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Blood—Brain Barrier Permeability in Healthy Rats and Rats with Experimental C6 Glioma after Fractionated Radiotherapy of the Brain

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Brain tumors are accompanied by blood-brain barrier (BBB) dysfunction. However, targeted drug delivery still remains difficult to achieve. **Objective.** To evaluate the effect of fractionated radiotherapy on permeability of the blood-brain barrier in healthy rats and rats with C6 glioma *in vivo*. **Materials and Methods.** An increase in BBB permeability in C6 glioma was assessed by dynamic MRI monitoring (glioma size before and after radiation therapy in combination with immunotherapy, *n*=30) and confocal microscopy (fluorescence imaging of tumor invasion boundaries in a dose-dependent experiment for the amount of injected antibodies). In healthy rats, BBB permeability to macromolecular substances (MMS) was assessed by ELISA (*n*=23, 192 plasma samples) and confocal microscopy (*n*=7). **Results.** It was shown that BBB permeability to biological macromolecules in blood—brain and brain—blood directions was increased after fractionated radiotherapy. Conclusion: Drug delivery to the brain can be improved using therapeutic doses of radiation treatment that affects the BBB and thus the risk of serious side effects that are often associated with drug dose can be minimized.

Keywords: *glioma, blood-brain barrier in norm and pathology, fractionated radiotherapy.*

The stability of the internal environment of the brain is normally ensured by the integrity of the blood—brain barrier (BBB). Brain tumors are accompanied by BBB dysfunction. However, targeted drug delivery still remains difficult to achieve. Accordingly, the efficacy of prescribed chemotherapy or immunotherapy in brain tumors is extremely low as opposed to treatment of tumors of internal organs. What is the reason for such selectivity?

In clinical practice, a temporary break of BBB is achieved in exceptional cases by systemic administration of osmotic diuretics (mannitol) and inflammatory mediators (bradykinin) [1—3]. There are recent data on positive results of the preliminary irradiation of brain tumors before chemotherapy, but the range of applied doses of fractionated radiotherapy is quite wide [4—6]. For example, most researchers [7—9] believe that radiation therapy doses of 20 to 30 Gy with fraction size of 2 Gy can be used to break the BBB without any side effects.

In the last decade, there is only fragmented information on the molecular changes in the cerebral endothelium, astroglial reaction, duration and timing of the opening of the blood—brain barrier during fractionated irradiation [10—15]. The problem of cognitive function status and quality of life of patients after a cycle of chemotherapy or radiotherapy accompanied by increased life expectancy still remains controversial and open question in modern neuro-oncology [16, 17]. As a result, the study of advisability of preventive radiation therapy before chemotherapy or immunotherapy for patients with brain tumors is important.

The objectives of the study included evaluation of temporal parameters of increase in BBB permeability to specific and non-specific macromolecular substances injected into the systemic blood circulation of healthy rats and rats with C6 glioma subjected to various doses of fractionated radiotherapy.

Materials and methods

Experimental animals and experimental design. The study was conducted on 60 Wistar rats. At the beginning of the experiment, the rats were 2 months old and weighed 200±20 g. Prior to the experiment, the animals were kept in laboratory vivarium in cages (8 animals in each cage) on a standard diet (not less than 2 weeks) with free access to water and normal light regime. Housing conditions for rats and all experimental procedures complied with international rules of animal handling (the European Community Directive 2010/63/EU of 24 September 2010 available at http://mslasa.ru/wp-content/uploads/Dir_2010_63_Rus-LASA2.pdf). Rats were randomized into 2 groups. Group 1 included animals with C6 glioma model (*n*=30), group 2 included intact animals (*n*=30). Each group was divided into three subgroups treated with radiotherapy: 1A (*n*=10), 1B (*n*=10), 1C (*n*=5) and 2A (*n*=10), 2B (*n*=15), 2C (*n*=5). Additionally, each group included two subgroups without radiotherapy (1d and 2d).

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Regimen of the rat brain irradiation. Irradiation of the rat brain was performed on PRIMUS linear accelerator (Siemens, USA) at the Burdenko Neurosurgical Institute using 6 MV bremsstrahlung with a dose rate of 2 Gy/min. The distance from radiation source to the plane of the rats was 100 cm. Four rats were irradiated simultaneously. The rats were placed into a foam mold with their noses directed towards the center in such a way that they rested against the wall of the mold and field boundary defined based on a half-height of dose distribution passed behind the brain with a margin of 5 mm. This ensured irradiation of the whole brain in all rats. Square field size at isocenter was 140 mm. Solid water layer with a thickness of 20 mm was used to provide electronic balance. Radiation field penumbra, which was determined based on the dose drop from 80 to 20%, was reduced to 4 mm by means of specially designed 60 mm thick frame of Wood's alloy. Irradiation was performed without general anesthesia. The rats were immobilized using restrainers (DecapiCones Braintree Scientific, Inc., USA). The animals were daily exposed to irradiation 5 times a week until a total dose of radiotherapy of 36 Gy was achieved (2 Gy×18 fractions (groups 1A and 2A), 4 Gy×9 fractions (groups 1b and 2b), 6 Gy×6 fractions (groups 1c and 2c), and 0 Gy×0 fractions (groups 1d and 2d).

The experimental protocol to assess BBB permeability in rats exposed to irradiation (normal and with glioma). In group 1, changes in BBB permeability for MMSs during fractionated radiotherapy of C6 glioma was evaluated by monitoring of magnetic resonance imaging (MRI) (morphometry of glioma before and after radiotherapy in combination with immunotherapy, n=30) and confocal microscopy (fluorescence imaging of tumor invasion boundaries in a dose-dependent experiment based on the amount of administered antibodies). In group 2 rats, BBB permeability to MMSs was assessed by ELISA (n=23; 192 blood plasma samples) and confocal microscopy (n=7).

Quantitative analysis of BBB permeability based on concentration level of glial fibrillar acidic protein (GFAP) in blood serum of rats exposed to irradiation by enzyme immunoassay (ELISA, Sandwich option). After the cycle of radiotherapy, 2 ml of the whole blood was sampled from the tail vein of each rat once per 7 days during 3 months. Blood corpuscles were sedimented by centrifugation. Plasma was collected and frozen at −80°C. Components for ELISA (PabGFAP, recGFAP, and MabGFAP) were obtained in the Laboratory of Immunochemistry of the Federal Medical Research Center of Psychiatry and Addiction Medicine of the Ministry of Health of the Russian Federation. Polystyrene plate was activated by MabGFAP (100 µl with concentration of 5 µg/ml) in 0.1 M bicarbonate buffer with pH 9.6 for 8 h at 4°C. The plate was washed for 3 times to remove the excess of MabGFAP and blood plasma samples containing the antigen (50 µl) were added followed by incubation at 20°C for 1 hour. After another triple washing, PabGFAP with dilution of 1:5000 was added to the plate and developed using a-rabbit-HRP with dilution of 1:200 (Goat antirabbit IgG-HRP, Sigma, USA). Antispecies antibodies were preincubated for 1 h with murine serum (1:200) to reduce their nonspecific adsorption. The reaction of the substrate mixture with 3,3',5,5'-tetramethyl-benzidine (Ready-to-use, Invitrogen, USA) was stopped by adding 0.1 N HCl. The numerical value of GFAP concentration was calculated based on the optical density using a calibration curve.

Qualitative analysis of BBB permeability based on accumulation of fluorescently labeled monoclonal antibodies in the brain of rats exposed to irradiation (BBB permeability for MMSs). Specific and non-specific immunoglobulins (Ig) were labeled with Alexa fluorescent label with different wavelengths using the protocol provided by manufacturer (Invitrogen, USA). Antibodies to GFAP (PabGFAP, MabGFAP), VEGF and connexin-43 (Cx43) — MabVEGF, were obtained at the laboratory of immunochemistry of the Federal Medical Research Center of Psychiatry and Addiction Medicine of the Ministry of Health of the Russian Federation. Additionally, commercial nonspecific Ig (Sigma, USA) and antibodies to von Willebrand factor (vWF) (Abeam, USA) were purchased.

The rats were injected with Ig labeled with Alexa 660 fluorescent label through the femoral vein under general anesthesia. Accumulation of labeled antibodies in brain structures *in vivo* was evaluated using confocal laser microscopy. To this end, 24, 48 and 72 hours after administration of the labeled antibodies, rats underwent transaortic perfusion of 4% paraformaldehyde under deep anesthesia and decapitation. The brain was then removed and 150—200 microns thick sections were prepared. Sections were exposed in the solution with polyclonal anti-GFAP (astrocytes), anti-vWF (endothelial cells) and immunodeveloped with antispecies antibodies (Invitrogen, USA) to visualize cell structures with a positive signal of Alexa 660.

Analysis of degradation of MabGFAP and IgG covalently bound to Alexa Fluor 488 in the blood. Binding of IgG and MabGFAP to Alexa Fluor 488 was performed according to the manufacturer's protocol (Invitrogen, USA). The fluorescence intensity was measured using VICTOR X3 Multilabel Plate Reader fluorometer in obtained samples with various dilutions. The labeled antibodies were diluted in blood plasma taking into account 5% loss of fluorescence. Incubation in blood plasma and sampling in 18, 24, 48 and 72 hours were performed followed by measuring the intensity of fluorescence in the sample. Centrifugation through a 30 kDa filter (Alexa Fluor 488 — 0.7 kDa) and measurement of flow through fluorescence intensity were carried out.

Statistical analysis. GFAP level was compared to the control using Dunnett's parametric test. The data were

represented by an average accumulation index and its standard deviation *(Mean±SD)*.

Results

Quantitative analysis of GFAP in plasma of group 2 rats subjected to fractionated irradiation was conducted in 23 rats. According to ELISA, GFAP level in the blood plasma increased 1.5—2-fold compared to the normal level. The highest values of protein level in rat serum were observed on 3—4th and 8—12th weeks. Six weeks after radiotherapy, GFAP levels returned to baseline normal values. On the 10th week, maximum GFAP plasma level was observed. No significant differences in plasma concentration of protein in the groups of rats that differ in a single dose of radiation therapy were detected **(Fig. 1)**.

The study of BBB permeability to high molecular weight biomolecules in the blood—brain direction was conducted 2 and 10 weeks after completion of radiotherapy cycle, focusing on quantitative GFAP values obtained by enzyme immunoassay.

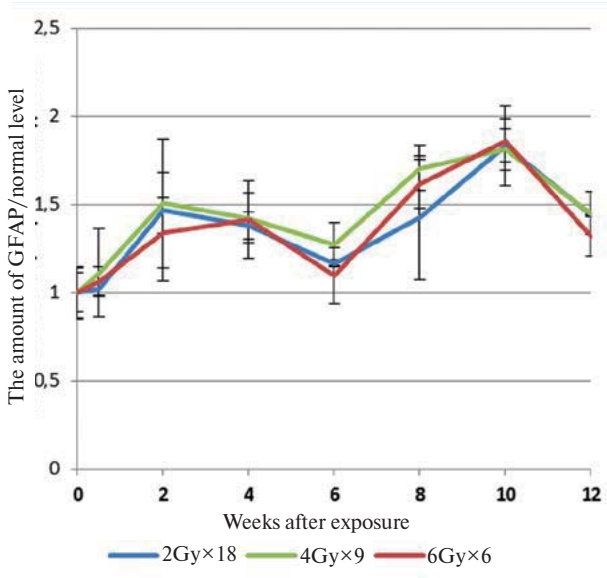
Given that increase in BBB permeability to biological macromolecules after radiation exposure may be not selective, it was important to differentiate between nonselective and selective accumulation of antibodies. For this purpose, specific and nonspecific Ig labeled with Alexa fluorescent label (Dye Alexa 488 absorb 495 nm, emit 519 nm; dye Alexa 660 absorb 663, emit 690 nm; Life Technologies Corporation, USA) were used in the experiment.

At the first stage, we evaluated accumulation of nonspecific Ig in the brain of rats after exposure *in vivo* 24, 48 and 72 hours after injection into the systemic circulation in each experimental group **(Fig. 2a, b)**. It was found that in 72 hours the signal from the fluorescence of non-specific Ig in the brain tissue almost disappeared, and occasionally preserved fluorescence was observed only in the vascular cavity **(see Fig. 2b)**.

In addition, the experiment with injection of labeled non-specific Ig have shown that BBB can be permeable to substances with a weight of over 150 kDa in all radiotherapy options that we applied (2 Gy×18 fractions, 4 Gy×9 fractions, and 6 Gy×6 fractions). Based on previously published data on the studies of cognitive functions after radiotherapy and positive effects of the latter against gliomas, a single fraction used in rats at the second stage was not more than 4 Gy. Thus, the fluorescent signal was recorded only 72 hours after injection of specific antibodies to VEGF, GFAP, and Cx43.

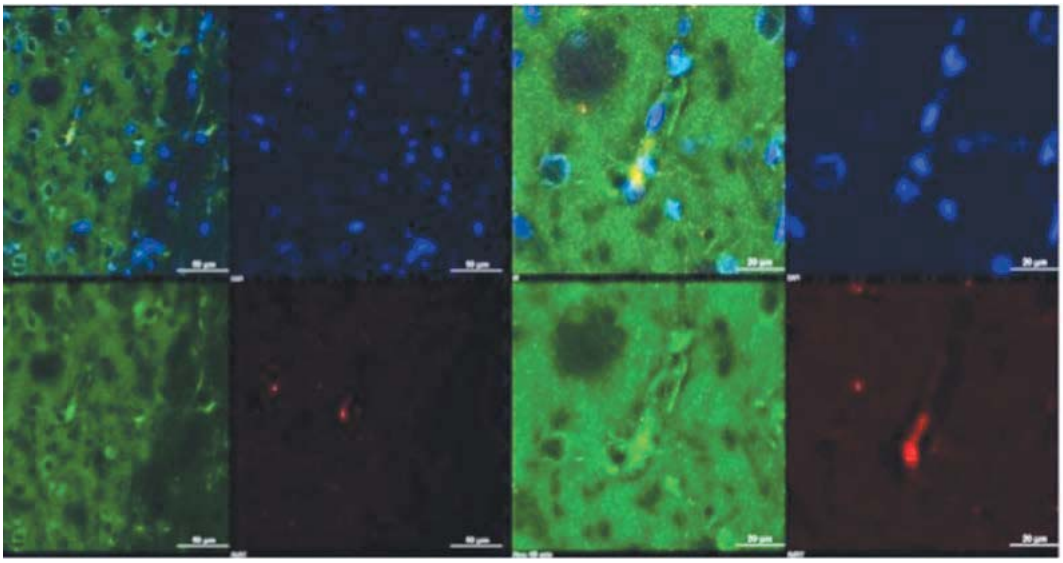
Target antigens for fluorescence imaging *in vivo* were selected based on the quantity of protein synthesis in the nerve tissue, specificity, and availability. The level of GFAP expression in astrocytes increases in reactive gliosis. Cx43 is a membrane protein for homologous and heterologous gap junctions between astrocytes, endotheliocytes, and tumor cells [18—21]. Synthesis of the vascular endothelial growth factor (VEGF) increases in response to hypoxia and inflammatory processes, including those caused by radiation exposure [22].

According to the data of microscopy, BBB permeability to monoclonal antibodies was increased for a long time. Two weeks after fractionated radiotherapy, we recorded BBB permeability to labeled Ig independent of single-fraction dose value. Ten weeks after



2Gy×18	0,5 weeks	0,992
	2 weeks	0,019
	4 weeks	0,096
	6 weeks	0,791
	8 weeks	0,044
	10 weeks	<0,01
	12 weeks	0,032
4Gy×9	0,5 weeks	0,849
	2 weeks	<0,01
	4 weeks	0,003
	6 weeks	0,130
	8 weeks	<0,01
	10 weeks	<0,01
	12 weeks	0,009
6Gy×6	0,5 weeks	0,913
	2 weeks	0,100
	4 weeks	0,004
	6 weeks	0,890
	8 weeks	<0,01
	10 weeks	<0,01
	12 weeks	0,183

Fig. 1. **The amount of GFAP in rat plasma after fractionated irradiation normalized to the basal level: the significance of differences from the normal GFAP level.**



a b

Fig. 2. **Microscopy of accumulation of labeled IgG in sections of rat brain 72 hours after intravenous administration of IgG Alexa 660 (red fluorescence). Sections were additionally immunodeveloped *ex vivo* using MabVEGF + Anti-mouse Alexa 488 (vessels — green). Nuclei were counterstained with DAPI (blue).**

a — survey microscopic picture of slices of the rat brain with glioma (scale bar — 10 mm), b — increasing microscopy resolution to 20 microns.

radiotherapy, permeability to monoclonal antibodies has not changed.

An intense fluorescence signal was observed on the sections of the anterior and middle portions of the brain and cerebellum after injection of monoclonal anti-GFAP and anti-VEGF antibodies into the systemic circulation of rats and 72 h blood circulation. When analyzing accumulation of Alexa 488 labeled monoclonal anti-GFAP antibodies in vivo, rat brain slices were further exposed in solution with polyclonal antibodies to GFAP and developed using Anti-mouse Alexa 594 antispecies antibodies. According to the results of confocal microscopy on localization of monoclones and polyclones to GFAP, monoclonal anti-GFAP antibodies were accumulated in reactive astrocytes **(Fig. 3)**.

To determine the species of the structural elements, where the fluorescence signal from anti-VEGF antibodies injected into the bloodstream of rats was recorded, brain slices were counterstained with antibodies to vWF and Mab2B6. Immunohistochemical results demonstrate selective accumulation of anti-VEGF antibodies in vWF- and AMVB1-positive cerebral endotheliocytes **(Fig. 4, 5)**.

Thus, we have demonstrated that long-term increase in permeability of the blood brain barrier to MMSs was observed when the brain of healthy rats was exposed to fractionated radiotherapy.

Additional experiment for degradation of Alexa 660 labels covalently bound to antibodies in rat serum was conducted to ensure the reliability of the results on accumulation of labeled antibodies in the nervous tissue. When analyzing the intensity of fluorescence in various dilutions of labeled antibody preparations, linear relation between the fluorescence and sample dilution was observed **(Fig. 6)**. The background value measured in the normal blood plasma of rats was 724.8±223.2.

The minimum detectable value was calculated as the five-fold background value and it was 4740. In order to detect a 5% loss of fluorescence of labeled antibodies during incubation in blood plasma, this value was taken to be 5%. Consequently, the fluorescence intensity in the final sample should be at least 94,802. After dilution in blood plasma, fluorescence intensity was 126,473.6±1,385 and 131,541.6±1,694 for Alexa 488 and MabGFAP labeled antibodies, respectively. Fluorescence intensity in plasma sampled in 18, 24, 48 and 72 hours decreased over time.

Thus, fluorescence intensity in 72 hours was 111,814±9,245 and 112,340±9,552 for Alexa 488 IgG and MabGFAP labeled antibodies, respectively. The decrease in the fluorescence was 11.6 and 14.6% compared to the initial value for Alexa 488 IgG and MabGFAP labeled antibodies, respectively **(see Fig. 6, Table)**. After filtration of labeled antibody preparations through 30 kDa pore size filters, flow through fluorescence values were significantly reduced (size of labeled antibodies was greater than 150 kDa). Fluorescence intensity differed from the background value in 24, 48, and 72 h **(see Table)**.

Thus, the fluorescence intensity in the flow through samples taken at various time periods after the beginning of incubation did not exceed the background level. This indicates that there is no low molecular weight fluorescent components up to 30 kDa, indicating that cleavage of the

label from the antibody—Alexa 488 complex is not more than 5%.

When assessing the improvement of BBB permeability in experimental glioma for diagnostic and therapeutic agents during radiotherapy, we relied on the results of radiotherapy in dose-dependent experiments: the higher the single-fraction dose, the more evident antineoplastic effect [27]. In the case of 6-day long irradiation with a single dose of 6 Gy, glioma size did not increase from the beginning of radiotherapy. However, rats died of side effects within 2—3 days after completion of the experiment. In the group of rats with radiotherapy schedule 4 Gy×9 fractions, the survival after radiotherapy was 100% and efficacy against tumor growth was evident. However, a week later, tumor growth resumed and invasion into the surrounding tissues was so intensive that another week later rats died of intracranial dislocation **(Fig. 7a—c).**

When immunotherapy was added within a week after completion of irradiation, in particular with MabCx43, which previously demonstrated antitumor efficacy in monotherapy, we observed further inhibition of tumor growth **(see Fig. 7)** [27]. In this case, a single injection of 1 mg dose of antibodies resulted in increased lifespan of the animals up to 56 days. In the case of monotherapy with the same dosage, similar effect was observed when it was administered weekly.

In addition, the results of confocal microscopy study of brain slices with glioma are shown. Previously, not less than 500 μg of MabCx43 with covalently bound Alexa fluorescent label had to be injected into systemic circulation of a rat *in vivo* to visualize a peritumoral area. After radiotherapy (4 Gy×9 fractions and 2 Gy×18 fractions), administration of labeled antibodies at a dose of 40 μg enabled registering the fluorescence signal at the reactive astrogliosis area (around glioma) **(Fig. 8)**.

Summarizing the results, we can state that BBB permeability to diagnostic and therapeutic agents increases after fractionated radiotherapy.

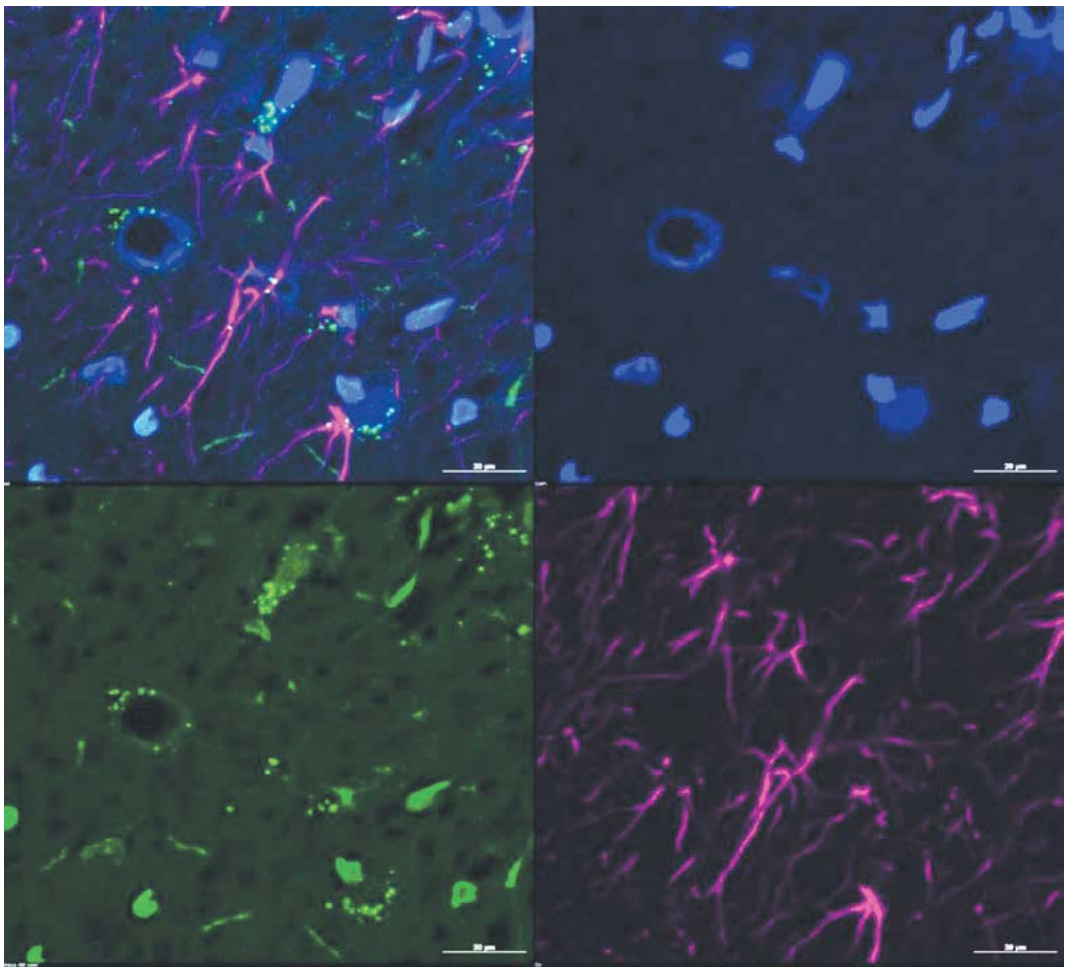


Fig. 3. **Microscopy of accumulation of Alexa 488 labeled MabGFAP in slices of the rat brain 72 hours after intravenous administration (corpus callosum). MabGFAP Alexa 488 (green fluorescence), slices were additionally immunodeveloped using PabGFAP + Anti-mouse Alexa 594 (purple). Nuclei were counterstained with DAPI (blue).**

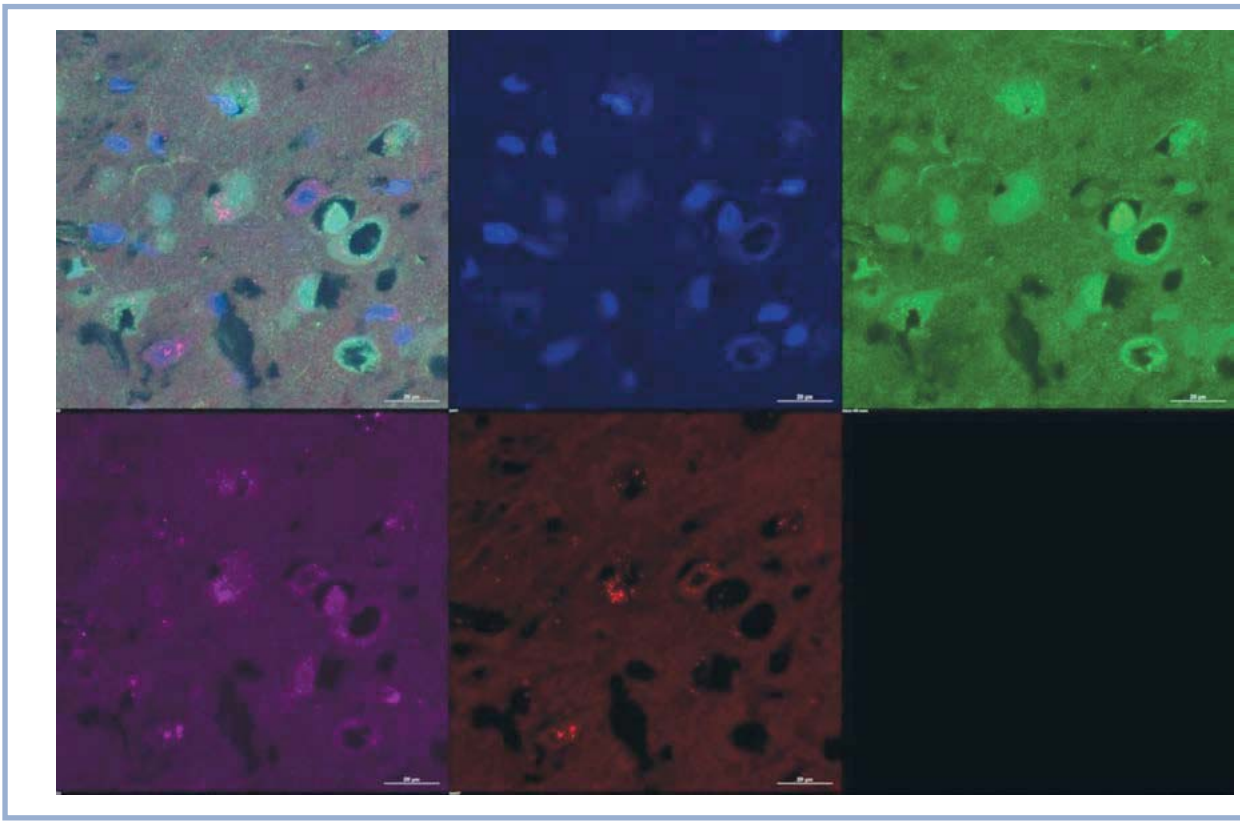


Fig. 4. **Analysis of accumulation of Alexa 660 labeled MabVEGF in the striatum of the rat brain 72 hours after intravenous administration of MabGFAP + Anti-mouse Alexa 488 (green fluorescence), MabVEGF + Alexa 660 (red). The slices were additionally immunodeveloped with PabvWF + Anti-rabbit Alexa 594 (purple). Nuclei were counterstained with DAPI (blue).**

Significance of differences from the background flow through fluorescence intensity value after centrifugation through 30 kDa

Incubation time of labeled Ig in serum, h	Specimens of fluorescently labeled specific and nonspecific Ig	Fluorescence intensity values, conventional units
18	IgG	0.001
	MabGFAP	<0.001
24	IgG	1.000
	MabGFAP	0.301
48	IgG	0.515
	MabGFAP	<0.001
72	IgG	0.137
	MabGFAP	0.646

Discussion

The problem of permeability of the blood—brain barrier to therapeutic and diagnostic agents is still relevant and it is being intensively studied by researchers and clinicians around the world. Previously, researchers used radioactive markers [23], MRI monitoring [24], immunohistochemistry [25], positron emission tomography, quantitative analysis of albumin [26], and other methods in their experiments to estimate the increase in BBB permeability caused by therapeutic doses in the radiotherapy of the brain. In individual cases, such studies are already being carried out on patients. Permeability has been shown to increase upon daily fractionation of 3 Gy until a total dose of 30 Gy in brain regions bordering the irradiation field. Increase was directly proportional to the dose of ionizing radiation [10]. Fractionation with a single daily dose of 2 Gy [27] is advisable to reduce long-term toxicity. Another study [12] showed that the fractionation dose of 2 Gy used to change BBB permeability can optimize the effect of chemotherapy. D. Qin et al. noticed that BBB

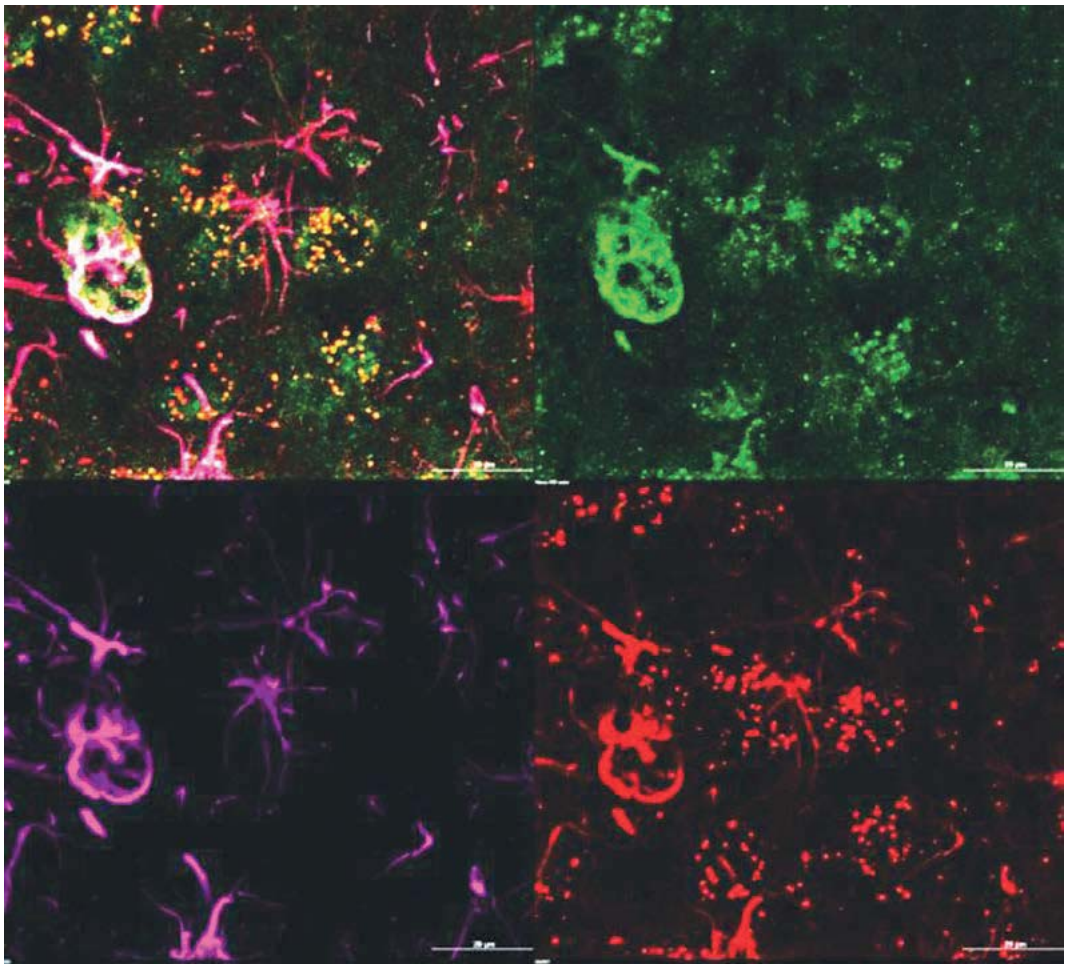


Fig. 5. **Microscopy of accumulation of Alexa 660 labeled MabVEGF in slices of the rat brain 72 hours after intravenous administration (corpus callosum) of Mab2B6 + Anti-mouse488 (green fluorescence), PabGFAP + Anti-rabbit Alexa 594 (purple), and MabVEGF + Alexa 660 (red).**

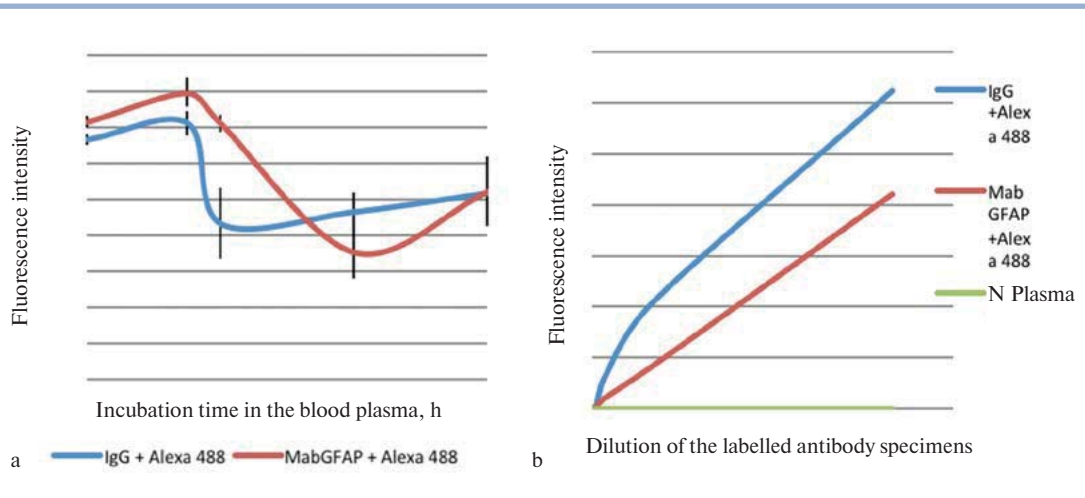


Fig. 6. **Time history of fluorescence intensity of labeled antibody specimens.**

a — during incubation of labeled antibodies in the blood plasma; b — in different plasma dilutions.

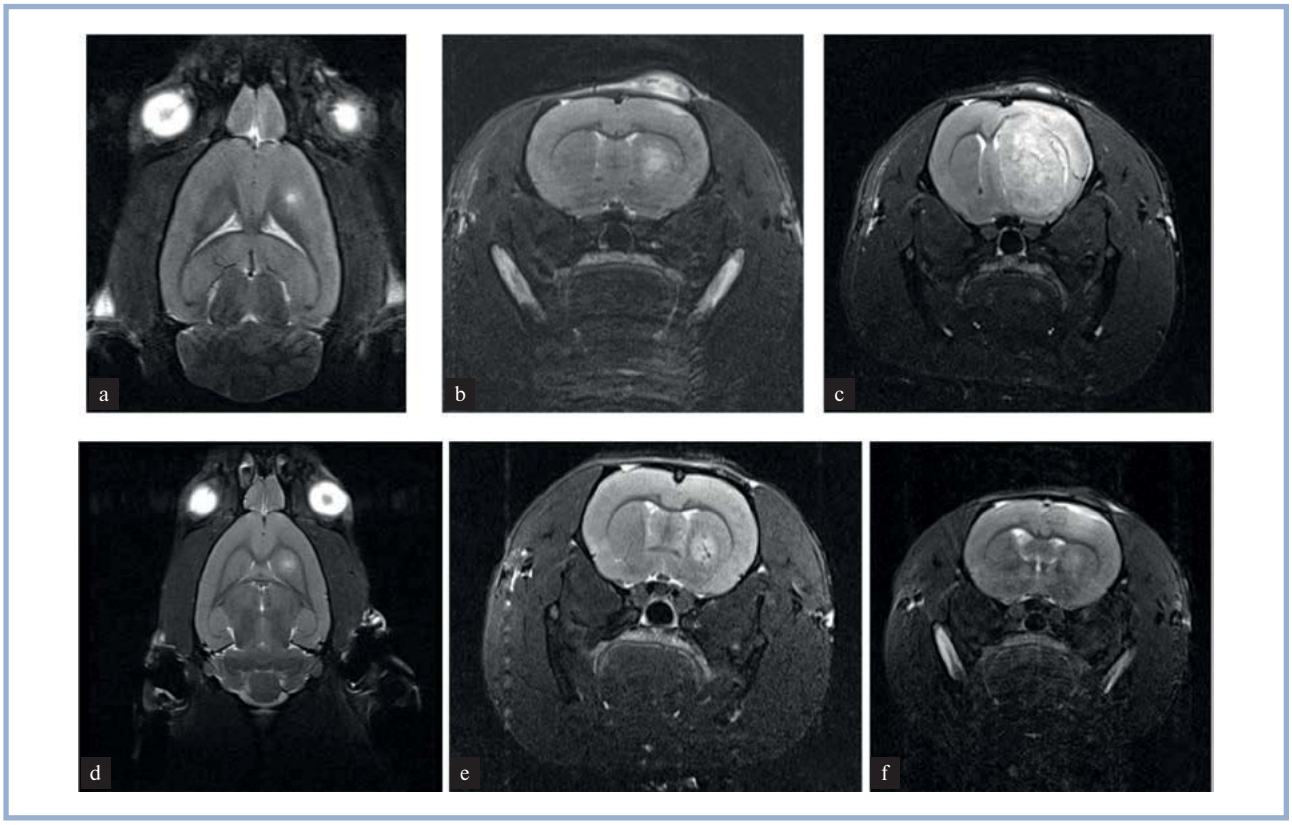


Fig. 7. **MRI view of the rat brain with C6 glioma after radiotherapy according to the schedule of 4 Gy×9 days.**

a, b, c — the 1st, 7th and 14th days after radiotherapy and after radiotherapy according to the schedule of 4 Gy×9 days followed by administration of MabCx43; d, e, f — the 1st, 7th and 14th days after the radiotherapy.

permeability within and around a tumor is only 20% higher than that in the normal brain tissue [12]. However, despite the investigations in this field, the use of radiotherapy aimed at enhancing BBB permeability and efficacy of chemotherapy is still limited [7, 28].

The use of ionizing radiation in clinical practice is based on differences in radiation sensitivity of tumor and normal tissues [7, 28]. Alternative processes of damage and recovery arise upon exposure to ionizing radiation. Normal tissue recovers from radiation injury more rapidly and completely than tumors due to stable neurohumoral interaction with the host. Using these differences and managing them, one can achieve total destruction of the tumor, while preserving normal tissues. Moreover, adequately chosen dose fractionation can result in significantly higher damage to the tumor.

In Russia, in the case of radical treatment, irradiation with fractions of 1.8—2 Gy 5 times a week once a day to total doses that are determined by morphological structure of the tumor and tolerance of the normal tissue in the irradiation area (typically 60—70 Gy). Standard regimen of the remote fractionated radiation therapy include irradiation of the bed of the resected tumor (or the tumor itself) plus 2 cm around to a total focal dose of 55—60 Gy in 25 to 30 fractions (1.8—2.0 Gy per fraction) during 5—6 weeks. Abnormal signal area is detected using T2-weighted MRI (or FLAIR mode in case of benign gliomas) [7]. Besides the conventional radiotherapy regimen, other regimens of dose fractionation can be used in patients with brain tumors [7]. Concomitant chemoradiotherapy resulted in median survival of 14.6 months compared to 12.1 months in the group where only radiation treatment was used [7]. In patients with severe somatic status, in some cases, chemotherapy is not performed (radiotherapy is not indicated to patients with intracranial hypertension, until it is resolved).

The study of various regimens of combined chemotherapy and radiation therapy in patients with malignant gliomas is an important clinical problem. In our experimental study, we have shown that increased BBB permeability was observed after radiotherapy in rats with C6 glioma. In hospital environment, it can facilitate permeation of high concentrations of chemotherapeutic agents into the tumor. It was previously shown that in clinical practice the break through the BBB can be achieved in patients with brain lymphoma using bolus intra-arterial administration of mannitol [1].

Study of BBB permeability in hospital environment is possible due to monitoring of blood levels of various neurospecific proteins that are present in plasma at low

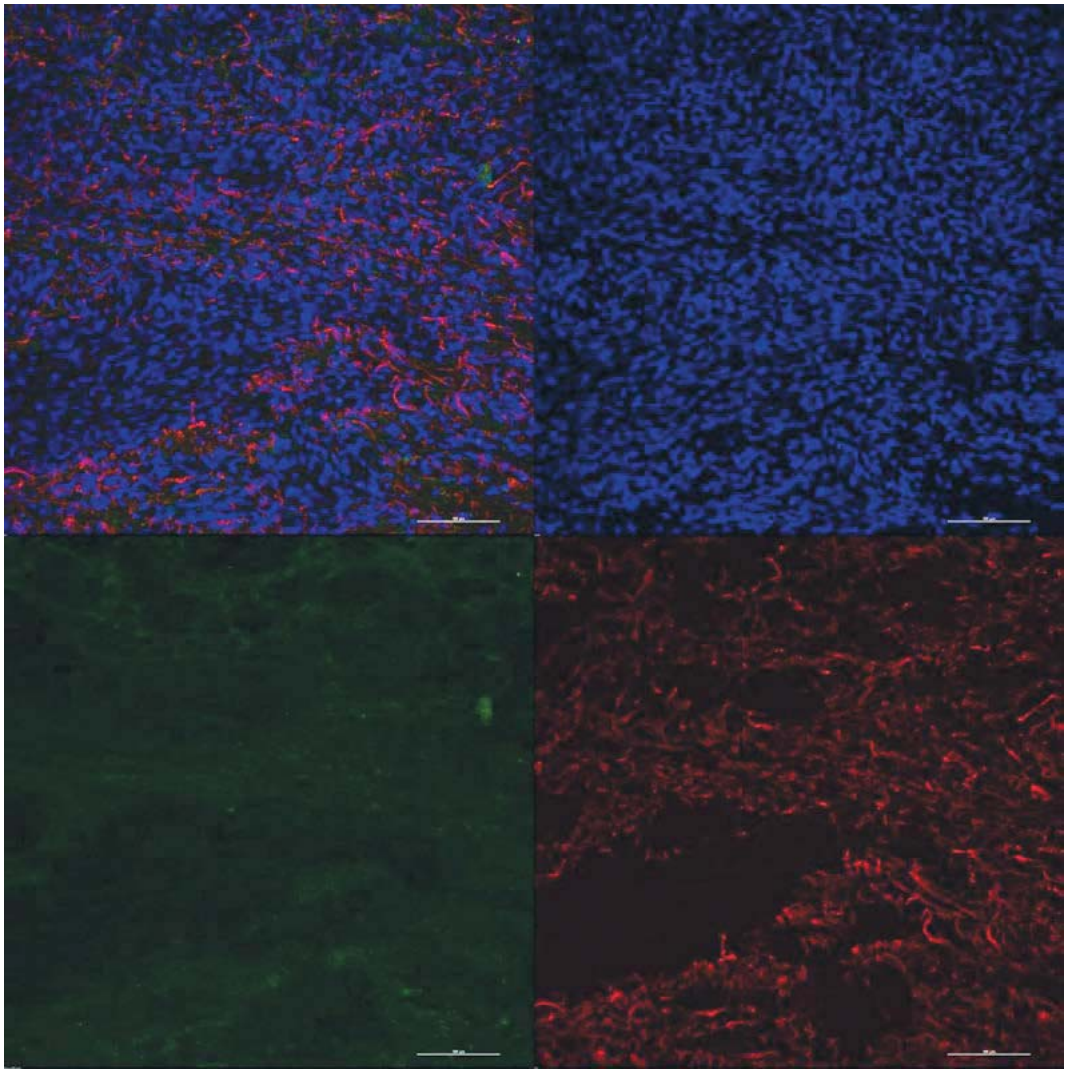


Fig. 8. **Microscopy of periglioma area using labeled MabCx43 at a dose of 40 μg injected into the systemic circulation of rats after fractionated radiotherapy with a single dose of 4 and 2 Gy.**

levels in health and significantly increase in CNS pathology due to increased BBB permeability. GFAP can play a role of such marker. Elevated level of GFAP is observed in experimental models of astrogliosis due to cryogenic brain injuries, stab wounds, experimental allergic encephalitis, hyperthermia, electric shock, toxic injuries, and upon exposure to ionizing radiation. Changes in GFAP expression are used for qualitative or quantitative assessment of the severity of brain injury, as well as in malignant tumors [22, 24, 25].

Increase in GFAP level in various brain structures and blood after a single exposure to γ-irradiation has been investigated by V.P. Chekhonin et al. [29]. Increased GFAP level in the blood plasma was observed, which is indicative of enhanced BBB permeability to neurospecific proteins in brain—blood direction. We repeated this experiment, exposing rats to the total irradiation of the entire body surface with a single dose of 30 Gy. In this experiment, we did not register the increase in BBB permeability to radiolabeled antibodies in blood—brain direction. In contrast to a single high-dose total irradiation of the brain, fractionated radiotherapy with small doses resulted in long-term increase in permeability of BBB capable of transmitting biological molecules with a molecular weight of 150 kDa.

Meanwhile, we have shown that the efficacy of fractionated radiotherapy of C6 glioma correlates with a single dose value [22]. The higher dose value results in faster inhibition of tumor growth. However, the number of side effects and the risk of rapid death of the animal also increases. There was virtually no increase in glioma volume upon irradiation with fractions of 4 Gy. However, tumor growth resumed at twice the speed after completion of irradiation [27, 31].

Neurophysiological tests on rats in the control group (without glioma) revealed cognitive deficit with all

radiotherapy options that we used (2 Gy×18 fractions, 4 Gy×9 fractions, and 6 Gy×6 fractions). The severity of cognitive disorders increases with increase in a single fraction dose [22, 29, 30]. For this reason, in the studies of BBB permeability in initially healthy rats (no glioma), we selected fractionation regimens that were shown to be effective in rapid stabilization of C6 glioma volume without severe side effects, namely 4 Gy×6 fractions and long-term irradiation with low doses of 2 Gy×9 fractions [31].

It has been shown that prolonged fractionated irradiation of the brain with small doses leads to BBB opening in blood—brain and brain—blood directions in healthy rats and improves permeability in experimental glioma. Total irradiation of the rat brain resulted in increased BBB permeability to macromolecular substances in all brain regions. In the case of targeted systems, increase in BBB permeability was observed at the area of presentation of the relevant antigen (i.e. there is selectivity of accumulation). In particular, we have shown that there was accumulation of labeled anti-GFAP and anti-Cx43 antibodies at astrogliosis area in response to radiation exposure. We also managed to visualize the vessels of the cortex, hippocampus, and cerebellum after injection of labeled anti-VEGF antibodies.

Conclusions

The results of confocal microscopy of glioma have shown that it is possible to visualize glioma after radiotherapy using a tenfold lower dose of labeled antibodies than that prior to irradiation. The amount of diagnostic label is also reduced. According to the results of quantitative analysis of GFAP in rat plasma, accumulation of intravenously injected fluorescently labeled antibodies in the brain of rats, and neurophysiological tests, BBB permeability to a greater degree depends on the duration of fractionated irradiation rather than magnitude of a single dose. In combination therapy of C6 glioma, preliminary radiotherapy allowed us to halve the dose of therapeutic antibodies. Thus, the preliminary exposure to therapeutic doses of irradiation that affects the BBB can improve the delivery of drugs and diagnostic agents to the brain and thereby minimize the risk of serious side effects associated mostly with drug dose.

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Commentary

Stability of the internal environment of the brain is normally ensured by the integrity of the BBB. Brain tumors are accompanied by BBB dysfunction. However, targeted drug delivery still remains difficult to achieve.

The study was aimed at assessing the timing of increase in BBB permeability to macromolecular substances *in vivo* after fractionated radiotherapy. The irradiation of the rat brain was performed on the PRIMUS linear accelerator (Siemiens, USA) at the Burdenko Neurosurgical Institute. Increase in BBB permeability in C6 glioma was assessed by dynamic MRI monitoring (glioma volume before and after radiation therapy in combination with immunotherapy; n=30) and confocal microscopy (fluorescence imaging of the boundaries of tumor invasion in a dose-dependent experiment for the amount of administered antibodies). In healthy rats, BBB permeability to macromolecular substances was assessed by ELISA (n=23; 192 plasma samples) and confocal microscopy (n=7). BBB permeability to biological macromolecules was shown to increase in blood—brain and brain—blood directions after fractionated radiotherapy. In addition, the conjugate stability during prolonged incubation in serum was assessed, i.e. the analysis of degradation of antibodies covalently bound to Alexa Fluor 488.

The problem of BBB permeability to therapeutic and diagnostic agents is still relevant. Similar studies are conducted in the laboratories around the world, in rare cases these studies are conducted in patients [5, 6, 14, 19, 20]. It has been shown that permeability increases upon daily fractionation of 3 Gy at the brain areas bordering the irradiation field [14]. The researchers showed that labeled anti-GFAP and anti-Cx43 antibodies are accumulated at atrogliosis area in response to radiation exposure and managed to visualize the vessels of the cortex, hippocampus, and cerebellum after injection of labeled anti-VEGF antibodies. In combination therapy of experimental glioma, preliminary radiotherapy allowed reducing the dose of therapeutic antibodies by a factor of 2.

Thus, in case of the need for combination therapy of glioblastomas, BBB exposure to therapeutic doses of irradiation may improve the delivery of drugs to the brain and thereby minimize the risk of serious side effects associated mostly with drug dose.

In summary, it can be stated that the problem of crossing the barrier is a topical issue and solution of this problem has clear prospects of practical implementation.

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