximab and temozolomide. *Neurology*. 2004;63:901–903.
20. Wong ET, Tishler R, Barron L, Wu JK. Immunochemotherapy with rituximab and temozolomide for central nervous system lymphomas. *Cancer*. 2004;101:139–145.
21. Neuwelt EA, Barnett P, McCormick CI, Remsen LG, Kroll RA, Sexton G. Differential permeability of a human brain tumor xenograft in the nude rat: the impact of tumor size and method of administration on optimizing delivery of biologically diverse agents. *Clin Cancer Res*. 1998;4:1549–1556.
22. Neuwelt EA, Barnett PA, Hellström KE, et al. Delivery of melanoma-specific immunoglobulin monoclonal antibody and Fab fragments to normal brain utilizing osmotic blood-brain barrier disruption. *Cancer Res*. 1988;48:4725–4729.
23. Soussain C, Muldoon LL, Varallyay C, Jahnke K, DePaula L, Neuwelt EA. Characterization and magnetic resonance imaging of a rat model of human B-cell central nervous system lymphoma. *Clin Cancer Res*. 2007;13:2504–2511.
24. Küker W, Nägele T, Korfel A, et al. Primary central nervous system lymphomas (PCNSL): MRI features at presentation in 100 patients. *J Neurooncol*. 2005;72:169–177.
25. Roman Goldstein SM, Goldman DL, Howieson J, Belkin R, Neuwelt EA. MR of primary CNS lymphoma in immunologically normal patients. *AJNR Am J Neuroradiol*. 1992;13:1207–1213.
26. Macdonald DR, Cascino TL, Schold SC, Cairncross JG. Response criteria for phase II studies of supratentorial malignant glioma. *J Clin Oncol*. 1990;8:1277–1280.

27. Küker W, Nagele T, Thiel E, Weller M, Herrlinger U. Primary central nervous system lymphomas (PCNSL): MRI response criteria revised. *Neurology*. 2005;65:1129–1131.
28. Magrath IT, Freeman CB, Pizzo P, et al. Characterization of lymphoma-derived cell lines: comparison of cell lines positive and negative for Epstein-Barr virus nuclear antigen. II. Surface markers. *J Natl Cancer Inst*. 1980;64:477–483.
29. Fulci G, Breymann L, Gianni D, et al. Cyclophosphamide enhances glioma virotherapy by inhibiting innate immune responses. *Proc Natl Acad Sci USA*. 2006;103:12873–12878.
30. Yamauchi K, Yang M, Hayashi K, et al. Induction of cancer metastasis by cyclophosphamide pretreatment of host mice: an opposite effect of chemotherapy. *Cancer Res*. 2008;68:516–520.
31. Wu YJ, Muldoon LL, Dickey DT, Lewin SJ, Varallyay CG, Neuwelt EA. Cyclophosphamide enhances human tumor growth in nude rat xenografted tumor models. *Neoplasia*. 2009;11(2): 187–195.
32. Batchelor T, Loeffler JS. Primary CNS lymphoma. *J Clin Oncol*. 2006;24:1281–1288.
33. Blay JY, Bouhour D, Carrie C, et al. The C5R protocol: a regimen of high-dose chemotherapy and radiotherapy in primary cerebral non-Hodgkin's lymphoma of patients with no known cause of immunosuppression. *Blood*. 1995;86:2922–2929.
34. Herrlinger U, Schabet M, Brugger W, et al. German Cancer Society Neuro-Oncology Working Group NOA-03 multicenter trial of single-agent high-dose methotrexate for primary central nervous system lymphoma. *Ann Neurol*. 2002;51:247–252.
35. Omuro AM, DeAngelis LM, Yahalom J, Abrey LE. Chemoradiotherapy for primary CNS lymphoma: an intent-to-treat analysis with complete follow-up. *Neurology*. 2005;64:69–74.
36. Dukic SF, Heurtaux T, Kaltenbach ML, Hoizey G, Laliemand A, Vistelle R. Influence of schedule of administration on methotrexate penetration in brain tumours. *Eur J Cancer*. 2000;36:1578–1584.
37. O'Connor PM, Wassdermann K, Sarang M, Magrath I, Bohr VA, Kohn KW. Relationship between DNA cross-links, cell cycle, and apoptosis in Burkitt's lymphoma cell lines differing in sensitivity to nitrogen mustard. *Cancer Res*. 1991;51:6550–6557.
38. Iwamoto Y, Fujita Y, Sugioka Y. YIGSR, a synthetic laminin peptide, inhibits the enhancement by cyclophosphamide of experimental lung metastasis of human fibrosarcoma cells. *Clin Exp Metastasis*. 1992;10:183–189.
39. Barnett PA, Roman-Goldstein S, Ramsey F, et al. Differential permeability and quantitative MR imaging of a human lung carcinoma brain xenograft in the nude rat. *Am J Pathol*. 1995;146:436–449.
40. Neuwelt EA, Barnett PA, Hellström KE, Hellström I, McCormick CI, Ramsey FL. The effect of blood-brain barrier disruption on intact and fragmented monoclonal antibody localization in intracerebral human carcinoma xenografts. *J Nucl Med*. 1994;35:1831–1841.
41. Kitzmann A, Pulido J, Mohny B, et al. Intraocular use of rituximab. *Eye*. 2007;12:1524–1527.
42. Ohguro N, Hashida N, Tano Y. Effect of intravitreal rituximab injections in patients with recurrent ocular lesions associated with central nervous system lymphoma. *Arch Ophthalmol*. 2008;126:1002–1003.
43. Angelov L, Doolittle ND, Kraemer DF, Siegal T, Barnett GH, Peereboom D, Stevens G, McGerger J, Jahnke K, Lacy CA, Hedrick NA, Shalom E, Ference S, Bell S, Sorenson L, Tyson RM, Haluska M, Neuwelt EA. Blood-brain barrier disruption and intra-arterial methotrexate-based therapy for newly diagnosed primary CNS lymphoma: a multi-institutional study. *J Clin Oncol*. In press 2009.