Cancer Therapy: Clinical

Complement Activation and Intraventricular Rituximab Distribution in Recurrent Central Nervous System Lymphoma

Cigall Kadoch, Jing Li, Valerie S. Wong, Lingjing Chen, Soonmee Cha, Pamela Munster, Clifford A. Lowell, Marc A. Shuman, and James L. Rubenstein

Abstract

Purpose: To elucidate the mechanistic basis for efficacy of intrathecal rituximab. We evaluated complement activation as well as the pharmacokinetics of intraventricular rituximab in patients who participated in two phase 1 multicenter studies.

Experimental Design: We evaluated complement activation as a candidate mediator of rituximab within the central nervous system (CNS). Complement C3 and C5b-9 were quantified by ELISA in serial cerebrospinal fluid (CSF) specimens after intraventricular rituximab administration. We determined rituximab concentration profiles in CSF and serum. A population three-compartment pharmacokinetic model was built to describe the disposition of rituximab following intraventricular administration. The model was derived from results of the first trial and validated with results of the second trial.

Results: Complement C3 and C5b-9 were reproducibly activated in CSF after intraventricular rituximab. Ectopic expression of C3 mRNA and protein within CNS lymphoma lesions was localized to myeloid cells. Constitutive high C3 activation at baseline was associated with adverse prognosis. A pharmacokinetic model was built, which contains three distinct compartments, to describe the distribution of rituximab within the neuroaxis after intraventricular administration.

Conclusions: We provide the first evidence of C3 activation within the neuroaxis with intraventricular immunotherapy and suggest that complement may contribute to immunotherapeutic responses of rituximab in CNS lymphoma. Penetration of rituximab into neural tissue is supported by this pharmacokinetic model and may contribute to efficacy. These findings have general implications for intraventricular immunotherapy. Our data highlight potential innovations to improve efficacy of intraventricular immunotherapy both via modulation of the innate immune response as well as innovations in drug delivery. Clin Cancer Res; 20(4); 1029–41. ©2013 AACR.

Introduction

Since the first serotherapy trial using a monoclonal antibody was performed in 1980, immunotherapeutic approaches based upon recombinant antibody technology have come to transform clinical practice in a variety of disciplines (1). In particular, the introduction of the anti-CD20 antibody rituximab has dramatically impacted the management of patients with B-cell malignancies and other disorders related to B-cell pathology (2). The now widespread utilization of recombinant antibodies to treat human disease has prompted significant interest in analyses of their safety, mechanisms of action and resistance, pharmacokinetics and distribution, and combinatorial use with other agents (3, 4).

Although agents such as trastuzumab and rituximab improve outcomes in systemic HER2-positive breast cancer and B-cell non-Hodgkin lymphoma (NHL), respectively, there is evidence that the blood–brain barrier significantly attenuates efficacy of immunotherapeutic approaches for brain tumors that involve systemic administration of protein macromolecules (5–9). For this reason, several investigators have evaluated intrathecal administration of antibodies such as rituximab or trastuzumab, to bypass the blood–brain barrier and induce responses of central nervous system (CNS) lymphoma or breast cancer brain metastases (10–27).

Our group recently performed the first-dose escalation studies of the intrathecal administration of a naked monoclonal antibody (rituximab). An initial study was performed in cynomologus monkeys, followed by two multicenter
Translational Relevance

Among the many impediments to the development of successful strategies to treat brain tumors are problems of drug delivery and an incomplete understanding of the tumor microenvironment, in particular, the innate and adaptive immune response within the brain. Using the brain ventricle as a window to this microenvironment, we explored for the first time the pharmacodynamics of complement activation within the cerebrospinal fluid (CSF) upon intrathecal rituximab administration. In addition, we applied data generated from two recent phase I trials of intraventricular rituximab in recurrent central nervous system lymphoma to develop and validate a novel, compartmental pharmacokinetic model that describes monoclonal antibody distribution after intraventricular administration in humans. We demonstrated rapid complement activation in CSF that may contribute to the lymphocytotoxic effects of intraventricular rituximab. Distribution of rituximab was measured via CSF and blood samples and supported a three-compartment pharmacokinetic model with rituximab penetration into the brain. We hypothesize that this information may provide insights into the basis for efficacy of intraventricular rituximab in the treatment of lymphoma within the neuroaxis, and may be of general relevance to the development of novel therapeutic strategies for brain diseases that are based upon the intra-CSF administration of therapeutic antibodies and other macromolecules.

In the first clinical study, we evaluated rituximab as monotherapy, administered primarily via the intraventricular route. In second, we evaluated combination rituximab plus methotrexate as immunochemotherapy. Intraventricular rituximab was well tolerated at the 10 and 25 mg dose levels, as monotherapy and in combination with methotrexate, and induced responses not only within the cerebrospinal fluid (CSF), but surprisingly, also within the intracocular compartment and in non-bulky lesions (<2 cm) within brain parenchyma. Activity was detected in patients with disease recurrent or refractory to intravenous rituximab, independent of steroid administration. Similar results with disease recurrent or refractory to intravenous rituximab were obtained with bevacizumab (31). Moreover, the intraventricular administration of neurotrophic proteins has reproducibly been demonstrated to induce neurogenesis in deep brain structures, including thalamus and substantia nigra in mouse models (32–34). Taken together, these findings implicate existence of selective mechanism(s) that facilitate transport of macromolecules from CSF or vitreous into neural tissues.

Because the CSF is specialized to bathe the brain and spinal cord and is not exposed to the systemic circulation, there is significant representation of CNS-relevant inflammatory cells and protein constituents within its approximate 150 mL volume. We previously described the first large-scale proteomic analysis of CSF to identify CSF proteins that reproducibly distinguish CNS lymphoma from benign conditions (29). Although a significant proportion of these peptides is associated with lymphoma invasion and extracellular matrix, most of the upregulated CSF peptides identified involve the innate immune response, including CD14, a marker of monocyte activation, as well as activating and inhibitory components of the complement cascade (29).

In addition, Pulido and colleagues recently demonstrated selective penetration of rituximab (molecular weight 145 kD) into full-thickness retina, in contrast with tissue plasminogen activator (70 kD), after intravitreal injection (30). Similar results were obtained with bevacizumab (31). Moreover, the intraventricular administration of neurotrophic proteins has reproducibly been demonstrated to induce neurogenesis in deep brain structures, including thalamus and substantia nigra in mouse models (32–34). Taken together, these findings implicate existence of selective mechanism(s) that facilitate transport of macromolecules from CSF or vitreous into neural tissues.

We previously described a pharmacokinetic model of rituximab disposition after intraventricular injection in cynomolgus monkeys, which predicts a three-compartment model with distinct rate constants for rituximab distribution from CSF to serum and from CSF to brain tissue. The goals of this study are to (i) define the pharmacodynamics of complement activation within the CSF upon intrathecal rituximab administration and (ii) utilize pharmacokinetic data from two recent phase I trials in CNS lymphoma to describe the disposition of rituximab after intraventricular administration in humans. We hypothesize that this information will yield insights into the basis for efficacy of intraventricular rituximab in the treatment of lymphoma within the neuroaxis, as well as will be relevant to the development of novel therapeutic strategies for brain diseases that are based upon the intra-CSF administration of therapeutic macromolecules.

Materials and Methods

Study population

CSF and blood specimens from subjects were obtained after informed consent in accordance with Institutional Review Board–approved protocols at the University of California, San Francisco (UCSF; San Francisco, CA). Ten subjects were treated in the first phase I trial of intraventricular rituximab (median age 55.5 years, range 28–82; seven males, three females). Fourteen subjects were treated in the second phase I trial of intraventricular rituximab plus methotrexate (median age 61 years, range 37–75; seven males, seven females). Traumatic CSF specimens were excluded.

Study population

These positive results have been somewhat surprising given the concept of the CNS as an immunologically privileged site, assumed to be devoid of significant concentrations of complement mediators (28) and immune effector cells and because of the presumption that macromolecules administered within the CSF are unable to penetrate brain parenchyma.
Immunohistochemistry
Frozen sections were immunostained with monoclonal anti-complement C3 (Quidel). Anti-Iba-1 antibody was from Abcam. Immunoreactivity was visualized as described (35). Confocal imaging was performed using a Zeiss LSM510 NLO Meta microscope with multi photon (800 nm), 488 nm and 543 nm laser excitation.

In situ hybridization
Full-length human complement C3 cDNA in pBlue-scriptSK(-) was from American Type Culture Collection and verified by resequencing. In situ hybridization was performed using digoxigenin-labeled riboprobes, as described (35).

ELISA
C3a ELISA: quantitative determination of C3a concentration was performed using C3a Enzyme Immuno Assay Kit (Quidel) for the detection of C3-desArg. Albumin ELISA was from Bethyl Laboratories.

Western blot analysis
CSF proteins were subjected to SDS-PAGE (10% Bis-Tris) under reducing conditions and transferred to nitrocellulose for immunoblot analysis using an anti-C3 mouse monoclonal antibody (Quidel), peroxidase-conjugated anti-mouse immunoglobulin G (IgG; Jackson Immunoresearch), and enhanced chemiluminescence (Amersham).

Flow-cytometric purification and gene expression analysis of CSF macrophages and B cells
After collection, CSF was centrifuged at 1,500 rpm, and supernatant was carefully removed. Cell pellets were resuspended in fluorescence-activated cell sorting (FACS) buffer (PBS, Ca²⁺/Mg²⁺-free, with 5% fetal calf serum) and incubated with anti-CD11b/Mac-1-APC (BD Biosciences), anti-CD14-AlexaFluor700 (BD Biosciences), and anti-CD19-PE (BD Biosciences) antibodies for 30 minutes, protected from light. Cells were washed twice and resuspended in FACS buffer with 4’,6-diamidino-2-phenylindole (DAPI). Cells were analyzed and sorted using BD FACS Aria II. Live cells were gated by DAPI exclusion, size, and granularity based on forward and side scatter parameters. Cells were sorted directly into Direct Lysis Buffer from NuGEN One-Direct kit and stored at −80°C. Samples were processed using a NuGEN FL-Ovation cDNA Biotin Module V2. Quantitative real-time PCR (RT-PCR) analyses were performed using human complement C3 probe and normalized to glycer-aldehyde-3-phosphate dehydrogenase (ABI).

Pharmacokinetic sampling
Serial CSF samples for pharmacokinetic analysis were obtained from Ommaya reservoir. During the first week of the trial, matched CSF and venous blood serum samples were obtained immediately before treatment and at 1, 2, 4, 8, 24, and 96 hours after dose. During the following 4 weeks, CSF and blood samples were obtained on day 1 and day 4 immediately before each dose and again 1 hour after dose. Blood samples were allowed to clot at room temperature for 45 minutes, then centrifuged at 1,300 g. CSF and serum were frozen within 1 hour of collection and stored at −80°C.

Bioanalysis
CSF and serum concentrations of rituximab were determined using a validated ELISA (36). The lower limit of quantification for rituximab was 0.250 μg/mL for CSF and 0.500 μg/mL for serum.

Pharmacokinetic data analysis
Rituximab CSF and serum concentration data were modeled simultaneously using nonlinear mixed effects modeling (NONMEM VII version 7.2.0, ICON). Graphical evaluation of NONMEM outputs was performed using S-PLUS 8.0 for Windows (Insightful). The first-order conditional estimation with interaction (FOCEI) method was used for population pharmacokinetic analyses. Pharmacokinetic parameters were derived using POSTHOC step in NONMEM. CSF and serum concentrations below the lower limit of quantitation were assigned as missing.

Results
Rapid lymphocytotoxic effects of intraventricular rituximab
During the course of both phase I trials, we observed rapid lymphocytotoxic efficacy of intraventricular rituximab in responding patients with cytologically positive leptomeningeal disease. Marked depletion of B-lymphoma cells within the CSF was demonstrated within hours of intraventricular rituximab therapy, by differential cell counts and cytologic analyses, performed and reported by the clinical laboratory at baseline and at timepoints after intraventricular rituximab. In addition, flow cytometry of B cells within CSF specimens at baseline and repeated at 1 hour reproducibly confirmed depletion of B cells 1 hour after rituximab administration. Notably, in several cases in which there was rituximab-associated B-cell lysis, the concentration of monocytes in serial CSF specimens increased in a time-dependent manner, demonstrating the selective anti-B-cell activity of rituximab after intraventricular administration (Fig. 1).

Complement activation in CSF
Given the rapid lymphocytotoxic effects of rituximab observed in the treatment of leptomeningeal lymphoma, we evaluated the possibility of activation of the complement cascade in CSF in patients with CNS lymphoma treated with intraventricular rituximab (13). Complement C3 is central to the activation of both the classical and alternative complement pathways; therefore, we measured the concentration of C3a anaphylatoxin as a biomarker of complement activation within the CSF. We used a quantitative ELISA for C3a des-arg to evaluate the temporal relationship between rituximab administration and complement activation in CSF.
Intraventricular rituximab monotherapy was reproducibly associated with complement activation in each of the nine study subjects examined, at each of the three dose levels: 10, 25, and 50 mg rituximab (13). Mean baseline C3a des-arg in CSF in these phase I trial subjects was 28.56 ng/mL (range 5.9–60 ng/mL). Within 1 to 2 hours of intraventricular rituximab administration, C3a des-arg achieved peak concentrations within CSF (mean 74.6 ng/mL, range 12–181 ng/mL) followed by a decline to baseline or below within 24 hours (Fig. 2A and 2D). Notably, the rapid onset of complement activation was observed to temporally correlate with antilymphoma cytologic effects in responding patients with leptomeningeal disease. Increased C3a generation was confirmed by immunoblot analysis using an anti-C3a–specific monoclonal antibody (Fig. 2B). In parallel, generation of the C5b–9 complex within the CSF was also detected after intraventricular rituximab, demonstrating propagation of the complement cascade at the level of C5 and downstream within the CNS lymphoma microenvironment (Fig. 2C). Serial measurement of C3a des-arg concentrations over the course of the 5-week study of twice weekly intraventricular rituximab confirmed increased C3 activation in CSF at 1 hour post rituximab administration in CSF with repeat dosing, and demonstrated that C3 protein did not rapidly become depleted (Fig. 2E).

**Complement C3 expression within CNS lymphoma**

Given the stable expression of complement proteins within CSF over serial treatment, we addressed the possibility that complement mediators such as C3 may be synthesized within the CNS lymphoma microenvironment. Immunohistochemical analysis demonstrated strong C3 expression in diagnostic specimens of primary CNS lymphoma (PCNSL), with particular C3 immunoreactivity associated with tumor vasculature (Fig. 3). In contrast, C3 protein immunoreactivity in normal brain was weak or undetectable. We used *in situ* hybridization with a C3-specific antisense riboprobe to demonstrate intratumoral mRNA expression of C3 by infiltrating cells surrounding tumor vessels. This finding was confirmed in each of five diagnostic specimens of PCNSL analyzed. There was no evidence for C3 gene expression by endothelial cells in these cases. C3 transcripts were minimally detectable in normal brain specimens (not shown). C3 expression within diagnostic specimens of PCNSL was confirmed by quantitative RT-PCR as well (Fig. 3). These results demonstrate for the first time the intratumoral production of C3 transcript and protein in the CNS lymphoma microenvironment and implicate complement activation as a potential means by which rituximab may mediate lymphocytotic responses within CSF and deep brain structures.

**Figure 1.** Rapid lymphocytotoxic effects within the CSF following intraventricular rituximab. A, forward versus side scatter FACS plots of CSF cells from a subject with recurrent CNS lymphoma, pre- (left) and 1 hour post (right) intraventricular rituximab monotherapy treatment (3 mL CSF analyzed/condition). B, anti-CD19 (PE-labeled)–positive populations in the CSF pre–(left) and 1 hour post (right) intraventricular rituximab in same patient as in Fig. 1A. (Continued on the following page.)
Although the majority of total complement C3 protein is synthesized by hepatocytes, a variety of evidence has suggested that inflammatory cells such as circulating monocytes may produce C3, particularly in pathologic states such as autoimmune disease (37). To test the hypothesis that monocytes within CNS lymphoma and the leptomeningeal compartment contribute to the elevated C3 and complement activation detected in CNS lymphoma, we used flow cytometry to isolate, and quantitative RT-PCR to profile, infiltrating leukocytes within the CSF for C3 expression. In three consecutive cases, we demonstrated that activated CD14$^+$ monocytes within the CSF selectively express C3 RNA transcripts, as demonstrated by quantitative RT-PCR. In contrast, C3 transcripts were not detectable in CD14$^-$/CD19$^+$ lymphoma isolated from CSF. Given our previous observation that infiltrating macrophages exhibit a perivascular distribution in PCNSL (38), these data demonstrate that intratumoral CD14$^+$ monocytes contribute to C3 expression within CNS lymphoma (Fig. 3). This conclusion is further supported by dual-color immunofluorescence staining and confocal microscopy (Fig. 3G–3J), which reproducibly demonstrated immunoreactivity of C3 protein that was localized to the cytoplasm of CNS lymphoma-associated macrophages and microglia, identified by Iba-1 expression (39, 40).

**Spontaneous complement activation in CNS lymphomas**

To further define the relationship between complement activation and the pathogenesis of CNS lymphoma, we compared baseline C3a des-arg concentrations in CSF obtained from 27 HIV-negative patients with nonmalignant inflammatory neurologic conditions, 31 patients with a new diagnosis of PCNSL, 26 patients with recurrent CNS lymphoma, and 13 patients with brain metastases. Compared with patients with nonmalignant neurologic conditions and brain metastases, patients with CNS lymphoma exhibited significantly higher spontaneous complement activation within CSF ($P = 0.05$), with the highest concentrations in CSF of patients with recurrent disease (Fig. 3K).
We noted a trend between high constitutive complement activation in CSF and shorter progression-free survival: among the 15 patients with CNS lymphoma with C3a levels in CSF above 30 ng/mL (more than two SD above mean C3a in nonmalignant neurologic controls), 13 exhibited tumor progression or death within 2 months of CSF collection. In particular, among 17 relapsed CNS lymphoma patients who were treated with intraventricular rituximab, those with elevated C3a measured in baseline, pretreatment CSF specimens, experienced shorter overall survival compared with patients with CNS lymphoma with lower C3a levels at baseline ($P < 0.01$) (Fig. 2A). Similar results were detected in an independent population of 35 newly diagnosed patients with CNS involvement of diffuse large B-cell lymphoma (DLBCL) in whom elevated C3a in CSF at pretreatment staging, identified in a small subset of patients, also was associated with shorter progression-free survival ($P < 0.019$; Supplementary Fig. S1). Taken together, these data demonstrate that the complement cascade is triggered within the CSF after intraventricular rituximab administration, and may contribute to rituximab-mediated cytotoxicity of CNS lymphoma. In addition, high constitutive levels of complement activation at baseline may be associated with diminished rituximab efficacy. One possible explanation for this paradoxical observation is that C3b, also generated by cleavage of C3, may attenuate the immune response.
response via disruption of natural killer cell activation and antibody-dependent cellular cytotoxicity (41). The determinants of rituximab response and/or resistance in CNS lymphoma may also relate to its pharmacokinetics and distribution upon intraventricular administration, parameters that have not previously been presented.

Intraventricular rituximab pharmacokinetics

Eighty-five rituximab serum samples and 135 CSF samples were collected from 10 patients in the first trial for pharmacokinetic analyses (13). Three patients (patient No. 7, 8, and 9), were excluded from the overall pharmacokinetic analyses due to the detection of high levels of existing rituximab concentrations at pretreatment. These data were visually inspected for outliers in which we identified and omitted six outlier datapoints from the final dataset. The final dataset contained data from seven patients (three from the 10 mg dose group and four from the 25 mg dose group), with 58 serum pharmacokinetic samples and 118 CSF pharmacokinetic samples.

Rituximab concentration in the CSF displayed a biphasic profile after intraventricular administration. A steady accumulation was observed for serum rituximab after multiple administrations. Representative rituximab concentration-time profiles in CSF and serum are presented in Fig. 4.

Pharmacokinetic model and parameters

A compartmental pharmacokinetic model was built to describe the rituximab concentration time profiles in both CSF and serum after intraventricular administration in seven patients. The model consists of three compartments: CSF, serum, and brain tissue compartments (Fig. 4). Clearance of drug from the CSF occurs by distribution clearance, $Q$, with the serum compartment, and another distribution clearance, $C_{ld}$, with the brain tissue compartment. This model assumes that rituximab elimination occurs only from the serum compartment, which is through FcRn recycling and catabolism for monoclonal antibodies, the same characteristics elimination mechanism as with intravenous injection. The concentrations of rituximab in each of the three compartments are described by the following set of first-order differential equations:

$$\frac{dA_1}{dt} = R_0(t) - (k_{11} + k_{12}) \cdot A_1 + k_{31} \cdot A_3$$

$$\frac{dA_2}{dt} = k_{12} \cdot A_1 - k_{20} \cdot A_2$$

$$\frac{dA_3}{dt} = k_{13} \cdot A_1 - k_{31} \cdot A_3$$

where $t$ is the sample time, $A_1$, $A_2$, and $A_3$ are the amount of drug in the CSF, serum and brain tissue, respectively. $k_{12}$, $k_{13}$, and $k_{31}$ represent the distribution rate constants from CSF to serum (12), from CSF to brain tissue (13), and from brain tissue to CSF (31), respectively. Potential efflux of the drug from the serum compartment to CSF was ignored in this model, justified on the basis of the experimental observation that the concentration of monoclonal antibody in the CSF after intravenous dosing is greater than 1,000 times lower than the serum concentration (42). Therefore, the potential contribution to the CSF concentration from serum compartment efflux to CSF is expected to be insignificant. A representative individual fit of the model to both CSF and serum concentration is depicted in Fig. 4 as well as the goodness-of-fit plots for the full dataset and the population-based compartmental pharmacokinetic model used (Fig. 4C and 4D). Overall, the three-compartmental pharmacokinetic model with the brain tissue compartment effectively describes both serum and CSF pharmacokinetic data.

Rituximab CSF and serum data after intraventricular dosing were fitted simultaneously to estimate critical pharmacokinetic parameters (Table 1). The estimated volume of distribution of rituximab in CSF ($V_1$) and in serum ($V_2$) were 62.2 [22.3% standard error of estimate (SEE)] and 9,250 mL [23.4% SEE], respectively. Distribution rate constants from CSF to serum and brain tissue in the model were 3.57/day [24.2% SEE] and 2.61/day [34.3% SEE], respectively, and the elimination rate constant from serum was 0.0329/day [57.4% SEE]. The calculated distribution clearance between CSF and serum ($Q$) and between CSF and brain tissue ($C_{ld}$) were 208 and 189 mL/day, respectively, and the calculated volume of distribution in brain tissue ($V_3$) was 320 mL. The elimination clearance from the serum compartment, $C_{l}$ was 332 mL/day, associated with median terminal elimination half-life from serum was 21.1 days, which was consistent with rituximab elimination half-life previously described after intravenous administration (43).

Pharmacokinetic evidence for rituximab penetration in brain

The observed biphasic disposition profiles in CSF provide supportive evidence for rituximab penetration in brain tissue. To better understand whether the biphasic curve is a consequence of rituximab penetration within brain tissue after intraventricular administration, the final compartmental pharmacokinetic model was used to perform a theoretical sensitivity analysis on the $V_3$ parameter. When $V_3$ is equal to 0 mL, meaning to assume that there is no brain tissue compartment, a much faster decline (i.e., mono exponential decline) of rituximab concentration is observed in CSF, indicating that there does not exist a second biologic compartment besides the CSF for drug disposition. As the $V_3$ increases, a second elimination phase (biphasic) in the CSF appears (Supplementary Fig. S2). The biphasic disposition profiles in CSF observed using this model also provide supportive evidence for rituximab penetration in brain tissue.

Validation of the pharmacokinetic model in a second phase I study

The utility of this three-compartment pharmacokinetic model is highlighted by the fact that it predicted an approximation of the pharmacokinetics of rituximab disposition following intraventricular administration in an independent cohort of seven patients treated at the
25 mg dose level in the second multicenter phase I trial of intraventricular rituximab plus methotrexate (Fig. 5; ref. 22). This result supports the general applicability of this model structure to the design of clinical interventions that use intraventricular monoclonal antibodies to treat disease within the CNS.

Discussion

Here, we provide the first demonstration of the activation of the complement cascade within the brain microenvironment after intra-CSF administration of rituximab, a monoclonal antibody containing IgG1 constant domains. These observations provide a potential mechanistic explanation for some of the immunotherapeutic responses of CNS lymphomas with intrathecal rituximab that have been described by many investigators. Notably, it was previously demonstrated that although intravenous rituximab induces significant C3 cleavage in serum, simultaneous activation of C3 in matched serial CSF specimens from a patient with CNS lymphoma could barely be detected (44). Our results therefore identify one mechanism by which intraventricular rituximab may overcome the blood–brain barrier to mediate cytotoxicity against otherwise drug-resistant recurrent NHL. Nevertheless, although the time course of complement activation correlates with the onset of B-cell lysis in CSF, other mechanisms of rituximab-mediated efficacy such as antibody-dependent cellular cytotoxicity also likely contribute in this setting.

In addition, we demonstrate upregulation of C3 protein not only within the CSF, but also within brain parenchyma. Given the pharmacokinetic evidence for brain penetration of rituximab, these results raise the possibility that rituximab-induced activation of the complement cascade within the brain may have the capacity to elicit antitumor
responses throughout the neuroaxis, a result that has implications for the intravenous route of administration as well. In support of this hypothesis is our demonstration of the expression of the C5b-9 membrane attack complex not only within the CSF, but also diffusely within three consecutive CNS lymphomas specimens analyzed (unpublished data). We also detected spontaneous complement activation within the CSF of patients with other types of brain tumors, including metastatic carcinoma and glioma (not shown).

Somewhat surprisingly, high levels of the C3 cleavage product C3a were found to be associated with inferior prognosis in independent populations of patients with CNS lymphoma. The activating signal for spontaneous complement activation within brain tumors is undefined. One possibility is that C3 convertase activity is mediated via the lectin pathway that may occur by presentation of mannose receptors expressed by alternatively activated macrophages or by tumor cell apoptosis. Nevertheless, our results demonstrate for the first time that complement components in the CSF can be reproducibly quantified in the clinical setting in CNS lymphoma and other brain tumors, and that C3a concentration in CSF may be a prognostic biomarker denoting high-risk disease. An emerging body of data implicates complement activation in the pathogenesis of multiple sclerosis and other neuro-inflammatory and/or neurodegenerative diseases (45–49). The fact that we demonstrated significantly higher levels of complement activation in CNS lymphoma compared with these and other nonmalignant neurologic conditions underscores the potential physiologic significance of C3 activation in CNS lymphoma and other brain tumors.

An important question that emerges from these studies is the mechanism by which constitutive activation of complement C3 cleavage might promote CNS lymphoma...
progression. In addition to the generation of C3b, which attenuates natural killer function (41), activation of the complement cascade at the level of C5a may potentiate intratumoral chemotaxis of myeloid suppressor cells, which may disrupt adaptive antilymphoma immunity (50). In addition, in experimental autoimmune encephalomyelitis, there is direct evidence that C3a itself promotes chronic inflammation in the CNS, as a chemoattractant (45). There is also evidence that complement C3 and C3a promote growth signals in cancer and potentiate CXCL12 signaling (51, 52). C3 and C3a may also regulate lymphoma-associated vascular permeability and/or angiogenesis, a possibility supported by recent observations about the antiangiogenic effects of C3a and C5a on retinal vasculature (53). Notably, in support of this hypothesis, we detected a significant correlation between C3a concentration in CSF and the contrast-enhancing volume of CNS lymphomas ($R^2 = 0.65$; data not shown).
The rapid decrease in rituximab concentrations in CSF indicates a rapid elimination and/or distribution in the cranial-spinal axis. At first, we modeled the CSF and brain tissue compartment as a single compartment. However, the necessity of treating the serum and brain tissue compartments separately became evident after unsuccessful attempts to fit the CSF and serum data, when $V_1$ and $V_3$ are combined. Such a model without a third compartment cannot explain the biphasic distribution of rituximab in CSF, as the distribution to serum was slow and serum levels were considerably lower than those measured in the CSF. The biphasic CSF rituximab indicates rapid distribution to a second biologic space, consistent with penetration into brain tissue. Nevertheless, additional studies are required to directly quantify brain tissue penetration of rituximab.

The parameters that regulate distribution of rituximab into this second biologic space are not defined but may relate to dynamics of vascular permeability (perhaps mediated by complement), dynamics of tumor cellularity and interstitial pressure, intraventricular CSF pressure, as well as hypothetical mechanisms of facilitated uptake of therapeutic molecules such as IgG1 into the brain. Despite these variables, the utility of this three-compartment pharmacokinetic model is underscored by its accurate prediction of the pharmacokinetics of intraventricular rituximab in the second study, as well as in independent multicenter phase I trials (22).

The emerging body of data about the physiology of the complement system within the CSF in CNS lymphoma suggests several possible interventions to improve efficacy or to overcome resistance to intraventricular rituximab, for example, via the coadministration of pharmacologic inhibitors of complement antagonists such as clusterin, which is upregulated within the CSF in CNS lymphoma, as a means to potentiate rituximab-mediated cytotoxicity (54). In addition, we provide the first pharmacokinetic model of the distribution of a naked monoclonal antibody after intraventricular administration in humans. We believe that this model may provide a framework for the design of future studies of the intraventricular injection, as well as infusion, of therapeutic biologic molecules within the CSF and neuroaxis.

Table 1. Population pharmacokinetic parameters for rituximab after intraventricular administration in patients with CNS lymphoma

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<th>Parameter</th>
<th>Population estimated (% SEE)</th>
<th>Interpatient variability (% SEE)</th>
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<tr>
<td>Volume of distribution in CSF ($V_1$), mL</td>
<td>62.2 (22.3)</td>
<td>50.6 (30.9)</td>
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<tr>
<td>Volume of distribution in serum ($V_2$), mL</td>
<td>9,250 (23.4)</td>
<td>33.6 (30.9)</td>
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<td>Distribution rate constant from CSF to serum ($k_{12}$), per day</td>
<td>3.57 (24.2)</td>
<td>61.6 (26.8)</td>
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<td>Elimination rate constant from serum ($k_{20}$), per day</td>
<td>0.0329 (57.4)</td>
<td>56.7 (37.0)</td>
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<td>Distribution rate constant from CSF to brain tissue ($k_{13}$), per day</td>
<td>2.61 (34.3)</td>
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<td>Distribution rate constant from brain tissue to serum ($k_{31}$), per day</td>
<td>0.638 (69.4)</td>
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<td>Residual error for CSF data (proportional), %</td>
<td>62.3 (14.7)</td>
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<td>Residual error for serum data (proportional), %</td>
<td>17.4 (39.1)</td>
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<tr>
<td>Elimination half-life from serum, day</td>
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Figure 5. A and B, rituximab concentration-time profiles in (A) CSF and (B) serum from seven patients in 25 mg dose group from the second phase I trial of intraventricular rituximab. Overall disposition of rituximab in the second study was successfully predicted on the basis of the three compartment model derived from analysis of rituximab pharmacokinetics in the first phase I trial of intraventricular rituximab. Open circles represent the original observations; shaded area represents the 95% prediction interval according to the model; the black lines represent the 97.5th, 50th, and 2.5th percentiles of the simulated data.
Disclosure of Potential Conflicts of interest

J. Li is employed in Genentech and MedImmune. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions

Conception and design: C. Kadoch, P. Munster, C.A. Lowell, M.A. Shuman, J.L. Rubenstein
Development of methodology: C. Kadoch, J. Li, V.S. Wong, P. Munster, J.L. Rubenstein
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): C. Kadoch, V.S. Wong, L. Chen, P. Munster, J.L. Rubenstein
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): C. Kadoch, J. Li, P. Munster, J.L. Rubenstein
Writing, review, and/or revision of the manuscript: C. Kadoch, J. Li, V.S. Wong, L. Chen, S. Cha, P. Munster, C.A. Lowell, J.L. Rubenstein

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