LETTER TO THE EDITOR

THE SUPPOSEDLY ATTENUATED HY-HK VARIANT OF HIGHLY VIRULENT HYPR STRAIN OF TICK-BORNE ENCEPHALITIS VIRUS IS OBVIOUSLY A STRAIN OF LANGAT VIRUS

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The pathogenesis of the disease caused by Tick-borne encephalitis virus (TBEV; the species *Tick-borne encephalitis virus*, the genus *Flavivirus*, the family *Flaviviridae*) (*1*) has been intensively studied in the 60s and 70s of the last century. Many of the classical studies were based on experimental attenuation of the virus and subsequent characterization of biological properties of the attenuated virus under *in vitro* as well as *in vivo* conditions.

In 1966 Mayer has reported a successful attenuation of the highly virulent strain Hypr of TBEV (*2*). The attenuated variant, designated Hy-HK, was obtained by passaging of the parental strain in cultures of a line of human amnion cells for more than 400 days, then in cultures in primary cultures of hamster kidney cells (HK cells; 28 passages), and finally in mouse brains (2 passages). Hy-HK formed small plaques in chick embryo cell cultures. In suckling mice, it did not cause a disease after subcutaneous (s.c.) administration, but it led to the development of encephalitis and death after intracerebral (i.c.) inoculation (*2*). More interestingly, Hy-HK exhibited strongly attenuated phenotype in *Macaca mullata* monkeys; intrathalamical inoculation of Hy-HK did not cause any obvious symptoms of disease for at least 48 days post infection (*2*).

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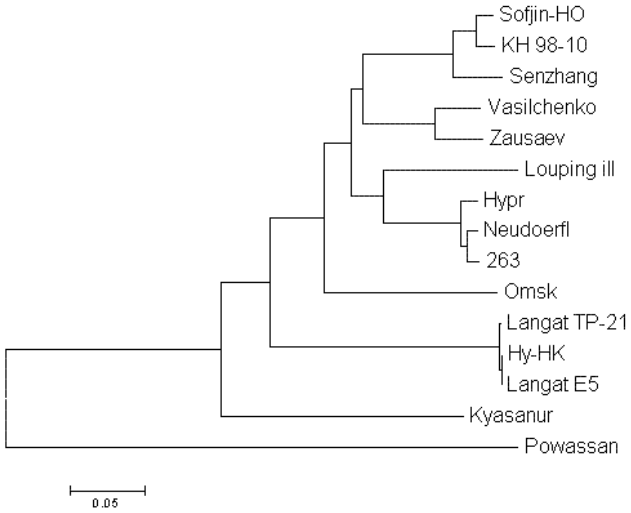
Abbreviations: i.c. = intracerebral; s.c. = subcutaneous; TBE = tick-borne encephalitis; TBEV = TBE virus

It has been believed that the comparison of this attenuated variant and the parental strain should bring important data about the biology of TBEV and the pathogenesis of tick-borne encephalitis (TBE). Therefore, Hy-HK represented an excellent tool for further research in this field.

Indeed, a number of studies have been done with Hy-HK using various laboratory animals (monkeys, sheep, and pigs) (*2–10*). All of the published results indicated that the Hy-HK could serve as an excellent live TBEV vaccine thanks to high level of attenuation, phenotypic stability and ability to induce protective antibodies. The vaccination at least of goats with Hy-HK with the aim to decrease the risk of TBE infection of men by alimentary route has been proposed (*2*).

Recently, the TBEV research has been focused mainly on the identification of mutations in different parts of viral genome responsible for changes of virulence. In this view, Hy-HK represented a useful tool because the comparison of a selected part of its genome sequence with that of virulent parental strain could lead to the identification of the changes responsible for the decrease of virulence. Of the entire genome we chose the gene encoding the envelope (E) protein because it represented an important antigenic determinant of the virus, was responsible for the host-cell interaction, and was considered the main determinant of viral virulence (*10*). Besides, it was possible to differentiate the parental strain from the attenuated variant using monoclonal antibodies against the E protein (*11*). We decided to compare



the E protein sequence of Hy-HK variant not only with that of the Hypr strain (both belonging to TBEV) but also with those of other flaviviruses available in GenBank.

We worked with a low pasage of Hy-HK in HK cells that we had obtained from Dr. V. Mayer, Institute of Virology, Slovak Academy of Sciences, Bratislava, Slovak Republic. Total RNA was isolated from 20% mouse brain suspension using the Qiagen QIAamp Viral RNA Kit (Qiagen). Subsequent synthesis of cDNA was performed using the RevertAid H Minus First Strand cDNA Synthesis Kit (Fermentas). Using PCR, two overlapping fragments representing whole E gene were produced. They were sequenced by means of the CEQ™ Dye Terminator Cycle Sequencing Kit and the automated Beckman CEQ 2000 DNA Analysis System (both from Beckman Coulter). The obtained nucleotide and deduced amino acid sequences were deposited in GenBank under Acc. No. DQ845244.

The phylogenetic tree based on deduced amino acid sequences of the E protein (see the figure) demonstrated that Hy-HK was most closely related to the Langat virus strain E5, a bit less related to the Langat virus strain TP-21, and unrelated to the Hypr strain of TBEV. This result was rather surprising because it meant that Hy-HK was not at all a strain/variant of TBEV, but a strain of Langat virus. It is probable that Hy-HK was a result of laboratory contamination of cell cultures with Langat virus rather than a product of attenuation of the parental virulent strain Hypr of TBEV. It is improbable, that the Hy-HK examined in this study was not identical with the originally derived Hy-HK (*2*) and investigated in later studies *(3–10)* because it had undergone only a few passages in our laboratory.

Langat virus (the species *Langat* virus, the genus *Flavivirus*) belongs together with TBEV to the group of tick-borne flaviviruses (*1)*. It was originally isolated from pools of *Ixodes granulatus* and *Haemaphysalis* spp. ticks in Malaysia and Thailand (*12*). Langat virus is completely non-virulent for adult mice after s.c. or intraperitoneal administration and for primates after i.c. inoculation. However, i.c. inoculation of the virus to mice leads to the development of encephalitis. More interestingly, Hy-HK carries a specific amino acid substitution, Asn389Asp that is located on the lateral surface of the domain III of the E protein, similarly to strain Langat E5 (*13*). This substitution is believed to be a molecular determinant of attenuation of TBEV and Murray Valley encephalitis virus (*14*). In describing the attenuation of the Hypr strain, Mayer (*2*) quoted as especially interesting that the attenuated Hy-HK variant showed a combination of phenotypic markers similar to that of the naturally attenuated strain TP-21 of Langat virus (*2*). Unfortunately, no appropriate method that would be able to discriminate between so closely related viruses was available that time.

In conclusion, our study showed that some of the classical studies (*2–9*) focused on the pathogenesis of TBE brought mistaken results because they were based on the experiments with Langat virus but not with TBEV. Consequently, now it is time to reexamine the general features of TBE pathogenesis, optimally by applying modern techniques of molecular biology. To emphasize the assignment of the Hy-HK variant to Langat virus we propose to use for it a new name, e.g. Langat Hy-HK.

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