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STUDY OF THE VIRULENCE OF TICK-BORNE ENCEPHALITIS VIRUS. XI. GENETIC HETEROGENEITY OF THE VIRUS FROM NATURALLY INFECTIOUS *IXODES RICINUS* TICKS

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Summary. — Clones of tick-borne encephalitis (TE) virus (Western subtype), isolated directly from individual, naturally infectious *Ixodes ricinus* ticks or after their first mouse intracerebral (ic) passage, were studied. The data concerning the character of five genetic markers were completed with values of virus invasivity indices and values obtained by the method of the average death time estimation (in mice). Clones, showing the termolabile *(t)* character prevailed over thermostable *(t^+)* ones among viruses isolated recently from the nature. The *t* marker was found to be a genetically fixed property. The urea-sensitivity *(u^s)*, in the majority of the clones studied was associated with the *t* marker. A considerable heterogeneity in the distribution of s^+ and *s* markers, without any apparent relation to the other markers investigated, was observed. The invasivity indices coincided well with the average death time values in individual clones, showing thus a correlation between the rate of lethal activity manifested after ic administration and the ability to invade the nervous system after peripheral administration of the virus.

In general, a markedly pronounced genetic heterogeneity of TE virus isolated recently from the nature was in contrast to the genetically homogeneous laboratory-maintained virus strains. A detailed marker analysis allowed to demonstrate the effect of a single mouse brain passage on the properties of the virus obtained from viruliferous ticks as compared to the same virus not propagated in mouse brain after isolation.

Introduction

The mutual relations between the virus and susceptible cells, experimental pathogenicity and questions dealing with virus ecology belong to the best studied fields of TE research. The most frequently employed material in these studies has been the laboratory-kept TE virus, "adapted" by numerous serial ic mouse or in vitro cell culture passages. It has been represented by "strains" of TE virus isolated, as a rule, from suspensions prepared from

a definite number of ticks or less often from clinical, pathological or other specimens, collected e.g. in the course of ecological investigations.

Individual viruliferous ticks were shown to contain the virus in relatively high titres expressed in plaque forming units (PFU) (Kožuch *et al.*, 1970). In the isolation experiment, the material containing suspended virus particles, is administered to a susceptible animal host or cell cultures. Quantities of the virus, measured in infectious units or PFU, usually vary from $10—10^3$, or even less, thus creating relatively favourable conditions for selection. It can be assumed that, owing to the selection pressure of the environment exerted on the virus propagation, one particular sub-population usually becomes prevalent. Therefore, laboratory strains, in particular those at higher passage levels, represent — depending also on the host system used — virus populations which a) are heterogeneous in a low degree (Mayer *et al.*, 1967); and b) possess properties — we mean biological and physico-chemical — very likely distinct from those found in TE virus circulating in the nature, or at least with quite different distribution.

The work concerned with the study of genetic markers of TE virus (Mayer, 1966, Mayer *et al.*, 1967) and with the way of obtaining clones of TE virus, made possible a certain analysis from these aspects. Based on the preceding extensive data on the laboratory strains of TE virus of the Western and Eastern subtype (Blaškovič, 1967) or other viruses of the TE complex, we attempted to obtain information on the character of TE virus isolated by cloning directly from naturally viruliferous *Ixodes ricinus* ticks (or isolated by the same method after 1 ic mouse passage and to test in this way the assumped diversity in the character of the "wild" and of the laboratory-maintained TE virus populations.

It should be mentioned, however, that some spontaneous mutants occurring in the population of TE virus circulating in the nature might have been conditionally lethal for the isolation environment used. The detection systems employed (see Materials and Methods) are considered as belonging to the most sensitive ones known at present (Libíková *et al.*, 1962; Kožuch *et al.*, 1970). Nevertheless, the reliability of our data about the actual character of TE virus, harboured by the natural vectors and/or reservoirs, is fully proportional to the adequacy of the procedures used.

Materials and Methods

Ticks. Adult *Ixodes ricinus* ticks were collected in early spring in a region known as an elementary focus of TE at the southern slopes of the Tribeč mountains (South-west Slovakia).

Isolation experiments. Before starting the experiments aimed to obtain the cloned TE virus, individual *I. ricinus* ticks were assayed for virus. One-tick suspensions were prepared in 5% heated newborn calf serum (without specific antibodies against TE virus) in Hank's solution containing 0.5% lactalbumin hydrolysate. Half volume of each suspension was frozen in solid CO_2 and the second half was used in pilot experiments. In the latter, the presence of the virus was demonstrated by inoculating the undiluted material (after slight centrifugation) ic to newborn mice and into tube cultures of chick embryo cells (CEC). The isolation of the virus in newborn mice was performed by routine methods, including its identification by specific immune serum against TE virus. In the CEC, the presence virus was proved by interference with Western equine encephalomyelitis virus (Vilček, 1960).

The virus was found in 3 out of more than 100 suspensions prepared from individual ticks. By the plaque method (Mayer, 1962), we succeeded to demonstrate the virus in tick suspension designated *Ix-285*, in a titre as high as 1.6×10 PFU/ml. From the same part of this suspension, the virus was also recovered in the first ic mouse passage; this was designated M_1-*285*. The plaque titre of the M_1-*285* material amounted to 1.83×10^8 PFU/ml of 10% mouse brain suspension. The frozen parts of tick suspensions *Ix-296* and *Ix-297* were thawd to repeat the pilot experiments (cannibalism of mice), but attempts to recover the virus from them by the plaque method failed. However, two TE virus strains, designated M_1-*296* and M_1-*297* were isolated from these materials in the first ic mouse passage. The amounts of virus found in the mouse brains were 2.7×10^8 and 1.19×10^9 PFU/ml of 10% brain suspension in case of M_1-*296* and M_1-*297*, respectively.

Considerable difficulties of technical character were encountered in the preparation of the small amounts of suspensions from individual ticks and their storage. Moreover, all the work was complicated by the lability of the virus, demonstrated in the following experiments. These circumstance undoubtedly affected the recovery and cloning of the virus.

Materials for genetic studies on the TE virus clones. The starting material for the proper experiments, aimed at obtaining data on the natural distribution of genetic markers of the virus isolated, was prepared as follows: The center of a developed and well isolated plaque was sucked into a Pasteur pipette and then suspended in 0.5 ml of 5% heated calf serum. This suspension was inoculated into two litters of newborn mice. From the brains of TE-infected moribund mice we prepared a) 10% suspensions in 5% heated calf serum; these were stored in small amounts at −28° C as stock suspensions, and b) suspensions in borate buffer (Clarke and Casals, 1958), pH 9, supplemented with 0.2% of the Vth fraction of bovine albumin. This latter suspension was subjected to high speed centrifugation (Nozima *et al.*, 1964) before used in studies on the sensitivity of the virus to the action of urea (Mayer and Sabó, 1966).

Genetic marker studies. The characters of the five genetic markers of TE virus investigated have been described and defined in detail in previous communications (see Introduction). Therefore only the essential working criteria will be repeated here.

The ability of the virus to kill 6—8 g mice after ic inoculation up to high titres (their levels equalling those obtained by titration in CEC cultures and by back inoculation of the culture fluid to mice — Libíková *et al.*, 1962) is designated ic^+.

The ability of the virus to kill 6—8 g mice after sc inoculation up to high titres, with levels maximally 3 log units lower than the titres of the same virus suspension determined by ic inoculation of mice, is designated sc^+. The virus is designated as *sc*, when the difference between ic and sc titres corresponds to at least 6.5 to 7 $\log_{10}$ units, the titre itself usually being less than 10 LD_{50}/0.1 ml. The virus is designated as $sc^\pm$, when its titre after sc administration is intermediate between these two extreme values.

Viruses, forming plaques of about 3 mm diameter under the standard conditions described, are designated as s^+, while viruses forming plaques of about 1 mm are designated as *s*. Viruses forming plaques of intermediate size are designated as $s^\pm$.

Viruses, the infectivity titres of which decrease after heating at 50° C for 12 minutes by less than 1.5 $\log_{10}$ units, are designated t^+ and viruses, the titres of which decrease by more than 1.5 and less than 2.9 $\log_{10}$ units, are designated $t^\pm$. Virus is designated as *t* (thermolabile) when its infectivity titre decreases after identical treatment at least by 3 $\log_{10}$ units.

Viruses are designated as urea-sensitive (u^s), when treatment by 2M urea at 35° C for 30 minutes causes a decrease in the infectivity titre of at least 3 $\log_{10}$ units. Viruses, the infectivity of which decreases under the same conditions of treatment by less than 1 $\log_{10}$ unit, are designated as urea-resistant (u^r). Viruses, yielding values intermediate between those mentioned, are designated as $u^\pm$.

Number and passage history of the clones studies. The origin and the passage history of the clones selected from the *Ix-285* material can be schematically represented as follows: *Ixodes ricinus female* → *plaque* → M_1 (used for preparation of working and stock suspensions). Eight such virus clones were studied. In the case of M_1-*285*, M_1-*296* and M_1-*297* viruses, the origin and the passage history of the material under study was as follows: *Ixodes ricinus (female or male)* → → M_1 → *plaque* → M_1 (used for preparation of working and stock suspensions). The clones were isolated by the standard procedure described (Mayer, 1962) from the highest dilutions of the materials titrated. From the *Ixodes ricinus* — M_1 materials we isolated 15, 14 and 11 virus clones, respectively. The clones from the M_1-*297* virus were used only in comparative studies on the distribution of s^+ ($s^\pm$) *s* markers, however.

Table 1. Naturally occurring variants of TE virus

Designation of clones	Character	ADT*	Index of invasivity	log ic LD_{50}/0.1 ml
Ixodes ricinus → Plaque → M 1 (No. 285)				
1/285/0	ic^+ sc^+ s^+ t $u^{\pm}$	180	−1.2	7.0
2/285/0	ic^+ sc^+ $s^{\pm}$ $t^{\pm}$ $u^{\pm}$	226	−2.0	7.0
3/285/0	ic^+ sc^+ s^+ t u^s	198	−2.5	6.0
4/285/0	ic^+ sc^+ s t $u^{\pm}$	177	−0.2	6.7
5/285/0	ic^+ sc^+ s $t^{\pm}$ u^s	181	−1.2	8.2
6/285/0	ic^+ sc^+ s t $u^{\pm}$	224	−2.5	8.0
7/285/0	ic^+ sc^+ s t u^s	224	−1.5	6.0
8/285/0	ic^+ sc^+ s $t^{\pm}$ $u^{\pm}$	194	−2.7	7.0
Ixodes ricinus → M 1 → Plaque → M 1 (No. 285)				
1/285	ic^+ sc^+ s^+ t u^s	200	−1.5	7.3
2/285	ic^+ sc^+ $s^{\pm}$ t u^s	219	−1.2	7.2
3/285	ic^+ sc^+ $s^{\pm}$ t u^s	203	−2.3	8.0
4/285	ic^+ sc^+ s^+ t u^s	177	−1.0	7.2
5/285	ic^+ sc^+ s^+ t u^s	183	−1.5	8.0
6/285	ic^+ sc^+ s^+ t u^s	170	−1.2	7.7
7/285	ic^+ sc^+ s^+ t $u^{\pm}$	222	−1.0	8.2
8/285	ic^+ sc^+ s^+ t $u^{\pm}$	222	−1.5	8.0
9/285	ic^+ sc^+ s t u^s	229	−1.8	8.0
10/285	ic^+ sc^+ s t u^s	179	−0.7	7.2
11/285	ic^+ sc^+ s $t^{\pm}$ u^s	182	−0.0	8.0
12/285	ic^+ sc^+ s $t^{\pm}$ u^s	180	−0.0	8.0
13/285	ic^+ $sc^{\pm}$ s^+ t u^s	186	−3.5	8.0
14/285	ic^+ sc^+ s^+ t u^s	224	−2.8	8.0
15/285	ic^+ sc^+ s^+ t u^s	186	−0.3	7.0
Ixodes ricinus → M 1 → Plaque → M 1 (No. 296)				
1/296	ic^+ sc^+ s^+ t^+ u^s	178	−0.0	7.2
2/296	ic^+ sc^+ s^+ t u^s	219	−1.5	8.0
3/296	ic^+ sc^+ s^+ $t^{\pm}$ $u^{\pm}$	190	−0.5	7.2
4/296	ic^+ $sc^{\pm}$ s^+ t^+ $u^{\pm}$	189	−3.3	7.8
5/296	ic^+ sc^+ s^+ $t^{\pm}$ $u^{\pm}$	183	−1.0	8.5
6/296	ic^+ sc^+ s^+ t u^s	175	−0.5	7.0
7/296	ic^+ sc^+ s^+ t u^s	179	−2.0	7.2
8/296	ic^+ sc^+ s^+ t u^s	175	−1.3	8.0
9/296	ic^+ sc^+ s $t^{\pm}$ u^s	206	−1.3	8.0
10/296	ic^+ sc^+ s t u^s	203	−1.5	8.7
11/296	ic^+ sc^+ s $t^{\pm}$ u^s	214	−1.5	8.7
12/296	ic^+ $sc^{\pm}$ s t u^s	214	−0.8	8.0
13/296	ic^+ sc^+ s t^+ u^s	222	−2.3	9.0
14/296	ic^+ sc^+ s t u^s	224	−1.5	8.0
Controls (known virus clones)				
P III-E	ic^+ sc^+ s^+ t^+ u^r	146	−1.0	8.0
Hy-HK28"2"	ic^+ sc s t u^s	206	−7.0	7.3

* Average death time in hours.

The experiments on the characterization of the different clones were checked by parallel investigations on cloned model TE virus variants, with already known properties, namely the variant P III-E (with a marker set of the $ic^+ sc^+ s^+ t^+ e^+ u^r$ character) and the variant Hy-HK28"2" (with a marker set of the ic^+ *sc s t e* u^s character) (Mayer, 1966).

Mice. Subadult white mice of the Děčín breed weighing 6—8 g, known to be highly susceptible to TE virus administered ic and sc, were used. For virus titration, the mice were inoculated with serial tenfold dilutions of the suspensions, using 0.03 ml for ic and 0.1 ml for sc inoculation. Each dilution, if not stated otherwise, was inoculated into groups of 4 mice. The results were read 14 days after inoculation and the titres calculated according to Reed and Muench.

Average death time. Differences among the viruses could be noticed when comparing the time interval within which the particular virus strains were able to kill mice. This time interval may be considered as an expression of one attribute of virulence, namely of the rate of lethal activity manifested. The differences observed between the incubation period and the death time of mice ic inoculated with various TE virus variants were discussed earlier (Mayer, 1964). We attempted now to make the differences mor pronounced and measure them more precisely. For this reason, groups of 10 mice each were inoculated with given, rather high, virus dilutions (e.g. 10^{-6}, 10^{-7}, 10^{-8} and 10^{-9}) and the dead animals were counted at intervals of 12 hours. The group, in which all animals died (usually mice inoculated with approximately 100 ic LD_{50}), was used as the basis for determination of "average death time" (ADT), using the following formula:

$$ADT = \frac{\text{sum total of death times of mice given the minimal virus quantity lethal for all mice inoculated}}{\text{number of mice}}$$

The index of invasivity (Ii) expresses the amount of mouse ic LD_{50} needed to cause lethal encephalitis in mice after peripheral administration. Although numerous factors (such as, e.g., the degree of virus multiplication outside of the central nervous system) remain unevaluated when using this way of characterization of a particular strain, it has been widely employed (Libíková *et al.*, 1964; Pogodina and Savinov, 1964). The formula for Ii calculation is as follows:

$$Ii = \frac{\log \text{mouse sc } LD_{50}}{\log \text{mouse ic } LD_{50}}$$

Ii uses to be of negative value, since, as a rule, log ic LD_{50} > log sc LD_{50}.

Results

The description of individual clones selected from materials designated *Ix-285*, *M_1-285* and *M_1-296*, based on the estimated character of 5 genetic markers, was completed with data on ADT and Ii values.

All the clones studied (Table 1) displayed marked differences in the characters investigated, as compared to the characters of known laboratory-maintained clones, lines and strains of the Western or Eastern subtype of TE virus (Table 2). It is true that the new clones were ic^+ and sc^+ $(sc^{\pm})$,

Table 2. Character of TE virus mantained by intracerebral passages in subadult mice under laboratory conditions

TE virus	Number of strains*	Number of passages	Genetic character
Western subtype	16	18— 54	$ic^+ sc^+ s^+ t^+ e^+ u^r$
Eastern subtype	8	15—100	$ic^+ sc^+ s^+ t^+ e^+ u^r$

* Data compiled from Mayer (1966) and Mayer *et al.* (167).

Table 3. Characters of naturally occurring variants of TE virus

Virus	Number of clones investigated	Characters												
		ic^+	sc^+	$sc^\pm$	sc	s^+	$s^\pm$	s	t^+	$t^\pm$	t	u^r	$u^\pm$	u^s
Ix-285	8	8*	8	0	0	2	1	5	0	3	5	0	5	3
		100**	**100**			25	12.5	**62.5**		**37.5**	**62.5**		**62.5**	**37.5**
M_1-285	15	15	14	1	0	9	2	4	0	2	13	0	2	13
		100	**93.3**	6.7		60	13.3	**26.2**		13.3	**86.6**		13.3	**86.6**
M_1-296	14	14	13	1	0	8	0	6	3	4	7	0	3	11
		100.0	**92.9**	7.1		57		**42.6**	**21.3**	**28.5**	**50**		14.2	**78.7**
M_1-strains, total	29	29	27	2	0	17	2	10	3	6	20	0	5	24
		100	**93.1**	6.8		**58.6**	6.8	**34.4**	10.2	20.4	**68.8**		17.2	**82**
All strains, total	37	37	35	2	0	19	3	15	3	9	25	0	10	27
		100	**94.6**	5.4		**51.3**	8.1	**43.2**	8.1	24.3	**67.5**		**27**	**73**

* Number of clones.
** % of the total number of clones derived from the given strain.

but as distinct from viruses at higher mouse passage levels, of which 99% were shown to be s^+, the distribution of s^+, $s^\pm$ and s in the first plaque passage of the *Ix-* and M_1-strains was markedly different (e.g., in the M_1-*297* virus, of 11 plaques from the highest dilution 27.3% were of $s^\pm$ and 72.7% of s character). The distribution of the plaque diameter marker, in relation to other virus characters, is presented in Table 3.

Table 4. Stability of the thermolabile character of TE virus (Western subtype) clones
(Passage history: *Ixodes ricinus* → M 1 → plaque → M 1)

Designation and original character of the virus clones		Resulting character of the virus clones after 14 successive passages			
		in chick embryo cells		intracerebrally in mice	
2/285	t*	−3.5**	t	−4.5	t
6/285	t	−4.8	t	−3.3	t
7/285	t	−3.0	t	−3.0	t
8/285	t	−3.0	t	−3.4	t
10/285	t	−4.7	t	−4.2	t
14/285	t	−3.0	t	−4.6	t

* The symbol for thermolability (see Materials and Methods).

** The $\frac{N}{N_o}$ value: N = If D_{50}/ml of the original virus; N_o = If D_{50}/ml of the same virus suspension heated at 50° C for 12 minutes.

But the most pronounced differences, as compared to laboratory strains, were observed in the character of thermostability and sensitivity to the action of urea. Ninety-two per cent of clones had t (67.5%) and $t^\pm$ (24.3%) characters. The estimated values qualifying several clones as $t^\pm$ approached closely those defining the t character. Not a single clone had an u^r character, but 73% of clones exhibited an u^s, and 27% had an unclear, intermediate $u^\mp$ character.

Ix-285 virus was relatively the least homogeneous. As compared to M_1-*285* and M_1-*296* viruses, we observed a proportionally more frequent incidence of $t^\pm$ and $t^\pm$ markers in the *Ix-285* virus.

A set of separately conducted experiments was performed to ascertain whether the observed termolabile character of the virus breeds true. Out of the 20 clones under study showing the t character, 6 clones, originating from the M_1-*285* material, were selected and then propagated in parallel by two different methods:

a) in succesive passages in CEC tube cultures, i.e. on a substrate known to favour the prevalence of thermolabile variants in a given, genetically heterogeneous virus population; and

b) intracerebrally in subadult mice. The mouse nervous system was shown, in opposite to the in vitro cultivated cells (for references see Introduction), to favour the prevalence of thermostable variants in non-cloned TE virus

"strains" (Western or Eastern subtype) propagated in this way, as also witnessed by data presented in Table 2.

The character of the selected thermolabile clones, investigated after propagation under the conditions just mentioned, remained unchanged (Table 4), indicating its genotypic fixation.

The presence of true t^+ variants also was unequivocally demonstrated (Table 1) in 3 out of the 29 virus clones studied, in addition to 6 variants,

Table 5-I. Characters of the naturally occurring variants of TE virus. Incidence of character pairs

Group of characters	*I. ricinus* → → Plaque → M 1		*I. ricinus* → M 1 → Plaque → M 1				M 1 strains total	
	No. 285		No. 285		No. 296			
	Number	%	Number	%	Number	%	Number	%
sc^+ t	5	*62.5*	12	*80.0*	7	*50*	14	*65.3*
sc^+ $t^\pm$	3	*37.5*	2	*13.3*	4	*28.5*	6	20.4
sc^+ t^+					2	14.2	2	6.8
$sc^\pm$ t			1	6.7			1	3.4
$sc^\pm$ $t^\pm$								
$sc^\pm$ t^+					1	7.1	1	3.4
sc^+ s	5	*62.5*	5	*33.3*	6	*42.6*	11	*37.9*
sc^+ $s^\pm$	1	12.5	2	13.3			2	6.8
sc^+ s^+	2	25	7	*46.6*	7	*50*	14	*48.2*
$sc^\pm$ s			1	6.7			1	3.4
$sc^\pm$ $s^\pm$								
$sc^\pm$ s^+					1	7.1	1	3.4
sc^+ u^s	3	*37.5*	10	*66.6*	11	*78.5*	21	*72.4*
sc $u^\pm$	5	*62.5*	2	13.3	2	14.2	4	13.6
$sc^\pm$ u^s			2	13.3			2	6.8
$sc^\pm$ $u^\pm$					1	7.1	1	3.4
Clones investigated	8		15		14		29	

showing an intermediate type $(t^\pm)$ of their thermostability. This satisfactorily proved the mixed nature of the M_1-virus population.

In evaluating the clones according to the incidence of different combinations of markers, we followed the relations between sc^+ and $sc^\pm$ markers on the one hand, and those of t^+, $t^\pm$, t, and s^+, $s^\pm$, s or u^s, $u^\pm$ on the other (Table 5-I). The most frequent combinations were sc^+t, sc^+s, sc^+s^+, sc^+ u^s, and $sc_+u^\pm$. Even though the number of *Ix-285* virus clones was not high, the relatively more frequent incidence of $sc^+u^\pm$ combinations (found in 62.5% of cases) as compared to M_1-*285* and M_1-*296* viruses (where they represented 13.6%) seems to be of interest. The viruses subjected to one mouse passage before cloning exhibited approximately a twice greater incidence of the sc^+s^+ combination than *Ix-285* virus (Table 5-I).

Evaluation of the incidence and of relations of other pairs of markers (Table 5-II) revealed an apparent analogy of combinations found in 29 clones of M_1-*285* and M_1-*296* viruses, expressed in a relatively frequent incidence of s^+t, s^+u^s, $s\,u^s$ and $t\,u^s$ combinations. The $s\,t$, $t\,u^+$ and $s\,u^+$ combinations were more frequent with *Ix-285* virus (though not as frequent as the aforementioned combinations) and only rare with other clones.

Table 5-II. Characters of the naturally occurring variants of TE virus. Incidence of character pairs

Group of characters	*I. ricinus* → → Plaque → M 1		*I. ricinus* → M 1 → Plaque → M 1				M 1 strains total	
	No. 285		No. 285		No. 296			
	Number	%	Number	%	Number	%	Number	%
s^+ t	2	25	9	*60*	4	*28.5*	13	*44.8*
s^+ $t^\pm$					2	14.2	2	6.8
s^+ t^+					2	14.2	2	6.8
$s^\pm$ t			2	13.3			2	6.8
$s^\pm$ $t^\pm$	1	12.5						
$s^\pm$ t^+								
s t	3	37.5	2	13.3	3	21.3	5	17.2
s $t^\pm$	2	25	2	13.3	2	14.3	4	13.6
s t^+					1	7.1	1	3.4
t^+ u^s			2		2	14.2	2	6.8
t^+ $u^\pm$					1	7.1	1	3.4
$t^\pm$ u^s	1	12.5	2	13.3	2	14.2	4	13.6
$t^\pm$ $u^\pm$	2	25			2	14.2	2	6.8
t u^s	2	25	11	*73.4*	7	*50.0*	18	*63.0*
t $u^\pm$	3	*37.5*	2	13.3			2	6.8
s^+ u^s	1	12.5	7	*46.6*	5	*35.7*	12	*41.3*
s^+ $u^\pm$	1	12.5	2	13.3	3	21.3	5	17.2
$s^\pm$ u^s			2	13.3			2	6.8
$s^\pm$ $u^\pm$	1	12.5						
s u^s	2	25	4	*26.6*	6	*42.6*	10	*34.4*
s $u^\pm$	3	*37.5*						
Clones investigated	8		15		14		29	

Interesting differences suggesting that the character of variants prevailing in M_1-viruses (variants isolated from randomly chosen plaques, which developed after inoculation with the highest virus dilutions) might have been influenced by even a single newborn mouse brain passage, were reflected in a more frequent incidence of $s\,t$, $t\,u^\pm$, $s\,u^\pm$ variants of *Ix-285* virus, as compared to $s^+\,t$, $t\,u^s$ and $s^+\,u^s$ combinations found e.g. with the M_1-*285* virus.

The aforementioned heterogeneity of the *Ix-285* virus clones was even more pronounced on percentual evaluation of coincidence of three markers

(Table 6). A marked prevalence of s^+ t u^s and s t u^s combinations was observed with M_1-viruses. This incidence seems to indicate that the character of the plaque size is not affected by the t u^s combination, since the incidence of s^+ and s markers was independent of the character of the pair mentioned.

To compare the ADT and Ii, we started from the hypothesis that a virus less virulent for weaned mice after peripheral administration would multiply

Table 6. Characters of the naturally occurring variants of TE virus. Incidence of character triplets

Group of characters	*I. ricinus* → → Plaque → M 1		*I. ricinus* → M 1 → Plaque → M 1				M 1 strains total	
	No. 285		No. 285		No. 296			
	Number	%	Number	%	Number	%	Number	%
s^+ t u^s	1	12.5	7	*46.6*	4	*28.5*	11	*37.9*
s^+ $t^\pm$ u^s								
s^+ t u^s					1	7.1	1	3.4
s^+ t $u^\pm$	1	12.5	2	13.3			2	6.8
s^+ t^+ $u^\pm$					2	14.2	2	6.8
s^+ t^+ $u^\pm$					1	7.1	1	3.4
$s^\pm$ t u^s			2	13.3			2	6.8
$s^\pm$ $t^\pm$ u^s								
$s^\pm$ t^+ u^s								
$s^\pm$ t $u^\pm$								
$s^\pm$ $t^\pm$ $u^\pm$	1	12.5						
$s^\pm$ t^+ $u^\pm$								
s t u^s	1	12.5	2	13.3	3	*21.3*	5	*17.2*
s $t^\pm$ u^s	1	12.5	2	13.3	2	14.2	4	13.6
s t^+ $u^\pm$					1	7.1	1	3.4
s t $u^\pm$	2	*25.0*						
s $t^\pm$ $u^\pm$	1	12.5						
Clones investigated	8		15		14		29	

less and perhaps more slowly after ic inoculation (i.e. that it does not exert a marked lethal effect upon mouse cells). We expected, therefore, a distinct differentiation in the dynamics or modes of multiplication after ic administration between the clones possessing high or lower peripheral virulence.

Studies on the clones of 3 virus materials investigated yielded relations that were to a certain extent in accordance with the hypothesis mentioned.

We should mention that only 2 out of all the clones examined displayed a difference from the ic titre $>3.0 \log_{10}$ units after sc administration. In most cases, there were reproducible but not very marked differences within the limits of sc^+ definition. We observed that the greater the difference between log ic LD_{50} and log sc LD_{50} average values, the more pronounced

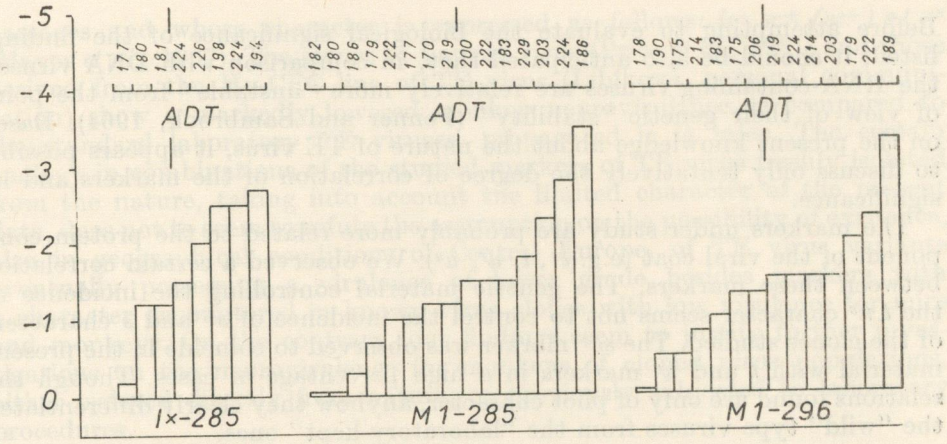

Fig. 1.

Relation of invasivity index values to the average death time (ADT, expressed in hours) in individual clones of *Ix*- and M_1-virus

Ordinate: invasity index values

tendency to ADT protraction (Fig. 1). If we agree that Ii = —1.5 represents a border value, the relation mentioned was as follows:

	ADT of viruses		
	Ix-285	M_1-*285*	M_1-*296*
Ii > —1.5	179	189	188
Ii < —1.5	213	206	207

The differences in the ADT values listed were in average 34 hours with *Ix-285* clones, and 17—19 hours with M_1-viruses. It seems likely that they may contribute to the illustration of the effect of even a single mouse passage on the virus virulence.

Discussion

An analysis of 29 virus clones of the type *I. ricinus* → M_1 → *Plaque* → M_1, and of 8 virus clones of the type *I. ricinus* → *Plaque* → M_1, revealed differences not only among variants of the group mentioned, but also among known clones of "laboratory" TE viruses of Eastern and Western subtype, respectively.

Moreover, our investigations pointed out a definite genetic heterogeneity of the virus isolated from both viruliferous *I. ricinus* ticks and the type M_1 virus.

As already mentioned, we assume that we worked only with those virus mutants which were isolated because they were not conditionally lethal for the detection system used. Therefore, it cannot be excluded with certainty that the material studied by the methods described, does not necessarily reflect the character of a "wild" TE virus population in its completeness.

Before attempting to evaluate the biological significance of the findings listed, it should be also anticipated that, in comparison with DNA viruses, the RNA-containing viruses are relatively more "unstable" from the point of view of their genetic "stability" (Fenner and Sambrook, 1964). Based on the present knowledge about the nature of TE virus, it appears possible to discuss only tentatively the degree of correlation of the markers and its significance.

The markers under study are probably more related to the protein component of the viral coat (e.g. t^+, t, u^r, u^s). We observed a certain correlation between these markers. The genetic material controlling the incidence of the $t\ u^s$ character seems not to control the incidence of s^+ and s characters of the clones studied. The sc^+ marker was observed to coincide in the present material with t and u^s markers in a high percentage of cases. Though the relations found are only of pilot character, anyhow they clearly differentiated the "wild" type viruses from the "laboratory-kept" ones.

Heterogeneity or the tendency to genetic "unstability" appears to be a marked property of a recently isolated TE virus population. On the other hand, we may assume that, especially thanks to this variability, the survival of TE virus, in conditions of the circulation in nature, is possible only in those variants which are competent to multiply in markedly distinct types of cell in different vertebrate hosts and even in those of ticks. Such a virus could represent a mixture of fractions, present in varying proportions, consisting of particles showing definite, but different, behavioural capacities. The various specific selection pressures probably influence the character of a given resulting wild type population, recorded in a definite period. The existing heterogeneity could be probably manifested also by the prevalence of definite population components, according to the propagation of a certain virus strain in different artificial laboratory conditions.

Moreover, our results seem to touch also another field of considerable importance, namely the questions of the presence in, and recovery from, nature of a virus with attenuated virulence for man, since obtaining of such a virus solely by laboratory procedures is not merely a question of systematic work. The model Hy-HK28"2" virus, the description of which was the subject of our previous work (for references see Mayer, 1966), demonstrates that a monkey-attenuated TE virus (Western subtype) may in principle exist. On the other hand, there is the Langat virus, strain TP-21 (Smith, 1956), the only virus of the TE complex known to possess virulence naturally lowered to a certain degree (Price et al., 1961; O'Reilly et al., 1965; Mayer and Rajčáni, 1967). The results of the study on the marker character of the TP-21 virus clones pointed out variants of $ic^+\ sc^+\ s^\pm\ t\ e\ u^s$ and $ic^+\ sc^\pm\ s^\pm\ t\ e\ u^s$ type (Mayer *et al.*, 1967). With the *Ix*-285, M_1-*285* and M_1-*296* types of virus, the same combination and character of at least 5 markers was observed as with the Langat virus (markers of ic and sc mouse virulence, plaque appearance, thermostability, and urea sensitivity). Furthermore, there also is a striking resemblance to the combinations and characters of markers observed in the clones of the Hy-CEC line (Libíková and Stanček, 1965) which had undergone several hundred CEC culture

passages and whose character is expressed as follows: *ic*$^{+}$ *sc*$^{\pm}$ *(sc*$^{+}$*) s t u*^{s} (Mayer *et al.*, 1967). The clones of Langat virus (Price *et al.*, 1961) and those derived from the Hy-CEC line of TE virus (Libíková, personal communication) show a markedly lowered monkey neurovirulence as compared to the standard laboratory TE viruses, propagated ic in mice. The certain analogy in combinations of the studied markers of TE virus freshly isolated from the nature, taking into account the limited character of the present data, does not to seem to refute the assumption on the possibility of existence, also in geographical conditions of Central Europe, of TE virus variants eventually possessing a virulence of lower grade besides markers with a character encountered in known virus clones with low virulence for mice and monkeys. On the contrary, our findings seem to justify further investigations on the neurovirulence for monkeys of cloned virus populations, either isolated directly from nature, or previously subjected to laboratory procedures.

References

Blaškovič, D. (1967): Public health importance of tick-borne encephalitis in Europe. *Bull. Wld Hlth Org.* **36**, Suppl. 1, 5.

Clarke, D., and Casals, J. (1958): Techniques for hemagglutination inhibition with arthropod-borne viruses. *Amer. J. trop. Med. Hyg.* **7**, 571.

Fenner, F., and Sambrook, J. F. (1964): The genetics of animal viruses. *Ann. Rev. Microbiol.* **18**, 47.

Kožuch, O., Mayer, V., and Nosek, J. (1970): A quantitative study of the tick-borne encephalitis virus in individual *Ixodes ricinus* ticks. *Acta virol.* (in press).

Libíková, H., Řeháček, J., and Mayer, V. (1962): Comparison of different in vitro and in vivo methods for the detection of small quantities of various tick-borne encephalitis complex viruses, p. 201. In H. Libíková (ed.): *Biology of viruses of the tick-borne encephalitis complex.* Academic Press, New York.

Libíková, H., Řeháček, J., Mayer, V., Kožuch, O., and Ernek, E. (1964): Tick-borne encephalitis viruses recoverd by different methods from *Ixodes persulcatus* ticks. *J. Hyg. Epidem. (Praha)* **7**, 7.

Libíková, H., and Stanček, D. (1965): Characterization of three different lines of a single strain of tick-borne encephalitis virus. *Acta virol.* **9**, 481.

Mayer, V. (1962): Study of the tick-borne encephalitis virus — chick embryo cell system by the plaque method. *Acta virol.* **6**, 309.

Mayer, V. (1964): Study of the virulence of tick-borne encephalitis virus. II. Pathogenetic properties of two variants of a single virus strain. *Acta virol.* **8**, 14.

Mayer, V. (1966): A mutant of tick-borne encephalitis (TE) virus with lost neurovirulence for monkeys. *Acta virol.* **10**, 561.

Mayer, V., and Rajčáni, J. (1967): Study of the virulence of tick-borne encephalitis virus. VI. Intracerebral infection of monkeys with clones experimentally attenuated virus. *Acta virol.* **11**, 321.

Mayer, V., and Sabó, A. (1966): Study of the virulence of tick-borne encephalitis virus. V. Effect of urea on thermolabile and thermostable virus variants. *Acta virol.* **10**, 486.

Mayer, V., Slávik, I., and Libíková, H. (1967): Study of the virulence of tick-borne encephalitis virus. VIII. Differentiation by chromatography on hydroxylapatite of clones possessing distinct biological properties. *Acta virol.* **11**, 407.

Nozima, T., Mori, H., Monobe, Y., and Yamamoto, S. (1964): Some properties of Japanese encephalitis virus. *Acta virol.* **8**, 97.

O'Reilly, K. J., Smith, C. E., McMahon, D., Wilson, A. L., and Robertson, J. M. (1965): Infection of sheep and monkeys with Langat virus: cross protection against other viruses of the Russian spring-summer complex. *J. Hyg. (Lond.)* **63**, 213.

Pogodina, V. V., and Savinov, A. P. (1964): Variation in the pathogenicity of viruses of the tick-borne encephalitis complex for different animal species. I. Experimental infection of mice and hamsters. *Acta virol.* **8**, 424.

Price, W. H., Lee, R. W., Gunkel, W. F., and O'Leary, W. (1961): The virulence of West Nile virus and TP-21 virus and their application to a group B arbor virus vaccine. *Amer. J. trop. Med.* **13**, 403.

Smith, C. E. G. (1956): A virus ressembling Russian spring-summer encephalitis virus from an Ixodid tick in Malaya. *Nature (Lond.)* **178**, 581.

Vilček, J. (1960): Interference between tick-borne encephalitis and Western equine encephalomyelitis viruses in chick embryo tissue cultures. *Acta virol.* **4**, 308.