Using Human CD20-Transfected Murine Lymphomatous B Cells to Evaluate the Efficacy of Intravitreal and Intracerebral Rituximab Injections in Mice

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PURPOSE. The treatment of primary central nervous system lymphoma (PCNSL) and its subset, primary intraocular lymphoma (PIOL), remains of limited efficiency, and salvage therapies are often used without prior testing in adequate animal models. Most PCNSL/PIOL are aggressive B-cell malignancies. Two animal models that closely mimic the human situation were established to evaluate the efficiency of intravitreal and intracerebral anti–CD20 monoclonal antibody (rituximab) injections.

METHODS. Human CD20-transfected murine B-lymphoma cells (38C13 CD20+) were inoculated in the vitreous through the pars plana or in the caudate nucleus with the use of a stereotaxic frame in immunocompetent syngenic mice. Animals were monitored clinically and by funduscopic and histologic examination. Rituximab was injected intravitreally or intracerebrally. Occurrences of exophthalmia, neurologic disturbance, and weight loss were monitored over 2 months.

RESULTS. Inoculation of 38C13 CD20+ cells in the eye or the brain resulted in tumor occurrence after a median of 15 days or 22 days, respectively, with histologic characteristics closely resembling those of PIOL and PCNSL. Local rituximab injections eradicated tumor colonization in more than half the graft recipients and inhibited tumor progression significantly in the others compared with progression in mice that underwent grafting with the control 38C13 cell line (no human CD20 expression) and in mice that underwent grafting with 38C13 CD20+ cells that received local injections of an irrelevant antibody (trastuzumab).

CONCLUSIONS. Inoculation of native or human CD20-transfected murine 38C13 cells in the vitreous or the brain of immunocompetent mice provides useful novel models for evaluating the biology and treatment of PIOL and PCNSL. Intravitreal and intracerebral rituximab injections reduced tumor occurrence and growth in each model. (Invest Ophthalmol Vis Sci. 2008;49:4738–4745) DOI:10.1167/iovs.07-1494

Primary central nervous system lymphoma (PCNSL) and primary intraocular lymphoma (PIOL) are closely related diseases involving two immunoprivileged sites. PCNSL is an aggressive malignancy that accounts for 1% to 4% of primary brain tumors1–4 and approximately 1% of all non-Hodgkin lymphomas. PIOL is a subset of PCNSL in which lymphoma cells invade the subretinal pigment epithelial space, vitreous cavity, and optic nerve, with (25%) or without CNS involvement at diagnosis.3–5 Approximately 95% of PCNSL5,6 and 98% of PIOL5,4 are diffuse large B-cell lymphomas that express CD19 and CD20. In the past two decades, the incidence of PCNSL and PIOL has risen threefold in immunocompromised and immunocompetent patients.1–4 The prognosis for patients with PCNSL or PIOL remains poor, and the median overall survival is only 16 to 40 months.5,6–14

The chimeric anti–CD20 monoclonal antibody (rituximab) is approved for the treatment of B-cell lymphomas and, used alone or in combination with chemotherapy, has greatly improved the prognosis of diffuse large B-cell lymphomas without central nervous and intraocular involvement.15–19 However, PCNSL/PIOL typically shows no evidence of systemic involvement, and the efficacy of intraventricular rituximab is restrained by the intact blood-brain barrier/blood-retinal barrier, which prevents antibodies from effectively penetrating the CNS and the posterior segment of the eye.16 Anecdotal experiences suggest that intraventricular and intraocular use of rituximab has potential activity in PCNSL and PIOL.11,18–23

To investigate the efficiency of local rituximab therapy for PCNSL and PIOL, animal models can be useful. In this study, we have developed two novel murine models that closely mimic human PCNSL and PIOL. Because clinical, epidemiologic, and prognostic characteristics of PCNSL/PIOL differ substantially between immunodeficient and immunocompetent patients, immunocompetent adult syngeneic mice were chosen as recipients. The use of a murine B-cell lymphoma cell line24,25 stably transfected with human CD2026 allowed us to evaluate the therapeutic potential of anti–CD20 rituximab. Regression of induced brain and ocular tumors was obtained after intracerebral and intravitreal injection of rituximab, respectively.

MATERIALS AND METHODS

B-Cell Lines

The 38C13 cell line is a carcinogen-induced large B-cell lymphoma of C3H murine origin24,25 (kindly provided by Kris Thielemans, Laboratory of Physiology, Vrije Universiteit Brussel, Brussels, Belgium). The 38C13 cell line stably transfected by human CD2026 (38C13 CD20+) was a generous gift of Josec Golay (Laboratory of Cellular and Gene
Flow Cytometric Analysis

To control murine B-cell lineage and human CD20 expression, 2 × 10⁵ 38C13 or 38C13 CD20⁺ cells were stained directly using conjugated antibodies against mouse CD19 and human CD20 according to the manufacturer’s protocol (Beckman Coulter, Fullerton, CA). To prevent any nonspecific binding, the Fcγ receptors CD16, CD32, and CD64 were saturated in a first step using an anti–CD16/32 antibody (BD Biosciences, Pharmingen, San Diego, CA), an anti–CD64 antibody (BD Biosciences), and a control chimeric human/mouse antibody (trastuzumab; Herceptin; Roche Laboratory, Paris, France). To control the binding of rituximab to 38C13 CD20⁺ cells, indirect immunostaining was performed with 10 μg rituximab (MabThera; Roche Laboratory) for 30 minutes at 4°C. The cells were washed twice with phosphate-buffered saline (PBS) supplemented with 1% bovine serum albumin and incubated for 30 minutes at 4°C with fluorescein isothiocyanate-conjugated rabbit anti-human F(ab’)₂ (1:10 dilution; DakoCytomation, Glostrup, Denmark). After two washes, at least 5000 cells were analyzed with a flow cytometer (Epics XL; Beckman Coulter). Before injections to mice, cultured tumor cells were washed once in PBS, and cell viability was evaluated using the appropriate settings for forward and side scatter. Flow count fluorospheres added to the sample were simultaneously acquired to adjust the concentration to precisely 1250 viable cells/μL.

Mice

Animals were 6-week-old C57/HeN mice (Janvier Laboratory, Genest, France) weighing 15 to 18 g. Mice were provided food and water ad libitum and were kept on a 12-hour light/12-hour dark cycle. All procedures conformed to the principles for laboratory animal research defined by the European Communities Council Directive (86/609/EEE) and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. All experimental procedures were approved by the local animal care and use committee and were periodically controlled by a veterinarian.

Tumor Inoculation

In preliminary experiments, a minimum of 500 lymphoma cells (either 38C13 or 38C13 CD20⁺ cells) yielded 100% of subcutaneous tumor development in mice. A dose of 500 cells in 0.4 μL PBS was thus used for all brain and ocular inoculations. Because tumor cell viability was greater than 90% in PBS after 4 hours at room temperature, all injections were performed within 3 hours after cytometric count.

Under general anesthesia (44 mg/kg ketamine and 10 mg/kg xylazine injected intraperitoneally), lymphoma cells were injected in the right caudate-putamen using a stereotactic frame (Kopf Instruments, Los Angeles, CA). A 30-gauge needle was used to drill a hole at 2.5 mm lateral to the bregma. Injections were made with a 10-μL syringe (Hamilton, Reno, NV) and a 32-gauge needle at the depth of 5 mm from the skull surface and the rate of 0.3 μL/min (Hamilton injector). The needle was left in place 1 minute before and after the injection.

For intravitreal inoculation, local complementary anesthesia was performed using topical tetracaine. The injection was administered under microscopic magnification and surgical aseptic conditions through the pars plana into the right eye. Cells were slowly injected at the rate of 0.4 μL/min using the syringe and needle described. Then rifamycin was topically administered to the injected eye.

Rituximab Injections

For therapeutic experiments, groups of mice were implanted with 38C13 CD20⁺ cells on the same day and were split into one subgroup injected with rituximab and another subgroup receiving the vehicle control. Rituximab (25 μg in 2.5 μL PBS, pH 7.4) or PBS (2.5 μL) was directly injected into the brain tumor bed at the speed of 0.3 μL/min 1 day after the inoculation of 38C13 CD20⁺ cells in the right putamen. Mice implanted with 38C13 CD20⁺ cells in the eye underwent intraocular injection on 1, 3, and 5 days after tumor inoculation, each with 5 μg rituximab in 0.5 μL PBS or with 0.5 μL PBS only. The initial puncture site was easily seen under microscopic magnification of the eye, which allowed therapeutic injections to be performed close to the location of the tumor cell graft.

To rule out a possible nonspecific effect of the chimeric rituximab antibody, we performed two complementary in vivo experiments. First, intravitreal or intracranial control injections of an irrelevant humanized antibody (trastuzumab), an antibody against human epithelial receptor type 2 in 38C13 CD20⁺ recipients, were administered according to a regimen of injections similar to that used for rituximab in frequency, antibody concentration, and volume. Second, groups of mice were underwent grafting with the untransfected 38C13 cell line and were injected intravitreally or intracranially with rituximab according to the same regimen used for mice implanted with 38C13 CD20⁺ cells.

Clinical Evaluation

Each animal was examined daily. As a general measure of toxicity, body weights were recorded twice a week. Animals were killed if neurologic disturbance, cervical lymphatic hypertrophy, or difficulties in self-feeding occurred or if they lost more than 25% of their body weight. For ocular grafts, funduscopic examinations were performed twice a week under general anesthesia as long as the lens remained clear, but they had to be stopped when cataract formed. Animals were killed when exophthalmia occurred.

Histology and Immunohistochemistry

For evaluation of pathologic conditions, mice were deeply anesthetized at specified time points and were perfused transcardially through a blunt cannula with 20 mL PBS and then with 4% paraformaldehyde (40 mL) in PBS. Brains or eyes were harvested, postfixed in the same fixative, embedded in paraffin, cut in 5-μm sections (brains were cut in the coronal plane and eyes in the sagittal plane), and stained with hematoxylin and eosin or with a mouse anti-human CD20 monoclonal antibody (Immunootech, Marseilles, France) diluted 1:600 in PBS, using a biotinylated secondary rabbit anti-mouse antibody (Vector Laboratories, Burlingame, CA) and avidin-biotin-peroxidase complex (Vector Laboratories). Finally, sections were stained by dianamobenzidine (Merck, Darmstadt, Germany) and Mayer hemalin (Fluka, Buchs, Switzerland).

Statistical Analysis

Survival curves were constructed according to the Kaplan-Meier method. Logrank test was used to determine the effect of rituximab treatment on tumor development. Differences between treated and control groups were tested with the Yates χ² test. All analyses were performed using commercial software (SPSS; Chicago, IL).

RESULTS

Expression of Human CD20 by the 38C13 CD20⁺ Cell Line

As expected for a mouse B-cell lymphoma, the native 38C13 cell line was positive for the mouse pan-B-cell marker CD19 and the three types of Fcγ receptors (CD16, CD32, CD64) but was negative for human CD20 (data not shown). The transfected 38C13 CD20⁺ cell line was strongly positive for human CD20 after direct immunostaining (data not shown) and with rituximab as a primary antibody (Fig. 1). The level of human CD20 expression by 38C13 CD20⁺ cells was as high as that of...
human PIOL cells collected by intraocular taps and were stained with rituximab using the same protocol (data not shown).

**Induction of Murine Intracerebral and Intraocular Lymphomas Resembling Human PCNSL and PIOL**

In four separate experiments, 27 of 29 (93%) animals implanted in the right putamen with 38C13 CD20⁺ lymphoma cells developed tumors in the brain parenchyma, whereas in one experiment 20 of 20 tumors developed with 38C13 cells. Symptoms leading to mouse kill (weight loss greater than 25% or neurologic disturbance) occurred after a median of 22 or 20 days, respectively (range, 16–34 or 16–31 days). Some recipients were killed at days 7, 14, 21, and 28 after tumor cell graft for pathologic evaluation. Tumor cells were identified microscopically in brain parenchyma as noncohesive cells containing little cytoplasm and few pleomorphic nuclei. The earlier detectable lesion was composed of tumor cells in arachnoid forming lymphomatous meningitis. The mural tumor cell layer in arachnoid was shallow on day 14 and became thicker with tumor evolution. Multiple lymphoma nodules were seen in brain parenchyma 3 weeks after inoculation (median, 21 days). The typical aspect was small, multiple, nonconfluent lesions around blood vessels (Fig. 2). Lesion aspect and tumor cell appearance were thus very close to those of human PCNSL.

Intraocular lymphoma developed in 28 of 29 (97%, two experiments) and 19 of 20 (95%, one experiment) mice inoculated with 38C13 CD20⁻ or 38C13 cells, respectively. Mild exophthalmia occurred after a median of 16 or 15 days, respectively (range, 14–22 or 13–22 days). Funduscopic examinations revealed cataract or severe vitreous haze 1 to 4 days before exophthalmia (as funduscopic controls, 10 other animals were unilaterally injected with 1 μL PBS, but none developed cataract). Some recipients were killed after 7, 14, and 21 days for pathologic evaluation. Tumor cells were easily recognized; they were small and had scant cytoplasm and pleomorphic nuclei. Early tumors showed lymphoma cells present mostly in the posterior vitreous appended to the internal retina (Fig. 3A). After vitreous involvement, tumor cells focally invaded the retina (Fig. 3B) and then the subretinal space (Fig. 3C), which is a hallmark of human PIOL. At later stages, tumor invaded the anterior part of the eye and the entire conjunctival tissue in the orbit. Tumor enlargement could rapidly lead to major exophthalmia and corneal involvement and was not killed 24 to 48 hours after the first sign of exophthalmia. No brain metastases were observed on systematic pathologic examination.

Except for human CD20 expression by immunohistochimistry (Figs. 2C, 3D), pathologic features, tumor location, and tumor growth did not differ after inoculation of 38C13 or 38C13 CD20⁻ cells.

**Rituximab Efficacy to Treat In Vivo PCNSL/PIOL**

In the model of 38C13 CD20⁺ cell-induced intracerebral lymphoma, 24 mice were implanted in groups of 8. In each group, 4 mice received rituximab at day 1, and the other 4 mice received PBS. Incorrect body position during injection led to apnea and death of 3 mice in the vehicle subgroups. After single intracerebral injection of rituximab, tumor occurrence decreased in frequency: 4 of 12 mice (33%) compared with 7 of 9 (78%) or 8 of 9 (88%) control mice injected with PBS or irrelevant antibody (Yates χ² test; P < 0.04 or P < 0.01, respectively). In the 4 rituximab-treated mice that developed clinical manifestations, symptoms were delayed (median, 27.2 days) compared with a median clinical onset of 20 or 18 days in mice receiving PBS or irrelevant antibody only. The corresponding Kaplan-Meier curve is depicted in Figure 4A. Time before symptom occurrence was analyzed by Kaplan-Meier product limit and log-rank test comparing 38C13 CD20⁺ and rituximab injections to 38C13 CD20⁺ and control antibody (trastuzumab) or PBS injections (P < 0.05 or P < 0.02, respectively). To verify that no tumor was slowly growing in mouse brain, the recipients were systemically killed at day 50 (3 animals) and day 90 (5 animals). Pathologic evaluation evidenced no sign of tumor.

In the model of 38C13 CD20⁺ cell-induced intraocular lymphoma, 28 mice were implanted in the eye, all on the same day. After receiving intravitreal injections on days 1, 3, and 5, significantly fewer mice (9/19; 47%) in the rituximab-injected group developed intraocular tumors with exophthalmia than in the PBS or irrelevant antibody groups (9/9 or 7/7; 100%; Yates χ² test; P < 0.03 or P < 0.04, respectively). Symptom occurrence was not delayed by the treatment (medians, 15 days for rituximab and PBS groups and 14 days for the irrelevant antibody group). The corresponding Kaplan-Meier curve is depicted in Figure 4B. Exophthalmia occurrence in mice receiving 38C13 CD20⁻ cells and rituximab injections and mice receiving 38C13 CD20⁻ cells and the control antibody (trastuzumab) or PBS injections was compared by log-rank test (P < 0.04 or P < 0.02, respectively). Two of the remaining rituximab-treated recipients were killed at day 60 without tumor evidence by pathologic evaluation. The other 8 were still asymptomatic by day 180. Repeated intraocular injections resulted in traumatic cataract in all animals (both the rituximab-treated and the PBS-control group), making funduscopic evaluation unachievable.
As a control for local tolerance of rituximab, brain and eye sections were examined after intracerebral and intravitreal injections of rituximab, respectively, in mice that had not been inoculated previously with lymphoma cells. Neither brain or retina attrition nor inflammatory cell recruitment was observed after histologic examination compared with histologic sections from animals receiving only PBS injections.

As a complementary control for the specificity of rituximab action, recipients of intravitreal and intracranial 38C13 CD20<sup>−</sup> (native) lymphoma cells were given the same regimen of rituximab injections as were 38C13 CD20<sup>+</sup> recipients. Most intravitreal recipients developed exophthalmia (11/14; 79%) by a median of 15 days (range, 14–22 days). All the intracranial recipients (5/5; 100%) were killed for symptom occurrence by a median of 18 days (range, 16–28 days). Results with an irrelevant antibody did not differ from results obtained after PBS (vehicle) injections (Figs. 4A, 4B).

**DISCUSSION**

Animal models of PCNSL and PIOL may be useful for elucidating disease pathophysiology and evaluating therapeutic strategies. By intracerebral and intravitreal injections of B-cell lymphoma cells in immunocompetent mice, we established two murine models of primary lymphoma that closely mimic PCNSL and PIOL, respectively, in humans. After using human CD20-transfected murine B-cell lymphoma cells for tumor inoculation, these models allowed evaluation of the therapeutic potential of locally injected anti-human CD20 rituximab antibody.

The large B-cell lymphoma cell line (38C13) of C3H murine origin was injected into syngeneic C3H adult mice. After a single inoculation of 500 lymphomatous B cells per animal, local tumor resulting in clinical manifestations within 2 (intravitreal inoculation) and 3 (intracerebral inoculation) weeks developed in 78% to 100% of recipients. Considering the respective sizes of human and murine vitreous (4 mL and 7/9262 L, respectively), this would correspond to the inoculation of 300,000 tumor cells in a human eye. Analyzed histologically, tumor cells were large pleomorphic B cells with scant cytoplasm and hyperchromatic nuclei, which are the typical features of human PCNS/PIOL cells. In the brain parenchyma, all large tumor nodules induced by 38C13 or 38C13 CD20<sup>+</sup> cells comprised blood capillaries surrounded by malignant lymphocytes, demonstrating a striking angiocentric pattern. Arachnoid involvement, a frequent tropism in human PCNSL, was constant in our model. We cannot formally exclude that meningeal involvement results from cell migration by mechanical reflux along the needle trajectory; however, we used the lowest inoculation speed reported in the literature to minimize this effect. Furthermore, meningeal involvement was never observed in early pathologic sections (7 days after inoculation). Systemic spread is rare in PCNSL, and, accordingly, we never observed epidural or subcutaneous tumors after intracerebral inoculation. After intravitreal inoculation, tumors induced by 38C13 or 38C13 CD20<sup>−</sup>
cells developed primarily in the vitreous cavity and then the retina, the subretinal space, and the choroid. The location of tumor cells in the vitreous cavity and the subretinal space is the classic characteristic of human PIOL. Clinical cervical lymphatic hypertrophy corresponding to pathologic involvement can occur at late-stage PIOL (after major exophthalmia if animal kill was delayed) and for 10% of intracranial inoculations. Spleen involvement was never observed.

Currently, there is no murine model of primary central nervous or ocular lymphoma. Direct intravitreal inoculation of a murine B-cell lymphoma cell line into syngeneic immunocompetent mice has recently been described to induce intracocular tumors. Although it provides useful information on T cells infiltrating the tumor, this model does not allow direct investigation of the therapeutic potential of monoclonal antibodies available against human tumors. Conversely, intravitreal inoculation of human lymphomatous B cells has been reported, but ocular tumors can develop only in immunodeficient mice. Compared with these experimental models, the use of a murine lymphoma cell line stably transfected to express human proteins at high levels, as presented in this study, combines the advantages of having immunocompetent syngeneic recipients, suitable for testing agents that could stimulate antitumoral responses, and allowing the testing of the therapeutic effect of antibodies against human molecules.

The CD20 cell surface marker is overexpressed in 95% of human PCNSL/PIOL. The 38C13 CD20\(^+\) cell line used in this study stably expresses human CD20 at levels as high as on primary human follicular lymphoma cells. Tumors developed similarly after intracerebral and intravitreal inoculation of either 38C13 or 38C13 CD20\(^+\) cells. The expression of human CD20 by 38C13 CD20\(^+\) cells was verified by flow cytometry and was as dense as on the surfaces of cells collected from human PIOL. Expression of human CD20 was obvious by immunohistochemical staining of intracocular tumors. The retinal pigment epithelium is dark and is completely infiltrated by lymphoma cells. Sections were incubated with anti-human CD20 antibody and then stained by diaminobenzidine and Mayer hematin.

**Figure 3.** Histopathology of intraocular lymphoma after intravitreal injection of 38C13 CD20\(^+\) cells into syngeneic mice. (A) Vitreous colonized by deposits of lymphoma cells in a mouse eye presenting without clinical event, collected 10 days after inoculation. Photomicrograph showing vitreous (V) involvement by small lymphoma cells (arrows) close to the superficial retina (R), which is thickened without obvious tumoral infiltration (hematoxylin and eosin; original magnification, \(\times400\)). (B) Retina colonization and invasion by lymphoma cells in an eye harvested 21 days after inoculation. Numerous lymphoma cells in the vitreous cavity (blue arrows) and the retina (blue arrowheads). (Hematoxylin and eosin staining; original magnification, \(\times400\)). (C) Late stage of lymphoma development. The eye was harvested from an animal killed 48 hours after exophthalmia. Lymphoma cells invaded the retinal pigment epithelium and the choroid. A spontaneous hemorrhage (H) occurred in the vitreous (V). Tumoral cells were also localized in the vitreous. The split between retina (R) and subretina is a histologic section artifact that was frequently observed (hematoxylin and eosin; original magnification, \(\times100\)). (D) Immunohistochemistry of intraocular tumor. The retinal pigment epithelium is dark and is completely infiltrated by lymphoma cells. Sections were incubated with anti–human CD20 antibody and then stained by diaminobenzidine and Mayer hemalin. Human CD20\(^+\) positive cells are strongly stained (brown; original magnification, \(\times400\)).
FIGURE 4. Kaplan-Meier survival analysis of clinical events after inoculation of 38C13 CD20− lymphoma cells or native 38C13 lymphoma cells and then in situ injection of rituximab monoclonal antibody or control injections. Intracranial rituximab injections. Mice were inoculated in the right caudate nucleus with native murine 38C13 CD20− negative lymphoma cells (dashed lines) or with its variant transfected by human CD20 (38C13 CD20+, solid lines). One day after inoculation, the recipients were injected intracranially with rituximab (25 µg in 2.5 µL, thick black line), a vehicle control (2.5 µL PBS, gray line), or an irrelevant antibody (25 µg in 2.5 µL trastuzumab, thin black line). Neurologic disturbance, 25% loss of body weight, and cervical lymphatic hypertrophy were used as end points. Time before symptom occurrence was analyzed by Kaplan-Meier product limit and log-rank test comparing 38C15 CD20− and rituximab injections to 38C15CD20− and control antibody (trastuzumab) or PBS injections ($P < 0.05$ or $P < 0.02$, respectively). No significant difference was observed between PBS and the control antibody group. The surviving recipients in the 38C13 CD20− rituximab group were systematically killed at day 50 (three mice) or day 90 (five mice). Pathology evaluation evidenced no sign of tumor development. (B) Intravitreal rituximab injections: Mice were inoculated in the right eye with native murine 38C13 CD20− lymphoma cells (dotted lines) or with its variant transfected by human CD20 (38C13 CD20+, full lines). At days 1, 3, and 5 after inoculation, mice were injected intravitreally with rituximab (5 µg in 0.5 µL, large black line), vehicle control (0.5 µL PBS, gray line), or an irrelevant antibody (5 µg trastuzumab in 0.5 µL). Exophthalmia occurrence was analyzed by Kaplan-Meier product limit and log-rank test comparing 38C15 CD20− and rituximab injections with 38C13CD20− and control antibody (trastuzumab) or PBS injections ($P < 0.04$ or $P < 0.02$, respectively). No significant difference was observed between PBS and control antibody groups. Two surviving recipients in the rituximab group were killed at day 60 without any sign of tumor development; the other eight rituximab-treated recipients were still asymptomatic on day 180.

Clinically effective therapies for treating PIOL and PCNSL are awaited. Lymphomas originating outside the central nervous system are routinely treated by rituximab, a mouse/human chimeric IgG1 monoclonal antibody that targets the CD20 antigen.15,16 The difficulty of monoclonal antibodies to cross the blood-brain and blood-retina barriers17–19,38 explains partially why intravenous rituximab has been of limited efficacy against PCNS/PIOL or PCNSL.39,40 Several reports of anti-CD20 antibody injections in cerebrospinal fluid have been published but are contradictory.20–25 The murine model using 38C13 CD20− lymphoma cells allowed evaluation of the therapeutic potential of rituximab injected locally. Rituximab was injected locally 1 day after inoculation of 38C13 CD20− cells because this cell line has a rapid proliferation rate (doubling time, approximately 10 hours) and rituximab has been given 1 day after tumor inoculation in other murine B-lymphoma models.26 In vitro, a concentration of 1 µg/µL rituximab was lethal to 38C13 CD20− cells (our unpublished observation, 2007). For intracranial tumor, to avoid animal loss during stereotaxic injections, a single injection was performed. The injected dose of rituximab (25 µg in 2.5 µL) was calculated from the dose preventing the development of tumors after systemic injection of 38C13 CD20− lymphoma cells in mice.26 For intraocular tumors, three intravitreal injections (5 µg in 0.5 µL) were performed because the vitreous size (7 µL) cannot accommodate large volumes. Furthermore, intravitreal injections are commonly repeated in clinical use.
After local inoculation of 38C13 CD20\(^+\) lymphoma cells, intracerebrally and intravitreally injecting rituximab significantly prevented tumor development in more than half the recipients. The antitumoral effect of rituximab can be attributed to its binding to human CD20 antigen expressed by 38C13 CD20\(^+\) cells because rituximab injections were not effective against tumors developing after inoculation of non-transfected 38C13 cells, though nonspecific inflammatory reactions or toxic effects were not observed after rituximab was injected in the eye or the brain of mice not inoculated beforehand with lymphoma cells. Antitumoral efficacy of rituximab in vivo stems from complement-dependent cytotoxicity,\(^3,4\) as demonstrated in a murine model using 38C13 CD20\(^+\) lymphoma cells inoculated intravenously.\(^5\) Antibody-dependent cellular cytotoxicity and release of inflammatory mediators can also be involved because murine Fcy receptors can bind the Fc fragment of human IgG1 in the chimeric antibody, and removing all activating murine Fcy receptors eliminates most rituximab activity against subcutaneous lymphoma xenografts.\(^6\) There are also indications of synergy between complement and Fc\(^+\) receptor-mediated effects.\(^5,5\) In normal brain and eye, complement protein levels are low,\(^4,5\) and recruitment of inflammatory cells can be limited by blood-brain and ocular barriers, which might explain why tumor development was not inhibited in all recipients.

Rituximab injections decreased tumor occurrence in the brain and in the eye, but the symptom-free period was prolonged only in the intracranial model. Local conditions may explain this difference. In the brain model, the cells were injected in a homogeneous parenchyma. In the PIOL model, the tumor has a two-step development: first a vitreous stage, then retinal involvement (Fig. 5). Rituximab was injected at the vitreous stage and can presumably prevent retinal involvement in some recipients. The lack of delay in symptom occurrence suggests that the treatment is more effective at the early stage than after retinal involvement.

In the brain model, 2 of 9 implanted 38C13 CD20\(^+\) recipients with PBS injection have not developed a brain tumor. A similar result was observed during model establishment (2/29 animals remained tumor free). In the PIOL model, only 1 of 29 38C13 CD20\(^+\) recipients did not develop intraocular tumor during model establishment and none 0 of 8 in the experimental PBS group. As discussed above, local conditions could explain such a difference. Brain parenchyma includes microglia that could be directly stimulated by 38C13 CD20\(^+\) cells injection and lead to complex immunoinflammatory response. In the PIOL model during the vitreal stage, 38C13 CD20\(^+\) cells are surrounded by fewer host cells. Immunoinflammatory stimulation, therefore, may be delayed until retinal involvement, and the host response would be overridden by a fast-growing tumor.

In spite of these limitations, and cognizant of the fact that studies are needed to test injections while symptoms are occurring, the two models presented here provide an experimental control for preclinical evaluation.

References


