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Evaluation of Immunological Efficiency Among Patients Vaccinated Against Tick-Borne Encephalitis

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1. Introduction

Tick-borne encephalitis virus (TBEV) widespread in the Eurasian continent is a member of the genus *Flavivirus,* family *Flaviviridae.* Human TBE cases are registered in more than 30 countries of the world [*Charrel RN et al.,* 2004]. TBEV is subdivided into three main subtypes according to its genetic structure and antigen specificity: European, Siberian, and Far Eastern ones [*Holzmann H, et al.1992; Ecker M. et al.* 1999]. Differences in nucleotide sequences of TBEV genome of these subtypes may reach about 20% [*Loktev VB* et al. 2007]. Probably, a high TBEV genetic diversity provides its differences in human pathogenicity. Human pathogenicity of European subtype TBEV is less marked; however, towards the east, a severity of infection increases as well as lethality [*Loktev VB* et al. 2007; *Pogodina VV et al. 2007*]. In the Russian Far East, the lethality caused by this infection remains at the highest levels [Pavlenko et al. 2010]; for the last 40 years it has reached, on average, 17,5% [*Leonova et al., 2006; Leonova, 2009*]. A vaccination is known to provide the most reliable and effective protection from many virus infections. Tick-borne encephalitis (TBE) as a very danger infection for humans is not the exception as well. Practical application of different vaccines against tick-borne diseases shows that vaccination of the population is a key link in terms of mass TBE prevention in highly endemic territories [*Kunz C., 2003; Onishchenko G.G., et al. 2007*].

Tick-borne encephalitis is considered to be the international health issue, because the number of risk areas and reported cases across Europe, Russia, and parts of Asia is increasing. The incidence of TBE has fluctuated considerably from year to year in many countries, but in the past decade the number of TBE cases has significantly increased in the Baltic states, the Czech Republic, and Germany, in addition to the countries previously considered to be free from TBE, such as Denmark (specifically the main island of Zealand), France, and Italy. A number of factors have been suggested to explain the growth of incidence including climate changes, extended travelling, and outdoor pursuits increasingly placing people in contact with infected ticks [Eckhardt Petri et al., 2010].

Development of vaccine against TBE has started since the discovery of causative agent in 1937. A group of discoverers, headed by already well known virologist prof. L.A. Zilber, understood that only vaccine can become a reliable protection from this dangerous disease.

As early as in 1938 a group of young virologists headed by E.N. Levkovich started working on a vaccine against tick-borne disease; they obtained the first formalin-inactivated brain vaccine from the Far Eastern TBEV strain Sofjin [*Vanda Pogodina*. 2001].

In 1939, the study aimed to investigate the vaccine effectiveness was conducted in Oborsk forestry (Khabarovsk Region). Lethality was not registered among the vaccinated patients. Despite the obvious epidemiological effectiveness of this vaccine, it caused serious side effects in vaccinated patients. In some years, number of postvaccinal encephalitis reached 1:20000 of vaccinated patients. Reduction of concentration of brain tissue from white mice in the vaccine up to 2.5% did not make it better [3]. Thus, a search for safer approaches to the development of new vaccine was necessary. In 1960, development of the vaccine against TBE using chick-embryo cell culture for the virus reproduction was a significant step to improve quality of the vaccine preparation. TBE strain Sofjin was reproduced in a cell culture of chick fibroblasts, inactivated by formaldehyde in a concentration of 1:2000, and virus antigen was absorbed on aluminium hydroxide. However, vaccination of patients showed that not only reactogenicity, but also protective characteristics of this vaccine were lower in comparison with formolated brain vaccine [*Vorobyova MS et al., 2007*].

At the same time, at the Tomsk Scientific Research Institute of Vaccines and Sera, a cultural vaccine was developed using western TBEV subtype. It's immunological activity and protective characteristics were also insufficient [*Leonova GN, 1997; Vorobyova MS et al., 2007*]. From 1964, this vaccine was introduced in the Far East. It's immunologic effectiveness in hemagglutination-inhibition reaction (HAIR) was 70% with low immunity stress in HAIR (GTM 1:15). Soon, on the Primorye Territory, single TBE cases were reported among the patients vaccinated three times and revaccinated [*Dorokhova V.S. & Tatarinova L.G, 1969*]. In 1967, rate of vaccinated persons didn't exceed 1.8% of urban population and 13.2% of villagers. A cumbersome vaccination protocol and short-term humoral immunity in the patients vaccinated with a formalin-inactivated liquid tissue culture vaccine constantly initiated research work on production immunologically more active vaccines.

In the middle of 1980s at the Institute of Poliomyelitis and Viral Encephalitides, Academy of Medical Sciences of the USSR, a group of scientists headed by L.B. Elbert developed a new preparation technique of concentrated vaccine based on the TBE strain Sofjin of Far Eastern subtype. A specific activity of the obtained vaccine considerably exceeded activity of non concentrated preparation [*Vorobyova MS et al., 2007*]. Nowadays, this vaccine is widely used on endemic territories of the Russian Federation as a dry concentrated vaccine produced by M.P. Chumakov Institute of Poliomyelitis and Viral Encephalitides (IPVE) of Russian Academy of Medical Sciences (RAMS). The TBE strain 205 isolated in 1973 from ticks I.persulcatus on Khabarovsk Territory [*Vereta et al., 1990*] is now used as a master seed virus for Tomsk vaccine. From 1984, liquid tissue culture vaccine against TBE has being manufactured by scientific production association (SPA) "Virion" (affiliated with Federal State Unitary Enterprise (FSUE) SPA "MicroGen") on the base of this strain which is a typical representative of Far Eastern subtype of TBE. From 2003 it has being produced as liquid tissue culture inactivated, purified, concentrated, and absorbed vaccine "EnceVir".

Additionally, in 1970s, the development of live vaccine based on the attenuated strain TP-21 of the Langat virus and antigenically similar naturally attenuated Elantsev strain, isolated from human blood on the territory of Siberia, provoked a great response in scientific world [*Smorodintsev A.A.& Doubov A.V* , 1986]. Although these developments did not have any practical application, the authors outlined scientific approaches to creation of such vaccines.

According to these authors, the prime advantage of such vaccines consisted in absence of clinical and pathomorphological signs of central nervous system damage after intracerebral administration to monkeys.

In 1971, the first foreign inactivated tissue culture vaccine against TBE was developed in Austria on the base of the Neudorfl strain (western subtype of TBE) isolated from ticks I. ricinus. In 1980s, a new technique was implemented; this enabled to obtain a preparation up to 99% free of heterologous protein and to concentrate TBEV antigen. The vaccine was called "FSME-Immun-Inject" [*Kunz et al. 1980*]. In 1993, this vaccine produced by "Baxter Vaccines" (Austria – USA) was registered in Russia for adults (0.5 mL), and in 2006-2007 – for children 1-16 years old at half the volume (0.25 mL) [*Vorobyova MS et al., 2007*].

In 1998, another TBE vaccine manufactured by the German company "Chiron Behring" (currently "Novartis Vaccines and Diagnostics GmbH & Co KG") was registered in Russia. This vaccine was based on the strain K-23 (western subtype of TBE) isolated from ticks I. ricinus. In Russia, the vaccine is used in two variants: "Encepur® Adults" and "Encepur® Children". This high-purity vaccine, free of homologous and heterologous proteins, is practically a non-reactogenic preparation which is very important for evaluation of its safety [*Zent et al*. 2003]. The vaccine variants for adults and children differ only by the concentration of the virus antigen (1.5 mg/0.5 mL and 0.75mg/0.25 mL, respectively). So far, there have not been registered any TBE cases both in European countries and the Russian Federation among those vaccinated with this vaccine, unlike the other ones described above.

Availability of the above TBEV strains for vaccine preparations to protect from the current virus population including different subtypes of TBEV was reported in several papers [*Holzmann H et al., 1992; Leonova GN, et al., 2006; Leonova GN et al., 2007*]. On the other hand, new genetically different variants within the Far Eastern subtype of TBEV were found. They were Oshima, Senzhang, Glubinnoe/2004-like strains, and the strain of hemorrhagic form of TBE [*Loktev VB* et al. 2007; *Leonova GN & Pavlenko EV,* 2009]. Genetic diversity of TBEV was detected, when all three main TBEV genotypes could circulate simultaneously on the same territory [*Pogodina VV. et al., 2007*]. Thereupon, it is often a problem for public health in geographically different Eurasian regions to decide what vaccine among those consisting of different genovariant strains would be the most effective for protection of the population against TBE.

2. Characterization of the immune response to Encepur® Adult vaccine

Vaccine Encepur® Adult is produced using strain K23, which belongs to European subtype of TBEV (Bock HL, et al. 1990). Nucleotide sequences of Far Eastern and European TBEV subtypes have only 78–82% similarity. This naturally raises the question whether the vaccine based on the European subtype of TBEV is protective against the Far Eastern subtype of the virus. We sought to evaluate specific humoral immune response in volunteers immunized with vaccine Encepur® in relation to Far East strains TBEV.

We characterized genetic and antigenic variation among TBEV strains P-73, P-202 and P-69, isolated in Far Eastern region of Russia. Locations, dates and sources of isolation Far Eastern strains used in this study are presented in Table 1. Genotyping of strains P-73, P-202, and P-69 unambiguously demonstrated that they belong to Far Eastern subtype of TBEV (Leonova G. et al. 2007).

TBEV strain	Year of isolation	Source of isolation	Place of isolation	Reference
Primorye-73 (P-73)	1973	Human brain (lethal case)	Dal'nerechensk region, Primorsky krai	Leonova GN et al. 2004
Primorye-202 (P-202)	1997	Leukocytes of healthy human after tick bite	Ussuriisk region, Primorsky krai	Leonova GN et al. 2004
Primorye-69 (P-69)	2000	Leukocytes of healthy human after tick bite	Nadezhdinsk region, Primorsky krai	Leonova GN et al. 2004

Table 1. TBEV strains used in the study

The strains P-73, P-202, and P-69 are highly virulent for suckling mice. The invasivity index for these strains (the difference between $\log_{10}$ LD50 for intracerebral and subcutaneous challenge) were found to be 2.0, 1.4, and 0.7, respectively. Strains P-73 and P-69 were replicating efficiently in PEK cells (7.5 and 6.0 log TCID50/0.1 ml, respectively). However, strain P-202 was not able to replicate effectively in PEK cells — the titer did not exceed 4.75±0.25 log TCID50/0.1 ml. An attempt to adapt P-202 to cell culture by several passages in PEK cells was unsuccessful.

We also measured humoral immune response against these strains after complete vaccination of volunteers with Encepur® Adult. We have shown significant differences in levels of antibodies and the number of NT-positive sera against strains P-73, P-202, and P-69 1 year after the second immunization (Table 2). After the third immunization, key parameters of the immune response increased considerably and the number of sera positive in NT with strain P-202 reached 95.5±3.1 and 97.6±2.3% with strain P-73. The strain P-69 behaved differently, as the number of sera positive in NT was only 63.9±7.2%, which is statistically different from the corresponding data for the other strains.

TBEV strain	No. of positive human sera (%)		Maximal titer		Average titer[a]	
	Before	After	Before	After	Before	After
P-73 (a)	56.8	95.5	80	640	19.7	128
P-202 (b)	52.3	97.6	40	320	15	34
P-69 (c)	27.3	63.9	20	80	11.3	28

[a] Mean geometric titers of neutralizing antibodies.

Table 2. Titers of neutralizing antibodies to TBEV strains P-73, P-202, and P-69 in volunteer's sera before and after the third immunization with the vaccine Encepur® Adult ($n = 44$)
Note: Reverse titer values are shown. Statistical significance: before immunization for (a–b) $P < 0.05$; for (a–c) and (b–c) $P < 0.01$, after immunization for (a–b) and (a–c) $P < 0.001$; for (b–c) $P < 0.01$.

The most complete immune response to immunization with the German vaccine Encepur® Adult was achieved against highly virulent Far Eastern strain P-73. The volunteers were also

seemed to be protected against the two other TBEV strains. Induction of neutralizing antibodies at titers considerably exceeding the minimal protection level was demonstrated after both double and triple immunizations.

3. Comparative evaluation of immunologic activity of vaccines against TBE used in the Russian Federation

In Russia, the following vaccines are certified and widely used: "FSME-IMMUN-Inject" (Austria), "Encepur® Adults" (Germany), and two local vaccines – the TBE vaccine manufactured by M.P. Chumakov Institute of Poliomyelitis and Viral Encephalitides (Moscow, Russia) and "EnceVir" manufactured by SPA "MicroGen" (Tomsk, Russia) [*Vorobyova MS*, 2007].

We have performed cohort and simultaneous study of immunologic effectiveness of the four vaccines against TBE in relation to several contemporary Far Eastern TBEV strains on 290 volunteers [*Leonova GN & Pavlenko EV*, 2009]. We evaluated immunologic activity of the vaccines against TBE according to the intensity of antibody response one and two years later after full course of immunization with the vaccines. Postvaccinal antibody response was evaluated by a level of virus neutralizing antibodies, total antivirus antibodies, and their avidity regarding different TBEV strains.

All volunteers were vaccinated three times during one year according to application instructions of the vaccines. The exception was made for a combined vaccination which consisted of alternate use of the Russian and foreign vaccines.

The mean age of the vaccinated patients was almost the same, and in the first period of study it varied from 32.6 to 47.3 years, in the second one – from 34.7 to 47.4 years. Blood sera of the volunteers vaccinated against TBE were studied by enzyme-linked immunosorbent assay (ELISA) and neutralization test (NT) with the strain Primorye-73 (P-73) of Far Eastern TBE subtype.

We have concluded that all studied vaccines are able to induce neutralizing antibodies against contemporary TBEV strains [*Leonova GN & Pavlenko EV*, 2009]. Levels of seroconversion for the vaccine "FSME-IMMUN-Inject" (Austria) were 88.2% in the year after full course of immunization and 78.1% of cases – two years later, "Encepur® Adults" (Germany) – 100% and 100%, the vaccine manufactured by IPVE RAMS (Moscow) – 100% and 94.1%, "EnceVir" (Tomsk) – 88.2% and 83.9%, and during the combined vaccination – 100% and 92.7% of cases, respectively. Use of different types of vaccines during the combined vaccination of a patient provided a high and stable level of seroconversion according to NT, which is important for revaccination with different vaccines available in order not to interfere with the recommended vaccination protocols (Table 3).

As a rule, antibodies with high affinity and avidity to surface proteins of TBEV virions are necessary for manifestation of neutralizing antibody activity. Only these antibodies do not allow the virus to interact with receptors and enter the cell. We have shown a variability of common and avid antibodies to different TBEV strains in vaccinated patients in the year of vaccination and two years later. Not all sera of those vaccinated with different vaccines had high-avid antibodies to the studied TBEV strains even in the year of vaccination.

Moreover, not only the proportion of patients having antibodies and the general GMT of antibodies, but also GMT of avid antibodies to all TBE strains decreased at remote observation time (two years later after the vaccination course). It has been shown using

ELISA (Fig. 1) that in the year of vaccination, GTM of antibodies to TBEV reached 6.5 $\log_2$; two years later, GMT decreased up to 5.5 $\log_2$.

Vaccine	Observation time point	Number of examined vaccinated persons	Mean age		ELISA (IgG %)	NT (Ab %)
			years	p		
FSME-Immune Inject («Baxter Vaccine», Austria)	After the primary vaccination course	51	32.6 ± 2.3	0.592	86.3	88.2
	2 years after vaccination	32	34.7 ± 3.3		75	78.1
Encepur® Adults («Novartis Vaccines», Germany)	After the primary vaccination course	6	36.8 ± 6.0	0.128	83.3	100
	2 years after vaccination	11	46.4 ± 3.0		100	100
Vaccine by Chumakov Institute of Poliomyelitis (Moscow, Russia)	After the primary vaccination course	30	41.2 ± 3.3	0.274	90	100
	2 years after vaccination	17	47.4 ± 4.7		94.1	94.1
EnceVir («Microgen» Tomsk, Russia)	After the primary vaccination course	17	45.8 ± 4.5	0.971	76.5	88.2
	2 years after vaccination	56	45.6 ± 2.7		66.1	83.9
Combined vaccination*	After the primary vaccination course	29	43.2 ± 3.9	0.596	100	100
	2 years after vaccination	41	45.6 ± 2.6		92.7	92.7

* The first two inoculations have been made by foreign vaccines consisting of the TBEV strains of European subtype, the subsequent booster vaccination − by the Russian vaccines consisting of the TBEV strains of Far Eastern subtype.

Table 3. Comparative assessment of the immune response in patients vaccinated with different TBE vaccines after the primary vaccination course and the two years later after the primary vaccination course.
Note: NT, neutralization test; Ab, the antibodies to TBEV strain p-73; *p,* significance at the confidence interval of 95%
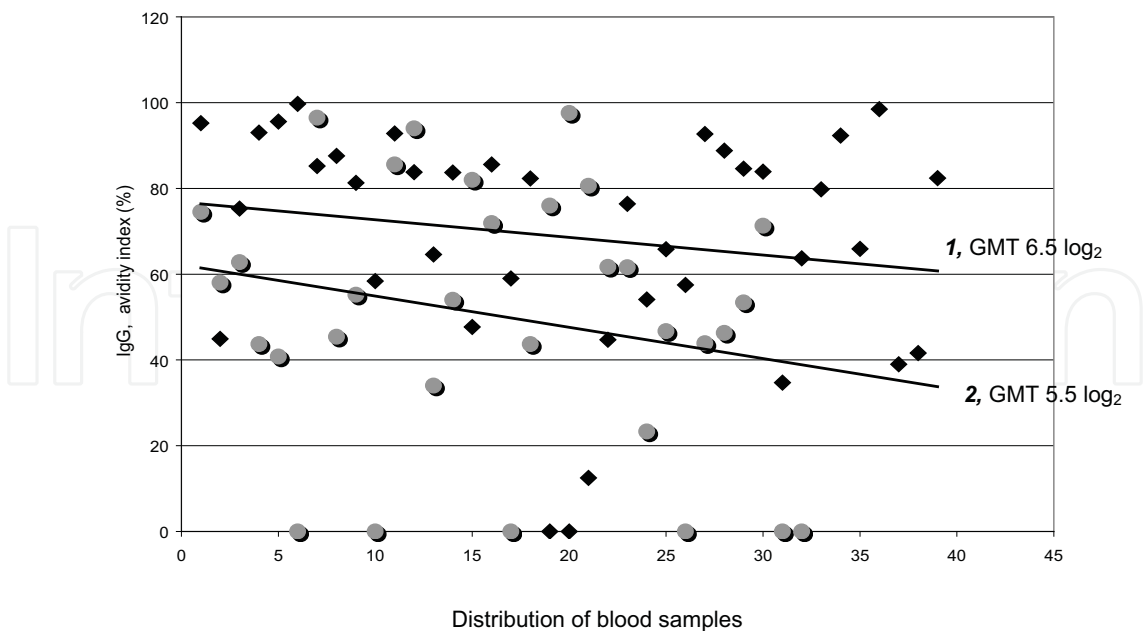
Fig. 1. Distribution of IgG avidity index in the patients vaccinated against TBE after the primary vaccination course and two years later after vaccination: ♦, blood samples after the primary vaccination course; ○, blood samples two years later after vaccination; trend line *1*, after the primary vaccination course; and trend line *2*, two years later after vaccination

Immune response of the patients vaccinated against TBE to different strains is known to vary [Holzmann al, 1992; Klockmann U. Et al,, 1999]. Figure 2 depicts comparative data on blood sera of the patients vaccinated against TBE in the first and second year after full course of vaccination studied by ELISA and indirect immunofluorescence assay (IFA). It is obvious that during both observation periods, antibodies of the vaccinated patients showed a closer immune relation to the strain P-73 in comparison with the other strains (P-202 and P-69) isolated from blood of the patients with inapparent TBE form. These data gave evidence that vaccinated patients had immunoprotection not only to pathogenic TBEV strain associated with focal form of infection with fatal outcome, but also to all other TBEV strains widely circulating on the territory of the Far Eastern region.

Therefore, all four vaccines against TBE can be recommended for wide use in the Russian Far East (possibly in China and Japan as well), where the most pathogenic TBEV strains circulate. Virus-specific antibody avidity test have a special significance for more adequate evaluation of a stress of postvaccinal immune response. Avidity of specific IgG antibodies characterizes immune response of the vaccinated patients more precisely. It is also important to study functional antibody activity of the vaccinated patients for evaluation of their protection, when it is necessary to determine immunity stress of these patients regarding TBEV, for example, after tick bite. Indeed, such studies are necessary for determination of immunity duration in vaccinated patients and time of remote revaccinations. Use of different types of vaccines for immunity formation in a patient also provided a high and stable seroconversion according to NT. These data have a practical importance to determine time of remote revaccinations using another type of vaccine against TBE and consider interchangeability of all four types of vaccines without any interference with recommended vaccination protocol. Such approach can also provide

formation of a more diverse immunity against the strains of western and Far Eastern subtypes of TBEV.

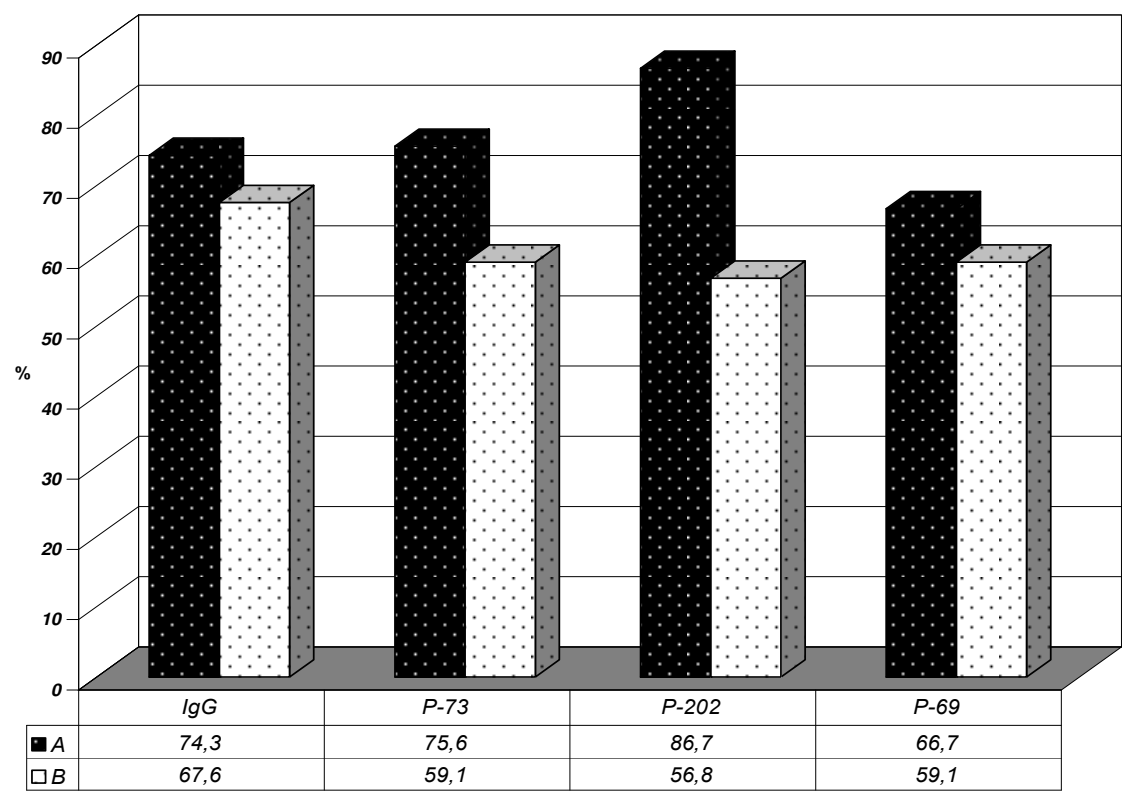|	IgG	P-73	P-202	P-69
■ A	74,3	75,6	86,7	66,7
□ B	67,6	59,1	56,8	59,1

Fig. 2. Rate of avid antibodies in patients vaccinated against tick-borne encephalitis after the primary vaccination course and two years later: IgG according to ELISA and the antibodies to P-73, P-202, and P-69 strains according to IFA; *A* − percentage of avid antibodies after the primary vaccination course; *B* − percentage of avid antibodies two years later after vaccination

4. Protective antibody titer and level of immunological memory in patients vaccinated against TBE

The notion of protective antibody titer appeared during the studies on evaluation of vaccinal immunological activity. Preventive effectiveness of vaccines against some infections was considered to be evaluated by a protective level of immunological characteristics [Briko NI. 2004]. In 1980 Kunz et al. [1980] suggested that the protective titer of hemagglutinating antibodies for TBEV vaccine is above 1:10. At present time the most of diagnostic laboratories use ELISA as the main standard detection method of specific IgG antibodies.

In the course of time, despite the decrease in characteristics of immune response, some vaccinated patients showed the ability to preserve antibodies for a long time [*Kunz C. et al.. 2003; Heinz FX, et al,. 2008*]. To determine a revaccination time, we have obtained the experimental evidence of protective effect of different antibody titers in patients vaccinated against TBE. Immunological characteristics obtained during examination of the vaccinated patients, allowed us to indirectly judge a possible epidemiological effectiveness of vaccinal prevention.

4.1 Experimental studies (experiment №1)

We performed the set of experiments using the specific anti-TBE immunoglobulin (titer from 1:6400 in ELISA, 1:160 – in IFA, and 1:320 – in NT). To find an optimum alternative of experimentation, we ranged experimental components by IFA: different dilutions of immunoglobulin containing antibodies of IgG class, different TBEV doses and time parameters. Fig. 3 (*A, B, C)* shows influence of different TBEV doses (1, 2, 3 lg TCID) on the specific antibodies with titers ranging from 1:160 to 1:10 (via IFA) at 5, 15 and 30 min. In experimental samples, virus titers had from two- to four-fold decrease depending on virus dose employed in experiment. The most convincing data on the decrease of virus titer can be observed in Fig.3 *C*. With the virus dose 3 lg $TCID_{50}$, the characteristics decreased significantly at all exposition times (5 – 30 min).

Further studies were carried out with samples containing different dilutions of immunoglobulin and only a virus dose of 3 lg $TCID_{50}$. Similar results were observed in ELISA and neutralization test (NT). While observing the antibody contact at different dilutions (antibody titers from 1:6400 to 1:12.5) with TBEV (3 lg $TCID_{50}$), we singled out the sample containing IgG with a titer of 1:400, in this sample the antibodies completely bounded to the virus antigen (Tabl. 4).

Virus antigen in this sample decreased to minimal values (K = 0.4). During the experiment, K values of virus antigen were ambiguous: with high titre values (1:6400 and 1:1600) K antigen was positive (3.9 and 1.6, respectively), then K antigen decreased up to 0.4. However, the further decrease of IgG titers led to the increase of K antigen up to the positive value (1.0). RT-PCR data were positive in all samples. Occurrence of free infective virus of 0.9 and 1.0 lg $TCID_{50}$ was an evidence of unfavourable conditions for a specific protection in samples with IgG titer less than 1:100. Similar results were observed in neutralization test, when the minimal protective threshold having the starting titer was analysed with that of 1:20.

ELISA		Neutralization test		Antigen K via ELISA	TBEV titration on PEK cells	RT-PCR
Initial IgG titer	Residual IgG titer	Initial IgG titer	Residual IgG titer			
6400	1600	320	80	3,8	0	+
3200	800	160	80	1,6	0	+
1600	400	80	80	0,8	0	+
800	200	40	40	0,5	0	+
400	**0**	**20**	**20**	**0,4**	**0**	**+**
200	0	10	0	1.0	0	+
100	0	5	0	0,8	0	+
50	**0**	2,5	**0**	**0,7**	**0,9**	**+**
0	**0**	**1,22**	**0**	**0,9**	**1,0**	**+**

Table 4. Experimental substantiation of protective IgG titer threshold under the influence of TBEV exposure (3 lg $TCID_{50}$) based on ELISA, Nt, virus titration, RT-PCR, and antigen detection via ELISA
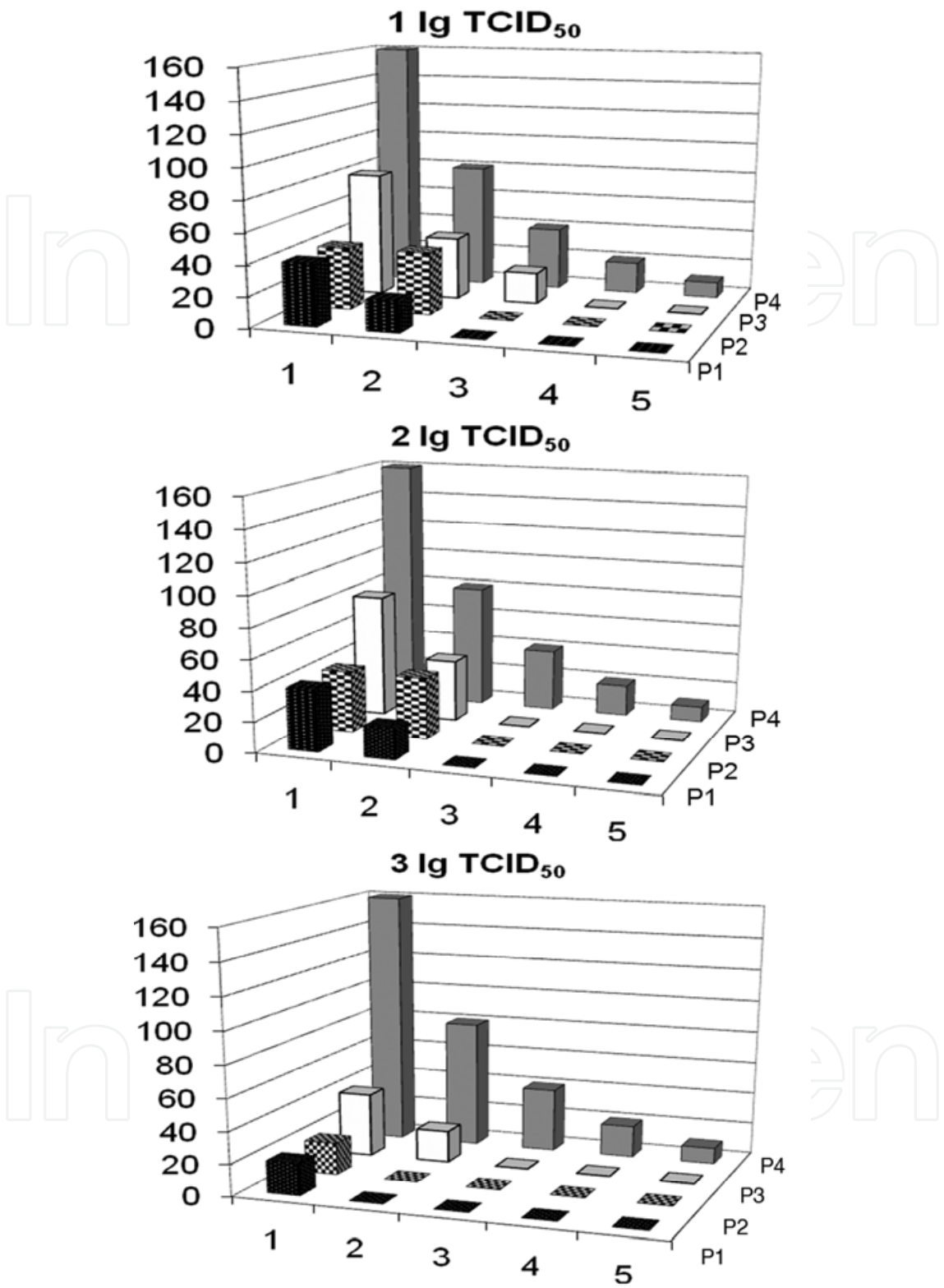
Fig. 3. Influence of different TBEV doses (A- 1 lg, B- 2 lg, C- 3 lg $TCID_{50}$) on IgG titer in 30 min (P1), 15 min (P2) and 5 min (P3), (P4 – control two-fold IgG dilutions 1:160-1:10) via IFA X-axis – sample number; Y-axis (left) – IgG titer via IFA TBEV vaccine was above 1:10. At present, most of diagnostic laboratories use ELISA as the main standard method of specific IgG antibodies detection

Fig. 4 depicts two-fold decrease of specific IgG *(2)* titers under the TBEV influence at a dose of 3 lg $TCID_{50}$. In addition, via IFA, it shows a gradual decrease in antigens *(3)* from the sample with IgG titer of 1:3200 to the one of 1:400 under the TBEV influence (3 lg $TCID_{50}$).
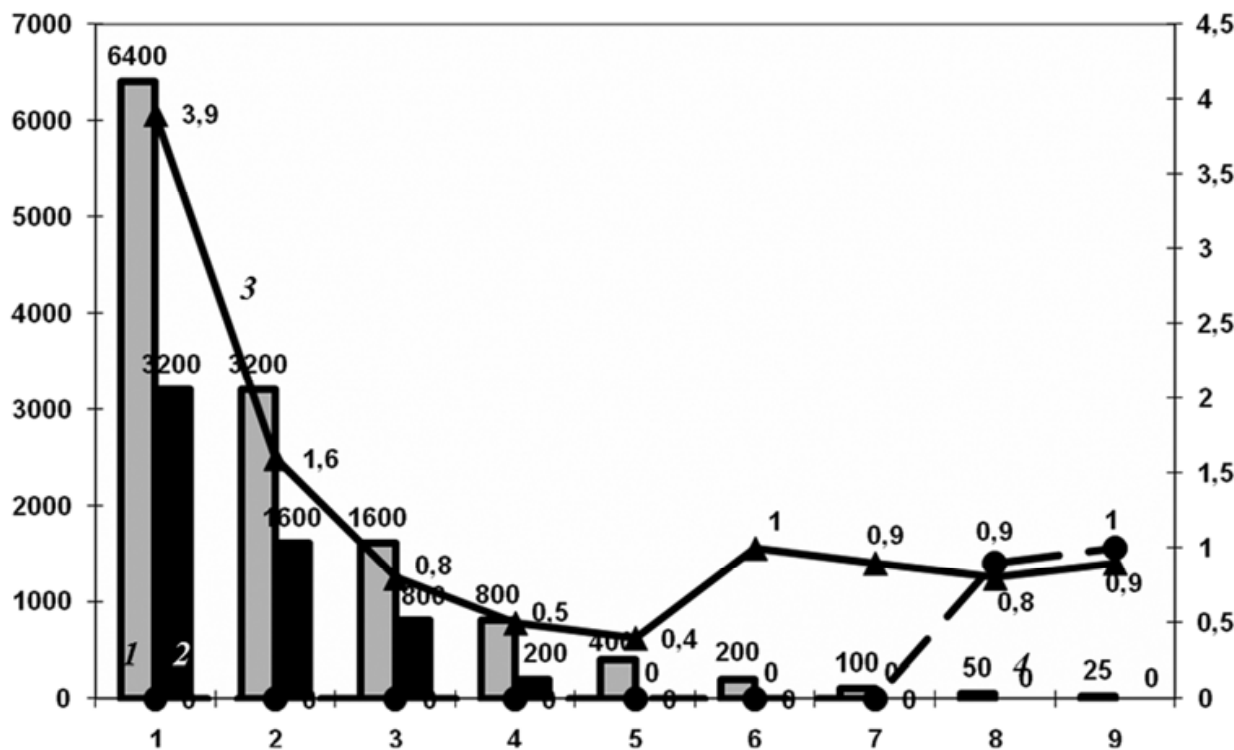
Fig. 4. Initial IgG titer (*1*), decrease of IgG titer (*2*), decrease of antigen titer (*3*), neutralization of TBEV
3 lg $TCID_{50}$ (*4*) in samples with serial two-fold IgG dilutions.
X-axis – sample number
Y1-axis (left) – IgG titer via ELISA, Y2-axis (right) – antigen titre via ELISA

There is also the virus neutralization from the sample with IgG titer of 1:3200 to the one of 1:100. With the further antibody dilution (1:50 and 1:25) on the monolayer of the pig embryo kidney (PEK) cell culture, a cytopathic effect of non-neutralized virus with a titer of 0.9 and 1.0 lg $TCID_{50}$ was detected in the samples.

4.2 Experimental studies (experiment №2)
To confirm the obtained data, we performed the second set of experiments (in vivo and in vitro). TBEV doses from 1 to 8 $TCID_{50}$ were compared via neutralization by immunoglobulin (titers 1:3200 and 1:400). Titration in PEK cells (Fig. 5 *A*) showed that IgG (titer 1:400) entirely neutralized 3 lg $TCID_{50}$ of TBEV as in the first set of experiments, and IgG (titer 1:3200) neutralized 4 lg $TCID_{50}$. Moreover, after IgG treatment (titer 1:3200), initial virus titer (5-6 lg $TCID_{50}$ or 7-8 lg $TCID_{50}$) was reduced by 3-4 lg $TCID_{50}$ or 1-2 lg $TCID_{50}$, respectively.
Titration on mice (Fig. 5 *B*) showed that IgG (1:400) entirely neutralized TBEV at 3 lg LD_{50} and decreased TBEV titer by 3-4 lg LD50 in comparison with the initial 4, 5, 6 lg LD50, but was unable to neutralize 7 and 8 lg LD_{50}. IgG with titer of 1:3200 entirely neutralized 5 lg LD_{50} and decreased TBEV titer of 6, 7 and 8 lg LD_{50} by 5.5 lg LD_{50}, 3 lg LD_{50}, and 2 lg LD_{50} of TBEV, respectively.
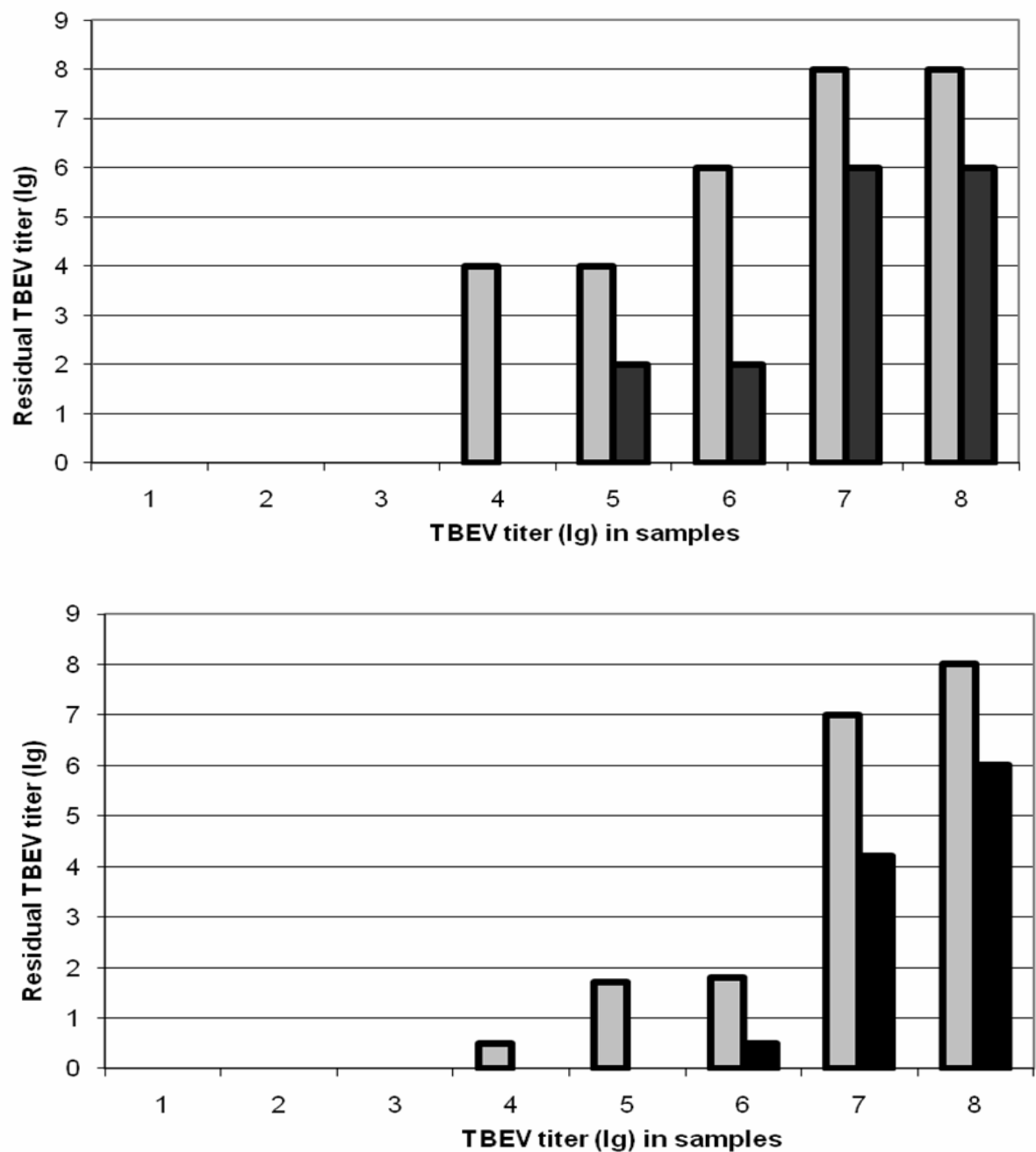
Fig. 5. IgG protective effect with titers of 1:400 (1) and 1:3200 (2) against TBEV (1-8 lg TCID$_{50}$ (A) & 1-8 lg LD$_{50}$(B)
A – PKE cells (*in vitro*); *B* – white mice (*in vivo*);
X-axis – sample number

According to our data on neutralization of different IgG antibody titers with one dose of TBEV (3 lg TCID$_{50}$), we considered a lower threshold of antibody protective level to be 1:400 in ELISA and 1:20 in NT. The second set of experiments confirmed that in ELISA IgG titer of 1:400 should be considered as the lower threshold of antibody protective level, and this threshold can be used for evaluation of immunological effectiveness of vaccines used for TBEV prevention.

Therefore, the set of experiments on neutralization of different antibody titers of IgG class with one TBEV dose (3 lg $TCID_{50}$) allowed us to consider a minimal protective antibody threshold level to be at 1:400 via IFA and 1:20 via NT. Additional evidence was obtained during neutralization of TBEV with different titers using immunoglobulin having maximal (1:3200) and minimal (1:400) antibody titers. These experiments confirmed the suggestion that IgG titer of 1:400 for IFA should be considered the minimal protective antibody threshold level. It can be used for evaluation of immunologic effectiveness of vaccines used to prevent from TBE. Additionally, antibody titer of 1:100 can be considered as a lower threshold of immunological memory.

Here we used TBEV at suboptimal doses of 1, 2, 3 lg $TCID_{50}$ which are usual for tick bites on humans. As a rule, such doses occur in nature not only in ticks (*Ixodidae*), but also in Muridae rodents as their main hosts. The viremia in these rodents may overweight infectivity threshold (2.7 – 3.0 lg LD_{50} of TBEV) and, thus, ensure the virus transfer to ticks, as well as the stable virus circulation in environment [*Chunikhin et al.* 1985]. Nevertheless, cases of tick infection with TBEV over the mentioned threshold, if compare with theoretical possibility, are rather rare. It is, in turn, a protective factor which provides a relatively low number of TBE cases.

Thus, at epidemiologically crucial virus dose of 3 lg $TCID_{50}$, IgG protective effect was observed in experimental samples from the titer of 1:6400 to 1:100. Hence, we consider that the antibody titer of 1:100 not as a protective threshold level according to Sanitary and epidemiologic rules and regulations of the Russian Federation [Sanitary and epidemiologic rules and regulations SP 3.1.3.2352-07. 2008], but a lower threshold of immunological memory which allows a vaccination course to be continued. At the same time, patients vaccinated against TBE and having antibody titer of 1:100 in their blood can not to fall ill, provided that after a tick bite, the virus titer penetrated into blood was very low. However, a possibility of TBE disease still remains. Moreover, in the group of vaccinated patients, there are tick bite cases followed not only by febrile TBE form, but also sometimes by a clinical picture of brain damage. In addition, it has been shown that high IgG titers (1:3200) actively neutralized TBEV and, obviously, were able to provide a reliable protection from this disease. In the second set of comparative experiments on neutralization of different TBEV doses (from 1 to 8 lg) with immunoglobulin and antibody titers of 1:400 and 1:3200, we obtained additional evidence that the IgG titer of 1:400 is a minimal protective antibody threshold level. The obtained data can be used at any postvaccinal time for observation on immunological effectiveness of vaccinal prevention. Antibody titers of 1:400 and below indicate that revaccination is necessary.

4.3 Evaluation of immunological effectiveness of vaccine Encepur® Adults

In our earlier studies [Leonova et al. 2006; Leonova, 2009], we noticed that during the whole course of three-fold vaccination, antibodies of IgG class were observed in all blood samples, in 100% of cases, when the patients had specific antibodies (seropositive group) before undergoing full course of immunization. In seronegative group, antibodies of IgG class increased dynamically during the whole course of vaccination. However, characteristics of immune response stress according to NT data were of special interest. In seropositive group, titers of the virus neutralizing antibodies after the third vaccine were significantly behind the ones in seronegative group (GTM 1:208). Such lag was evidence that the patients of this group had got an excessive antigen stress on the immune system which resulted in a

considerable exhaustion and decrease in activity of immune response. This data should be taken into account when determining time of revaccinations.

We evaluated immunological effectiveness of vaccine Encepur® Adults in remote periods after the primary course of vaccination. We analyzed blood sera of the patients (n=10) three and five years later after the vaccination. ELISA data (Fig. 6) showed that IgG titers in the patients were high three years later after full course of vaccination: 1:3200 – six cases, 1:1600 – one case, 1:800 – one case and 1:400 – two cases. The avidity index of these antibodies was high or had a mean value. Five years later, characteristics of immune response decreased: IgG 1:3200 remained in three cases, 1:1600 – two cases, 1:800 – two cases, 1:400 – two cases, and 1:200 – one case. The avidity index also decreased: it had the mean value in five cases and was lower in another five cases.
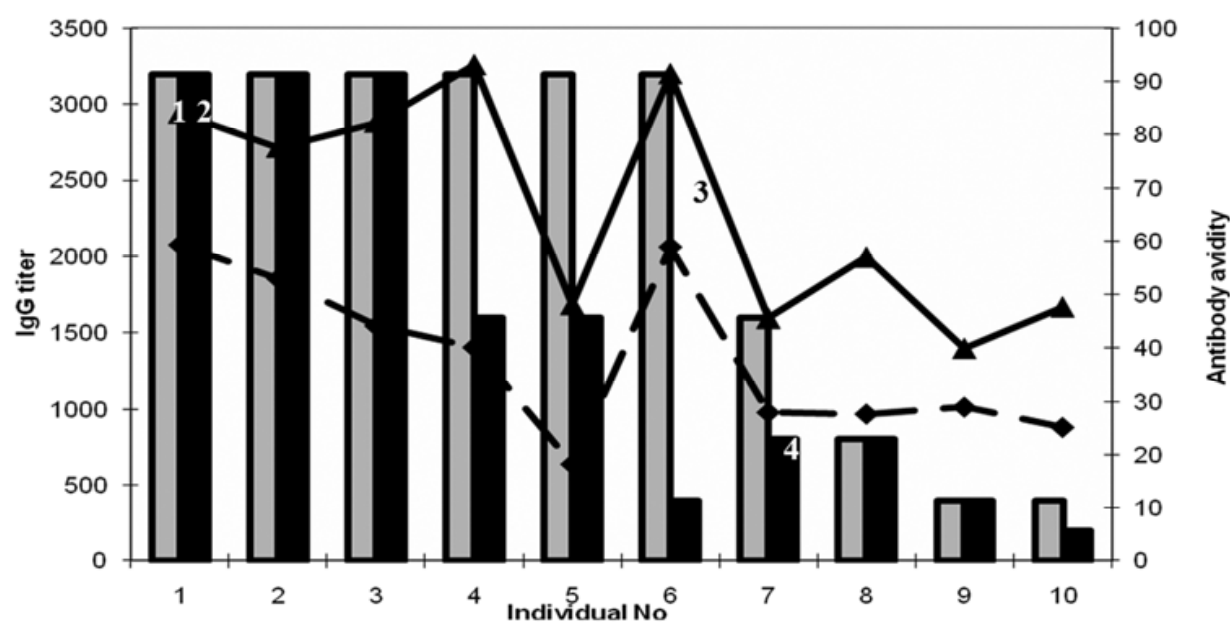
Fig. 6. The level of specific antibodies of patients immunized by Encepur® Adults
1 – IgG level three years later after immunization;
2 – IgG level five years later after immunization;
3 – antibody avidity later three years after immunization;
4 − antibody avidity five years after immunization
X-axis – sample number
Y-1 axis (left) – IgG titer via ELISA, Y-2 axis (right) – IgG titer via NT

Then, we continued studying immune response in this group of patients (n=15) in the last observation period, seven years later after full course of immunization, and one month later after the first revaccination (RV_1). It was determined that before RV_1, the percentage of patients with IgG antibodies according to IFA was 93.3% and GMT − 1:1120. Avid antibodies were found in 80% of the vaccinated patients, avidity index (AI) ranged from 45.2 to 91.4%, and was 69.6±4.5% at the mean. After RV_1, the values of immune response increased in these patients. We found specific antibodies in 100% of the patients. The stress of immune response was: IgG GMT – 1:3200 and AI – 83.6±4.8% (p=0,047).

The results of this study gave us evidence on reasonability of a selection of TBEV antigen stress in the vaccine Encepur® Adults having not only high values of immune response, but

also a long-term protective effect [*Zent et. al.* 2003; 2005; 2005]. Additionally, using IFA, we performed the comparative analysis of immunogenic activity of the vaccine Encepur® Adults to the TBEV strains P-73 (Far Eastern subtype) and Kolarovo (Siberian subtype) isolated from the ticks I. pavlovskiy. Before revaccination, the percentage of patients with antibodies to these strains was 100% while GMT to the strain P-73 was 1:91 and to the Kolarovo strain – 1:158. After revaccination, GMT to the strain P-73 was 1:239 and to the Kolarovo strain − 1:447. These data are evidence of a higher level of antibodies to the Siberian TBEV subtype in the vaccinated patients in comparison with that of the strain P-73 of Far eastern TBE subtype, thus, making a good reason to recommend using this vaccine not only in European countries and Far East, but also on the whole focal territory of Siberia.

For the first time, we obtained evidence for the lower threshold level of protective IgG titer in different experiments. Therefore, at the virus dose of 3 lg $TCID_{50}$, protective effect of IgG was detected with titers ranging from 1:6400 to 1:100. Cytopathic effect was not registered in the PEK cell culture infected with these samples. We considered a lower threshold of protective IgG activity to be 1:400 according to ELISA data, and 1:20 according to NT. Although this sample was positive according to RT-PCR, we did not identify virus antigen in ELISA, and antibodies in the examined sample eliminated completely. With the further decrease of antibody titer, we observed an increase in characteristics of K values via antigen-detection ELISA. Moreover, free infective virus up to 1lg TCD_{50} appeared in the samples with IgG titer lower than 1:100 (ELISA data). Due to this fact, we considered that antibody titer of 1:100 can not be called a threshold of protective level as it was reported by [*Leonova G.N.,* 2009], but a lower threshold of immunological memory enabling further vaccination. Patients vaccinated against TBE with antibody titer of 1:100 in blood cannot fall ill after a tick bite, if they receive a relatively low virus dose. However, the possibility of TBE infection still remains. In addition to febrile forms of disease, there are also some cases of severe damage of nervous system occurring among immunized patients. Moreover, high IgG titers (1:3200) actively neutralized TBEV and were probably capable of ensuring a safeguard against this disease. The second set of comparative experiments on neutralization of different TBEV doses (from 1 to 8 lg) with IgG titer of 1:400 and initial antibody titer of 1:3200 gave the additional evidence that IgG titer 1:400 is a minimal level of protective antibody activity. These data can be used at any time of observation after vaccination of the patients to evaluate the immunological effectiveness of vaccines used for TBEV prevention. Antibody titers of 1:400 and below indicate the necessity of revaccination.

Taking into account the experimental data, we analyzed the individual immune response of ten patients vaccinated with Encepur® Adults three and five years later after a course of three vaccine injections. We determined that this vaccine enables long-term production of antibodies which remains for up to seven years (observation time). As a rule, repeated vaccination contributes to formation of high-avidity antibodies [Leonova *GN & Pavlenko EV,* 2009] which usually have high affinity to specific antigen sites and are able to eliminate antigen actively. A considerable part of immune sera of vaccinated patients contained avid antibodies with high AI three years later after a full course of vaccination, and moderate AI five years later after vaccination. It reached the mean value in half of cases, and a lower value in the rest ones. In other words, patients with antigen titer of 1:400 and below, as well as those with low avidity should be revaccinated in five years after full course of vaccination. This approach to vaccination time was confirmed by the work of Heinz FX et al. [2008], who recommended revaccinations for all patients vaccinated against TBEV, and five

years later, revaccination was not recommended only to the aged patients. Therefore, the data obtained on the threshold level of antibody titer (1:400) and determination of a minimal level of their immunological memory (1:100) helps to determine an immunological protectiveness of the vaccinated patients and duration of postvaccinal protective immunity and, thus, specify revaccination schedule. To obtain the maximal immunological and epidemiological effectiveness of vaccinal prevention against TBEV, individual approach to each patient is appropriate. Moreover, it may have medical and economic importance.

5. Conclusion

The most important characteristics of a vaccine preparation are its immunogenic activity and capability of a long-term preservation of antibodies in vaccinated patients. These characteristics should be taken into account when determining time of revaccinations. Immunization course with different vaccines made from western and Far Eastern TBEV subtypes, as well as combined vaccination with different preparations provided the high and stable seroconversion according to IFA and NT. In accordance with the above experimental data, we have analysed an individual immune response in the patients vaccinated with Encepur® Adults three, five, and seven years later after the three-fold course of vaccination. It has been shown that this vaccine enables production of antibodies which are being preserved for a long time, up to seven years. As a rule, repeated vaccination contributed to formation of high-avidity antibodies [*Leonova G.N., 2009*] which usually had a high affinity level to specific antigen sites and were capable of active antigen elimination. Considerable part of immune sera from vaccinated patients contained avid antibodies which AI was still high three years later. Five and seven years later after full course of vaccination, AI decreased. High values of immune response after RV_1 seven years later after full course of vaccination gave us strong evidence that the obtained data on antibody avidity, protective antibody titer threshold (1:400) and minimal level of immunological memory (1:100) are considered to be the crucial parameters in detection of immunoprotection of vaccinated patients. Based on these data, we suggest that revaccination schedule should be individual for a vaccinated patient for obtaining the maximal immunological and epidemiological effect of vaccinal TBE prevention. Such approach to determination of revaccination time is of economic and medical importance.

6. Acknowledgements

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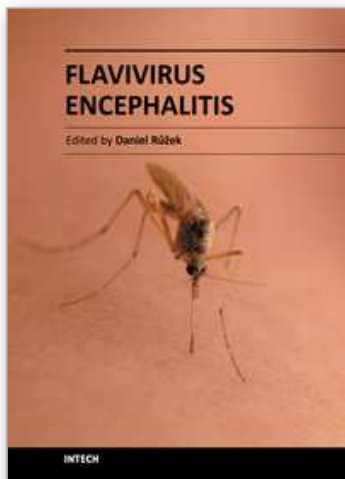
Flavivirus Encephalitis

Edited by Dr. Daniel Ruzek

Encephalitis is an inflammation of the brain tissue associated with clinical evidence of brain dysfunction. The disease is of high public health importance worldwide due to its high morbidity and mortality. Flaviviruses, such as tick-borne encephalitis virus, Japanese encephalitis virus, Murray Valley encephalitis virus, or St. Louis encephalitis virus, represent important causative agents of encephalitis in humans in various parts of the world. The book Flavivirus Encephalitis provides the most recent information about selected aspects associated with encephalitic flaviviruses. The book contains chapters that cover a wide spectrum of subjects including flavivirus biology, virus-host interactions, role of vectors in disease epidemiology, neurological dengue, and West Nile encephalitis. Special attention is paid to tick-borne encephalitis and Japanese encephalitis viruses. The book uniquely combines up-to-date reviews with cutting-edge original research data, and provides a condensed source of information for clinicians, virologists, pathologists, immunologists, as well as for students of medicine or life sciences.

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