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A protective chimeric antibody to tick-borne encephalitis virus

Ivan K. Baykov[a,*], Andrey L. Matveev[a], Oleg V. Stronin[b], Alexander B. Ryzhikov[c], Leonid E. Matveev[a], Marat F. Kasakin[a], Vladimir A. Richter[a], Nina V. Tikunova[a,*]

[a] Institute of Chemical Biology and Fundamental Medicine, Siberian Branch of Russian Academy of Sciences, prosp. Lavrentieva 8, Novosibirsk 630090, Russia
[b] Federal State Unitary Company "Microgen Scientific Industrial Company for Immunobiological Medicines" of the Health Ministry of The Russian Federation, Branch in Tomsk, ul. Ivanovskogo 8, Tomsk 634040, Russia
[c] Federal State Research Institution State Research Center of Virology and Biotechnology "Vector", Koltsovo, Novosibirsk Region 630559, Russia

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ABSTRACT

The efficiency of several mouse monoclonal antibodies (mAbs) specific to the tick-borne encephalitis virus (TBEV) glycoprotein E in post-exposure prophylaxis was assessed, and mAb14D5 was shown to be the most active of all those studied. It was proven that the hybridoma cell line 14D5 produced one immunoglobulin H chain and two L chains. They were used to construct chimeric antibodies ch14D5a and ch14D5b, the affinity constants of which were 2.6×10^{10} M^{-1} and 1.0×10^{7} M^{-1}, respectively, according to the SPR-based ProteOn biosensor assay. The neutralization index (IC_{50}) of ch14D5a was 0.04 μg/ml in the focus reduction neutralization test. In *in vivo* experiments, ch14D5a at a dose of 10 μg/mouse resulted in a 100% survival of the mice infected with 240 LD_{50} of TBEV. This chimeric antibody is promising for further development of prevention and therapeutic drugs against TBEV.

1. Introduction

Tick-borne encephalitis virus (TBEV), a member of the *Flaviviridae* family, is the causative agent of one of the most dangerous human neuroinfections [1,2]. This virus is transmitted by *Ixodidae* ticks and is endemic in many European countries, Russia, northern Kazakhstan, Mongolia, and China [3,4]. A single-stranded positive RNA genome (~10–11 kb) of TBEV contains a single open reading frame (ORF) flanked by 5′ and 3′ untranslated regions. The ORF encodes a polypeptide (~3400 amino acid residues) consisting of three structural proteins (C, prM, and E) and seven non-structural proteins NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5 (RNA-dependent RNA polymerase). This polypeptide is cleaved and co- or post-translationally processed by host- or virus-specific proteases [5]. Surface glycoprotein E mediates receptor binding; it is a major target for neutralizing antibodies and induces the host protective immune response [2,5–7].

This virus causes several thousands of cases of tick-borne encephalitis every year, with over half the cases recorded in Russia [8]. Several vaccines have been designed for prevention of this disease [9,10], namely, FSME-Immun (Baxter, Austria), Encepur (Novartis Vaccines, Germany), EnceVir (Microgen, Russia), and Anti-TBEV Vaccine Preparation and Klesch-E-Vak (Chumakov Institute of Poliomyelitis and Viral Encephalitides, Russia); administration of these vaccines has demonstrated their high efficiency. However, the rate of vaccinees in many regions of Russia still remains <10%, while the number of persons bitten by ticks in Russia is relatively high (400,000–600,000 per year) [11].

Currently, there are no specific therapeutic strategies approved for use in Europe [12]. Specific anti-TBE serum immune globulin (Encegam, FSME-Bulin), which was previously applied for post-exposure treatment, is not used in Europe now [13]. However, infections caused by the European subtype of TBEV, which is common in European countries, are mostly mild, whereas the infections caused by Far-Eastern and Siberian subtypes of TBEV are more severe with a higher mortality rate [14]. For this reason, in spite of the risk of possible side effects, specific serum immunoglobulin (TBE immunoglobulin) produced from the blood plasma of donors living in natural TBEV foci, is used for post-exposure

Abbreviations: TBEV, tick-borne encephalitis virus; ADE, antibody-dependent enhancement; RU, response unit.

* Corresponding authors. Tel.: +7 383 363 51 57; fax: +7 383 363 51 53.
E-mail addresses: ivan_baykov@mail.ru (I.K. Baykov), guterus@gmail.com (A.L. Matveev), stronin@virion.tomsknet.ru (O.V. Stronin), ryzhik@vector.nsc.ru (A.B. Ryzhikov), vam@niboch.nsc.ru (L.E. Matveev), kassakinm@gmail.com (M.F. Kasakin), richter@niboch.nsc.ru (V.A. Richter), tikunova@niboch.nsc.ru (N.V. Tikunova).

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prophylaxis and treatment in Russia and Kazakhstan [13,15,16]. The TBE immunoglobulin demonstrates a good therapeutic effect, but has certain disadvantages, similar to other preparations from donor blood. Administration of the donor blood products is always accompanied by a certain biological risk. In addition, TBE immunoglobulin is in deficiency in some epidemic seasons due to limitations in the amount of available donated blood.

The shortcomings of serum antibodies require the development of alternative immunological preparations. As for most mouse-derived monoclonal antibodies (mAbs), only the first treatment is efficient and subsequent doses are rapidly eliminated due to the human anti-mouse antibodies response. To counter this problem, chimeric antibodies containing more human and less immunogenic sequences have been developed. In these recombinant immunoglobulin molecules, the mouse variable domains, providing specificity and antiviral properties, are combined with the constant domains of human antibodies [17,18]. Previously, chimeric antibodies against TBEV were constructed and one of them was shown to successfully inhibit TBEV infection *in vitro* [19]. This article describes the development of a new chimeric antibody able to protect mice from a lethal challenge with TBEV *in vivo*.

2. Materials and methods

2.1. Viruses

TBEV strain 205 (Far-Eastern subtype) and TBEV strain Absettarov (European subtype) were obtained from repositories at the State Research Center of Virology and Biotechnology Vector, Koltsovo and Tomsk branch of the Federal State Unitary Company "Microgen", respectively. All the experiments with live TBEV were conducted under BSL-3 conditions.

2.2. Construction of plasmids encoding chimeric antibodies

Total RNA was isolated from 5×10^6 murine hybridoma cells 14D5 [20] using an "RNeasy Mini kit" (QIAGEN) according to the manufacturer's instructions. The cDNA was synthesized and the genes encoding variable domains of the mAb heavy (V_H) and light (V_L) chains were amplified using a "Titan one tube RT-PCR kit" (Roche) with the primers described by Wang et al. [21]. Two plasmid DNAs, pCH2 g and pCL2 h [22], derived from pcDNA 3.1 plasmid (Invitrogen), were used as expression vectors for antibody production. After amplification, the V_H and V_L fragments were sequenced and re-amplified using the primers designed for cloning into the plasmids pCH2 g and pCL2 h: 5′-GAG GTG CAG CTG CTC GAG TCTGG-3′, 5′-GAC GGT GAC CAG GGT ACCCTG GCC-3′, 5′-GG GAT ATCGTG ATG ACC CAG TCC CC-3′, and 5′-CCG TTT GAT CTC AAG CTT GGT CCC-3′. The V_H DNA fragment was purified, digested with *Xho*I and *Acc*65I endonucleases, and inserted into the *Xho*I/*Acc*65I-digested plasmid pCH2 g to generate the pCH2g-14D5 plasmid, which contains the heavy chain gene of the chimeric antibody. Similarly, the V_L DNA fragments were purified, digested with *Eco*RV and *Hin*dIII endonucleases, and inserted into the *Eco*RV/*Hin*dIII-digested plasmid pCL2 h to generate plasmids pCL2h-14D5a and pCL2h-14D5b containing the light chain gene. The cloning accuracy was verified by sequencing using an ABI 3130xl sequencer (Applied Biosystems).

2.3. Expression and purification of chimeric antibodies

The pCH2g-14D5, pCL2h-14D5a, and pCL2h-14D5b plasmid DNAs were isolated with a "PureYield Plasmid Midiprep System" (Promega) and pCH2g-14D5/pCL2h-14D5a or pCH2g-14D5/pCL2h-14D5b plasmid pairs were co-transfected into CHO-K1 cells for transient expression using Lipofectamine 2000 Reagent (Invitrogen). Cell supernatant was harvested and replaced daily for 6 days with a fresh Opti-MEM (Invitrogen) medium containing 2% fetal bovine serum with ultra-low IgG content. The chimeric antibody was purified according to general recommendations [23] with some modifications. Cell supernatant was clarified by centrifugation for 15 min at 12 000 g, filtered through a 0.22 μm polyethersulfone filter and loaded onto a 4 ml Protein A Sepharose column pre-equilibrated with PBS (phosphate buffered saline). After washing with 5 column volumes (CV) of PBS, the bovine antibodies present in the culture medium were eluted with 3 CV of 0.1 M citric buffer pH 5.0 and the chimeric antibody with 3 CV of 0.1 M citric buffer pH 3.0. Absence of bovine antibodies in the chimeric antibody preparation was confirmed by western-blotting using anti-bovine IgG polyclonal conjugate (Sigma A5295). The antibody was concentrated and buffer-exchanged against PBS containing 0.05% sodium azide using an Amicon Ultra-4 50 kDa (Millipore) filter. The final preparation was stored at a concentration of over 1 mg/ml at 4 °C.

2.4. Mass spectrometry analysis

Whole-molecule mass spectrometry analysis was performed using antibody purified on $ZipTip_{C18}$ tips (Millipore). The antibody (~5 μg) was co-crystallized with sinapinic acid matrix and the spectrum was recorded in a linear positive ion mode using an Autoflex speed MALDI-TOF mass spectrometer (Bruker).

For peptide mass fingerprinting, the heavy and light chains of the antibody were separated using 12% polyacrylamide gel electrophoresis (SDS–PAGE) under reducing conditions. The Coomassie-stained gel bands containing proteins were destained by incubation with 50 mM NH_4HCO_3 solution in 50% acetonitrile and then in 100% acetonitrile. An in-gel digestion with sequence grade trypsin (Promega) was performed for each chain according to the manufacturer's manual. Peptides were extracted from the gel with 50 μl of 25 mM NH_4HCO_3 solution, concentrated in a SpeedVac (Eppendorf), and purified using $ZipTip_{C18}$ tips (Millipore). The peptides were co-crystallized with 2,5-dihydroxybenzoic acid matrix to record the spectrum in reflective positive ion mode using an Autoflex speed MALDI-TOF mass spectrometer (Bruker).

2.5. Affinity constants measurement

Kinetic and thermodynamic parameters of the interaction were determined by a surface plasmon resonance (SPR) method using a ProteOn XPR36 System (Bio-Rad). The recombinant protein E [24] was covalently immobilized by conventional NHS-EDAC chemistry in 10 mM sodium acetate pH 4.5 at a 2700 response units (RU) level onto a GLC sensor chip surface, followed by blocking with 1 M ethanolamine–HCl. The reference surface was prepared similarly using PBS containing 0.005% Tween 20 (PBST) as a ligand solution. Serial two-fold dilutions of appropriate antibody in PBST starting from 50 nM were used as an analyte. The association step was performed at a flow rate of 25 μl/min for 10 min; dissociation was run at a flow rate 25 μl/min for 4 h. Response data was corrected using interspot referencing. Global analysis of experimental data using a single-site model or a heterogeneous analyte model was performed using the ProteOn Manager v. 3.1.0 software.

2.6. Focus reduction neutralization test (FRNT)

FRNT was performed according to Levanov et al. [19] using TBEV strain 205.

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2.7. In vivo antibody protection against TBEV

Antiviral activity of the monoclonal and chimeric antibodies was assessed using 10 g BALB/c mice (3 weeks old) intraperitoneally infected with 0.2 ml of TBEV strain Absettarov stock. This animal model was described previously [25]. One day after infection, the mice were treated intravenously with the antibody preparations. The mice were observed for 21 days. The actual LD_{50} was determined by the Reed–Muench method [26]. All animal procedures were carried out in accordance with the recommendations for the protection of animals used for scientific purposes (EU Directive 2010/63/EU). The protocols were approved by the Committee on the Ethics of Animal Experiments of the Administration of the Siberian Branch of the Russian Academy of Sciences.

3. Results

3.1. In vivo protection by mouse mAbs

Protective activity of mouse mAb14D5, mAb1B1, and mAb13D6, able to neutralize infectivity of TBEV *in vitro* [20], was studied in a TBE peripheral mouse model [25]. The animals were infected with approximately 500 LD_{50} of the virus; mAbs were injected 24 h later at a dose of 150 μg/mouse. This experiment demonstrated that the mouse survival rate provided by mAb14D5 was higher compared with mAb13D6 and mAb1B1 (Fig. 1).

3.2. Plasmid construction

RNA was isolated from the cells producing mAb14D5 and used for synthesizing the cDNA fragments encoding the variable domains of the antibody. One fragment, V_H14D5, was PCR-amplified for the heavy chain, while three fragments with different electrophoretic mobilities, designated V_L14D5a, V_L14D5b, and V_L14D5c, were synthesized for the light chain (Fig. 2). Subsequent sequencing demonstrated that the V_H14D5 sequence belonged to the IGHV1 family of mouse antibodies according to the IMGT classification (http://www.imgt.org/); V_L14D5a to the IGKV10 family; and V_L14D5b to the IGKV6 family. The V_L14D5c sequence belonged to the IGKV3-5 germline gene, but the insert of a genomic region leading to a frameshift mutation is present.

In order to clarify the number of light chains produced by the 14D5 hybridoma cell line, the fragments of trypsin hydrolyzed mAb14D5 light chains were analyzed by mass spectrometry. This showed that the preparation contained the antibody molecules

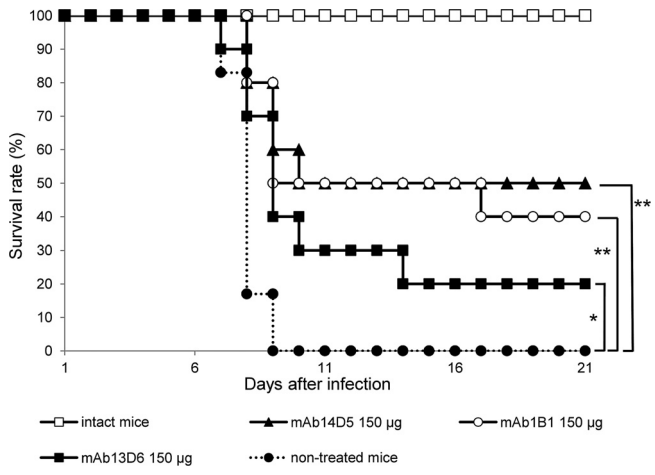

Fig. 1. Efficacy of anti-TBEV mouse mAbs post-exposure prophylaxis. Ten 10 g BALB/c mice were treated with 150 μg indicated mAbs *via* an i.v. injection one day after infection with 500 LD50 of TBEV. $^{*}p < 0.05$; $^{**}p < 0.005$ (log-rank test).

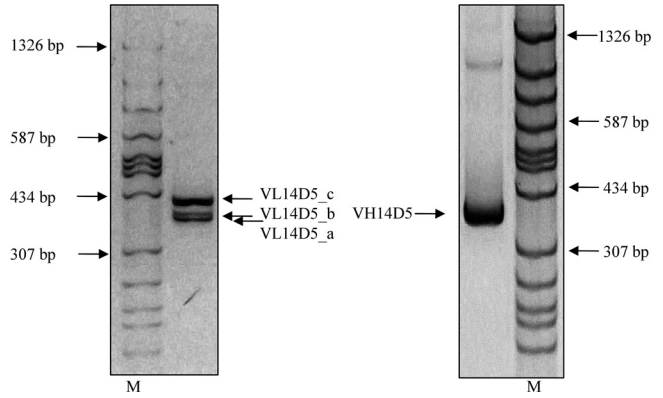

Fig. 2. Nondenaturing 6% polyacrylamide gel electrophoresis of cDNA fragments derived from mAb14D5 cells. M represents pQpE plasmid DNA digested with *Hae*III restriction endonuclease.

with light chains of two types: light_14D5a and light_14D5b (Fig. 3A). No peaks corresponding to the nonfunctional light_14D5c light chain were found in the spectrum. The height ratio of the peaks corresponding to light_14D5a peptide LLIYYTSR ($[M+H^+] = 1028.6$) and light_14D5b peptide LLIYSASYR ($[M+H^+] = 1085.6$ suggests that the molar ratio of light_14D5a and light_14D5b light chains in the preparation is approximately 1:3.

The DNA fragments V_H14D5, V_L14D5a, and V_L14D5b were used to construct expression plasmids. The V_H14D5 fragment was inserted into the plasmid pCH2 g [22], containing the fragments encoding a leader peptide, CH1, CH2, and CH3 constant domains of human IgG1 antibodies, and neomycin resistance gene. The final plasmid was designated pCH2g-14D5 (Fig. S1A). The fragments V_L14D5a and V_L14D5b were inserted into the plasmid pCL2 h [22], carrying the fragments encoding a leader peptide, the constant domain of human antibody kappa chains, and hygromycin B resistance gene. The final plasmids were designated pCL2h-14D5a (Fig. S1B) and pCL2h-14D5b.

Supplementary Fig. 1 related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vaccine.2014.05.012.

3.3. Production and purification of chimeric antibodies

Chimeric antibody ch14D5a was obtained by the co-transfection of a CHO-K1 cell line with the plasmids pCH2g-14D5 and pCL2h-14D5a and transient expression of the genes encoding the antibody light and heavy chains. The ch14D5a antibody with a molecular weight of approximately 150 kDa was isolated by chromatography on a Protein A Sepharose column; this was confirmed by mass spectrometry (Fig. 4A). Electrophoresis under reducing conditions showed that the protein contains both heavy and light chains with molecular weights of 50 and 25 kDa, respectively (Fig. 4B). Antibody ch14D5b was similarly obtained using plasmids pCH2g-14D5 and pCL2h-14D5b and then purified and characterized in the same manner.

Compliance of the produced ch14D5a and ch14D5b proteins with the initial genetic constructs was confirmed by mass spectrometry of trypsin-hydrolyzed antibodies (Fig. 3B–D). The ch14D5a light chain spectrum had a peak at 1028.6, which corresponded to the peptide LLIYYTSR, and the ch14D5b light chain spectrum, a peak at 1085.6, corresponding to the peptide LLIYSASYR. Sequence coverage of 80% was obtained for the ch14D5a antibody.

3.4. Determination of affinity constants

The kinetic parameters and affinity constants for the interaction between the antibodies and recombinant protein E were

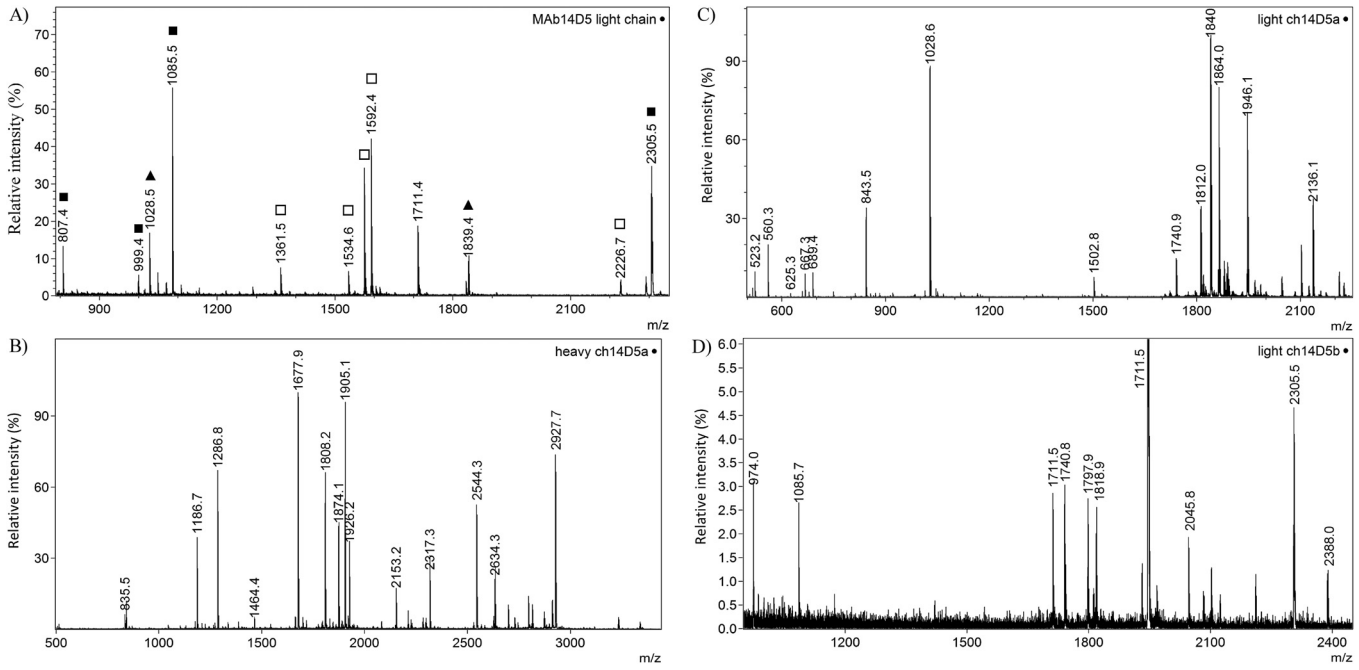

Fig. 3. Mass spectrometry characterization of mAb and chimeric antibody preparations. MALDI-TOF mass spectra of tryptic digest of mAb14D5 light chains (A), ch14D5a heavy chain (B), ch14D5a light chain (C), and ch14D5b light chain (D) in a reflective positive mode using DHB matrix. Peaks labeled with ▲ correspond to light_14D5a, while those labeled with ■c orrespond to light_14D5b. Peaks labeled with □ correspond to constant region of murine kappa chains.

determined in a label-free biosensor assay using a ProteOn XPR36 system (Fig. 5 and Table 1). A simple single-site model failed to provide a good description for the interaction between mAb14D5, containing different light chains, and protein E, leading to poor data fitting. However, a global analysis using the heterogeneous analyte model gave a good quality fit. Since ch14D5a antibody, compared to ch14D5b, displayed a considerably higher affinity for recombinant protein E, further experiments were performed only with this antibody.

3.5. *In vitro neutralization of TBEV by ch14D5a and mAb14D5*

Serially diluted ch14D5a and parental mAb14D5 were analyzed for the neutralizing activity against TBEV strain 205 by FRNT.

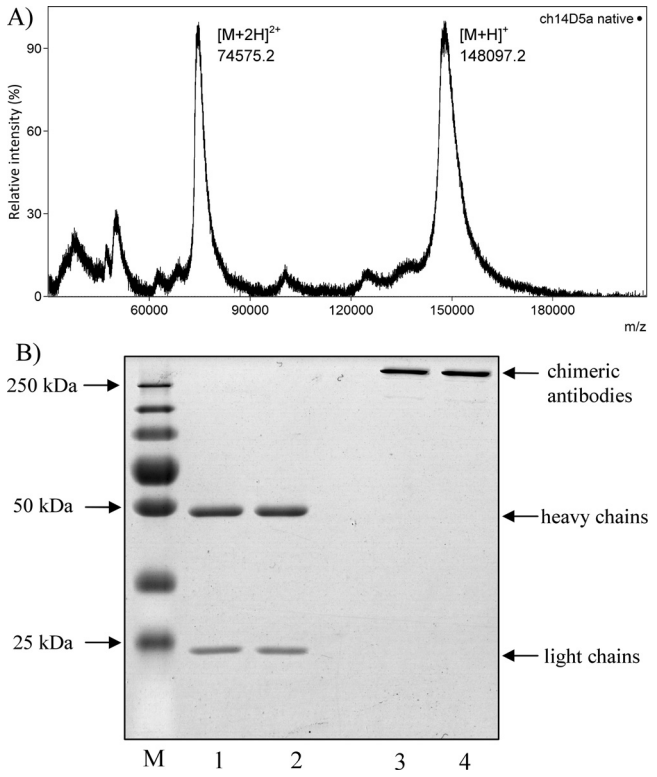

Fig. 4. Electrophoretic and MS analyses of recombinant antibodies (A) MALDI-TOF mass spectrum of whole antibody ch14D5a in linear positive mode using sinapinic acid matrix. Baseline correction was performed. (B) Coomassie blue stained 12% SDS–PAGE analysis of purified ch14D5a in reducing (lane 1) and nonreducing (lane 3) conditions as well as purified ch14D5b in reducing (lane 2) and nonreducing (lane 4) conditions. M represents protein molecular weight marker

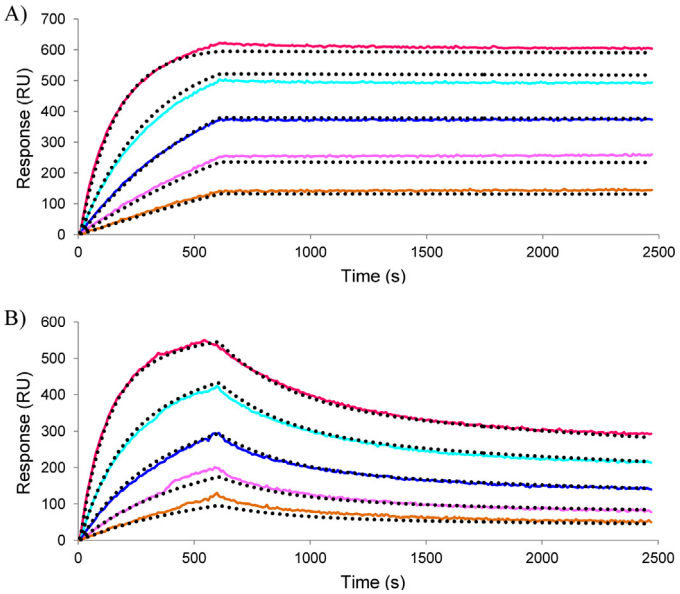

Fig. 5. Comparison of the ch14D5a or mAb14D5 binding to recombinant protein E. Binding progress of the soluble ch14D5a (A) or mAb14D5 (B) at analyte concentrations of 3.12, 6.25, 12.5, 25 and 50 nM, to recombinant protein E immobilized to GLC sensor chip surface. Global kinetic analysis using a single-site model (A) and single-site heterogeneous analyte model (B) (dotted lines).

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Table 1
The kinetic parameters and affinity constants of antibodies.

Antibody	Dissociation rate constant k_d (s^{-1})	Association rate constant k_a ($M^{-1}\ s^{-1}$)	Equilibrium affinity constant K_A (M^{-1})
ch14D5a	$4.99 \pm 0.55^{a} \times 10^{-6}$	$1.30 \pm 0.00 \times 10^{5}$	2.6×10^{10}
ch14D5b	$2.11 \pm 0.03 \times 10^{-3}$	$2.10 \pm 0.01 \times 10^{4}$	1.0×10^{7}
mAb14D5[b]	$1.24 \pm 0.05 \times 10^{-4}$	$1.05 \pm 0.01 \times 10^{4}$	8.5×10^{7}
	$3.21 \pm 0.06 \times 10^{-3}$	$1.57 \pm 0.01 \times 10^{4}$	4.9×10^{6}

[a] Mean ± standard error.
[b] Data shown for high and low affinity sites of mAb14D5.

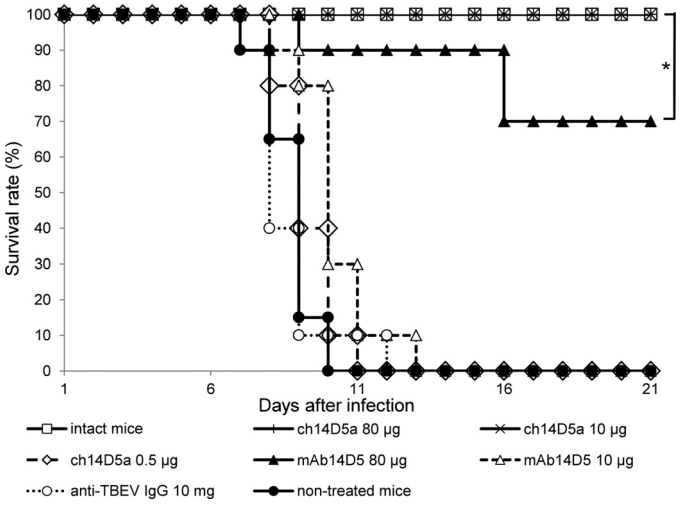

Fig. 6. Efficacy of anti-TBEV ch14D5a, mAb14D5, and TBE immunoglobulin post-exposure prophylaxis. Ten 10 g BALB/c mice were treated with indicated antibody preparations *via* an i.v. injection one day after infection with 240 LD50 of TBEV. * $p = 0.067$ (log-rank test).

Obtained data suggested that both mAb14D5 and the ch14D5a derived from the parental mAb14D5 were able to neutralize TBEV *in vitro*. The 50% inhibition doses of ch14D5a and mAb14D5 were 0.04 μg/ml and 0.5 μg/ml, respectively.

3.6. *In vivo* protection against TBEV infection by ch14D5a and mAb14D5

The protective properties of ch14D5a were assessed using 10 g BALB/c mice. The animals were infected with 240 LD_{50} of TBEV strain Absettarov, and the antibody preparations were administered 24 h after infection. The mouse mAb14D5 and human serum immunoglobulin against TBEV with a titer of 1:160 (Microgen, Russia) were also tested. In this experiment, treatment with ch14D5a resulted in a 100% survival rate at a dose of both 80 μg/mouse and 10 μg/mouse, while mouse mAb14D5 provided a 70% survival rate only at a dose of 80 μg/mouse (Fig. 6). The experiment has shown that the protective activity of ch14D5a is higher than that of both mAb14D5 and human serum immunoglobulin, which is currently used for TBE post-exposure prophylaxis and therapy. In the case of the low dose of ch14D5a (0.5 μg/mouse), the mean survival time was 8.3 ± 1.0 days compared to 7.7 ± 0.7 days for non-treated mice. According to a log-rank test, there was no significant difference ($p = 0.095$) between these two survival curves.

4. Discussion

Recombinant antibodies intended for specific therapy and prevention of various viral infections have been constructed [27]. Similar antibodies are being designed for flaviviruses, such as West Nile virus [28–30], dengue virus type 2 [31], yellow fever virus [32], and Venezuelan equine encephalitis virus [33]. Commercially available antibodies for TBE post-exposure prophylaxis and treatment are currently absent, and development of such antibodies that would be produced in cell cultures under standard conditions is an important challenge.

Previously, a panel of mouse mAbs specific to envelope glycoprotein E was developed. Some of these mAbs were proven to inhibit TBEV infection *in vitro* [20]. We have assessed the post-exposure prophylaxis activity of several most promising mAbs in *in vivo* experiments with a mouse model. MAb14D5 demonstrated the highest ability to protect mice from a lethal challenge of TBEV, and so its variable domains were used to construct a chimeric antibody.

Cloning and sequencing of mAb14D5 V_H and V_L genes have shown that hybridoma 14D5 produces two different light chains with variable domains V_La and V_Lb, and that the ratio, according to mass spectrometry, of these chains in a purified preparation of mAb14D5 is 1:3. Using the genes encoding the V_H, V_La, and V_Lb domains of mouse mAb14D5 and the C_H and C_L genes of human immunoglobulin IgG1/kappa, we constructed two chimeric antibodies: ch14D5a and ch14D5b. The contribution of light chains to the affinity of these antibodies appeared to be considerable, and their affinity constants differed by over two orders of magnitude.

The chimeric antibody ch14D5a is more efficient compared to ch13D6, which was constructed earlier [19]. The affinity of ch14D5a for glycoprotein E was two orders of magnitude higher compared to ch13D6. In *in vitro* experiments, ch14D5a more efficiently inhibited TBEV infectivity as compared with ch13D6 (IC_{50} for ch14D5a is 0.04 μg/ml *vs.* 4.5 μg/ml for ch13D6). In addition, ch14D5a, at a dose of 80 and 10 μg/mouse, resulted in a 100% survival of the mice infected with 240 LD_{50} of TBEV, whereas any protective activity of ch13D6 was not observed.

Moreover, ch14D5a displayed better antiviral properties as compared with the parental mAb14D5. According to FRNT, the inhibition of TBEV infectivity provided by ch14D5a was over ten-fold more efficient as compared with mAb14D5. A higher efficiency of ch14D5a is explained by the hybridoma cell line 14D5 synthesizing two types of light chains, L_a and L_b. Random joining of light and heavy chains suggests that the mAb14D5 preparation may contain the immunoglobulin molecules of L_aHHL_a, L_aHHL_b, and L_bHHL_b types, while ch14D5a contains only a high affinity antibody.

There are some concerns that passive immunization can induce antibody-dependent enhancement (ADE) of infection [34,35]. It is believed that ADE is associated with sub-neutralizing concentration of protective antibodies or the presence of non-protective antibodies in the preparation [13,36]. ADE has been observed in infections caused by the dengue virus [37]. In *in vitro* experiments, ADE has been also detected in infections with the Japanese encephalitis virus, Murray Valley virus [13], West Nile Virus [38], and TBEV [39]. However, no enhancement of infection with TBEV has been found in *in vivo* experiments [13,39]. In our *in vivo* experiments, ADE was not observed when mice were treated with a low ch14D5a dose (0.5 μg/mouse), which did not protect animals from lethal TBEV doses but also did not reduce their average lifespan (Fig. 6).

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When designing recombinant therapeutics, the degree of humanization of protein molecules is of importance. The ch14D5a homology to human antibodies computed using the Abysis database (http://www.bioinf.org.uk/abysis/) is 98.3%. The glycosylation profile of the antibodies produced in CHO cells is similar to that of human antibodies [40,41].

To date, the most effective way of preventing TBE infection is *via* active vaccination [2]. Despite the availability of effective anti-TBE vaccines, the overall vaccination rate in Russia is still low, resulting in thousands of TBE cases, including severe infections [9]. Even in European countries with a high vaccination rate, severe TBE infections still occur [4]. For the treatment of such cases new drugs or therapeutic strategies should be developed. Thus, the possibility of using high doses of intravenous immunoglobulin (IVIG) for the treatment of severe TBE infections is discussed [42]. Application of high dose IVIG in the treatment of flavivirus infections including severe TBE case provided some improvements; however, this preparation caused a number of side effects, including aseptic meningitis, renal failure, *etc.*, reviewed in [42]. For this reason, a highly protective antibody against TBEV or cocktail of such antibodies could be more preferable for therapy of TBE infections. If the ch14D5a antibody demonstrates efficacy in a later post-infection application, it might be further used in designing potential anti-TBE therapeutics.

Acknowledgments

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