Antitumor Effects of Monoclonal Antibodies to Connexin 43 Extracellular Fragment in Induced Low-Differentiated Glioma

G. M. Yusubalieva[1,2], V. P. Baklaushev[1,2], O. I. Gurina[1], M. V. Gulyaev[3], Yu. A. Pirogov[3], and V. P. Chekhonin[1,2]

Translated from *Kletochnye Tekhnologii v Biologii i Meditsine*, No. 1, pp. 51-57, January, 2012
Original article submitted July 29, 2011

We studied the effect of intravenous administration of monoclonal antibodies to the second extracellular loop of connexin 43 (MAbE2Cx43) on the dynamics of glioma growth and survival of experimental animals. Morphometric analysis of magnetic resonance imaging data showed that weekly intravenous administration of MAbE2Cx43 in a dose of 5 mg/kg significantly reduced glioma volume starting from day 21 after tumor implantation. By day 29, the mean volume of glioma in the experimental group (therapy with specific antibodies) was 2-fold lower than in controls. Deceleration of glioma growth in rats receiving MAbE2Cx43 was accompanied by a significant prolongation of rat lifespan (according to Kaplan–Meier test) and even led to complete recovery without delayed relapses in 19.23% animals. The mechanism of tumor-suppressing effects of antibodies can be related to inhibition of specific functions of connexin 43 in glioma cells in the peritumoral zone.

Key Words: *connexin 43; glioma C6; monoclonal antibodies*

According to recent data, the median survival in patients with multiform glioblastoma is about 14 months despite the use of all available means of combined therapy [1]. This high mortality is primarily determined by high invasiveness of multiform glioblastoma and rapid dissemination of the tumor in perivasal and perineural spaces [8,10]. Glioma cells deeply penetrating into the peritumoral zone cannot be completely removed by surgical methods. They are also resistant to chemo- and radiotherapy, because these cells do not proliferate during invasion [9].

In perivasal invasion, mobilized glioma cells spread along astrocytes surrounding microvessels. This process is always accompanied by opposite migration of reactive astrocytes towards the pathological focus with the formation of peritumoral astroglial hurdle [8,9]. Apart from the major proteins involved in migration and invasion (metalloproteinases 2 and 9, integrin-αvβ3, EphB2, and L1CAM [10,16]), a certain role in these processes was hypothesized to be played by heterologous gap junctions between astrocytes and Cx43-positive (Cx43: connexin 43) glioma cells [13]. This assumption is confirmed by the data that Cx43-positive glioma cells are more mobile and are primarily localized in the peritumoral zone [8,12,14]. In low differentiated glioma cells, the overall expression of gap junction proteins and the number of functionally active gap junctions decrease in comparison with those in high differentiated gliomas [15].

We previously obtained monoclonal antibodies to the second extracellular Cx43 fragment (MAbE2Cx43) [11] and showed that intravenous injection of these antibodies led to their accumulation in the peritumoral zone [3]. *In vitro* experiments showed that MAbE2Cx43 can inhibit structural organization of gap junctions [1].

[1]Department of Fundamental and Applied Neurobiology, V. P. Serbskii State Research Center of Forensic and Social Psychiatry, Ministry of Health and Social Development of the Russian Federation; [2]Department of Medical Nanobiotechnologies, N. I. Pirogov National Research Medical University, Ministry of Health and Social Development of the Russian Federation, Moscow; [3]Center of Magnetic Resonance Tomography and Spectroscopy, M. V. Lomonosov Moscow State University, Russia. ***Address for correspondence:*** gaukhar@gaukhar.org. G. M. Yusubalieva

0007-4888/12/1531-0163

Here we studied the development of low differentiated glioma against the background of repeated intravenous injections of MAbE2Cx43 in a high concentration.

MATERIALS AND METHODS

Hybrid cells producing MAbE2Cx43 were previously obtained by us [2]. Aliquots of hybrid cells ($3\text{-}5\times10^6$) from the cryobank were defrosted and injected intraperitoneally to BALB/c mice. After 10 days the animals were narcotized and ascitic fluid containing specific monoclonal antibodies in a concentration of 3-4 mg/ml was collected. MAbE2Cx43 were isolated from the ascitic fluid by affinity chromatography on agarose with immobilized protein G (Invitrogen) by the manufacturer's protocol and concentrated to 3-5 mg/ml in phosphate buffer; aliquots (1 mg) were stored at -70°C.

Isotyping of antibodies to Cx43 was performed by indirect immunoenzyme assay using ISO kit (Sigma). Affinity constant was determined by the method of J. D. Beattyetal *et al*. [5]. Calculations were performed by the formula:

$$K_{aff}=1/(4[Ab']-2[Ab]),$$

where [Ab'] is antibody concentration corresponding to 50% binding with antigen in concentration [Ag] and [Ab] is antibody concentration corresponding to 50% binding with antigen in concentration 2 [Ag].

Immunocytochemical analysis was performed on live and fixed glioma cell cultures and on cryostat brain sections of rats with experimental glioma perfused with 4% paraformaldehyde. In experiments with live cultures, antibodies to Cx43 were added to the medium to a concentration of 5 μg/ml and incubated for 1 h; then the cells were washed with physiological saline, fixed in 4% paraformaldehyde, washed again, and incubated with second antibodies to mouse immunoglobulins labeled with AlexaFluor 488 (Invitrogen). Immunocytochemical analysis and histochemical analysis of fixed preparations of cell cultures and brain sections were performed using standard immunocytochemical protocol. MAbE2Cx43 and polyclonal antibodies to GFAP were used as the primary antibodies. For double immunofluorescent staining we used a cocktail of the specified second antibodies to mouse immunoglobulins and antibodies to rabbit immunoglobulins labeled with Alexa Fluor 633 (Invitrogen). Cell nuclei were post-stained with DAPI (Invitrogen). The preparations were examined under a fluorescent microscope (LeicaDMI 6000) and a TCS SP5 scanning laser confocal microscope (LeicaMS) at N. K. Kol'tsov Institute of Developmental Biology, Russian Academy of Sciences.

The study was performed on 52 mature female Wistar rats weighing 200-220 g (at the start of the experiment); glioblastoma multiforme was modeled by stereotactic implantation of 5×10^5 glioma C6 cells into the striatum as described earlier [7].

The rats with experimental glioma were randomized into two groups. Group 1 rats (experiment, n=26) weekly received intravenous injections of MAbE2Cx43 in a dose of 5 mg/kg for 4 weeks starting from day 5 after implantation. Group 2 rats (control, n=26) received injections of unspecific immunoglobulins (IgGm) by the same scheme.

Immunoglobulin preparations were sterilized by filtering (Millex filter, 0.22 μ, Millipore Bedford) and injected into the femoral vein under ketamine narcosis (100 mg/kg body weight). Injections of specific and unspecific antibodies were stopped after death of all control rats (on days 25-30).

Dynamic magnetic resonance imaging (MRI) of the brain was performed weekly starting from day 5 after glioblastoma modeling on a BioSpec 70/30 tomograph (Bruker) at 7 T. For obtaining images, a linear transmitting coil with an inner diameter of 150 mm and a surface receiving coil for detection of the radiofrequency signal were used. T2 RARE (rapid acquisition with relaxation enhancement) sequences were applied (multiecho regimen). In RARE sequences, different phase-encoding gradients act during each echo signal, which allows using echo signals for construction of different lines in the same scan. The main RARE parameters were as follows: TR=6000 msec, TE=55 msec, echo number 8, section thickness 0.5 mm, array 156×156, resolution 0.167×0.167 mm/pixel.

Morphometric analysis of glioma in dynamics was performed using MRIcro 1.4 software. Survival analysis for rats treated with specific and unspecific immunoglobulins was performed by Kaplan–Meier method followed by data processing using Statistics 7.0 software. Functions of descriptive statistics were analyzed using MS Excel software.

RESULTS

In our previous studies MAbE2Cx43 were characterized by immunochemical methods [2]. Biodistribution of antibodies after injection into systemic circulation was evaluated using radioisotope and fluorescent labels [12]. *In vitro* experiments showed that MAbE2Cx43 block transport of fluorescent dyes through gap junctions by preventing organization of new channels [13].

Immunochemical analysis of MAbE2Cx43 (isotyping) showed that they belong to IgG2a subclass. Dissociation constant (Kd) of antigen–antibody complex for these antibodies inversely proportional to K_{aff} is 0.8×10^{-9} mol/liter.

Purified MAbE2Cx43 and IgGm prepared for the analysis were preliminary tested on fixed and live rat and human glioma cells (C6 and U251, respectively; Fig. 1).

The results of immunocytochemical analysis of Cx43 with MAbE2Cx43 on live rat and human cells suggest that the obtained antibodies are specific to Cx43. Positive fluorescence in both cultures attests to the absence of strict species specificity in the antigenic determinant recognized by the obtained antibodies.

Immunohistochemical analysis of Cx43 and GFAP on brain sections with experimental glioma showed that Cx43 was almost exclusively localized in the peritumoral zone of glioma invasion (Fig. 2); it should be noted that both GFAP-positive astrocytes (Fig. 2, *e*) and GFAP-negative cells (Fig. 2, *f*) were seen among Cx43-positive cells.

The data on potential role of Cx43 in glioma invasion [8,12,13], specificity of MAbE2Cx43 to native extracellular Cx43 fragments in glioma cells and reactive astrocytes, and overexpression of this protein in the zone of glioma invasion allowed us to hypothesize that intravenous injection of these antibodies in a high concentration can produce a suppressive effect on the development of experimental glioma C6.

The efficiency of MAbE2Cx43 therapy in our experiment was evaluated by the volume of glioma (weekly MRI data) and lifespan of experimental animals. Glioma volume was calculated on serial MRI scans of the brain with 0.5 mm intervals (36 images per layer). The absolute (in mm^3) and relative volume of glioma (% of brain volume) for each rat were determined.

According to the results of morphometric analysis, deceleration of glioma growth was observed in

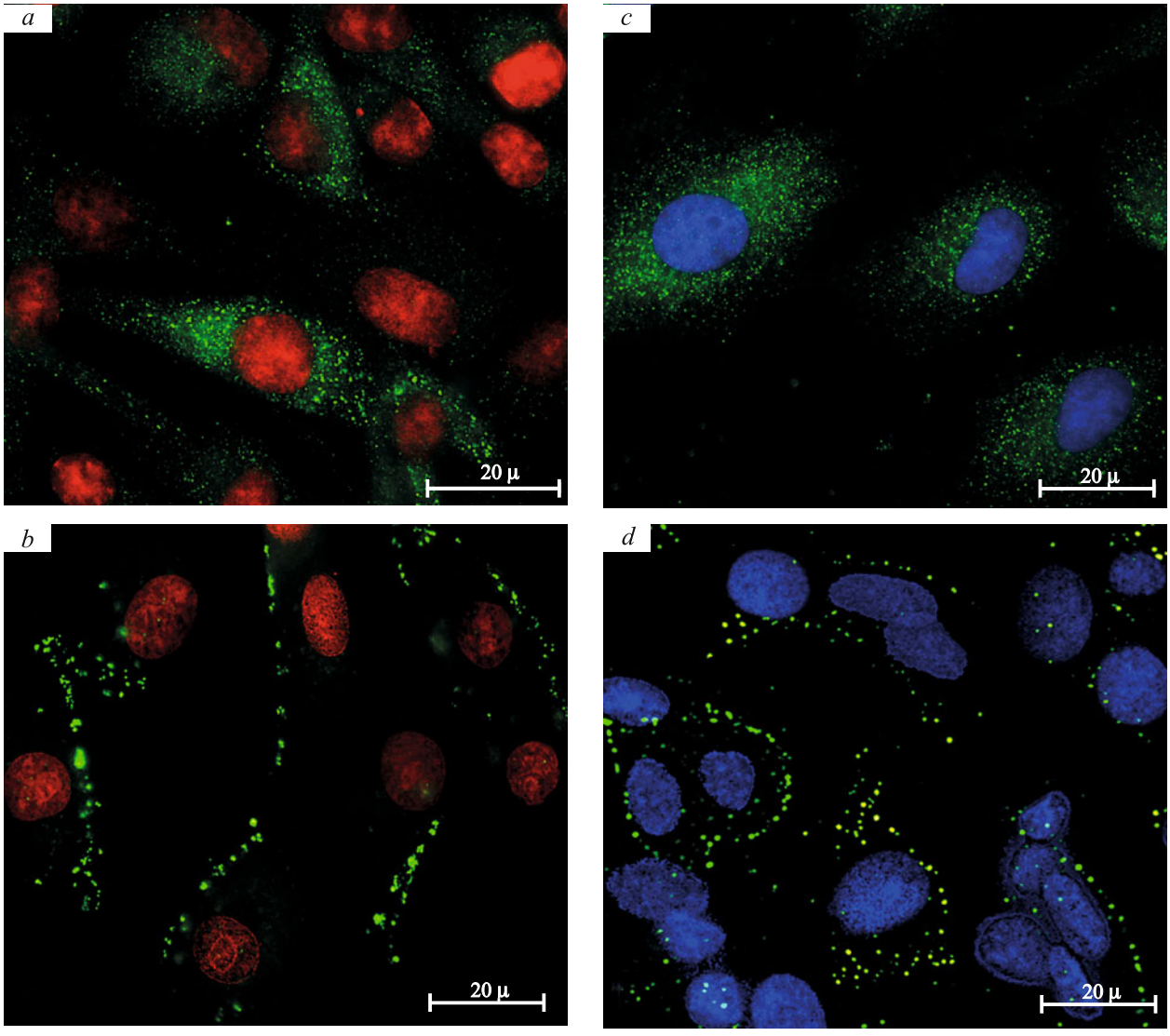

Fig. 1. Immunocytochemical analysis of Cx43 on fixed (*a*,*c*) and live (*b*,*d*) preparation of glioma C6 (nuclei are post-stained with TOTO 633, red fluorescence; *a*, *b*) and U251 (nuclei are post-stained with DAPI, blue fluorescence; *c*, *d*).

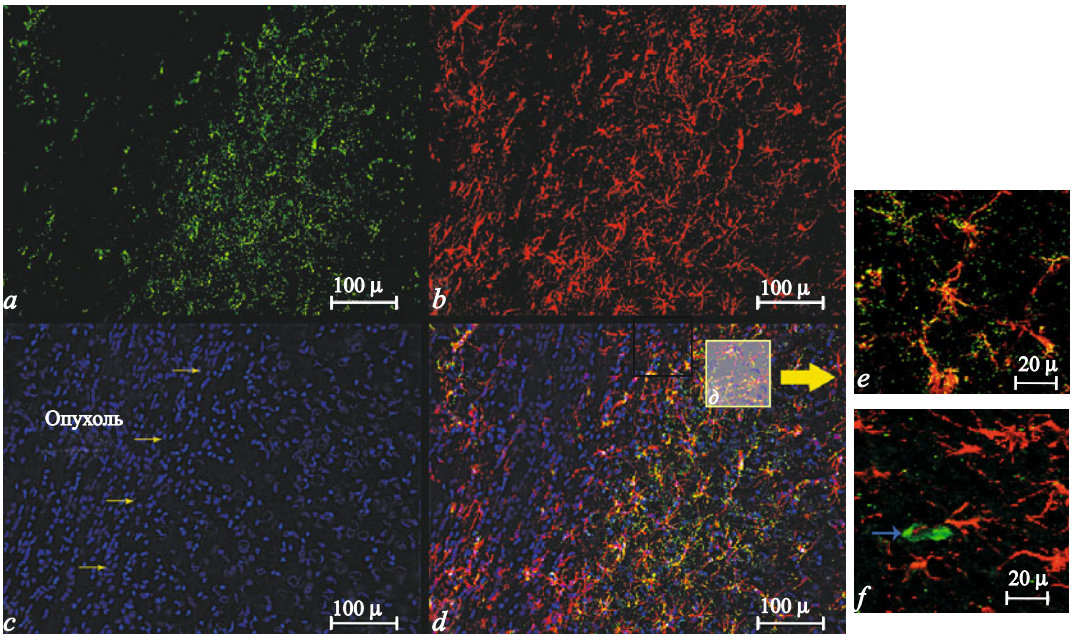

Fig. 2. Immunocytochemical analysis of Cx43 and GFAP on brain sections with experimental glioma C6. *a*) MAbE2Cx43+antimouse antibodies labeled with Alexa 488; *b*) rabbit polyclonal antibodies to GFAP+antibodies to rabbit immunoglobullins labeled with Alexa 633; *c*) cell nuclei are post-stained with DAPI, tumor boundaries are shown with yellow arrows; *d*) combined image; *e*) fragment demonstrating Cx43 and GFAP-positive reactive astrocytes at high magnification; *f*) fragment demonstrating Cx43-positive GFAP-negative cells in astroglial hurdle at high magnification (blue arrow).

the group of rats receiving MAbE2Cx43 therapy in comparison with the control starting from day 14 and became significant by day 21 after glioma implantation (Fig. 3). By day 29, the mean volume of glioma in the experimental group (therapy with specific antibodies) was 2-fold lower than in the control group (204.98±37.67 and 431.00±56.48 mm^3, respectively, p=0.003). The relative volume of glioma in the experimental and control groups was 12.4±2.7 and 23.6±3.1%, respectively). All rats of the control group died after 29 days, therefore quantitative morphometric analysis was not performed, but MRI in survivors of the experimental group was repeated until the 6th month postimplantation.

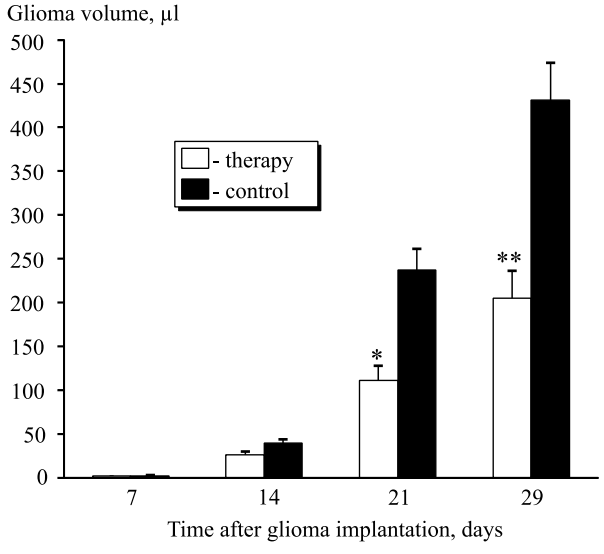

Fig. 3. Dynamic morphometry of glioma (MRI data). *p<0.05, **p<0.01 in comparison with the control.

According to Kaplan–Meier survival analysis, the rats with experimental glioma receiving MAbE2Cx43 therapy in a dose of 5 mg/kg lived significantly longer than controls (Fig. 4). In the control group, 100% animals died; survival median was 28 days. In the experimental group, 5 of 26 rats with C6 glioma (19.23%) completely recovered and produced offspring in 6 months after glioma modeling (by now, 10 months elapsed after glioma transplantation in survivors). In these rats, MRI-verified glioma completely regressed and no relapses were detected throughout the observation period despite pregnancy and delivery. The survival median in experimental rats was 38 days (p>0.01 in comparison with the control).

The results of dynamic brain MRI in survivors showed that glioma regressed and a gliomesodermal cicatrix or a cyst appeared (Fig. 5).

According to published data, both types of glioma, glioma C6 and human glioblastoma U251, are low differentiated gliomas (stage IV) characterized by reduced expression of Cx43 and other gap junction proteins [15]. The results of our experiments do not confirm published data on the absence of gap junction proteins in malignant gliomas, but agree with the

results of recent studies, where not only Cx43 expression in C6 glioma cells was demonstrated, but also Cx43-positive glioma strain was generated [4].

Cases of spontaneous recovery of rats with glioma C6 [17] were described, but our experience of glioma C6 modeling on more than 300 animals shows that animals with documented engrafting of implanted cells do not recover spontaneously and die within 4-5 weeks after glioma modeling irrespective of the dose of implanted cells. Standardized procedure of stereotactic implantation of glioma cells leads to their 100% engrafting and 100% animal death [7]. Taking into account these facts and published data that glioma C6 is one of the most aggressive and resistant to therapy, complete recovery of 19.23% rats (of 26 rats in the group) after MAbE2Cx43 therapy can be regarded as an encouraging result. Further studies of the mechanisms underlying the effect of antibodies to extracellular fragment of Cx43 can shed light in intimate mechanisms of glioma invasion and increase the efficiency of antiglioma therapy.

Experiments with intravenous administration of MAbE2Cx43 labeled with radioisotopes and fluorescent labels to rats with experimental glioma C6 demonstrated that antibodies injected into systemic circulation are accumulated in the peritumoral zone, where hyperexpression of Cx43 is observed. We found that both Cx43- and GFAP-positive reactive astrocytes and a pool of spindle-shaped GFAP-negative Cx43 positive cells were visualized [3]. The latter can be either differentiated astrocytes (astroblasts) losing expression of specific intermediate filament proteins, or glioma cells expressing gap junction proteins that migrate into the peritumoral zone [3]. To confirm this assumption and prove that intravenously injected antibodies bind to Cx43-positive cells originating from glioma, we carried out a special experiment with labeled glioma cells. Membrane tracer Dil characterized by high-intensity fluorescence with a peak at 560 nm was chosen as the label. Preliminary experiments showed that glioma C6 cells treated with 10 μM Dil do not lose their tumorigenic properties for at least 18 days and despite inevitable tracer dilution during cell proliferation retain sufficient amount of the fluorophore for their reliable identification.

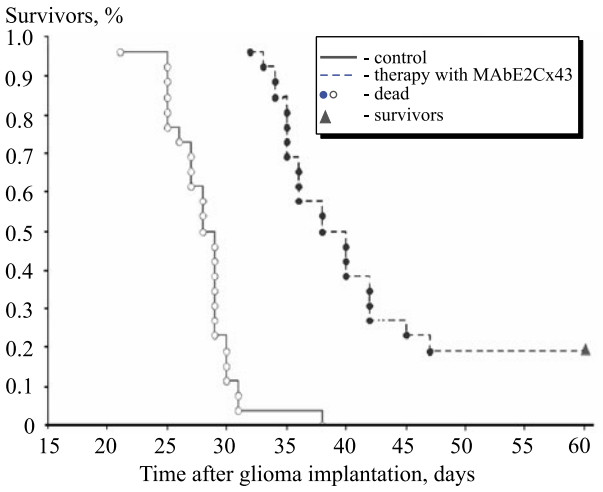

Fig. 4. Analysis of rat survival (Kaplan–Meier method).

Antibodies to Cx43 were labeled with succinimide ester of AlexaFluor 488 (Invitrogen) and injected intravenously on day 14 after transplantation of Dil-labeled glioma C6 cells. Fluorescent analysis of freshly prepared cryostat sections of the brain with glioma showed co-localization of Alexa 488 and Dil in cells of the peritumoral zone and even in normal

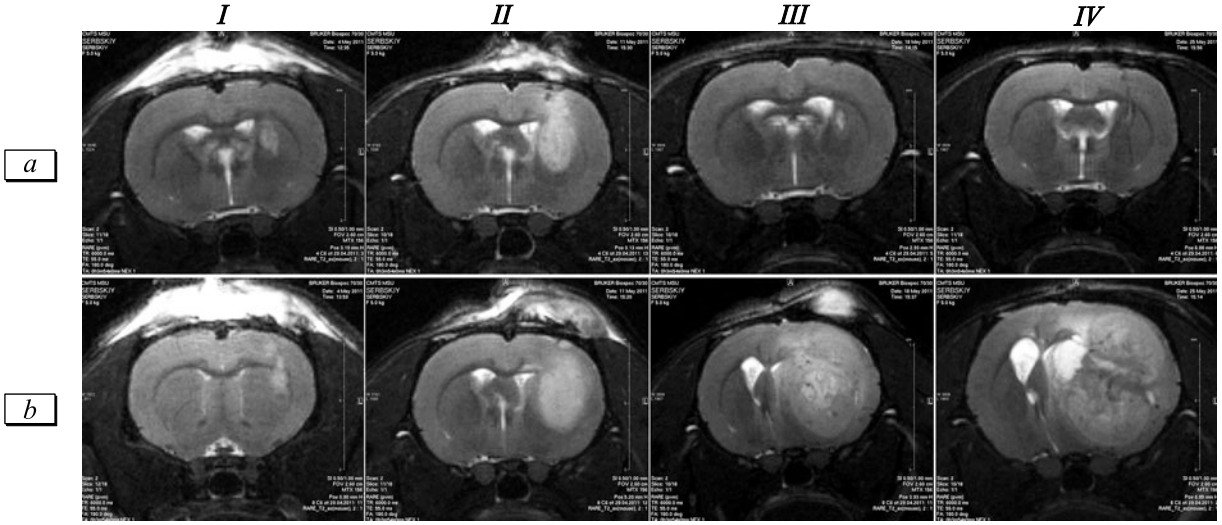

Fig. 5. MRI dynamics of glioma growth in animals receiving specific (*a*) and unspecific (*b*) treatment over 27 days after implantation. I: day 7, II: day 14, III: day 21; IV: day 28.

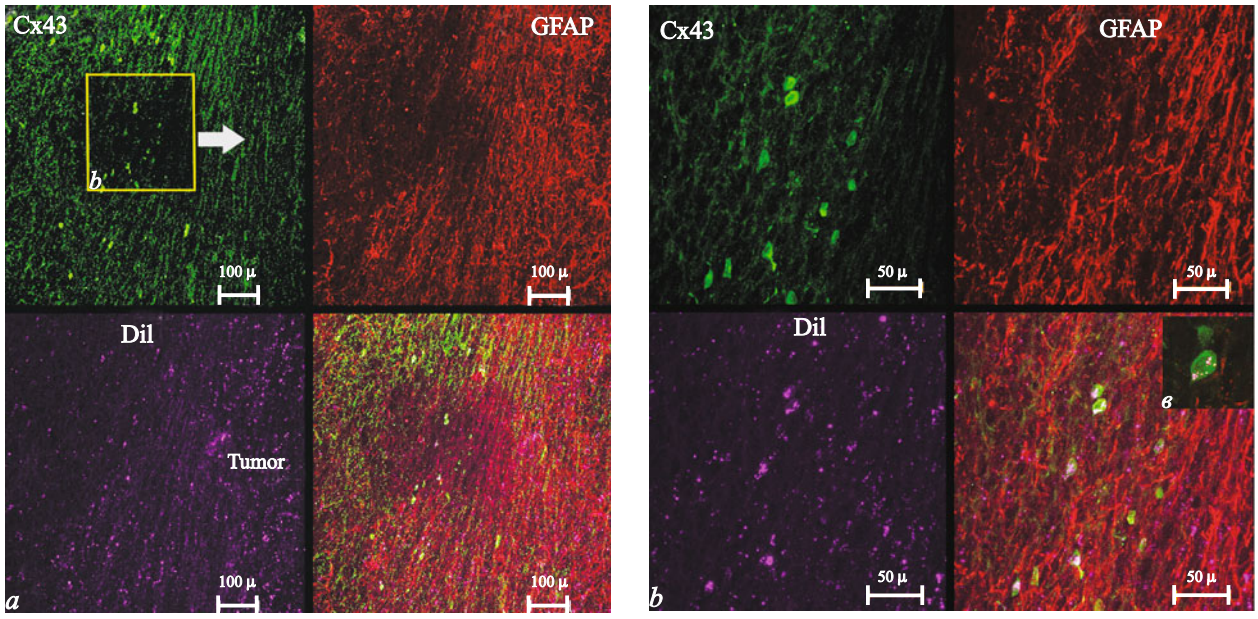

Fig. 6. Immunofluorescent analysis of peritumoral zone with intravenously injected labeled antibodies to Cx43. *a*) peritumoral zone at low magnification; *b*) area scanned at higher magnification. Arrows show sites of co-localization of Dil (primary labeled glioma cells) and Alexa 488 (intravenously injected antibodies to Cx43); c) fragment of Dil-positive glioma cell bound antibodies to Cx43 at 2-fold higher magnification.

tissue outside the peritumoral hurdle 48 h after their administration (Fig. 6). This proves that intravenously injected antibodies to Cx43 recognize Cx43-positive glioma cells migrating from glioma periphery.

These results suggest that the mechanism of tumor-suppressive effect of antibodies can be related to inhibition of migration and/or signaling in Cx43-positive glioma cells. In particular, blockade of extracellular Cx43 fragments with MAbE2Cx43 can hamper the formation of heterologous gap junctions with astrocytes whose role in invasion was experimentally demonstrated [6]. This assumption was confirmed by some other reports [8,12]. Two ways of glioma cell invasion into rat brain parenchyma were clearly demonstrated *in vitro* and *in vivo*: parenchymatous and along tumor blood vessels [9]. It was also noted that Cx43-positive cells can form heterologous contacts with astrocytes, which enables their migration along perineural spaces [12]. However, later experiments showed that Cx43 can intensify migration by activating p38 MAP-kinase and this process is not related with the formation of gap junctions [6]. Thus, both Cx43 functions in glioma invasion process and the mechanism of tumor-suppressive effects of antibodies to its extracellular fragment require further investigation.

Lengthening of animal lifespan against the background of MAbE2Cx43 therapy is most likely related to deceleration of the growth of implanted glioma due to hampered invasion of Cx43-postive cells. However, this hypothesis cannot explain complete recovery of animals with glioma after therapy with antibodies to Cx43. It can be hypothesized that in this case more complex antibody-associated immunological mechanisms are activated, which requires further investigation.

Thus, intravenous injection of MAbE2Cx43 obtained by us in a dose of 5 mg/kg to rats with glioma C6 at 7-day intervals significantly inhibited the growth of glioma C6 implanted into the brain and significantly increased animal lifespan and in 19.23% cases led to complete recovery without relapses within at least 6 months.

Authors are grateful to Prof. I. V. Viktorov and N. F. Grinenko, Cand. Biol. Sci., for their help in experiments with cell cultures, E. B. Tsitrin, Head of the Group of Optical Studies, N. K. Kol'tsov Institute of Developmental Biology, for his help in scanning laser confocal microscopy studies, M. A. Abakumov, postgraduate student, for determination of antibody affinity constant measurement, and Ya. A. Zorkina for her help in statistical analysis of the experimental results.

REFERENCES

1. V. P. Baklaushev, N. F. Grinenko, E. B. Tsitrin, *et al., Br. J. Med. Medic. Res.*, **1**, No. 2, 35-44 (2011).
2. V. P. Baklaushev, O. I. Gurina, G. M. Yusubalieva, *et al., Bull. Exp. Biol. Med.*, **148**, No. 4, 725-730 (2009).
3. V. P. Baklaushev, G. M. Yusubalieva, E. B. Tsitrin, *et al., Drug Deliv.,* **18**, No. 5, 331-337 (2011).
4. D. C. Bates, W. C. Sin, Q. Aftab, and C. C. Naus, *Glia*, **55**, No. 15, 1554-1564 (2007).
5. J. D. Beatty, B. G. Beatty, W. G. Vlahos, *J. Immunol. Methods*, **100**, Nos. 1-2, 173-179 (1987).
6. J. Behrens, P. Kameritsch, S. Wallner, *et al., Eur. J. Cell. Biol.,* **89**, No. 11, 828-838 (2010).

7. V. P. Chekhonin, V. P. Baklaushev, G. M. Yusubalieva, *et al., Bull. Exp. Biol. Med.*, **143**, No. 4, 501-509 (2007).
8. T. Demuth and M. E. Berens, *J. Neurooncol*., **70**, No. 2, 217-228 (2004).
9. J. Dietrich, E. L. Diamond, and S. Kesari, *Expert. Rev. Anticancer Ther*., **10**, No. 5, 171-175 (2010).
10. F. B. Furnari, T. Fenton, R. M. Bachoo, *et al., Genes Dev.*, **21**, No. 21, 2683-2710 (2007).
11. F. Lefranc, M. Rynkowski, O. DeWitte, and R. Kiss, *Adv. Tech. Stand. Neurosurg.,* **34**, 3-35 (2009).
12. J. H. Lin, T. Takano, M. L. Cotrina, *et al. J. Neurosci.*, **22**, No. 11, 4302-4311 (2002).
13. R. Oliveira, C. Christov, J. S. Guillamo, *et al., BMC Cell Biology,* **6**, No. 1, 171-175 (2005).
14. N. Prochnow and R. Dermietzel, *Histochem. Cell. Biol*., **130**, No. 1, 71-77 (2008).
15. L. Soroceanu, T. J. Manning Jr., and H. Sontheimer, *Glia*, **33**, No. 2, 107-117 (2001).
16. T. Suzuki, S. Izumoto, Y. Fujimoto, *et al., J. Clin. Invest*., **58**, No. 2, 166-171 (2005).
17. G. H. Vince, M. Bendszus, T. Scweitzer, *et al., Exp. Neurol.*, **190**, No. 2, 478-485 (2004).