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PURIFICATION AND MORPHOLOGY OF TRIBEČ ARBOVIRUS

M. GREŠÍKOVÁ, E. MRENA, A. VACHÁLKOVÁ

Institute of Virology, Czechoslovak Academy of Sciences, Bratislava

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Summary. — Tribeč virus was purified from infected suckling mouse brain suspension by differential centrifugation. The morphology of Tribeč virus was examined in the electron microscope by the negative staining technique. The average particle size was 70—75 nm. The particles were roughly hexagonal; the surface of the particles was not damaged. The structure of Tribeč virus was altered by differential centrifugation and by streptomycin or polymyxin sulphate treatment. Even after sucrose density gradient centrifugation of a clarified suspension, great differences in virion structure have been found under the electron microscope. Tribeč virus was shown to possess a shape, size and structure characteristic of arboviruses.

Introduction

Tribeč virus has been characterized as an RNA-containing virus (Žemla *et al.*, 1968), belonging to the Kemerovo group of arboviruses (Casals, unpublished data). This virus shares some characteristics with other better known arboviruses; it is sensitive to ether and sodium deosycholate (Grešíková and Vachálková, to be published). Further attempts at its classification will have to await more detailed morphological studies. In the present work the negative staining technique was used to determine the morphology of Tribeč virus.

Materials and Methods

Preparation of virus suspension. The strain of Tribeč virus isolated from *Ixodes ricinus* tick (Grešíková *et al.*, 1965) was used. Brains were removed from infected suckling mice before death and frozen at —60° C. Frozen infected brains were homogenized in phosphate buffered saline (PBS) pH 7.2.

Streptomycin and polymyxin sulphate treatment. A 20% mouse brain suspension was centrifuged at 13,000 × g for 60 minutes. One tenth volume of streptomycin sulphate was added to the supernate to a final concentration of 1%. After keeping it for 15 minutes in the cold, the precipitate was removed by centrifugation at 13,000 × g for 30 minutes. The same procedures were carried out with 0.1% of polymyxin sulphate.

Centrifugation. The supernatants were centrifuged at 75,000 × g for 120 minutes. The pellets were carefully resuspended in PBS pH 7.2, containing 0.2% bovine albumin, homogenized and then centrifuged at 8000 × g for 30 minutes. Two such cycles of ultracentrifugation were carried out.

Cell cultures. Monolayer chick embryo cell cultures were infected with Tribeč virus at an input multiplicity of 1000 LD_{50} per cell. After 2 hours of adsorption at 37° C, the fluid with nonadsorbed virus was removed and fresh medium 199 was added. Inoculated bottle cultures were held at 37° C for 24 hours; at this time complete destruction of the cultures was observed.

The culture fluid was collected and centrifuged for 15 minutes at 2000 rev/min. The virus was concentrated by vacuum dialysis for 24 hours at 4° C.

Sucrose density gradient centrifugation was performed with both materials, mouse brain suspension and concentrated tissue culture fluid. Two tenths ml of the material was layered on the top of the gradient made by mixing 5 and 60% sucrose, dissolved in PBS, pH 7.2. Centrifugation was carried out in the SW 39 rotor of the Spinco L centrifuge at 30,000 rev/min for 2 hours. Fractions of 5 drops each were collected; each fraction was assayed for virus infectivity, complement-fixing (CF) activity and optical density. The density of each fraction was determined by weighing 100 μl in a calibrated micropipette.

Titration of infectivity and CF activity. Infectivity was assayed by intracerebral inoculation of suckling mice with serial 10-fold dilutions of each fraction. The LD_{50} values were calculated using the Reed and Muench formula. The CF antigen was titrated by the methods of Okuno *et al.* (1958).

Electron microscopy. All materials were prepared on carbon coated perforated formvar grids and stained with 2% phosphotungstic acid adjusted to pH 7.2 (Brenner and Horne, 1959). Micrographs were taken by a JEM 6 C electron microscope at an instrumental magnification of × 50,000

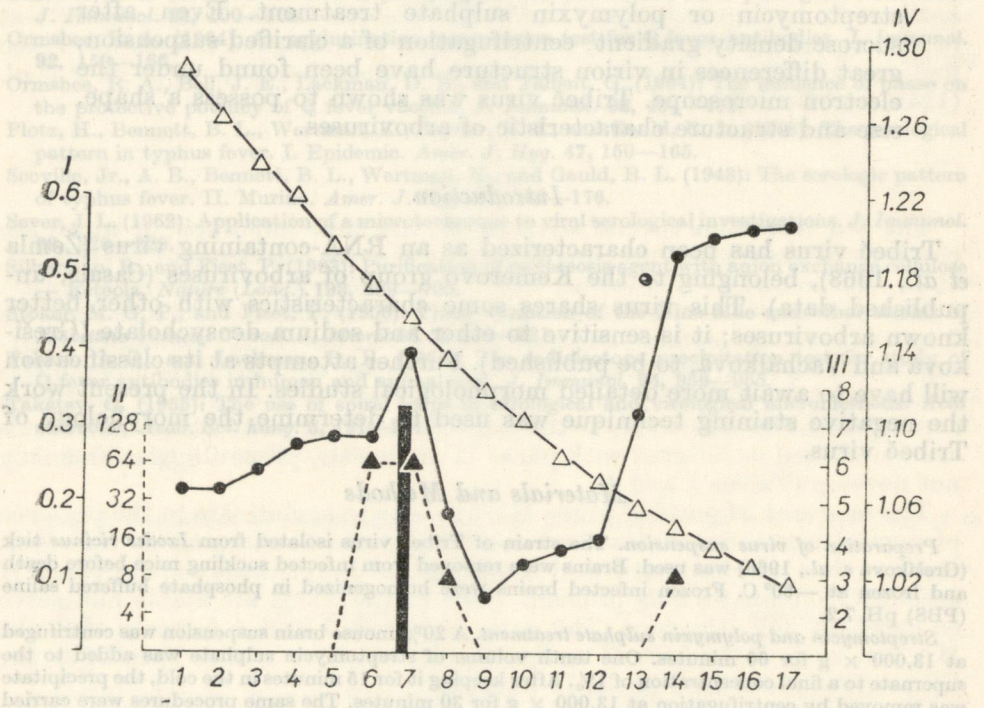

Fig. 3.

Distribution of infectivity and CF activity of Tribeč virus after centrifugation in sucrose density gradient.

Abscissa: fraction number
Ordinate I: absorbance at 260 nm (●)
Ordinate II: CF activity (▲)
Ordinate III: infectivity in log LD_{50}/0.01 ml values (black column)
Ordinate IV: density (△)

Results

Tribeč virus was purified from mouse brains by differential and sucrose density gradient centrifugation. All steps of the purification procedure were controlled by electron microscopy.

In the preparations made from virus-containing brain suspensions clarified at 10 000 rev/min for 60 minutes, a moderate number of viral particles 70 to 75 nm in diameter were found (Fig. 1).

Proteins contained in brain homogenates were partially removed by addition of streptomycin sulphate or polymyxin sulphate. No significant loss of infectivity after streptomycin sulphate was observed but the virion structure was altered as indicated by electron microscopy. Fig. 2 shows the shape and structure of Tribeč virus after polymyxin sulphate treatment and differential centrifugation. The figure suggests that a complete viral particle might be damaged. Great lability and fragility during the concentration procedure were the properties of Tribeč virus.

Since artificial disruption of viral particles after differential centrifugation was observed, clarified suspension or concentrated tissue culture fluid was subjected to sucrose density gradient centrifugation. Fig. 3 illustrates the distribution patterns of O.D. at 260 nm, infectivity and CF activity of Tribeč virus after the centrifugation of concentrated tissue culture fluid. The infectious particles were found in the first peak with the density of 1.16 g/ml. The CF activity was evenly distributed with some tendency to accumulate in the fraction of highest infectivity. No viral particles could be demonstrated under the electron microscope. In the material from infected suckling mouse brains after homogenization, clarification, and sucrose density gradient centrifugation, viral particles were observed, but maarked differences in virion structure as compared with original material were found. The average particle size was 64 $\pm$ 4 nm; on the surface of the particles spiky projections were visible (Fig. 4). The tendency of the virus particles to accumulate in clumps was observed under the electron microscope. A slight pleomorphism was seen, but the majority of the particles were roughly spherical.

Discussion

In the present study we tried to use a purification schedule commonly used for example with Japanese (Nozima *et al.*, 1964) and tick-borne (Slávik *et al.*, 1967) encephalitis viruses. It was shown that repeated differential centrifugation and especially the treatment with polymyxin and streptomycin, resulted in revealing new and probably artificial structures. This fact led us to search for intact particles in samples of original material after homogenization and clarification at low speed centrifugation. We rarely but significantly demonstrated intact particles in infected material which never occurred in uninfected preparations. These particles were very distinct in our samples, appearing in electron dense islands with the typical regular hexagonal shape and clearly visible substructure. Measurement of their size revealed very close values of 70 $\pm$ 1 nm for the distance between

opposite sides of the hexagon and 75 ± 1 nm in perpendicular direction. Rarely we also found empty capsids of the same dimensions.

After gradient centrifugation we found serious alterations in virion structure. The virions showed an "empty" core having 49 ± 3 nm in diameter. The outer zone revealed irregular structures and sometimes simulated spiky projections well known in other viruses. The extimated total average diameter of these virions was 64 ± 4 nm. The particles showed no sign of a hexagonal shape. In many cases we observed also damaged particles.

From the present results it appears that the intact particles of Tribeč virus could be demonstrated only in original clarified preparations. Chemical treatment with polymyxin or with streptomycin altered the substructure of the virions. Sucrose density gradient centrifugation revealed particles with smaller diameter, and probably artificial spiky projections sometimes suggesting the presence of "secondary coat" or structure which may also be interpreted as a viral membrane.

It may be concluded that virions of Tribeč virus are extremely sensitive to physico-chemical treatment similarly to other arboviruses, and that its morphology can hardly be derived from treated preparations studied by electron microscopy.

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Explanations of Electron Micrographs:

Fig. 1. Virions of Tribeč virus in clarified 10% mouse brain suspension.

Fig. 2. Virions of Tribeč virus after polymyxin sulphate treatment and differential centrifugation.

Fig. 4. Particles of Tribeč virus subjected to sucrose density gradient centrifugation.

Length of scale: 100 nm.