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PATHOGENICITY OF THE SKALICA STRAIN (FROM THE TICK-BORNE ENCEPHALITIS COMPLEX) FOR WHITE MICE

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Summary. — Outbred white mice of different age were inoculated by subcutaneous (s. c.) route with the Skalica strain of tick-borne encephalitis virus (TBE). In the CNS of 3-days-old mice diffuse necrotizing encephalitis, abundant cytoplasmic fluorescence of viral antigen in nearly each neuron and high levels of virus (8.8 log LD_{50}/mg tissue) were found. In 10-days-old mice, the extent of encephalitis and that of immunofluorescence in neurons were less widespread; the peak titre of the virus did not exceed 5.5 log LD_{50}/mg brain tissue. In the CNS of 21-days-old mice the infectivity titre was either very low (1.5 $\log_{50}$/mg on day 3 post infection) or the virus was not detected at all. A few neurons revealed positive fluorescence of viral antigen in the basal ganglia in 1 out of 2 mice examined by day 3 post infection (p. i.). No virus was isolated from the CNS of 2-months-old mice observed for 53 days. In the CNS of 3 out of 10 juvenile mice examined histologically within 8 days post infection, minimal inflammatory changes were seen; foci of neurons showing positive immunofluorescence were not found. The failure to recover infectious virus from cultured brain tissue fragments coming from these mice confirmed the negative outcome of direct virus isolations. It is concluded that the Skalica strain was not pathogenic for juvenile mice when administered by s. c. route.

Key words: *pathogenicity*; *immunofluorescence*; *organ culture*; *Skalica strain of tick-borne encephalitis virus*

Introduction

Variations in pathogenicity for mice of the viruses of tick-borne encephalitis (TBE) complex are of great practical interest. Pogodina and Savinov (1964) found that out of 18 strains tested, the lowest pathogenicity after peripheral inoculation displayed the Langat TP-21 strain. This strain isolated by Smith (1956) is so far the best characterized naturally occurring TBE strain not lethal for juvenile mice after peripheral administration. Thind and Price (1966) used it to prepare the low virulent strain TP 21 "E" by chick embryo

serial passages. The clone TP 21 "E 14" derived from this strain is apathogenic for young mice when given by subcutaneous (s.c.) route (Mayer and Mitrová, 1977). There is of interest, whether another TBE virus strains revealing similar properties exist in nature. According to our previous findings (Grešíková and Szekeyová, 1979, 1980) the Skalica strain from the TBE complex strongly differs from the prototype Hypr strain by its biological behaviour in mice, since it is not pathogenic for young mice after s.c. inoculation. These findings were extended in the present investigations.

Materials and Methods

Virus and animals. The Skalica strain belonging to the TBE complex was isolated from free living rodents (Grešíková et al., 1976). It was used in the 7th mouse passage (kept at −70° C). Outbred white mice at the age of 3, 10, 21 days and 2 months were infected by s. c. route with $10^{7.5}$ LD_{50} of the Skalica strain in 0.01 ml inoculum. By 1, 2, 4 and 6 days p. i., baby mice were succumbed; blood, spleen, liver, muscles, brain and spinal cord were sampled for virus titration and morphological examinations. Brains of 2 months old mice were investigated on days 3, 8 and 53 p. i.

Virus isolations. Direct isolations were performed from 10% organ suspensions (prepared with serum-free BEM) and from heparinized blood diluted 1 : 10 (2 U heparin per ml). Samples from brain cortex, basal ganglia, cerebellum and brain stem of the 2-months-old mice were minced in medium CMRL-1415 containing 10% fetal calf serum and cultured as described (Rajčáni *et al.*, 1975). The cultures were refed on day 4 and harvested on day 8 in culture. Fragments coming from the same area of the brain were collected and after grinding inoculated into newborn mice by intracerebral route.

Histology and immunofluorescence. The transversal blocks prepared from brains of 3, 10 and 21 days old mice succumbed on days 3 and 6 p. i. and the brain samples of 2-months-old mice autopsied on days 3, 8 and 53 p. i., were fixed in cold Carnoy solution and quickly embedded into paraffin (Albrecht *et al.*, 1966). The blocks were cut at room temperature; deparaffinized sections were alternatively stained with hematoxylin and erythrosin (H. E.) or by the indirect immunofluorescence (IF) technique. Guinea pig immune serum to TBE virus strain Hypr was used in dilution 1 : 8; the SwAGp conjugate was purchased from SEVAC, Prague and used in dilution 1 : 4 for staining.

Results

Isolation of the virus from organs and tissues

Outbred white mice of defined age groups were inoculated with $10^{7.5}$ LD_{50} of the Skalica strain by s. c. route. As shown in Table 1, the virus multiplied to high titres in the visceral organs, in muscles and in the CNS of 3-days-old mice. Relatively high levels of viraemia were detected from the 1st day p. i. (Table 1). As shown in Table 2, the virus multiplied also in peripheral tissue as well as in the CNS of 10-days-old mice, but the peak titres were lower as compared to those found in 3-days-old mice. Viraemia was detected only between days 1-4 p. i. and its levels were lower than in the previous age group. Extracellular replication of the Skalica strain was highly limited in 21-days-old mice (Table 3). The levels of virus in blood were low and its penetration to CNS was confirmed only at a single interval (day 3 p. i.). In 2-months-old mice infected by the s. c. route virus was not isolated from the CNS at either of intervals tested. Suspensions of brain fragments

Table 1. Multiplication of the Skalica strain in organs of 3-days-old mice

Days p. i.	Virus titre					
	Blood	Spleen	Liver	Muscles	Brain	Spinal cord
1	4.3*	2.5**	2.0	2.7	1.2	0.2
2	4.5	4.3	4.0	4.6	3.9	4.0
3	5.0	4.5	4.6	5.7	8.6	8.5
4	4.8	6.4	5.8	5.8	8.5	8.8
5	3.8	5.5	5.5	5.7	8.7	8.8
6	3.8	5.4	5.3	5.7	8.7	8.8

* Per μl of undiluted blood (3 mice per each interval)
** Per mg of tissue (3 mice per each interval)

kept in culture for 8 days were inoculated intracerebrally to baby mice. Nevertheless, attempts to reisolate virus from the suspensions of brain explants remained unsuccessful.

Morphological findings in mice infected by s. c. route

When sections from the brains of 3-days-old mice autopsied by day 3 p. i. were stained by indirect IF, the great majority of neurons showed positive IF in cerebellum (Purkyně cells), brain stem, brain cortex and striatum. On day 6, widespread fluorescence of the TBE antigen was seen in all areas of CNS and in each section examined. Due to advanced cytolysis and necrosis of neurons, the antigen was present mainly in debris of damaged neurons. Parallel sections stained with H. E. showed prominent oedema, cytolysis, eosinophilic necrosis of neurons accompanied by dilatation of vessels, polymorphonuclear and mononuclear infiltration. Such changes were diffuse, i. e. they were seen in cerebellum (Fig. 1), brain stem, basal ganglia and in brain cortex, especially in g. hippocampi.

In 10-days-old mice cytoplasmic fluorescence in neurons was found on day 6 p. i. Groups of neurons showed positive IF in g. hippocampi (Fig. 2 and 3),

Table 2. Multiplication of the Skalica strains in organs of 10-days-old mice

Days p. i.	Virus titre					
	Blood	Spleen	Liver	Muscle	Brain	Spinal cord
1	2.5	< 1	1	2.5	< 1	< 1
2	3.7	1.5	1.6	2.1	< 1	< 1
3	2.5	1.5	1.6	3.8	< 1	< 1
4	1.5	2.4	3.8	2.1	1.8	1.4
5	0	2.5	0	1.5	5.5	3.5
6	0	1.8	1	0	5.5	2.5

For explanations see Table 1.

Table 3. Multiplication of the Skalica strain in organs of 21-days-old white mice

Days p. i.	Virus titre					
	Blood	Spleen	Liver	Muscle	Brain	Spinal cord
1	2.5	2.0	1.5	1.5	0	0
2	1.5	1.8	1.8	0	0	0
3	1.5	1.0	1.5	0	1.5	1.0
4	1.5	1.0	0	0	0	0
5	1.5	0	0	0	0	0
6	0	0	0	0	0	0

For explanations see Table 1.

in the cerebellar cortex (Purkyně cells, Fig. 4), in the parietal cortex (Fig. 5), striatum, pallidum, thalamus and in the brain stem. Histological examination on day 3 p. i. showed mononuclear and polymorphonuclear infiltration in the vicinity of dilated vessels and in the meninges. On day 6 p. i., eosinophilic necrosis of neurons, oedema and cytolysis were prominent and were accompanied by inflammatory changes in all areas revealing the presence of TBE antigens.

In 21-days-old mice only patchy mononuclear infiltrates were seen occasionally in meninges. A few vessels in basal ganglia and brain cortex showed endothelial swelling and minimal mononuclear infiltration. Occasionally nodular infiltrates of mononuclear leukocytes were seen. On day 3 p. i. a few neurons in the striatum revealed positive fluorescence of TBE antigen (in a single section of 10 sections viewed from one of 2 mice examined on day 3 p. i.). All other areas of the brain of this particular animal including the cerebellar cortex were free of antigen.

No viral antigen was found in the CNS of subadult mice at any of given intervals. In the CNS of 3 out of 10 mice examined on days 3 and 8 p. i., minimal histological changes were found characterized by scarce round cell meningeal infiltration and perivascular cuffings (Fig. 6, 8). Occasionally moderate swelling of the capillary endothelium along with scarce cuffings were found (Fig. 7). Focal nodular pleomorphic infiltrates were also seen. Such scattered changes were present in the nc. amygdalae corpus callosum, striatum and parietal cortex.

Discussion

In the course of extensive field studies, the Skalica strain of TBE virus was isolated from organs of *Clethrionomys glareolus* (Grešíková *et al.*, 1976). It was shown that this strain was not pathogenic for adults and subadult mice when administered by s. c. route (Grešíková and Sekeyová, 1980) The Skalica strain dipslayed the following characteristic markers: ic^+, sc^-, t^- and Cg^-. In this respect it behaved similarly to certain "attenuated" Hypr strains (Mayer, 1966) and to the "attenuated" Langat strain "E 15 14" prepared by serial passages in chick embryo cells (Mayer, 1974). The latter

replicates to a different extent in extraneural tissues of suckling mice after s. c. administration (Mayer *et al.*, 1974). Nevertheless, the peak titres of low virulent strains in CNS of baby mice infected by s. c. route in general were lower as compared to the virulent ones. Thus the pathogenicity for baby mouse of the naturally occuring low virulent Skalica strain is intermediate between the behaviour of the arteficially prepared low virulent Langat strain on one hand and the virulent Hypr strain on the other. The low virulence of the Skalica strain did change neither during its passaging in mice (7 passages) nor by storage at −70°C for 5 years.

The age dependency of the sc marker seems to be a complex phenomenon. The replication of less virulent strains in nonneural tissues is more limited (Albrecht, 1968) and also their clearance by macrophages is more active (Jahrling *et al.*, 1977). As shown with the vaccination strain 17D of yellow fever virus, the survival time and the age dependance characteristics are indicators for the balance between the events at different pathways of virus spread and the host defence (Fitzgeorge and Bradish, 1980). Several flavivirus strains (Semliki forest virus, Venezuelan equine encephalomyelitis virus and the 17D-yellow fever virus) may be differentiated by criteria like infectious dose, lethal dose, and protection dose which all represent age dependent host functions (Bradish and Fitzgeorge, 1977). Table 2 shows clearly that viraemia and nonneural replication of the Skalica strain decreased at the time of increased replication of the virus in the CNS. The age between 10-14 days may be critical for the maturation of the macrophage clearance function, which, of course, is not acting within the CNS tissues itself.

The failure to isolate any infectious virus from the CNS of 2-months-old mice infected by s. c. route with the Skalica strain is similar to the results found with the low virulent Langat TP-21 "E 14" clone (Mayer and Mitrová, 1977; Rajčáni *et al.*, 1982). The significance of the minimal histological findings in the absence of any virus replication in neurons and glial cells is unclear and deserves further investigation.

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Explanation of Micrographs (Plates XXXIV—XXXV):

Fig. 1. Cerebellar cortex of 3-days-old baby mouse 6 days p. i. by s. c. route. Widespread oedema cytolysis, disintegration of Purkyně cells. H. E., × 100.

Fig. 2. Specific fluorescence of TBE antigens in neurons of g. hippocampi. Brain cortex of 10-days-old mouse 6 dyays p. i. by s. c. route. Indirect method, × 140.

Fig. 3. Detail from Fig. 2, × 460.

Fig. 4. Purkyně cells in the cerebellar cortex showing positive fluorescence in cytoplasm and in axoplasm of neurites. Mouse 10 days of age, 6 days p. i. by s. c. route, × 400.

Fig. 5. Pyramidal neurons in the brain cortex of 10-days-old mouse 6 days p. i. with the Skalica strain by s. c. route; × 420.

Fig. 6. Mouse 2-months-old infected with the Skalica strain by s. c. route, 8 days p. i. Two venules in the striatum show cuffing by round cells. Minimal focal nodular infiltrates in the vicinity of capillaries consist of mononuclears. H. E., × 180.

Fig. 7. The same mouse; endothelial swelling, focal perivascular round cell infiltration in the brain cortex. H. E., × 400.

Fig. 8. Focal patchy infiltrate in meninges of the brain cortex. Single nodular infiltrate of mononuclear cells in the molecular zone. H. E., × 120.