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EXPERIMENTALLY SEGREGATED ATTENUATED TICK-BORNE ENCEPHALITIS VIRUS: ALTERED BEHAVIOUR AFTER PROLONGED STORAGE AT SUBZERO TEMPERATURES

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Summary. — In an attenuated variant of tick-borne encephalitis virus, western subtype (TBEV) with an *ic*$^+$ *sc s t e u*^{s} character and lost virulence for monkeys, a distinct inhomogeneity together with a decrease in titre was observed after 8.7—9.6 years of storage at 4 and —14 (±2) °C, with several interruptions due to failures in refrigeration systems. Proved by clonal analysis, the inhomogeneity concerned plaque polymorphism, increased thermoresistance and degree of virulence for mice and monkeys. Marker studies of materials stored for up to 14.5 years yielded comparable findings. The possibility of the presence in the virus stocks in addition to a prevailing attenuated component also of minor component(s) having the advantage for survival under storage conditions, is discussed taking into account the intrinsic mutational capacity of the virus and known differences in resistance to environmental stresses between attenuated and virulent TBEV variants.

Key words: tick-borne encephalitis virus; changes in attenuation

Introduction

In the course of investigations on the virulance of TBEV variants, a mutant attenuated for mice and devoid of killing capacity for monkeys was isolated (Mayer, 1963; Mayer and Rajčáni, 1967). Within the last two decades, considerable interest was paid in this laboratory to properties of TBEV particles, suitable to be studied as characters (markers), allowing, e.g., a differentiation of intrastrain variants. The genetic markers were not described for the genus *Flavivirus*, in particular for TBEV, before the above investigations (reviewed by Mayer and Mitrová, 1977).

In the virus attenuation research, there is a continuous and pressing need for genetic marker systems linked to virulence character. But in any virus studied so far, the known markers, their sets or mode of covariation, reflect the complex polygenic nature of the biological behaviour of a virus (see, e.g., for polioviruses Kańtoch, 1978; Nakano *et al.*, 1978). A similar view concerning flaviviruses was substantiated recently (Mayer, 1976). These findings

seem to correspond to the widely experienced „high capacity for variation" that greatly disturbs investigators working on attenuated virus mutants (see Clark, 1977). The marker studies seem rather to characterize the end-products of laboratory manoeuvres. Systematic studies demonstrated that laboratory-maintained lines of TBEV or strains isolated from the nature actually represent biologically and biophysically inhomogeneous populations of virions (reviewedby Mayer and Mitrová, 1977). Nevertheless, during serial passages in subadult mouse brains of single-plaque-derived attenuated TBEV clones, no changes in virus character were observed. On the other hand, when the same virus was reoriduced e.g. in developing chick embryos, changes in some important markers, accompanied also by an increase of the encephalitogenic potential for monkeys, were noted (Mayer and Rajčáni, 1967). The assembled data seem to suggest that at least *a*) in the virus being at a given attenuation level, the relative proportions between the assumed predominant avirulent and the minor, biologically distinct, component(s) may change according to the selective advantage offered by substrates used for virus propagation and influence the behaviour of the virus, or *b*) the virus mutates fairly readily in the course of 2—3 passages in certain cells with a resulting changes in the over-all character of the virus. But the latter possibility seems to be less probable.

The present paper analyzes the altered behaviour of an attenuated TBEV variant, observed when assayed after 8.7—9.6 years of storage, and of some other TBEV variants stored for longer periods.

Materials and Methods

Viruses. The TBEV variants studied were plaque-segregated from the extracellular virus produced by the AM-57 human epithelial cells, persistently infected (106th passage) with the virulent prototype TBEV strain Hypr (Mayer, 1964). The following materials, stored lyophilized under conditions as described in Table 2, were examined: *a*) a variant designated "K" in its 8th suckling mouse brain (sm) passage (Table 12); *b*) a variant derived from the "K" virus and designated "2" (Table 11), which had undergone 28 passages in primary hamster kidney epithelial cell cultures (HK) and one subadult mouse brain (M) passage; and *c*) a variant designated Hy-HK28"2" (Tables 2, 3) which had undergone two additional M passages after cloning from the "2"virus. The following viruses were included for comparison: the virulent variant P III-E, isolated from the prototype TBEV strain Hypr (Tables 1, 10); the man-attenuated E5"14 clone (Mayer *et al.*, 1976) derived from the TP 21 Langat virus; and the 17D variant of the yellow fever virus (YFV), originating from the commercial vaccine prepared in chick embryo (CE) cells (Burroughs Wellcome and Co., London; Yellow Fever vaccine B.P.-Leukosis free, lot 4512) after one sm passage of the reconstituted virus.

Experimental animals. SPF outbred mice ("H" strain) weighing 8—10 g were used throughout. Suckling mice were from the same breed (breeding station Černý Vůl). The virulence testing of the reconstituted stored TBEV variants in *Cercopithecus aethiops* monkeys were done by Drs. M. Stárek and K. Kubištová at the Institute of Sera and Vaccines, Prague, who also supplied the frozen samples of monkey brain suspensions.

Cell cultures. The PS line of pig kidney epithelial cells (obtained from Dr. J. S. Porterfield) and SPF cells were used. Their cultivation and the plaque assays were described (Mayer, 1964, 1976).

Storage and reconstitution of TBEV variants. Stock materials in 0.5 ml portions of 10 per cent mouse brain suspensions (or culture medium from HK in case of virus variant "2") were lyophilized in an electric centrifugal freeze drier; the glass ampoules were flame-sealed under normal

Table 1. Properties of TBEV particles used for characterization of plaque-cloned materials

Symbol	Description of the markers
ic^+	Capacity to kill 6—8 g mice after i.c. inoculation of high dilutions of the virus
sc^+	Capacity to kill 6—8 g mice after s.c. inoculation of virus dilutions not lower by more than 3 dex as that required to reach the and-point by i.c. titration
$sc^\pm$	S.c. titre values significantly between sc^+ and sc
sc	S.c. titre values by at least 6.5—7 dex lower than in the sc^+ variant
s^+	Clear plaques of $\geqq$3 mm diameter*
$s^\pm$	Plaques of a size intermediate between s^+ and s
s	Clear plaques of $\leqq$1 mm diameter*
t^+	Relative resistance to inactivation at 50 °C/12 min (decrease of infectious titre not exceeding 1.5 dex)
$t^\pm$	Virus variant with intermediate resistance
t	Virus variant readily inactivated at 50 °C (decrease of the infectious titre at least by 3 dex)
e^+	Variants in which >50 % of the total virus activity is eluted from hydroxylapatite columns by low (0.1 or 0.2 M) Na_2HPO_4 concentrations
e	Variants in which >50% of the total virus activity is eluted by high (0.3 or 0.4 M) concentrations of phosphate
u^r	Variant, the infectivity of which decreases not more than by 1 dex due to the action of 2 M urea (35 °C/30 min)
$u^\pm$	Variant with significantly intermediate resistance
u^s	Variant, the infectivity of which decreases at least by 3 dex under similar conditions
N^+	After a short incubation the virus paralyzes and kills adult monkeys inoculated intrathalamically even with low doses
$N^\pm$	After a long incubation period the virus produces a prolonged paralysing lethal illness in intrathalamically inoculated monkeys
N	Virus causing no clinical encephalitis after intrathalamic inoculation of at least 6 dex mouse $icLD_{50}$

Character of selected model virus variants:
virulent: $ic^+\ sc^+\ s^+\ t^+\ e^+\ u^r\ N^+$ (designated P III-E)
attenuated: $ic^+\ sc\ s\ t\ e\ u^s\ N$ (designated Hy-HK28"2")

For references and detailed description see Mayer and Mitrová (1977).
* Assayed in PS and CE cells, overlaid with agar (Difco), washed agar, methyl-cellulose (2000 cps), or agarose (BDH).

atmosphere and stored as indicated in Table 2. The well-preserved dry discs of the lyophilized materials were reconstituted with sterile distilled water and after 15 min standing at 4 °C inoculated under controlled sterile conditions intracerebrally (i.c.) in SPF mice (held in strict separation) or in cell cultures.

Marker studies. The TBEV markers investigated are defined in Table 1. The reproduction capacity of TBEV variants at incubation temperatures of 36 and 41 °C (*ts* marker) was investigated in PS cells (Mayer, 1976). To ascertain the results, the experiments were repeated several times.

Results

The investigations were centered mainly on the Hy-HK28"2" variant (Table 2) which maintained its exceedingly low virulence under conditions described in Table 3.

Table 2. Some properties of the original attenuated TBEV variant Hy-KH28"2"*

Character of the virus	Note
ic⁺ sc s t e u^s N	Titre (CPD_{50}, PFU) differences at 36 and 41 °C were 1.5—2.5 dex
5 or 6 dex mouse $icLD_{50}$ not lethal in 13 *M. mulatta* monkeys inoculated intrathalamically	Low virus titres detected in various parts of the CNS up to the 21th day p.i. Histopathology of inflammatory type dominated.
7 dex mouse $icLD_{50}$ not infectious for 9 *M. mulatta* monkeys by the intranasal route	Virus not detected in the CNS by virological, histopathological and immunofluorescence methods.

* Passage history: $HK_{28}M_1HK_1P_2M_2$ (M — i.c. passage in 6—8 g mice; HK — passage in primary hamster kidney epithelial cell culture; P = plaque-to-plaque passage in primary CE cell culture. For details see Mayer and Rajčáni (1967).
Virus was lyophilized in August, 1967 as a 10 per cent mouse brain suspension and kept for about 4.5 years at 4 °C and for more then 4 and 5 years at −14 (±2) °C with several interruptions due to failures in refrigeration systems.

The attenuated Hy-HK28"2" variant of TBEV stored for 8.7—9.6 years

a) Virus reproduced in mice after 8.7 years of storage and assayed after cloning

The reconstituted virus suspension was diluted 1 : 100 and inoculated i.c. in mice. From the brains of agonic mice a 10 per cent suspension was prepared (the "2/2" M_1 virus) and stored at −68 °C for 12 months. The virus

Table 3. Marker characteristics of the attenuated variant Hy-HK28"2" of TBEV virus after serial, heavy inoculum propagation in various host substrates

The ic⁺ sc s t e u^s *character of the attenuated virus remained similar:*

a) in the course of 9 passages in brains of subadult mice
b) in the course of 19 passages in primary hamster kidney epithelial cells
c) during repeated chromatography under conditions for elution of virulent variants
d) in two strains isolated from n. lenticulostriatus and from the cerebellum of a *M. mulatta* monkey on the 14th day after intrathalamic inoculation of 6 dex of the attenuated virus and showing no signs of clinical involvement*
e) in the course of 2 passages in infected *Ixodes ricinus* ticks during interstadial development. The virus was isolated by the plaque method from nymphs on the 264th (1st passage) and on the 292nd (2nd passage) day after infection of larvae by feeding on viraemic newborn mice, i.e. transstadial transmission of virus had occurred.

*Increase of the virulence for subadult mice after s.c. administration**:

a) after 10—11 passages in the newborn mouse brain
b) after 17 passages in primary CE cell cultures
c) after 2 passages in 7-day-old chick embryos inoculated into the yolk sac. Increase in the monkey i.c. virulence ($N \rightarrow N^+$) detected after 9—10 passages in the absence of changes in other markers investigated.

* For detailed description and references see Mayer and Mitrová (1977).

Table 4. Characteristics of the Hy-HK28"2" TBEV reconstituted after 8.7 years of storage and recovered as the "C8" cloned virus

Virus designation	Passage level		Character of the virus
Hy-HK28"2"		ic^+ *sc s t e* u^s *N*	Lyophilized in August, 1967, reconstituted in
"2/2"	M_1	ic^+ *sc*	April, 1976 and reproduced in subadult mouse brain, stored at −68 °C until March 1977, when
"2/2"M_1*	M_2	ic^+ *sc* $s^{\pm}$ *t* u^s	a further mouse brain passage was made
	Double selection in CE cells from plaques of ≦1 mm diameter		
"C8"	M_1**	ic^+ *sc* $s/s^{\pm}$	5% lethality in mice (n = 40) given 4 dex $icLD_{50}$ of the virus s.c.
	sm_1	ic^+ *sc*/$sc^{\pm}$ $s/s^{\pm}/s^+$ *t* u^s	Plaques in PS cells of 1−3 mm diameter. Titre differences (CPD_{50}, PFU) at 36 and 41 °C 0.8 to 1.8 dex

* "2/2"M_1 sm_1 virus in PS cells showed s^+ plaques in more than 30 per cent of the total plaque count.

** 6 dex $icLD_{50}$ of the virus caused protracted fatal encephalitis in *C. aethiops* monkeys after a long incubation period ($N^{\pm}$).

Table 5. Characteristics of the "921" brain isolate from a C. aethiops monkey inoculated with the originally monkey-attenuated TBEV* stored for 8.7 years and plaque-cloned after reconstitution

Virus designation	Passage level		Characteristics of the virus
"C8"			
"921"	*C. aethiops* monkey	$N^{\pm}$	
"921"M_1	M_1	ic^+ *sc* $s/s^{\pm}/s^+$ $t^{\pm}$ $u^{\pm}$	Virus recovered only in i.c. inoculated mice, not in cell cultures Plaque diameter 1−3 mm in PS, 1−2 mm in CE cells Thermal inactivation at 50 °C by 2.29 dex (n = 7, range 1.7−2.8 dex) Titre differences (CPD_{50}, PFU) at 36 and 41 °C 1.25−1.4 dex
Randomly selected clones from the "921" M_1 virus in their M_1		ic^+ *sc* $s/s^{\pm}/s^+$ *t* u^s	picked from a plaque 3 mm in diameter
		ic^+ *sc* $s^{\pm}/s^+$ $t^{\pm}$ $u^{\pm}$	picked from a plaque 1 mm in diameter
		ic^+ *sc* $s^{\pm}/s^+$ *t* u^s	picked from a plaque 1 mm in diameter

* Original character of the virus: ic^+ *sc s t e* u^s *N*.

The "921"M_1sm_1 was $s/s^{\pm}$ in CE cells; difference in titres (CPD_{50}, PFU) at 36 and 41 °C was 2 dex.

Table 6. Characteristics of the Hy-HK28"2" TBEV reconstituted after 9.6 years of storage and recovered as a non-cloned material ("Lyo M_1")

Virus designation	Passage level		Characteristics of the virus
Hy-HK28"2"		ic^+ *sc s r e* u^s *N*	Lyophilized in August, 1967, reconstituted in March, 1977 and reproduced in mouse brain.
"Lyo M_1"	M_1	ic^+ $sc^\pm$ $s/s^\pm$ $t/t^\pm$ u^s	25% lethality in mice (n = 40) given 4 dex $icLD_{50}$ of the virus s.c.
	↓		Titre differences (CPD_{50}, PFU) at 36 and 41 °C 0.8—1.6 dex
	sm_1	$s/s^\pm/s^+$	Plaque diameter 1—3 mm in PS cells

M_1: 6 dex $icLD_{50}$ of the virus caused protracted fatal encephalitis in a C. *aethiops* monkey after a long incubation period ($N^\pm$).

was *sc* in four serial M passages, reaching dex $icLD_{50}$/ml values from 8—8.5. After the time period indicated, the virus was subjected to one more M passage (the "2/2" M_2 virus). After double cloning in CE cells, a virus suspension, designated C8, was prepared from M brains (Table 4). Bright, specific immunofluorescence was seen in mouse neurons. Mice immunized subcutaneously (s.c.) with one dose of 10^4 $icLD_{50}$ of the C8 virus showed a protection index of 4.5. The markers of the C8 virus after it had undergone one additional sm passage differed markedly from those of the original Hy-HK28"2" virus, including also the $N \rightarrow N^\pm$ transition (Table 4).

From the brain of an agonic monkey (No. 921), the virus was isolated in mice to avoid an eventual $s \rightarrow s^\pm$ and $sc \rightarrow sc^\pm$ promoting effect of the sm environment. The virus (921 M_1) was isolated only from undiluted material (Table 5). It was inhomogeneous concerning the plaque phenotypes in both CE and PS cells (s/s^+). Other changes, never observed in the original Hy-HK28"2" and C8 viruses, were its increased thermoresistance ($t^\pm$) and an $u^\pm$ character. Both these properties suggest that virions with surface properties differing from those prevailing in the original virus before storage were present in the recovered 921 M_1 virus. The *ts* characters of the viruses studied did not differ significantly. Clonal analysis of the 921 M_1 virus confirmed its inhomogeneity (Table 5).

b) Virus reproduced in mice after 9.6 years of storage and assayed as non-cloned.

The reconstituted undiluted virus was inoculated i. c. in mice. On the 7th day after infection, a 10 per cent suspension was prepared from the brains of agonic mice ("Lyo" M_1) and stored in portions at —68 °C. This virus differed from the original Hy-HK28"2" material in its unusually high peripheral virulance for mice, plaque size polymorphism in CE and PS cells and by an increased thermoresistance at 50 °C. After a s.c. dose of 10^4 $icLD_{50}$ of the "Lyo" M_1 virus — identified as TBEV — the surviving mice showed a protection index of 5. This virus displayed also a $N^\pm$ property (Table 6).

Table 7. Characteristics of the "925" brain isolate from a C. aethiops monkey inoculated with the originally monkey-attenuated TBEV*, stored for 9.6 years and non-cloned after reconstitution

"Lyo M_1"			
	↓		
"925"	*C. aethiops* monkey	$N^{\pm}$	
	↓		
"925"M_1	M_1	ic^+ *sc* $s/s^{\pm}/s^+$ $t^{\pm}$ u^s	Virus recovered only in i.c. inoculated mice, not in cell cultures Plaque diameter 1—3 mm in PS cells Thermal inactivation at 50 °C by 2.23 dex (n = 9) Titre differences (CPD_{50}, PFU) at 36 and 41 °C 0.5—1.2 dex
	↓		
	sm_1	$s^{\pm}/s^+$	Plaque diameter 2—4 mm in PS cells
Randomly selected 3 clones from the "925"M_1 in their M_1 (all behaved similarly to each other)		ic^+ $sc/sc^{\pm}$ $s/s^{\pm}/s^+$ t u^s	Picked from plaques 1—1.5 mm in diameter. Titre differences at 36 and 41 °C 0.7 dex

* Original character of the virus: ic^+ *sc s t e* u^s *N*.

From the brain of an agonic monkey (No. 925), the virus was recovered in mice again only from undiluted material. The isolate, designated 925 M_1, similarly to the "Lyo" M_1virus, showed a marked plaque-size inhomogeneity, retaining clearly its $t^{\pm}$ character (Table 7). The *ts* character of the 925 M_1 virus approached this of the 921 M_1 virus. The $u^{\pm}$ character was not observed in the 925 M_1 virus; this finding remains obscure, although its direct relation to the complex $N^{\pm}$ property can be hardly expected.

Recovery of the stored attenuated TBEV variant from individual ampoules

Infectious virus was recovered from five of eight lyophilized samples assayed. The amounts of the infectious virus were unequal, but always significantly lower than the titre of the Hy-HK28"2" virus estimated immediately after lyophilization (Table 8). Virus was not recoverable in suckling mice in spite of repeated attempts. The virus from the ampoule „H" was subjected to clonal analysis to obtain at least a partial insight into the ivrus population that survived the storage. Seven clones were isolated from *s* plaques (representing about 85 per cent of the whole plaque counts, the remaining plaques being $s^{\pm}$). Individual clones were reproduced in one M passage (the 67 M_1 virus) and examined. A distinct inhomogeneity of their plaque size phenotypes ($s \rightarrow s^+$) was evident (Table 9). Besides this property, never observed during the years of study on the attenuated variant, three clones were consistently $t^{\pm}$ and two were virulent for mice after s.c. administration, although moderately and displaying a "prozone" pheno-

Table 8. Recovery of the attenuated Hy-HK28"2" TBEV stored at subzero temperatures for 8.7—10.3 years after lyophilization

Virus ampoule	Years of storage[1])	Virus titres (dex values per 0.1 ml)[2]) PS cells CPD$_{50}$	PS cells PFU	CE cells PFU	Mice subadult i.c. LD$_{50}$	Mice subadult s.c. LD$_{50}$	Mice newborn i.c. LD$_{50}$
A[3])	8.7	3.5			≥ 2		
B	9.6	0	0	0	0	0	
C[4])	9.6				+[5])		
D	10	0	0	0	0	0	0
E	10.2		0	4	≥ 4		
F	10.2	0					
G	10.3	≥ 4.5					
H	10.3	5.5	6		5.75	<1	

[1]) Lyophilized virus was kept for 4.5 years at 4 °C and for more than 4 and 5 years at −14 (±2) °C with several interruptions due to failures in refrigeration systems.
[2]) Dex icLD$_{50}$/0.1 ml of the virus (10 per cent mouse brain suspension) after lyophilization in 1967 was 7.3.
[3]) Source of the "C8" virus (see Table 4).
[4]) Source of the "Lyo M_1" virus (see Table 6).
5) Only undiluted suspension of the reconstituted virus was assayed.
0 = virus not recovered.

menon. Thus a deviation from the typical character of the original Hy-HK28'2" virus was apparent in the long-term stored virus after its reconstitution.

The study of the *ts* character in clones derived from the 67 M_1 virus (Table 9) by the CPD$_{50}$ method offered an information only about the reproduction of the given virus as a whole. As a further step in the analysis of

Table 9. Clonal analysis of the Hy-HK28"2" attenuated TBEV virus (ic$^+$ sc s t e u^s) after lyophilization and 10.3 years of storage

Clone	Characteristics of the virus	Plaque diameter in PS cells (mm)
m 1	ic^+ *sc* $s/s^\pm$ *t*	1—2
m 2	ic^+ *sc*/$sc^\pm$ $s^\pm/s^+$ *t*	2—4
m 3	*ic sc* $s^\pm/s^+$ $t^\pm$	1.5—3
m 4	ic^+ *sc*/$sc^\pm$ $s^\pm$ $t^\pm$	1.5—2.5
m 5	ic^+ *sc* $s/s^\pm$ *t*	1—2
m 6	ic^+ *sc* $s/s^\pm/s^+$ $t/t^\pm$	1—3
m 7	ic^+ *sc* $s/s^\pm/s^+$ *t*	1.5—4

Randomly selected *s* plaque-cloned virus from ampoule H (see Table 8) in its 1st mouse brain passage (designated "67"M_1).
Titre differences (CPD$_{50}$, PFU) at 36 and 41 °C in clones m 1—7 ranged from 0.7—1.8 dex.

Table 10. Genetic dimorphism correlated with differences in reproduction at 41 °C in the attenuated TBEV bearing an *s* character, as observed in reconstituted material after 10.3 years of storage; other viruses included for comparison

Virus designation	Plaquing in PS cells at 36 °C	Plaquing in PS cells at 41 °C
67 M_1 m 1[1])	$s/s^{\pm}$	pp[2])
m 2	$s^{\pm}/s^{+}$	pp
m 3[3])	$s^{\pm}/s^{+}$	**pp** $\approx 4.6 \times 10^5$ PFU/ml $s^{\pm}$ 5.9×10^4 PFU/ml
m 4	$s^{\pm}$	pp
m 5	$s/s^{\pm}$	pp
m 6	$s/s^{\pm}/s^{+}$	pp
m 7	$s/s^{\pm}/s^{+}$	pp
921 M_1	$s/s^{\pm}/s^{+}$ 1—3 mm plaques	intermediate pp → *s* plaques
925 M_1	$s/s^{\pm}/s^{+}$ 1—3 mm plaques	pp
YFV 17D	$s/s^{\pm}$	$s/s^{\pm}$
Langat E5"14"[4])	$s^{\pm}$ 1.5—2 mm plaques	no recognizable plaques
P III-E[5])	s^{+} 8—9 mm plaques	$s^{\pm}$ 7—8 mm plaques
63 M_1[6])	$s/s^{\pm}/s^{+}$	intermediate pp → *s* plaques
64 M_1	$s/s^{\pm}/s^{+}$	pp

[1]) For origin of m 1—7 clones see Table 9.
[2]) Pin-point plaques 1 mm in diameter.
[3]) Virus yielding dimorphous plaques.
[4]) Monkey- and human-attenuated TBEV (Mayer *et al.*, 1976).
[5]) Monkey-virulent (N^{+}) prototype strain Hypr, see Table 1.
[6]) For origin see Tables 11 and 12.

stored virus we studied the plaque size marker — reflecting the velocity of replication of a given mutant at incubation temperatures of 36 and 41 °C. The seven clones mentioned above showed at 36 °C a strongly expressed plaque-size polymorphism ($s/s^{\pm}/s^{+}$) but at 41 °C, barely visible, but unusually size-homogeneous plaques of a "pin-point" (pp) character were observed, except in the m 3 clone. In the latter, plaque size dimorphism was apparent at 41 °C, i.e. besides a majority of pp plaques, also $s^{\pm}$ phenotypes were clearly recognized, comprising about 13% of the total plaque count (Table 10). In these experiments, other flaviviruses were examined for comparison.

According to the plaquing efficiency at 41 °C, five behavioural groups were observed: a) viruses without significant differences in the plaque size at both temperatures; b) viruses forming *pp* plaques only; c) viruses yielding a dimorphous mixture, i.e. *pp* and $s^{\pm}$ phenotypes; d) viruses displaying a transient plaque size character (*pp* → *s*); and e) viruses forming no discernible plaques at 41 °C.

To complete the study of changing patterns of the long-term stored virus, we investigated two other virus lines, ancestors of the attenuated Hy-HK28"2" clone, but with another passage history and presumably also with other population traits.

Table 11. Characteristics and clonal analysis of an experimentally segregated attenuated TBEV* reproduced in HK cells and stored lyophilized for 13 years

Virus designation	Passage level	Characteristics of the virus	
"2"	$HK_{24}M_1HK_1$	ic^+ *sc s t*	Lyophilized in November 1964, initial dex $icLD_{50}$ per 0.1 ml 4.2
Reconstituted virus "64"		ic^+ $s^{\pm}/s^+$	Material reconstituted in November 1977, 3.2 dex $icLD_{50}$ per 0.1 ml 2 or 4.6 dex PFU/0.1 ml
"64" M_1	M_1	ic^+ $sts^{\pm}/s^+$ $t/t^{\pm}$	Plaques in PS cells at 41 °C of pinpoint character Titre differences at 36 and 41 °C 1.3 dex
	↓		
	M_2	ic^+ $sc^{\pm}$ $s/s^{\pm}$	Titre differences at 36 and 41 °C 2 dex
Randomly selected 3 clones (plaque diameter 2—3 mm) from the "64"M_1 virus in their M_1		ic^+ *sc* $s^{\pm}/s^+$ *t* ic^+ *sc* $s^{\pm}/s^+$ *t* ic^+ *sc* $s/s^{\pm}/s^+$ $t^{\pm}$	Titre differences (CPD_{50}) at 36 and 41 °C 1.5, 0.3 and 1.1 dex

* Parental virus for the Hy-HK28"2" virus.
For conditions of storage and other explanations see Table 4.

Characteristics of TBEV attenuated variant "2", stored for 13 years

The virus, behaving before the storage regularly as *s*, was distinctly $s^{\pm}/s^+$ when plated as reconstituted suspension. Reproduced in one M passage (during the recovery), designated 64 M_1 (Table 11), it was $s/s^{\pm}$. When examined after 5 months of storage at about −12 °C, its PFU titre was about 2 dex lower and the virus was predominantly s^+. At 41 °C it produced plaques of an uniform pp appearance. In the second M passage, a low degree of s.c. virulence for mice was observed, agains with a "prozone" phenomenon. Clonal analysis of the 64 M_1 virus revealed a marked plaque size inhomogeneity (plaque diameter 1—5 mm) and in one clone also the $t^{\pm}$ character was repeatedly established (Table 11).

Characteristics of the partially attenuated "K" variant of TBEV, stored for 14.5 years

The "K" virus underwent in 1963 eight sm passages, during which it regained the $sc^{\pm}$ trait. As such it was lyophilized and stored (Table 12). The virus titered relatively high in mice and in PS cells (6.6 dex PFU/ml). The lyophilized virus, plated as reconstituted suspension, formed an unusually high proportion of s^+ plaques (58% on the average), but was still *sc*. After one M passage, the virus (designated 63 M_1) reached almost an sc^+ character. At 41 °C, a polymorphous plaque mixture, ranging from *pp* to *s*, was noted. In its second M passage, the virus was clearly sc^+, with marked plaque in-

Table 12. Characteristics and clonal analysis of an experimentally segregated attenuated TBEV*, lyophilized in its 8th sm passage and stored for 14.5 years

Virus designation	Passage level	Characteristics of the virus	
"K"	sm8	ic^+ $sc/sc^{\pm}$ s	Lyophilized in March 1963, initial dex $icLD_{50}$/0.1 ml 7.2. Reconstituted in November 1977, dex $icLD_{50}$/0.1 ml ranged from 4.5—7
↓			
Reconstituted virus in 3 ampoules ("63")		ic^+ sc $s/s^{\pm}/s^+$	
"63"M_1	M_1	ic^+ $sc^{\pm}/sc^+$ $s/s^{\pm}/s^+$ t	Titre difference (CPD_{50}, PFU) at 36 and 41 °C 2.1 dex
↓			Plaques at 41 °C of intermediate pin-point — (s) character
	M_2	ic^+ sc^+ $s/s^{\pm}/s^+$ $t^{\pm}$	Titre difference at 36 and 41 °C 1.7 dex
Randomly selected 3 clones from plaques 1—3 mm in diameter of the "63" M_1 virus, in their M_1		ic^+ sc $s^{\pm}/s^+$ t	Titre differences at 36 and 41 °C on the average 2.2 dex
		ic^+ sc $s/s^{\pm}/s^+$ t	
		ic^+ $sc^{\pm}$ $s^{\pm}/s^+$ t	Plaque diameter 2—6 mm in $s^{\pm}/s^+$ materials

* Parental virus for the Hy-HK28"2" virus.
For conditions of storage and other explanations see Table 4.

homogeneity and increased thermoresistance ($t^{\pm}$), not observed before the storage. The heterogeneity of the 64 M_1 virus, emerging after storage and concerning the s.c. virulence for mice and plaque size (the plaque diameter reaching 6 mm) was proved by clonal analysis (Table 12).

Discussion

The molecular determinism of the unique specificity of individual viruses for certain host species as well as for the tropism for different cells within a host is poorly understood, the same as the genetic basis of attenuation. The latter is felt to be a multigenic phenomenon, related to the control of virus replication or determining specific virus-host cell interactions. Traditionally, host-range mutants, containing multiple mutations not easily amenable to genetic analysis and control, have been used the most often for live virus vaccines. The reacquisition of pathogenic potential by attenuated viruses has been frequently described (Clark, 1977). In this respect also differences in the degree of attenuation, observed in individual harvests during live virus vaccine production (Kańtoch, 1978) may be relevant. The possible increase in virulence. whether in the course of propagation or during prolonged storage of stock materials (e. g., Salk and Salk, 1977; Ilyenko, Grachev — personal communications) represent a constant concern in the live vaccine field.

One of the most challenging problems in the present study is the interpretation of the character changes, observed in attenuated variants of TBEV

when reconstituted after 8.7 — 14.5 years of storage. The plaque size approached often that of the virulent TBEV virus, its thermoresistance was clearly increased, but the $sc^{\pm}$ and $N^{\pm}$ traits were closer to those of the attenuated virus. The inhomogeneity findings seem to corroborate the concept of an eventual emergence of activity of minor subpopulation(s) originally present in the stored virus. Under non-permissive conditions, due to an overwhelming majority of attenuated particles, which in earlier studies determined the overall character of the freshly propagated virus, the minor component might not have been competent to express itself significantly in the biological systems studied, remaining thus unrecognized.

The present data neither delineated a mechanism involved in the expression of the mutants observed, nor determined whether more than one mechanism are operative. What our data showed, is:

a) a marked inhomogeneity in the material reconstituted, after a prolonged storage of an attenuated TBEV variant, concerning the $s \rightarrow s^{+}$, $t \rightarrow t^{\pm}$, $sc \rightarrow sc^{+}$ transitions, with an $N \rightarrow N^{\pm}$. Such situations were never noted in the virus passaged on specifically suitable substrates before storage;

b) a genetic dimorphism (plaque character at 41 °C) in the reconstituted stock virus, detected by cloning. In the break-through mutants it correlated probably with a shortened replication cycle (as compared to this of small-size plaque mutants), determining apparently the pathogenic effect observed; and

c) that a partial shift in the character of some genetic markers in an attenuated TBEV variant may signalize also other events, related e.g. to its neuroactivity.

Bearing in mind the limitations of the assay system available, it seems conceivable that in both virulent and attenuated TBEV variants the population heterogeneity is due to an intrinsic mutational capacity. Under specific selection pressures, as provided for the virus under study by the subadult mouse brain, the largely prevailing attenuated virions may interfere in vivo with a significant replication of minor subpopulation(s), exhibiting, e.g., a higher pathogenicity. The interference mechanism is still undefined, but it probably is unrelated to the inability to replicate at higher temperature. Attenuated TBEV virions, as already amply documented, are, in general, much less resistant to environmental influences and it appears acceptable that even their inactivation participated substantially in the observed drop in titre of the stored virus. The virulent particles are definitely more resistant to environmental stresses. Based on the present findings, it is temping to suggest that the minor components of the virus population studied had, under the conditions of storage, an advantage for survival and were thus detectable in the stored virus after reconstitution. To expand this last idea, one can imagine that the changed proportions between the major and minor components of the virus could then express in vivo also as changes in pathogenicity ("prozone" phenomena, $N^{\pm}$). The virus, recovered from brains of monkeys and showing traits different from the original attenuated virus (Hy-HK28"2"), may have resulted from a preferential enrichment of

the minor, more virulent virus component. Other possibilities of virulence regaining, as, e.g., the genetic interaction of two mutants with no or low virulence, by which more virulent recombinants may arise (Vollbracht *et al.*, 1979), could not be explored for methodological reasons. It is obviously difficult to answer the question about the mutational rate of the attenuated TBEV studied or that as to whether the changes observed were under control of separate loci. In the absence of a selective system for TBEV mutants, the question could be answered only by examining about $10^4 - 10^6$ plaque-isolated lines, considering the assumed mutation frequency of RNA viruses.

Recent evidence from oligonucleotide mapping, suggesting a high degree of mutability of RNA viruses mutability even under standard conditions of laboratory passages (Clewley *et al.*, 1977) seems to provide an additional, indirect explanation for the observations reported, although some puzzling incongruities remain. It is hoped that the data gathered so far may contribute to more rational studies of attenuation phenomena in flaviviruses.

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