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STUDIES ON THE VIRULENCE OF TICK-BORNE ENCEPHALITIS VIRUS
X. CHARACTER OF ATTENUATED Hy-HK28"2" VIRUS DURING PROPAGATION IN MONKEY BRAIN OR *IXODES RICINUS* TICKS

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Summary. — The character of 5 biological and physicochemical genetic markers of the highly attenuated clone Hy-HK28"2" of the Western subtype of tick-borne encephalitis (WTE) virus remained unchanged after multiplication of the virus in monkey thalamic and cerebellar tissue in vivo or after two passages in *Ixodes ricinus* ticks, throughout their interstadial development.

The ease or a certain readiness of a virus attenuated for certain hosts to increase the virulence may be considered as one of the main criteria measuring the degree of its attenuation. There are known several examples of the selection and mutation pressures exerted by the internal environment of the host, resulting in the changed character of the attenuated virus progeny. E.g., the attenuated poliovirus (types 1, 2 and 3) is known to restore a certain degree of monkey neurovirulence during passaging in the human gastrointestinal tract (Benyesh-Melnick and Melnick, 1959). Similarly, an increase in virulence for mice and change of the temperature *(T)* marker of the attenuated OCT-541 Japanese encephalitis virus strain in the course of its propagation in the central nervous system (CNS) of intracerebrally (ic) inoculated *Macaca mulatta* monkeys was described (Hammon *et al.*, 1963).

During previous work on WTE virus attention was mainly paid to the question of the stability of its certain individual characteristics (for references see Mayer *et al.*, 1967*b*; Mayer and Rajčáni, 1968).

The present material has been obtained with the aim to elucidate the question of the possible changes in the phenotypic expression of the highly attenuated Hy-HK28"2" virus after propagation under extremely different in vivo conditions. A set of 5 defined markers was investigated.

On this model virus, less virulent than any WTE virus observed hitherto, we

a) assayed its genetic stability during long-term contact with the monkey CNS ganglion cells, and

b) attempted to find out whether or not a reversion of the character of the markers investigated does take place in the tick vector.

The results of previous experiments revealed that the Hy-HK28"2" virus (ic passed in subadult mice) persists in the brains of *M. mulatta* monkeys

up to the 14th-21st day after intrathalamic administration, multiplying to low titres without causing clinical symptoms (Mayer and Rajčáni, 1967). This circumstance was extremely favourable for investigating the maintenance of the attenuated character of the virus, as the virus, deposited at the site of the inoculation lesion, was forced to multiply during this relatively long period of time under selection pressure exerted by the nerve cell environment.

A young male *M. mulatta* monkey was inoculated by the intrathalamic route with 10^6 mouse ic LD_{50}/0.1 ml of the Hy-HK28"2" virus clone. The virus had a clear *ic*$^+$ *sc s t e u*^{s} character (Mayer, 1966). After two weeks, during which no clinical symptoms were observed, the animal was killed by exsanguination, and its brain removed. The virus content in specimens, taken from different parts of the brain, was estimated in ic inoculated mice. In the motoric brain cortex, c. striatum and in the brain stem, only traces of virus were found. In the excisions from the central thalamic region and from the cerebellum, the virus titres were as high as 2.0 and 2.5 log LD_{50}/ml of 10% suspension, respectively. The virus used in the present experiments was then reisolated from specimens taken from the c. lenticulostriatum and from the cerebellar cortex (excision reaching up to the margins of n. dentatus).

Both isolations were performed by ic inoculation of suckling mice with the medium from chick embryo cell (CEC) tube cultures, which had been inoculated with either of the materials and then incubated for 3 days at 36° C. As already described, the propagation in CEC cultures and suckling mice, or both, represent advantageous conditions for the multiplication of potentially present virus particles with characters typically found in virulent TE virus strains (Price *et al.*, 1963; Mayer, 1964). The characters of the following markers were then studied (for methods see Mayer, 1966) in both isolates: virulence for ic or subcutaneously inoculated subadult mice, the plaque diameter in CEC cultures, the degree of thermostability at 50° C and the degree of sensitivity against the effect of 2 M urea, which was measured by the changes in infectivity titres of the test materials.

The virus isolated from both brain specimens had clearly the *ic*$^+$ *sc s t u*^{s} character. It should be stressed that, although the log ic LD_{50}/ml value of the 10% mouse brain suspensions was 8.2—8.7, we observed a complete avirulence for mice after subcutaneous administration.

Subcutaneous administration of the attenuated Hy-HK28"2" virus to small or great laboratory animals does not result in a developed viraemia. Even by the most sensitive methods known at present we failed to demonstrate the presence of virus in the blood from e.g. peripherally inoculated 6—8 g mice, goats, sheep and monkeys, the same as after ic or intranasal inoculation (Mayer, 1963; Mayer *et al.*, 1967*a*; Mayer and Rajčáni, 1968). The possibility, therefore, that the tick, the vector of WTE virus, becomes infected by sucking on an animal to which the attenuated virus nad been administered for immunization purposes, remains rather hypothetical. The infection of the ticks is quite impossible in practice, because the only animal known to be susceptible to subcutaneous inoculation is the suckling mouse. Nevertheless, to verify the relation of the attenuated WTE virus to the *Ixodes ricinus* tick during its interstadial development, we passed the virus two times successively in larvae which, while carrying the virus, underwent the metamorphosis.

I. ricinus larvae from a laboratory breed were allowed to feed for 3 days on viraemic newborn mice (Kožuch *et al.*, 1966), to which a Hy-HK28"2" virus suspension, containing 10^8 ic LD_{50} of virus per ml, had been simultaneously administered ic (0.01 ml) and intraperitoneally (0.03 ml). On the 264th day after feeding on the viraemic suckling mice (including the accomplished metamorphosis), individual nymphs were examined in isolation experiments. Forty nymph suspensions were tested by the plaque method (Mayer, 1962).

The virus was found only in suspension No. 27, its titre being extremely low (8 PFU/ml); the plaques were of the *s* character. We obtained 4 clones of the virus which, after isolation from single plaques, were ic passed once in suckling mice and once in mice weighing 6—8 g. All four clones, studied by the aforementioned methods, had uniformly an ic^+ *sc s t* u^s character. The starting infectivity titres of the virus suspensions examined were almost identical with those observed in the virus isolated from the monkey brain.

The second passage of the virus in *I. ricinus* ticks was performed by the same method as in the first passage. The suckling mice used for feeding of the larvae were infected with clone No. 2, isolated from the suspension prepared from *I. ricinus* nymph No. 27, in which the first virus passage was accomplished. The whole period, for which the ticks harboured the virus in its second passage (including metamorphosis of larvae to nymphs) lasted 292 days. The virus was isolated in suckling mice from suspensions prepared again from individual nymphs and had very clearly expressed the ic^+ *sc s t* u^s character. The experimental values, defining the individual markers, were similar to the values characterizing the original Hy-HK28"2" virus.

Although limited in number (due to the nature of the material investigated and methods used) and deserving further confirmation of their more general validity, the present data seem to shed some light on the nature and on the variability of the experimentally obtained attenuated WTE virus. The fact that the attenuated virus clone under study showed no change in the characters investigated, either following the relatively long-term propagation in the predilectionally sensitive cels of the thalamic nuclei and in the cells of monkey cerebellum or during the repeated passage throughout the interstadial development of *I. ricinus* ticks (being in the tick organisms for a total of 556 days) is interesting from the point of view of the biology of an avirulent variant, showing a rather low mutation rate under the experimental conditions used. Moreover, the absence of apparent deviations from the type of the original working stock of virus, including a character of such complex nature as the virulence *(sc)*, seems to indicate that a marked tendency to the reversion under certain defined conditions of some of characters, as observed e.g. in Japanese encephalitis virus (Hammon *et al.*, 1963) could be considered as a property helping to define the virus clone selected for the study rather than a generally valid attribute which can be expected to became manifest in attenuated subgroup B arboviruses.

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