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3 Revised

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6 Original Article

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12 **Treatment of central nervous system lymphoma in rats with intraventricular**
13 **rituximab and serum**

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19 Running head:

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22 CNSL therapy model with intraventricular rituximab and serum

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2
3 **Abstract**
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10 B-cell lymphomas often develop in the central nervous system (CNS). Although rituximab (RTX) has
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12 been widely used for most B-cell lymphomas, the efficacy for CNS lymphomas has yet to be elucidated.
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16 A major concern is that RTX might not reach lymphoma lesions, and either the antibody-dependent
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18 cellular cytotoxicity or complement-dependent cytotoxicity (CDC) might not substantially operate in the
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22 CNS environment. Here we investigated the potential usefulness of co-administering RTX and human
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25 serum intraventricularly in nude rats carrying human B-cell lymphomas in the CNS. Raji, a
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28 CD20-positive lymphoma cell line, was inoculated into the cerebrum of F344 (*rnu/rnu*) nude rats. After
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31 several days, RTX and human serum were delivered into the ipsilateral lateral ventricle via a cannula.
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35 Intraventricularly administered RTX was localized specifically at the lymphoma lesions, indicating that
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38 RTX penetrated the ependymal layer of the lateral ventricle to reach the tumor lesion, where it
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41 specifically bound to the lymphoma cells. The combination of RTX and serum (n = 12), but not RTX
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44 alone (n = 13), significantly extended the survival of the rats ($P=0.049$). Intraventricular administration of
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47 RTX and serum in a rat/human CNS lymphoma model might be a potential novel treatment for CNS
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51 lymphomas of B-cell origin. Clinical trials are warranted.
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57 Keywords: CNS lymphoma, rituximab, complement-dependent cytotoxicity, intraventricular
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3 **1 Introduction**
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10 Central nervous system (CNS) involvement is one of the serious manifestations of B-cell
11 lymphomas. It occurs as extranodal involvement of systemic lymphoma, often during relapse, or as a
12 primary CNS lymphoma, which is now termed “primary diffuse large B-cell lymphoma (DLBCL) of the
13 CNS” in version 4 of the WHO classification [1]. High-dose methotrexate with or without radiotherapy
14 is the current mainstay of treatment. However, side effects, such as severe cytopenia, renal insufficiency,
15 and delayed neurological damage are a concern, especially in heavily pretreated or elderly patients. Even
16 if these therapies are initially effective, long-term outcomes remain poor [2-4].
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31 Rituximab (RTX) is a monoclonal antibody against CD20, a four-transmembrane protein
32 whose expression is confined to precursor and mature B cells [5, 6]. RTX, which consists of the variable
33 region of mouse antibody and the constant region of human IgG1 [7], is widely used to treat systemic
34 B-cell lymphomas. RTX is considered to have its therapeutic effect through (i) antibody-dependent
35 cell-mediated cytotoxicity (ADCC), mainly mediated by natural killer cells [8-10]; (ii)
36 complement-dependent cytotoxicity (CDC) mediated by complement factors that are activated by
37 binding of the complement protein C1q to RTX [8, 10-14]; and (iii) induction of tumor cell apoptosis by
38 CD20 stimulation [15-17]. Despite the fact that intravenous RTX is effective against most B-cell
39 lymphomas, its efficacy against B-cell lymphomas developing in the CNS (hereafter designated CNSL)
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3 has not yet been shown. The presence of the blood–brain barrier might hamper intravenous RTX from
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6 reaching the CNSL, given that the concentration of RTX in the cerebrospinal fluid (CSF) is 1% or less
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9 of that in the blood when it is administered intravenously [18-20].
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12 CSF is produced at the choroid plexus in the lateral ventricle, flows through the third and
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14 fourth ventricle, and finally flows into the subarachnoid space. CSF also spreads from the lateral
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16 ventricle into the brain parenchyma through ependymal cells, which form a thin epithelial membrane
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19 ventricle into the brain parenchyma through ependymal cells, which form a thin epithelial membrane
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21 lining the cerebral ventricle [21]. Some cytokines and antibodies administered into the lateral ventricle
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23 do penetrate into the brain parenchyma [21-24]. In addition, the pia mater, which directly covers the
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25 brain surface, is also a leaky structure [21]. Therefore, intrathecal or intraventricular administration of
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28 RTX could be a viable choice of treatment for CNSL. However, another important concern about the
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30 potential effectiveness of RTX against CNSL is that the CNS environment lacks immune cells and
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32 complement, the two key factors needed for RTX to kill the target cells.
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41 To investigate whether RTX can be used to treat CNSL, we developed a human CNSL
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43 xenograft model in rats and evaluated the efficacy of intraventricular co-administration of RTX and
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3 **2 Materials and methods**
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10 2.1 Antibodies and serum
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12 RTX was kindly supplied by Zenyaku Kogyo Co. Ltd (Tokyo, Japan). Human polyclonal
13 immunoglobulin (Gammagard[®], Baxter, Deerfield, IL) was used as a control immunoglobulin for RTX.
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16 Human serum was separated from the clotted whole blood of a healthy volunteer by centrifugation at
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1600 × g and stored at −30°C. Inactivated serum was prepared by placing the human serum at 56°C for
30 minutes.

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2.2 Cell line and cell culture

Raji, a CD20-positive human Burkitt lymphoma cell line, was obtained from RIKEN
BioResource Center (Tsukuba, Japan) and was cultured in RPMI1640 medium (Sigma-Aldrich, St.
Louis, MO) supplemented with 10% fetal bovine serum (Sigma-Aldrich) and penicillin and
streptomycin (Sigma-Aldrich; used at 100 units/mL and 100 µg/mL, respectively) at 37°C with 10%
CO₂ in humidified air.

2.3 Complement-dependent cytotoxicity assay

Raji cells (1.0×10^5) were cultured in medium containing 20 µg/mL RTX and various

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3 concentrations of human serum in 96-well round-bottom dishes for 7 hours. After the incubation, 50 μ L
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6 of propidium iodide (PI; 7.5 μ g/mL) was added and analyzed by flow cytometry (FACSCalibur[®], Becton
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9 Dickinson, Franklin Lakes, NJ). The ratio of PI-positive dead cells to all analyzed cells was measured to
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13 evaluate complement-dependent cytotoxicity.
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19 2.4 CNSL model rats 20 21

22 Eight-week old F344 (*rnu/rnu*) nude rats were purchased from CLEA Japan (Tokyo, Japan)
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25 and were bred in Laboratory Animal Resource Center, University of Tsukuba. Experiments with rats
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28 described in this paper were approved by Laboratory Animal Resource Center, University of Tsukuba.
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31 The nude rats were anesthetized with pentobarbital (Dainippon Sumitomo Pharma, Osaka,
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34 Japan) and immobilized with a brain stereotaxic apparatus. To create a port to administer RTX and
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37 serum, a 23-gauge stainless steel tube with a removable internal stopper was cannulated into the
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40 ipsilateral lateral ventricle at 1.6 mm lateral and 0.8 mm caudal to bregma, and fixed at a 4.5 mm depth
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43 from the cranial bone surface. The cannula was secured to the cranial surface with dental resin cement.
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46 Proper cannulation was confirmed in brain sections from rats sacrificed immediately after the
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49 administration of trypan blue through the cannula (n = 2). While the cannulation was performed,
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52 exponentially growing Raji cells were harvested at a density of 1×10^6 to 1.5×10^6 cells/mL and washed
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55 once in phosphate-buffered saline without calcium and magnesium (PBS). After the cells were
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3 centrifuged and the supernatant was removed, the cell pellet was loosened by tapping, aspirated into a
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6 microinjector, and left on ice until inoculation. A 5- μ L aliquot of this pellet, which contained 1×10^6 to 3
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9 $\times 10^6$ cells, was inoculated into the deep frontal lobe of the cerebrum of the above-prepared nude rats at
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12 2 mm lateral and 3 mm rostral to bregma, and 4.5 mm deep to the cranial bone surface.
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19 2.5 Immunohistochemical analysis

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22 From days 9 to 11 after Raji cell inoculation, 100 μ g RTX or control immunoglobulin in 10 μ L
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24 of 0.154 M NaCl solution (saline) was administered with 10 μ L human serum once a day into the lateral
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From days 9 to 11 after Raji cell inoculation, 100 μ g RTX or control immunoglobulin in 10 μ L of 0.154 M NaCl solution (saline) was administered with 10 μ L human serum once a day into the lateral ventricle through the cannula. The animals were euthanized and the brains were surgically removed 24 hours after the last administration, embedded in OCT compound (Sakura Finetechnical, Tokyo, Japan), frozen at -80°C , sliced at 3 or 6 μ m with a cryostat, and immunohistologically analyzed following a standard protocol. In brief, after blocking nonspecific adhesion with BPT solution (1% bovine serum albumin [Sigma-Aldrich], PBS, and 0.1% Tween 20 [Sigma-Aldrich]), the sections were incubated with antibody in BPT solution. To confirm the presence of the Raji-cell tumor in the brain, the sections were stained with RTX and Fluorescein isothiocyanate (FITC)-conjugated rabbit anti-human IgG immunoglobulin or control FITC-conjugated rabbit immunoglobulin (DAKO, Glostrup, Denmark).

To evaluate whether intraventricular RTX reached the tumor cells, consecutive sections were directly stained with FITC-conjugated rabbit anti-human IgG immunoglobulin or control

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3 FITC-conjugated rabbit immunoglobulin. Rats administered control immunoglobulin and human serum
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6 were stained in the same manner as negative controls. After rinses, the samples were covered with
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9 VECTASHIELD Mounting Medium with DAPI (Vector Laboratories, Burlingame, CA) and observed
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12 with an Axioplan2 fluorescent microscope (Carl Zeiss, Oberkochen, Germany). Images were taken and
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15 analyzed with an AxioCam CCD camera and AxioVision4.6 image processing software (Carl Zeiss),
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25 2.6 Treatment of lymphomas in CNSL model rats 26 27 28 29 30 31

32 Once a day on day 5 through day 9 after Raji inoculation, CNSL model rats were
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35 intraventricularly administered with a mixture of 100 μg (10 μL) of RTX or control immunoglobulin
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38 with 10 μL of human serum, or a mixture of the same dose of RTX with saline. The survival data were
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41 collected from 10 independent experiments. The rats were observed and weighted once a day after
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44 intraventricular administration. When >25% weight reduction or obvious weakness was observed, the rat
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47 was euthanized, and this time point was defined as the end of survival. The brains were removed from
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51 all of the rats, regardless of whether death was natural or by euthanasia. If no lymphoma was found in
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54 the brain, the animal was excluded from the analysis. Survival of each group was analyzed with the
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57 Kaplan-Meier method and log-rank test with StatCell version 2 software (OMS, Saitama, Japan), and
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significant differences were assumed at P values <0.05 .

3 Results

We first analyzed the induction of CDC by RTX and human serum *in vitro* using cells of the CD20-positive human non-Hodgkin lymphoma B-cell line Raji as a target. Cytotoxicity was observed with the combination of RTX and serum, but not with the combinations of control immunoglobulin and serum or of RTX and heat-inactivated serum (Fig. 1). To investigate the efficacy of intraventricular RTX combined with serum, we generated a rat CNSL model in which Raji cells were inoculated into the deep white matter of the nude rat brain frontal lobe (Fig. 2). Tumor formation was confirmed histologically in brains removed 12 days after inoculation (Fig. 3). At the time of Raji cell inoculation, an indwelling cannula was implanted into the lateral ventricle on the ipsilateral side to use as the route of intraventricular administration (Fig. 2). Most rats recovered fully after the operation and those which did not recovered were excluded from the following experiments. Next, the rats were administered with RTX or control immunoglobulin and human serum via the cannula for three consecutive days beginning on day 9 after Raji inoculation. Rats recovered normally after general anesthesia and intraventricular administration of the study drugs. Twenty-four hours after the last administration, the brains were removed for analysis by immunostaining. We observed specific staining with an FITC-conjugated rabbit anti-human immunoglobulin (Fig. 4, upper panels), but not with a control FITC-conjugated rabbit immunoglobulin (middle panels); this staining was present only after the administration of RTX (Fig. 4,

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3 Exp. 1 and Exp. 2) and not after control human immunoglobulin [Fig. 4, Exp. 1 (control) and Exp. 2
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6 (control). Note that control immunoglobulin was not detected even in the high sensitivity with some
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9 background in Exp. 1 (control) and Exp. 2 (control)]. The staining area largely overlapped with the
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12 CD20-positive Raji cell tumor area (Fig. 4, lower panels). This demonstrates that intraventricular RTX
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15 penetrated the ependymal layer and brain parenchyma, and finally reached and bound to lymphoma cells.
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18 To evaluate the anti-lymphoma effect of intraventricular RTX and serum, the tumor-inoculated rats were
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21 treated with either RTX plus serum, RTX plus saline, or control immunoglobulin plus serum.
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24 Lymphoma development was confirmed histologically in all the rats examined. A single rat in the
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27 RTX-plus-serum group died from technical error during the treatment period and was excluded from this
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30 analysis. The RTX-plus-serum group survived significantly longer than the
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33 control-immunoglobulin-plus-serum group ($P = 0.049$; Fig. 5). The RTX-plus-serum group also
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36 appeared to survive longer than the RTX-plus-saline group, although the difference was not statistically
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39 significant ($P = 0.083$). The survival times of the RTX-plus-saline and
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42 control-immunoglobulin-plus-serum groups did not differ from each other ($P = 0.382$). Body weights
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45 transiently decreased immediately after the intraventricular treatment in all arms, but all the rats
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48 subsequently re-gained the weight, which began to decrease shortly before the death (Supplemental Fig.
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3 **Discussion**
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10 A few rodent CNSL models have been described previously in which lymphoma cells were
11 directly inoculated into deep cerebral white matter [25, 26]. On the basis of the information in these
12 articles, we established a CNSL model in nude rats bearing a tumor made from the Raji cell line, which
13 is widely used for CDC and ADCC assays with RTX *in vitro* [8, 10, 12] and *in vivo* [27, 28]. An
14 intracerebral Raji cell tumor established in athymic mice was recently reported to recapitulate the
15 histopathology and molecular features of primary CNSL [29]. Although the authors of that paper
16 emphasized that their model was suitable for preclinical testing of novel agents, mice are too small and
17 technically difficult to handle for many investigators. We therefore chose a nude rat as a host.
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35 In this paper, we showed that intraventricular RTX reaches the tumor through the ependymal
36 layer and brain parenchyma, although the fluorescence intensity of tumor-bound RTX was much weaker
37 than the positive control staining (Fig. 4). This indicates that only a fraction of RTX penetrated the brain
38 parenchyma, although the dilution associated with diffusion in the brain might also be attributed.
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47 Despite the low concentration of RTX into the tumor, our results indicate that the binding of
48 RTX to the Raji cell tumor induced tumor killing. Because this effect was dependent on human serum, it
49 is likely that the cell killing was actually due to CDC against the Raji tumor cells *in vivo*. It is also
50 possible that RTX and complement induced tumor cell phagocytosis by microglial cells, which are
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3 known to possess Fc and complement receptors [30, 31]. In addition, other effector cells such as
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6 monocytes and NK cells might also migrate to the tumor site after the initial CDC or phagocytosis.
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9 The relatively modest effect of intraventricular RTX and serum (Fig. 5) could be due to an insufficient
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12 dose of RTX at the tumor, given the low level of RTX binding to the tumor cells. Increasing the
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15 concentration of intraventricular RTX might therefore improve the efficacy in clinical settings.
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19 Alternatively, although clinical studies should be more complicated, directly administering monocytes or
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22 NK cells may be more advantageous to elicit continuous anti-lymphoma effect in the CNS.
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25 Intrathecal or intraventricular RTX, administered as a single agent, has been reported to be
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28 effective against CNS lymphomas in clinical settings. The efficacy, however, has mainly been described
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31 for leptomeningeal involvement of B-cell lymphomas rather than the lesions in the brain parenchyma
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34 [20, 32, 33]. These reports are largely consistent with our findings that the survival times of the
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37 RTX-plus-saline and control-immunoglobulin-plus-serum groups did not differ from each other (Fig. 5).
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41 Recently, a case of refractory primary CNSL that was treated with intraventricular RTX and
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44 autologous serum was reported [34]. The patient's condition initially deteriorated, but remarkable tumor
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47 regression occurred after 8 weeks. Our results provide experimental evidence in support of this clinical
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50 report. The authors of the clinical report discussed the possible induction of an adaptive immune
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53 response to explain the relatively slow clinical course, rather than the immediate effect expected from
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56 CDC. Because dendritic cells have been shown to emerge in the CNS in mouse CNS inflammation
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3 models [35], it is possible that, after CDC, CNSL-associated antigens were processed and presented on
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6 brain dendritic cells and adaptive immunity was initiated, leading to tumor regression.
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10 Our treatment model could be adapted for use in patients because intraventricular cannulation
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12 and Ommaya reservoir implantation are routinely performed by neurosurgeons. Given the possible slow
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14 effect, intraventricular RTX and autoserum treatment might be suitable as a consolidation or
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16 maintenance therapy for CNSL in clinical settings. Clinical trials are warranted.
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3 **Acknowledgments**
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10 We thank Dr. J. Usui and Ms. K. Noguchi (University of Tsukuba) for technical assistance with
11 immunohistochemistry; Drs. T. Yamamoto, W. Tsuruta, and T. Tsurubuchi (University of Tsukuba) for
12 technical advice with the rat surgical experiments and brain stereotaxic apparatus; and Drs. T. Sakurai
13 (current affiliation, Kanazawa University) and T. Matsuki (current affiliation, University of Texas
14 Southwestern Medical Center at Dallas) for technical assistance with the rat cannulation. This work was
15 supported by the Aichi Cancer Research Foundation (Y. O.) and Research Grant of the Princess
16 Takamatsu Cancer Research Fund (S. C.).
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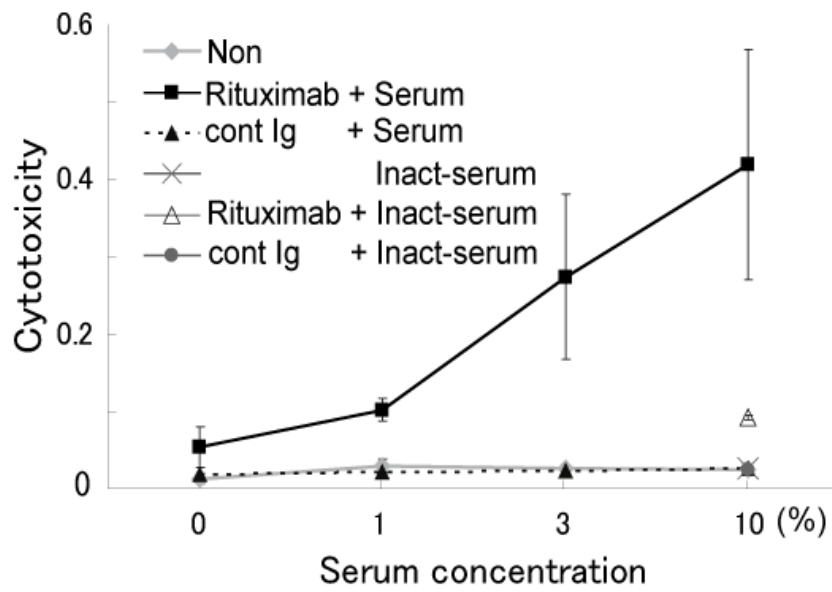
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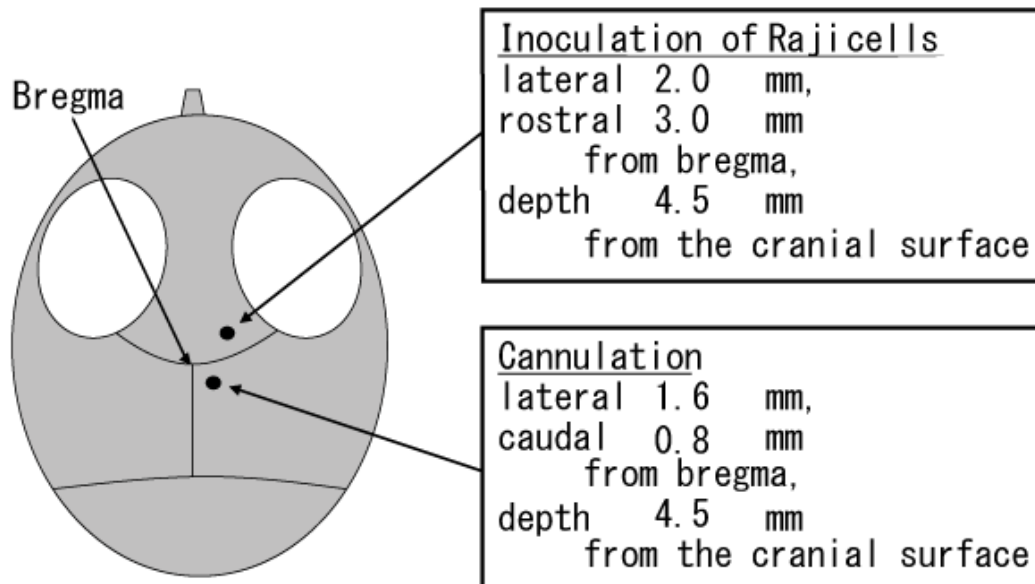
Fig. 1

**Complement-dependent cytotoxicity of rituximab and serum.**

Complement-dependent cytotoxicity of Raji cells treated with the indicated concentrations of human serum in the presence of rituximab or control human immunoglobulin (cont Ig) was measured.

Inact-serum, heat-inactivated serum. Data are representative of two independent experiments.

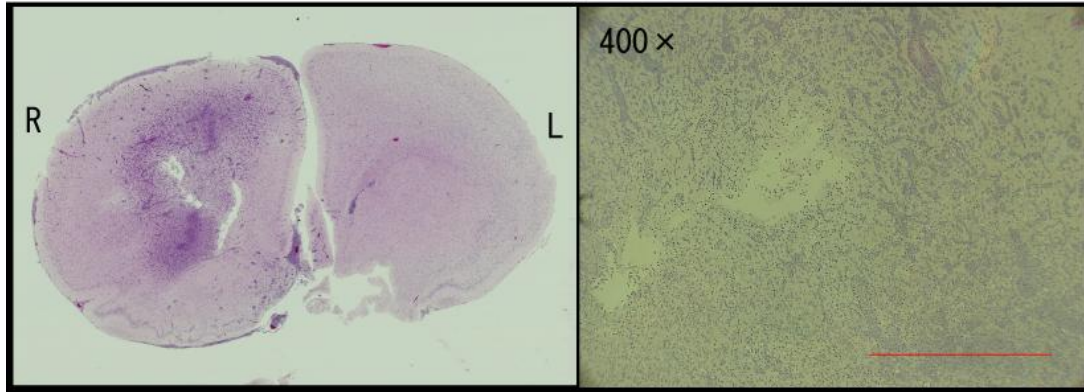
Fig. 2



Positions of intracerebral inoculation of Raji cells and intraventricular cannulation.

Raji cells were inoculated into the deep frontal lobe. The cannula was placed in the ipsilateral lateral ventricle.

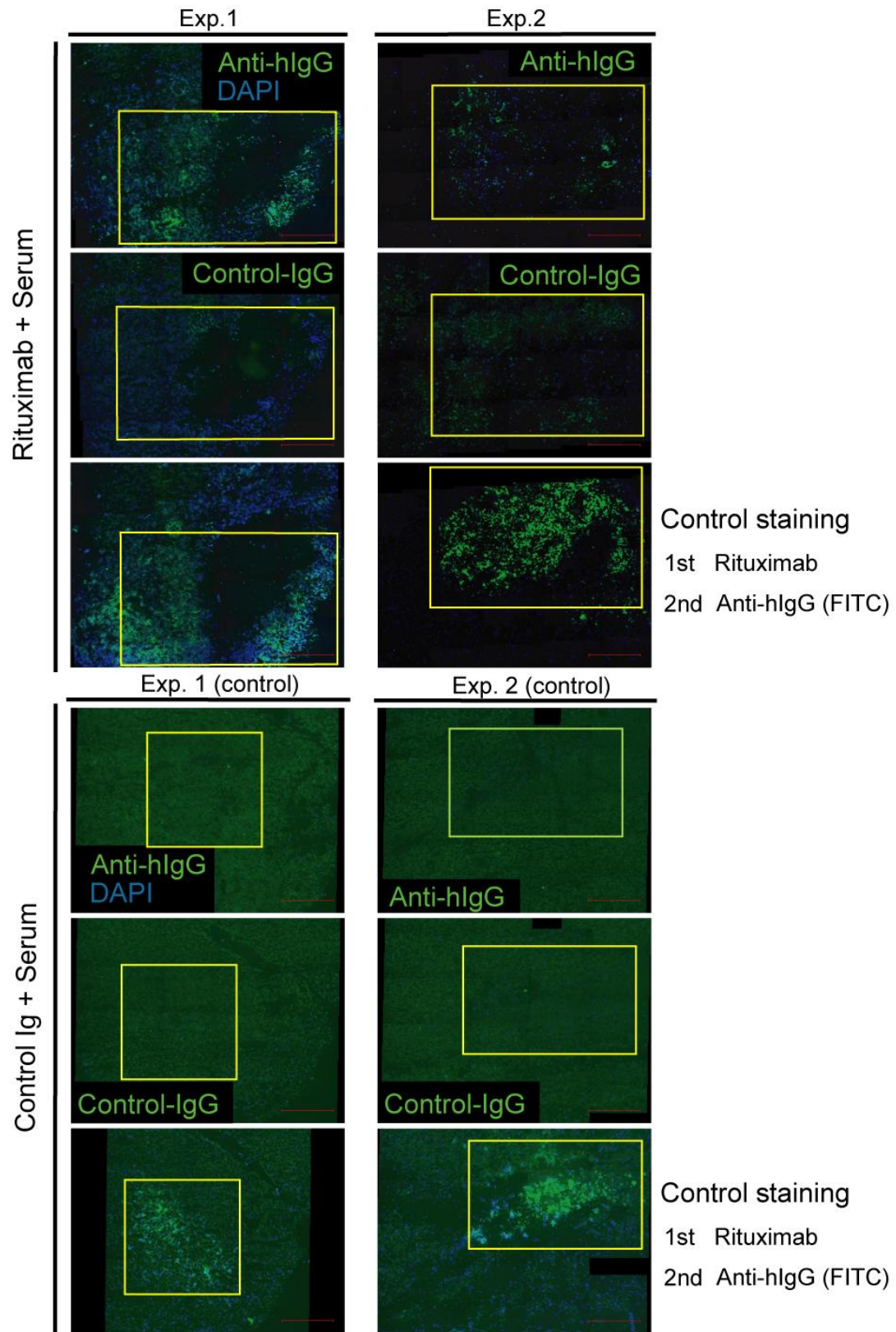
Fig. 3



Histological study of rat cerebrum after Raji cell inoculation.

A coronal section of nude rat cerebrum was stained with hematoxylin and eosin 12 days after Raji cell inoculation. Left panel: macroscopic finding. The Raji cell tumor is depicted by basophilic staining in the right hemisphere. Right panel: histology in a high-power field (400 \times). Raji cells diffusely infiltrated in the deep frontal lobe of the cerebrum. The scale bar indicates 1 mm.

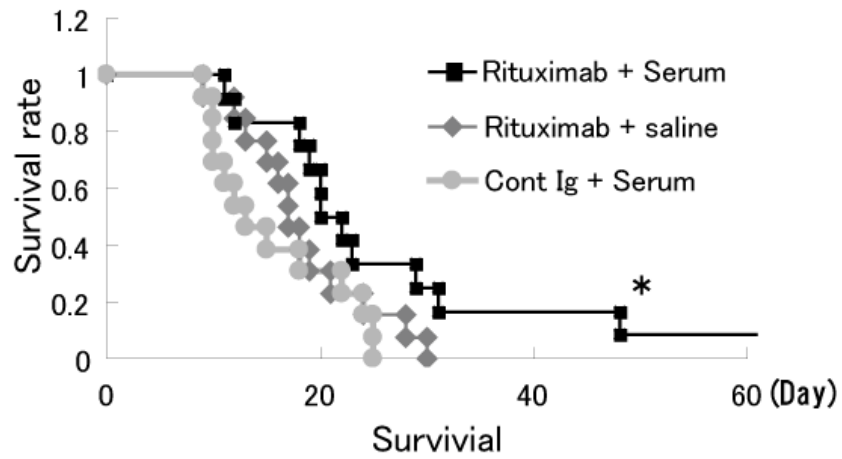
Fig. 4



Specific binding of rituximab to tumor cells in the cerebrum after intraventricular administration.

Rituximab plus serum (Exp. 1 and Exp. 2) or control immunoglobulin plus serum [Exp. 1 (control) and Exp. 2 (control)] was administered into the lateral ventricles of the rats from day 9 to day 11 after Raji cell inoculation. The animals were euthanized, and the brains were surgically removed and stained 24 hours after the last administration. Sections were stained with FITC-conjugated anti-human IgG (Anti-hIgG, upper panels) or FITC-conjugated control immunoglobulin (middle panels). To clarify the margin of the Raji cell tumor, the section was stained with rituximab as the primary antibody and FITC-conjugated anti-human IgG as a secondary antibody (lower panels). DAPI (4',6-diamidino-2-phenylindole) was used for nuclear staining. The yellow box depicts the area of the Raji cell infiltration and the scale bar indicates 500 μm . Data are representative of three independent experiments.

Fig. 5



Therapeutic effect of intraventricular administration of rituximab and serum.

Kaplan-Meier survival curves. Data were collected from 10 pooled experiments. The rats were treated with rituximab plus serum (Rituximab + Serum, $n = 12$), rituximab plus saline (Rituximab + saline, $n = 13$), or control immunoglobulin plus serum (Cont Ig + Serum, $n = 13$). The asterisk indicates a statistically significant difference between rituximab plus serum and control immunoglobulin plus serum ($P = 0.049$) in a log-rank test.

Supplementary Material

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