Acta virol. **30** : 243–248, 1986

STUDIES ON PREPARATION OF A TICK-BORNE ENCEPHALITIS (TBE) VACCINE FROM THE SKALICA STRAIN

M. GREŠÍKOVÁ, M. SEKEYOVÁ, K. MARCINKA, F. ČIAMPOR

Institute of Virology, Slovak Academy of Sciences, 817 03 Bratislava, Czechoslovakia

Received June 27, 1985

Summary. — Diethylether-treated vaccine against tick-borne encephalitis (TBE) represents a new type vaccine consisting of lipid-free and antigenically efficient components instead of whole virus particles. The TBE virus strain designated Skalica was used for vaccine preparation. This strain is thermosensitive, produces small plaques under agar overlay, is nonpathogenic for adult white mice following subcutaneous (s.c.) application and causes threshold viraemia in host animals. The vaccine was harmless and immunogenic as evidenced by experiments on white mice. Antibodies to TBE virus strain Ir 13 present in human healthy population of a natural TBE focus showed similar levels when tested with the Skalica strain. The Skalica virus strain can be recommended for preparation of the vaccine against TBE.

Key words: experimental vaccine; tick-borne encephalitis virus; Skalica strain; immunogenicity

Introduction

During field studies in West Slovakia a strain of TBE virus designated Skalica was isolated from the organs of a bank vole *(Clethrionomys glareolus)* (Grešíková *et al.*, 1976). Investigation of some its biological properties has revealed that Skalica strain is thermosensitive, nonpathogenic for adult white mice after s.c. inoculation (Grešíková and Sekeyová, 1980) and did not cause viraemia in *Cl. glareolus* adults (Kožuch *et al.*, 1981).

We decided to follow the antibody response in white mice experimentally infected with the Skalica strain and with the experimental vaccine prepared from this TBE virus strain.

In addition, we aimed at finding out whether healthy human population in the natural focus of TBE had antibodies to the strain Skalica, i.e. to elucidate whether this strain circulates in nature and is capable of inducing immune response in humans.

Materials and Methods

TBE virus strains. The strains used in our experiments were as fellows: prototype strain Hypr, strain Skalica and strain Ir 13 (Grešíková and Sekeyová, 1980).

Diethylether (DEE)-treated vaccine was prepared from TBE virus strain Skalica grown in cell culture. The cell culture was prepared from 11-day-old chick embryos (CE) from which the eyes

and viscera were removed. The cells were grown in basal Eagle's medium (BEM) containing 5% foetal calf serum and antibiotics in 1 liter Roux bottles at 37 °C. The Skalica strain was allowed to adsorb for 2 hr at 37 °C, nonadsorbed virus was removed and fresh BEM without serum was added. The cells were further cultivated at 37 °C. On days 1 and 2 post infection (p.i.) the medium was removed and cleared by centrifugation at 1120 × g for 10 min. Partial purification of the virus was performed by differentail centrifugation at 55 500 × g for 2 hr. Supernatant was removed and sediment was resuspended in 1/100 vol of isotonic phosphate buffered saline (PBS), pH 7.2. The virus titre was determined by haemagglutination test. The virus preparation was then treated 3 times with ether: first for 5 min at +4 °C, second for 18 hr at 4 °C and third again for 5 min. The ether phase was removed and nitrogen was bubbled through the virus suspension. The suspension obtained represented the DEE-treated vaccine. It was lyophilized in 0.5 ml vol and stored at 4 °C.

Formalin-treated vaccine. This vaccine was prepared from the Skalica strain in CE cell cultures by the same differential centrifugation procedure as described above. The virus suspension was treated with formalin diluted 1 : 2 000 and the mixture was held at +4 °C for 21 days. Formalin was then removed by adding 1.6 ml 0f 5% sodium hydrogene sulphite solution to 100 ml virus suspension. After mixing the sample was dialyzed against 8 l PBS at 4 °C for 20 hr. The final preparation was lyophilized.

Sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS/PAGE). Freeze-dried samples were dissolved in 62.5 mmol/l Tris-HCl buffer, pH 6.8, containing 2% (w/v) sodium dodecyl sulphate, 5% (w/v) 2-mercaptoethanol, 10% (v/v) glycerol, and 0.001% (w/v) bromophenol blue, 5 min boiled in a water bath, quickly cooled and subjected to electrophoresis. The samples were than electrophoresed in 14% polyacrylamide slab gel using the method of Laemmli (1970). The separated proteins were silver-stained according to the modified method of Marcinka (in preparation).

Electron microscopy. Diethylether treated vaccine was negatively stained with 2% phosphotungstic acid (PTA) at pH 7.5 and examined at 80 KV a Philips EM 300 electron microscope.

The sterility tests were performed on bacteriological media under aerobic and anaerobic conditions. The media remained sterile after cultivation for 14-days at 37 °C and 22 °C.

The safety tests. To prove that the vaccine was harmless, SPF white mice were inoculated intracerebrally with 0.01 ml volumes of the vaccine. After one week one half of mice were killed and from their brains 10% suspension in BEM containing 10% foetal calf serum was prepared and after clearing inoculated in a further series of white mice. The second and the third passage, respectively, were carried out again in one week interval. Nonkilled mice were further observed for the period of one month.

Immunization of mice. The first group of 15 g weighing SPF white mice of the Černý Vůl breed was immunized intraperitoneally with strains Hypr and Skalica (100 LD_{50}). The blood samples from immunized white mice were collected on days 5—7 after virus inoculation. The second group of mice was immunized with the DEE-treated vaccine. The levels of serum antibodies were determined on day 7 post-vaccination. (p.v.) On day 8 p.v., mice were challenged s.c. with prototype strain Hypr of TBE virus. The 3rd group of mice was immunized with the formaldehyde-treated vaccine.

Serological examination of white mice. White mouse sera were examined by heamgglutination-inhibition (HI) test according to Clarke and Casals (1958).

Serological examination of humans. Serological evidence of human exposure to the strain Skalica of TBE virus was obtained by HI tests. Sera were collected from randomly chosen inhabitants of lokality Jarok (Nitra district) in the proved natural focus of TBE. The sera were extracted by acetone; 4—8 haemagglutinating units of either Skalica strain and Ir 13 strain antigens were employed.

Results

In the first experiments antibody response in white mice inoculated with the Skalica strain and or with the prototype strain Hypr were compared. Whereas mean HI antibody titre in mice immunized with the Skalica strain was 1084 on day 7 post-inoculation, in mice immunized with strain Hypr it was only 77 at the same interval (Table 1).

Table 1. Levels of HI antibodies in mice immunized with one TBE virus dose of 100 LD_{50}

Serum No.	HI titre in mice immunized with the Skalica strain		Serum No.	HI titre in mice immunized with the Hypr strain	
	5 days p.i.	7 days p.i.		5 days p.i.	7 days p.i.
1	40	2 560	21	20	80
2	40	640	22	10	—
3	40	320	23	20	—
4	80	5 120	24	40	—
5	80	80	25	20	20
6	20	80	26	10	40
7	20	80	27	20	160
8	80	1 280	28	10	80
9	80	40	29	10	80
10	80	640	30	20	80
Average	56	1 084	Average	18	77

HI = Haemagglutination inhibiting
— = Death of mice

For preparation of the DEE-vaccine, diethylether treatment was performed under steady mixing and cooling. As shown on Fig. 1, PAGE of the vaccine preparation revealed a distinct band of apparent M_r of 60 000 corresponding to the structural envelope glycoprotein E (V3). No virus particles with the structure of TBE virus were detected by electron microscopy (Fig. 2) confirming hat the DEE-treated vaccine preparations contained glycoprotein E subunits and/or incomplete virions.

In mice immunized with the DEE-treated vaccine, the titres of HI antibodies found on day 7 post-inoculation varied from 10 to 80. The vaccine protected immunized mice against challenge with the Hypr strain (Table 2).

Table 2. Levels of HI antibodies in mice immunized with the diethylether-treated vaccine*

Serum No.	HI titre in mice immunized with the concentrated vaccine	HI titre in mice immunized with 10 times diluted vaccine	Survival of mice after s.c. challenge with 1000 LD_{50}**
1	40	±10	+
2	80	80	+
3	10	80	+
4	20	80	+
5	20	40	+
6	80	80	+
7	80	40	+
8	40	40	+

* Single dose administration
** Challenge with strain Hypr; 8 days after vaccination

Table 3. HI antibodies in white mice immunized* with the formaldehyde-treated vaccine

Mice No.	HI titre	Challenge with the Hypr strain (LD_{50})	HI titre after challenge	
			1st week	2nd week
1	40	5.0	320	160
2	80	5.0	640	80
3	40	5.0	320	40
4	320	4.0	320	40
5	160	4.0	160	320
6	320	3.0	160	40
7	320	3.0	160	40
8	80	3.0	80	80
9	80	3.0	80	40

HI = Haemagglutination inhibiting
* three doses

The results also demonstrated the immunogenicity of the formaldehyde treated vaccine (Table 3). As can be seen, the formaldehyde treated vaccine was antigenic and had a protective activity.

To find out whether the Skalica strain circulates in the natural foci of TBE, we collected 101 blood samples from permanent inhabitants of Jarok village and examined these sera in HI test. The results obtained showed that 37% of inhabitants had antibodies to TBE virus. When comparing antibody levels to antigens of strains Skalica and Ir 13 of TBE virus, it was found that they were either similar or varied only within one dilution range (Table 4).

Discussion

TBE in Czechoslovakia represents a serious public health problem. The first preventive measure against TBE was the use of formalinized vaccine prepared from mouse brains (Smorodintsev *et al.*, 1941). Because of the possibility of allergic encephalitis, further experimental vaccines were prepared in CE cells with subsequent formalin inactivation (Benda and Daneš, 1961; 1962). The vaccine prepared in this way is being produced and used in the U.S.S.R. (Levkovich, 1962) and in Austria (Kunz, 1978) for immunization of humans. A live attenuated vaccine from the Langat strain (strain of TBE isolated in Malaysia) has been also prepared and recommended for human use (Mayer and Mitrová, 1977). For prevention of TBE epidemics caused by drinking of infected milk experimental vaccination of milk-producing animals was performed (Blaškovič *et al.*, 1962; Grešíková *et al.*, 1962; Mayer *et al.*, 1976).

We succeeded in isolation of a thermosensitive Skalica strain of TBE virus from nature (Grešíková *et al.*, 1976), and attempted the preparation of an experimental vaccine from this strain. The vaccine was shown to be harmless and immunogenic. It protected immunized mice against challenge with the Hypr strain virus inoculated on day 8 post-vaccination.

Table 4. Levels of HI antibodies to TBE virus in healthy population from a natural focus*

Positive serum** sample No.	HI titre against		Positive serum** sample No.	HI titre against	
	IR 13	Skalica		IR 13	Skalica
2	80	80	61	40	40
5	80	80	64	20	20
9	40	40	68	20	10
11	160	160	69	80	40
18	80	80	74	20	20
27	320	320	76	80	80
29	20	10	77	40	20
30	40	40	79	160	80
32	10	20	80	320	320
33	20	10	82	20	10
39	40	40	85	40	20
41	40	20	87	20	20
44	40	40	88	40	20
45	20	10	92	80	40
47	160	320	93	40	40
48	40	40	95	20	20
50	40	40	97	40	40
54	80	80			
57	10	10			
60	40	20			

HI = Haemagglutination inhibition

* Locality Jarok (an other locality than the Skalica virus was isolated).

** 37 positive out of total 101 samples.

A further problem which remained to be solved was the question whether the thermosensitive strain Skalica circulated in natural foci of TBE in our country. The antigenic relationships between strains Ir 13 and Skalica were examined in kinetic complement fixation reaction (Grešíková and Sekeyová, 1979). The results have shown that the Ir 13 strain reacted with convalescent human serum already when incubated for 1 hr, while Skalica strain antigen reacted only after 18 hr incubation. At this incubation interval the antigens and serum titres were higher with the Ir 13 strain. Therefore, HIT was used to examine the sera of healthy inhabitants of Jarok village (Nitra district) for the presence of HI antibodies to the antigen of the Skalica strain. It was found that sera of humans from the village Jarok contained HI antibodies to a newly isolated virulent TBE virus strain coming from Tribeč natural focus of TBE (Grešíková and Sekeyová, 1980) as well as to the Skalica strain.

We conclude, therefore, that immunization of humans with the Skalica strain-derived DEE-vaccine may be promising, if other requirements for the vaccine for immunization of humans will be fulfilled.

Acknowledgements. Author thank V. Bieliková and M. Parobková for excellent technical assistance.

References

Benda, R., and Daneš, L. (1961): Study of the possibility of preparing a vaccine against tick-borne encephalitis, using tissue culture methods. V. Experimental data for the evaluation of the efficiency of formol treated vaccines in laboratory animals. *Acta virol.* **5**, 37—49.

Benda, R., and Daneš, L. (1962): Evaluation of the immunogenic efficiency of tick-borne encephalitis virus vaccine, pp. 345—357. In H. Libíková (Ed.): *Biology of Viruses of the Tick-Borne Encephalitis Complex.* Academia, Czechoslovak Acad. Sci., Praha.

Blaškovič, D., Libíková, H., Ernek, E., Grešíková, M., Mačička, O., Vilček, J., Mayer, V., and Řeháček, J. (1962): Results of experimental vaccination of demestic animals (in Slovak), pp. 76—9ě. In D. Blaškovič (Ed.): *Význam cielenej imunizácie domácich zvierat pre prírodné ohnisko kliešťovej encefalitídy.* Biologické Práce SAV, Bratislava.

Grešíková, M., Málková, D., and Slonim, D. (1962): Vaccines used and their preparation, (in Slovak), pp. 76—93. In D. Blaškovič (Ed.): *Význam cielenej imunizácie domácich zvierat pre prírodné ohnisko kliešťovej encefalitídy.* Biologické Práce SAV, Bratislava.

Grešíková, M., Mrciak, M., Brtek, V., and Sekeyová, M. (1976): Isolation and identification of tick-borne encephalitis virus from a bank vole *(Clethrionomys glareolus)* trapped in the vicinity of Radimovsky forest (Western Slovakia), pp. 105—110. *International Arbeitskolloquium über Naturherde von Infektionskrankheiten in Zentraleuropa*, Graz 25. 2.—28. 2. 1976.

Grešíková, M., and Sekeyová, M. (1979): Some aspects of biological and antigenic properties of freshly isolated tick-borne encephalitis virus strains, pp. 150—161. In Ch. Kunz (Ed.): *Tick-Borne Encephalitis.* International Symposium Baden/Vienna, 19th—20th October 1979.

Grešíková, M., and Sekeyová, M. (1980): Characteristics of some tick-borne encephalitis virus strains isolated in Slovakia. *Acta virol.* **24**, 72—75.

Kožuch, O., Chunikhin, S. P., Grešíková, M., Nosek, J., Kurenkov, V. B., and Lysý, J. (1981): The ecological marker "Cg" of tick-borne encephalitis virus in different rodent species. *Acta virol.* **25**, 219—224.

Kunz, Ch. (1978): Aktive und passive Immunoprophylaxe der Frühsommer-Meningoenzephalitis (FSME). *Arzneim.-Frsch. (Drug Res.)* **28** (II), 1806.

Laemmli, U. K. (1970): Cleavage of structural proteins during the assembly of the head of bateriophage T4. *Nature (Lond.)* **226**, 680—685.

Levkovich, E. N. (1962): Experimental and epidemiological bases of the specific profylaxis of tick-borne encephalitis, pp. 317—330. In H. Libíková (Ed.): *Biology of Viruses of the Tick-borne Encephalitis Complex.* Czechoslovak Academy of Sciences, Prague.

Mayer, V., Ernek, E., Blaškovič, D., Kožuch, O., and Nosek, J. (1967): Study of the virulence of tick-borne encephalitis virus. VII. Immunogenicity of attenuated virus (clone Hy HK28 "2) for goats, cattle and sheep. *Acta virol.* **11**, 334—345.

Mayer, V., and Mitrová, E. (1977): Low-virulent mutants of flaviviruses. Basic biological studies and concepts. *Biologické Práce SAV* **6**, 23, 5—153.

Smorodintsev, A. A., Kagan, U. V., Levkovich, E. N., Dankovskii, N. L. (1941): Experimenteller und epidemiologischer Beitrag zur aktiven Immunisierung gegen die Frühling-Sommer-Zecken-Encephalitis. *Arch. ges. Virusforsch.* **3**, 1—25.

Explanations to Figures (Plates XXII—XXIII):

Fig. 1. PAGE of the DEE-treated Skalica strain vaccine
Lane I — Skalica strain vaccine
Lane II — TBE vaccine manufactured by Immuno-AG, Wien (Austria) used as positive control
Lane III — Non-infected cells (negative control)
Lane IV — Standards (M_r control)

Fig. 2. Electron micrographs of the Skalica vaccine. DEE-treated vaccine was negatively stained with 2% phosphotungstic acid (PTA) at pH 7.5 and examined at 80 kV in a Philips EM 300 electron microscope.