

EXPERIMENTAL PATHOGENICITY OF TRIBEČ ARBOVIRUS FOR MICE

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Summary. — The pathogenicity of Tribeč virus for mice was studied by infectivity titrations, immunofluorescence and histological examination. After intracerebral (ic) inoculation of virus to suckling mice, infection appeared to start at the site of inoculation; the virus multiplied in the glial cells and neurons and reached high levels in the brain. Small amounts of virus were demonstrated in the blood and visceral organs on the 2nd day after inoculation. After ic inoculation of adult mice the virus persisted only in the brains in low titres. The fluorescent antibody (FA) technique revealed no viral antigen in neurons. A lymphocytic meningitis was detected histologically, while the animals showed no signs of clinical illness. After subcutaneous (sc) infection of suckling mice, the virus was detectable at first in the subcutaneous tissues. Thereafter it appeared in the blood and brain. The FA technique failed to detect both the primary site of viral multiplication and the invasion of the central nervous system (CNS).

Introduction

The Tribeč arbovirus was first isolated from *Ixodes ricinus* ticks in the Tribeč region in Czechoslovakia (Grešíková *et al.*, 1965). The virus produced fatal encephalitis in suckling mice and a mild meningitis in *Macaca rhesus* monkeys (Grešíková *et al.*, 1966). It is cytopathic for chick embryo cell cultures (Rajčáni and Grešíková, 1967) and immunologically related to the Kemerovo virus group (Libíková *et al.*, 1964). In the present study we investigated in detail its pathogenicity for suckling and adult mice by infectivity titrations and histological and immunofluorescence methods.

Material and Methods

Virus. The strain of Tribeč virus used has been maintained by ic inoculation in 2 days old Swiss albino mice. The brains were harvested when the infected animals showed signs of encephalitis. A 10% brain suspension was prepared in medium 199 containing 5% foetal calf serum and lyophilized after centrifugation at 2000 rev/min for 20 minutes. The titre of stock virus determined in suckling mice was $10^{8.5}$ LD₅₀/0.01 ml.

Experimental animals. Suckling mice 4—5 days old were infected ic with 0.01 ml or sc with 0.1 ml of undiluted stock virus suspension. Adult mice weighing 6—8 g were infected ic with 0.03 ml of the same material. Organ samples for infectivity titrations and FA and histological examinations were collected daily for 10 days or until the animals died. Two mice were used at each interval.

Virus assay. The brains, livers, spleens and other tissues were homogenized in medium 199 containing 5% calf serum and 1000 units of penicillin and 1000 μ g of streptomycin per ml. After centrifugation, the supernatants were diluted in 10-fold steps and inoculated ic into 2–5 days old Swiss albino mice. The LD₅₀ values were calculated according to Reed and Muench.

FA technique. The brains from suckling and adult mice were fixed in Carnoy's solution at 4° C for 3–4 hours, passed through chloroform overnight at 4° C and embedded into paraffin according to the procedure described by Albrecht *et al.* (1966). All fluids used were previously absorbed with charcoal to eliminate nonspecific fluorescence. The sections cut at room temperature were dried for 30 minutes at 37° C and after the removal of paraffin by xylene and passage through an ethanol series stained by the indirect method.

Hyperimmune sera against Tribeč virus were prepared in mice and rabbits; the neutralization index of serum, calculated by constant virus-serum dilution method was 10⁶. The sera diluted 1 : 4 and the conjugates (Anti-mouse and Anti-rabbit, obtained in swine, produced by the Institute of Sera and Vaccines, Prague), were absorbed with 20% control mouse brain suspension before use. Nonspecific staining properties of the conjugates were checked by staining parallel sections with the conjugates only.

The carcasses of the suckling mice (adult mice were not examined) were quickly frozen in liquid propane-butane, cut in a cryostat in horizontal planes to obtain representative sections through the organs of the thoracic and abdominal cavities. The same sera and conjugates as mentioned above were absorbed with a 20% suspension of control carcasses and the indirect method of staining was performed after acetone fixation. The preparations were mounted into neutral glycerol and examined with a Reichert's "Zetopan mit Binolux" microscope.

Histological methods. The bodies of suckling mice and brains from adult mice were cut in 3–4 mm thick sections, fixed in Bouin's solution at least for 24 hours and embedded into paraffin. The sections were stained with haematoxylin-erythrosin. From the brains, semiserial sections were prepared at 300 μ -distance (8–10 from a block).

Results

Intracerebral inoculation

Virus multiplication. The signs of encephalitis and death occurred in suckling mice on the 4th day after inoculation (p.i.). The virus, however, was detectable in their brains beginning from the first day p.i., and reached the highest titres 2, 3 and 4 days p.i. (Table 1). On the 2nd day, the virus was also found in the blood, spleen and liver.

Table 1. Intracerebral infection of suckling mice with Tribeč virus

Material examined	Virus titres (log LD ₅₀ /0.01 ml) on indicated days p. i.			
	1	2	3	4
Blood	0	1.5	0	0
Spleen	0	4.0	0	0
Liver	0	3.0	0	0
Brain	2.6*	6.5**	7.0**	6.5**

* = Specific fluorescence in some glial cells and mononuclears.

** = Specific fluorescence in numerous neurons and glial cells.

0 = No virus detected.

After ic inoculation into adult mice, no signs of illness were observed. The virus appeared first in the brain, persisting there for a 10-day period. No virus was found in the blood or visceral organs. The highest virus titre

in the brains of adult mice was detected on the first day p.i., ($10^{5.5}$ LD₅₀/0.01 ml); the titre subsequently decreased: on days 2, 3, 5, 6 and 10 it reached values of 2.5, 3.4, 4.3, 2.7 and 3.8 log LD₅₀/0.01 ml, respectively. On day 7, no virus was demonstrated in the brains.

Histology and immunofluorescence. The histological changes in suckling mice resembled those described previously (Grešíková *et al.*, 1966). Widespread neuronal damage and abundant polymorphonuclear infiltration were found especially in the cornu ammonis (Fig. 1).

Bright specific fluorescence of viral antigen was found in the same parts of CNS, where the histological changes developed, namely in the cortex (Fig. 2), in the caudate nucleus (Fig. 3) and putamen, in the thalamus and hypothalamic nuclei, and in the brain stem on the bottom of the IVth ventricle. In the cornu ammonis nearly all neurons contained virus and the majority of them showed severe damage or necrosis (Fig. 4). In the cerebellar cortex the specific fluorescence of viral antigen was found only in a few Purkyně cells. The majority of them showed neither specific fluorescence nor cytological changes when stained with haematoxylin-erythrosin. The involvement of the CNS was extensive between the 2nd and 4th day p.i. On the 1st day only a few glial cells showed the specific fluorescence around the needle track.

No fluorescence was detected in the visceral organs of ic infected suckling mice. The histological examination of this material also yielded negative results.

In adult mice, lymphocytic meningitis was found from the 1st through the 6th day p.i. The meningeal infiltrate consisted predominantly of lymphocytes and macrophages. Near the site of inoculation, haemorrhage, tissue necrosis, oedema, polymorphonuclear leucocyte exudation and later microglial proliferation were observed. In the white matter and cortex of the hemispheres, a few cuffs of lymphocytes and macrophages were found between the 4th—6th day p.i., but no neuronal damage could be detected (Fig. 6).

The histological changes corresponded closely to areas in sections stained by the FA technique, namely near the site of inoculation (thalamic nuclei or striatum) and below the ependymal layer in the walls of the lateral ventricles where a few mononuclear and glial cells were found to contain viral

Table 2. Subcutaneous infection of suckling mice with Tribeč virus

Material examined	Virus titres (log LD ₅₀ /0.01 ml) at indicated intervals p. i.				
	15 hr	1 day	2 days	3 days	4 days
Skin	1	0	0	0	0
Blood	0	2.6	0	0	0
Spleen	0	0	0	0	0
Liver	0	0	0	0	0
Brain	0	0	3.0	0	0

0 = No virus detected.

antigen. The slight specific fluorescence in these cells was located in the cytoplasm in the perinuclear area. No fluorescence in the neurons could be demonstrated (Fig. 5). The visceral organs of adult mice were not examined.

Subcutaneous inoculation

The infected animals showed no signs of clinical illness. The virus was first detected in the skin at the site of inoculation, 15 hours p.i. Soon after its appearance here, the virus was found in the blood. In the brains it was demonstrated 48 hours p.i. As shown in Table 2, the infectivity titres were

Table 3. Comparison of the pathogenicity of Tribeč virus for suckling and adult mice after intracerebral inoculation

Finding	Age of animals	
	Suckling	Adult
Clinical signs	Death in 4 days	Survival
Histological diagnosis	Encephalomeningitis	Meningitis
Cells forming the infiltrate	Neutrophils	Mononuclears
Neuronal damage	Abundant	None
Immunofluorescence	Positive in numerous neurons and glial cells	In some glial and mononuclear cells
Virus in the brain	Increases to high levels	Slowly decreases and persists at low levels

lower than those after ic infection. The FA studies in this group of animals did not yield any positive results. Fluorescence in some cells of subcutaneous and interscapular adipose tissues should be regarded as nonspecific in view of similar findings with heterologous conjugates. No inflammatory changes developed at the site of injection as revealed by histological examination.

The striking differences between the reaction of suckling and adult mice after ic inoculation with Tribeč virus are summarized in Table 3.

Discussion

After ic infection of suckling mice, Tribeč virus multiplies in glial cells and numerous neurons. During the first 48 hours, the virus reaches in the brain high levels and a picture of fatal encephalitis with widespread neuronal damage develops. Similar changes were demonstrated by histological examination of lesions induced by Kemerovo virus (Libíková *et al.*, 1965). On ic inoculation into adult mice, viral antigen was not visible in neurons of the CNS. The finding of specific fluorescence in some mononuclear and glial cells in the walls of the lateral ventricles indicates that the cerebrospinal fluid may be the path of virus spread. Studies on pathogenicity of Tribeč virus in monkeys confirmed that, after ic inoculation, the virus appeared in the cerebrospinal fluid only.

The differences in susceptibility of suckling and adult mice could probably be explained by increased resistance of neurons to infection with increasing age.

Another possibility is that the macrophages prevailing in the inflammatory exudate of adult mice may serve as a barrier in the spread of Tribeč virus to susceptible cells.

After sc inoculation, the virus was detectable in the subcutaneous tissue and subsequently in the blood and brain. The titre of virus was low and the failure to detect viral antigen in the sections was most likely due to low virus content in the tissues examined.

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Explanation of Micrographs:

- Fig. 1.* The cerebral cortex of a suckling mice infected ic with Tribeč virus. Necrosis and cytolysis of numerous neurons 48 hours p.i. Haematoxylin-erythrosin, $\times 35$.
- Figs. 2—4.* Bright specific fluorescence of Tribeč virus antigen in neurons and glial cells of the cortex (2), n.c. caudatus (3) and cornu ammonis (4) of suckling mice on the 3rd day p.i. Indirect FA method, paraffin-embedded material.
- Fig. 5.* Slight specific fluorescence in some glial and mononuclear cells in the wall of the brain ventricle. Adult mouse, 24 hours p.i.
- Fig. 6.* The cerebral cortex of an adult mouse on the 4th day after ic infection with Tribeč virus. Perivascular lymphocytic infiltration and slight microglial proliferation. No neuronal damage. Haematoxylin-erythrosin, $\times 300$.