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A new approach to development of live vaccine against tick-borne encephalitis

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Tick-borne encephalitis (TBE) virus, like many other arboviruses, is very stable in genetic characteristics such as neurovirulence. Extreme difficulty has been encountered in efforts to reduce its pathogenicity artificially and therefore many attempts in the U.S.S.R. to attenuate the virus and to develop a live vaccine for mass vaccination have been unsuccessful. A killed formol-vaccine has therefore been widely used since 1939 (Smorodintsev, Levkovich & Dankovsky, 1940; Smorodintsev *et al.* 1941), at first made in mouse brain and later in tissue culture (Smorodintsev & Ilyenko, 1961). Unfortunately such vaccines have to be given annually and a more effective and convenient live vaccine would be a considerable advantage if safe and immunogenic vaccine strains could be found.

Our first step in this direction was the study of the Langat (TP 21 strain) virus (Smith, 1956) because of its close antigenic relationship to TBE and absence of known natural disease caused by it in humans in Malaya. The mouse-adapted variant of Langat virus can be sharply differentiated from typical TBE virus on the basis of its inability to infect mice by intraperitoneal (IP) or subcutaneous (SC) inoculation and rhesus monkeys by intracerebral (IC) injection. The original Malayan strain grows poorly in chick embryo cells (CEC) at 40° C. and does not grow at 41·5° C. The original mouse brain virus contains a mixture of strains more or less pathogenic for mice, monkeys and man. The proportion of more virulent virus in the original mouse brain virus is very small but increases very sharply after five or more passages in CEC. Using terminal dilutions (which cause less than 50 % mortality in mice infected IC) thirty-eight pure clones of Langat virus including two virulent and thirty-six avirulent variants have been isolated from mouse-brain virus. These two main types of variant were genetically stable and were not changed in virulence by prolonged passage at 36° or 40° C. in tissue culture or by prolonged mouse-brain passage.

Table 1 compares the immunogenic activity of a standard killed TBE vaccine with live vaccines produced from variants of Langat virus. After three doses of standard formol-vaccine, 124 (84 %) of 148 susceptible persons developed neutralizing antibodies: titres in 11 % were low, in 26 % medium and in 47 % high. Live tissue-culture vaccine from the avirulent Langat strain gave much poorer immune responses than the corresponding live brain vaccine (70 % success, 30 % negative or low titres). This probably depended on the differences in virus concentration

in the vaccines: 8·0 log LD50/0·03 ml. in brain vaccine but only 5·5–6·5 log. LD50/0·03 ml. in tissue culture vaccine. With only one dose of a live tissue culture vaccine, made from virulent Langat virus, the antibody responses were best of all.

Table 1. *Neutralizing antibody responses 1½ months after vaccination with killed tick-borne encephalitis or live Langat vaccines*

Vaccine	No. of doses	No. vaccinated	Neutralization test positive		Percentage with log. neutralization index			
			Total	%	Neg. 0–2·0	Low 2·1–3·0	Medium 3·1–5·0	High >5·0
Killed tissue culture vaccine from biphasic encephalitis virus	3	148	124	84	16	11	26	47
Live brain vaccine from avirulent Langat strain	2	76	67	88	12	22	20	46
Live tissue culture vaccine from avirulent Langat strain	2	66	46	70	30	45	25	0
Live tissue culture vaccine from virulent Langat strain	1	62	60	97	3	3	32	62

Table 2. *Changes in virus neutralizing antibody titres 1½ months and 3 years after immunization of volunteers by killed biphasic or live Langat vaccine*

Vaccine	No. of doses	Log. LD50 per dose	No. vaccinated	Time after vaccination (months)	Number with log. neutralization index			
					Neg. 0–2·0	Low 2·1–3·0	Medium 3·1–5·0	High >5·0
Killed tissue culture vaccine from biphasic virus	3	9·0	24	1½	0	0	2	22
				36	17	6	1	0
Live brain vaccine from avirulent Langat virus	2	9·0	34	1½	0	10	9	15
				36	4	14	11	5
Live tissue culture vaccine from avirulent Langat virus	2	5·5–6·5	23	1½	—	8	15	0
				36	6	6	11	0

Table 2 shows the result of repeat tests 3 years after the immunization with killed and live avirulent vaccine, on those volunteers who had shown a distinct serological conversion 1½ months after vaccination. Volunteers vaccinated with killed vaccine showed a pronounced fall in antibody level, while those immunized with live brain or tissue culture vaccine showed more stable levels. In the first group (three doses killed vaccine) 17 (71 %) of 24 originally seropositive persons became negative; in the second group (two doses live brain vaccine) only 4 (12 %) of 34, and in the third group (two doses live tissue culture vaccine), only 6 (26 %) of 23 became negative. Thus immunity lasts longer after two doses of avirulent live vaccines than after three doses of formol-vaccine.

Studies of more than 1000 people vaccinated with the avirulent clone of Langat strain show that it is safe and does not provoke any general clinical or neurological reactions. Vaccination was not followed by viraemia except in a few subjects

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during the first few days. Tissue culture vaccine from an avirulent variant of Langat strain is thus very promising as a safe and more potent substitute for killed formalinized vaccine.

However, as the immunizing activity of this avirulent variant of the Langat strain is limited, especially after one dose, a vaccine strain with higher immunogenic potency is desirable. Naturally attenuated strains were therefore sought among TBE viruses isolated directly in tissue culture from the ticks *Ixodes persulcatus* or *I. ricinus*. Such strains were isolated in West Siberia and Kirgiz Republic, where human disease is rare but infection of man by ticks is very intensive.

Pronounced differences are known between the severity of the disease and the mortality rate from the paralytic form of TBE found in the Far East, and the milder form of disease found in the European part of the U.S.S.R., as well as between the paralytic and biphasic forms of the disease observed in U.S.S.R. and other European countries.

Widely different biological variants of TBE virus can be isolated from infected ticks but not from the brains of patients. Among the tick strains a group has been found which are only slightly pathogenic for mice by SC inoculation and non-pathogenic for rhesus monkeys by IC inoculation. Strains of this type have not been isolated from clinical cases of TBE whether of the severe paralytic or the much more benign biphasic meningoencephalitis form.

So far seventy-seven strains have been studied of the tick-borne and biphasic meningoencephalitis viruses isolated from various sources in different geographical zones from the Far East to Austria, and eight strains non-pathogenic for rhesus monkeys by IC inoculation have been isolated in Western Siberia (Isetsk district of the Tyumen region), in Kirghizia (Kamenetak district), and in European U.S.S.R. (Novgorod and Vologodsk regions). All these latter areas have high infection rates in ticks but very little clinical tick-borne encephalitis—an average of 10 % of the individual larval ticks were infected. These non-pathogenic strains cause little or no disease in monkeys and complete recovery is the rule. Besides these, strains were also recovered which caused persistent brain infections in monkeys and which frequently provoked chronic or progressive disease similar to the rarely observed chronic form in human beings. These strains may provide a laboratory model in monkeys for the study of the pathogenesis of severe complications of TBE in man.

Strains from an area with a high infection rate but a low level of tick-borne encephalitis morbidity in the subzone of aspen and birch woods of Western Siberia (Tyumen region) were compared with TBE strains from foci with a high morbidity (Yashkin district of the Kemerovo region) of typical TBE.

MATERIALS AND METHODS

Thirteen strains from Western Siberia were selected for detailed study (all from ticks except one from blood of a healthy human being): 4 from Kemerovo (K 1-1 to K 1-4) and 1 from Novorsibirsk (N 1-1); and 8 from Tyumen (T 2-1 to T 2-5, T 3-1 to T 3-3). T 3-1 is the Elantsev strain (from human blood), T 3-2 the Isetsk-237 strain and T 3-3 the Isetsk-9 strain.

These strains, which had been stored at −20° C. in mouse brain for not more than 2–3 days, were each inoculated into mice and CEF cultures. Their pathogenicity was studied in mice of 10–12 g. and in rhesus monkeys. Mouse brains were removed when symptoms appeared. For titrations mice were inoculated with volumes of 0·03 ml. IC and 0·25 ml. SC. Monkeys were inoculated IC with 0·5 ml. CEF tubes were inoculated with 3·0 log. LD 50/0·03 ml. virus, then incubated at 30°, 36° or 40° C. During 5 days, at intervals of 24 hr., 7–10 tubes were taken from each incubator and the pooled culture fluid titrated IC in mice.

RESULTS

As a result of these tests the strains could be classified into three pathogenicity groups: (I) strains with a high extraneural (SC) pathogenicity for mice and a high neurovirulence for monkeys; (II) strains with a high extraneural pathogenicity for

Table 3. *The pathogenic activity of various strains of tick-borne encephalitis virus tested in mice*

		LD 50 ± S.E.			
		Intracerebral Inoculation		Subcutaneous inoculation	
Pathogenicity group	Strain	Freshly isolated	Mouse brain adapted	Freshly isolated	Mouse brain adapted
I (Kemerovo)	K 1-1	9·5 ± 0·2	9·4 ± 0·13	7·5 ± 0·32	7·5 ± 0·2
	K 1-2	8·8 ± 0·34	8·7 ± 0·19	5·0 ± 0·3	5·1 ± 0·2
	K 1-3	9·6 ± 0·23	9·6 ± 0·1	6·5 ± 0·39	6·6 ± 0·1
	K 1-4	9·5 ± 0·3	9·5 ± 0·12	6·0 ± 0·2	6·1 ± 0·2
	N 1-1	9·2 ± 0·3	9·3 ± 0·2	7·6 ± 0·35	7·6 ± 0·2
	Mean	8·3 ± 0·08	9·3 ± 0·08	6·5 ± 0·23	6·6 ± 0·2
II (Tyumen)	T 2-1	6·1 ± 0·3	9·0 ± 0·12	4·2 ± 0·21	5·3 ± 0·1
	T 2-2	6·3 ± 0·2	9·3 ± 0·9	6·6 ± 0·28	6·8 ± 0·19
	T 2-2(?)	7·0 ± 0·24	9·1 ± 0·11	4·3 ± 0·3	6·1 ± 0·19
	T 2-5	6·5 ± 0·21	8·9 ± 0·13	4·7 ± 0·3	5·9 ± 0·17
	Mean	6·9 ± 0·58	9·0 ± 0·04	4·9 ± 0·19	5·8 ± 0·15
III (Tyumen)	T 3-1	8·6 ± 0·3	7·8 ± 0·08	2·0 ± 0·19	2·2 ± 0·25
	T 3-2	8·0 ± 0·3	8·2 ± 0·1	2·2 ± 0·21	2·2 ± 0·27
	T 3-3	8·3 ± 0·25	8·5 ± 0·13	2·4 ± 0·22	2·3 ± 0·2
	Mean	8·2 ± 0·13	8·3 ± 0·1	2·2 ± 0·13	2·3 ± 0·16

mice, but with irregular monkey mortality, and (III) strains with a low extraneural virulence for mice and causing no clinical symptoms after IC inoculation of monkeys. In addition growth indices in CEF cultures at optimal (36°), above optimal (40°) and below optimal (30° C.) temperatures were used for classification.

The SC susceptibility of mice was one of the important and stable properties for differentiation (Table 3). After adaptation to mouse brain, viruses in groups I and II had not only a high IC but also high SC virulence. By these markers strains of groups I and II could not be differentiated either from one another or from the

most typical highly pathogenic strains isolated from the brains of paralysed patients in the Far East. The degree of SC virulence distinguished the strains in group III. After mouse adaptation group III strains still had a very stable low

Table 4. *Clinical symptoms in rhesus monkeys infected intracerebrally by viruses of different neurovirulence*

'atho-nicity roup	Strain	The doses (log LD 50/ml.)	Clinical symptoms	Viraemia	Result
I	K 1-1	7·5	Paralysis of limbs	+	Death
	K 1-2	7·0	Paralysis of limbs	+	Death
	K 1-3	7·6	Paralysis of limbs	+	Death
	K 1-4	7·5	Paralysis of limbs	+	Death
	N 1-1	7·2	Paralysis of limbs	+	Death
II	T 2-1	8·4	Ataxia	+	Death
		9·7	Ataxia	±	Death
	T 2-2	7·5	Ataxia		Recovery
	T 2-3	9·7	Ataxia	±	Death
		8·0	Ataxia	+	Death
		5·0	Ataxia	+	Death
		8·5	Ataxia	+	Recovery
		3·0	Nil	+	No illness
	T 2-4	6·8	Ataxia, left hemiparesis		Death
		8·7	Ataxia, paraplegia	+	Recovery
	T 2-5	8·2	Ataxia, paraparesis left leg	+	Recovery
		8·5	Ataxia	+	Recovery
III	T 3-1	3·9	Nil	±	No illness
		6·9	Nil	−	No illness
		7·5	Nil	−	No illness
		7·7	Nil	±	No illness
		8·4	Nil	±	No illness
	T 3-2	3·5	Nil	±	No illness
		6·8	Nil	±	No illness
		7·7	Nil	−	No illness
		8·4	Nil	−	No illness

Table 5. *Final titres in primary chick embryo cultures at* 40°, 30° *and* 36° C.

Patho-genicity group	Strain	40° C.	30° C.	36° C.
I	K 1-1	5·8 ± 0·51	5·9 ± 0·42	5·9 ± 0·49
	K 1-2	5·7 ± 0·52	5·6 ± 0·43	5·9 ± 0·46
	K 1-3	6·2 ± 0·4	5·3 ± 0·41	6·2 ± 0·39
	K 1-4	6·2 ± 0·37	6·0 ± 0·42	6·3 ± 0·58
	N 1-1	5·9 ± 0·4	5·9 ± 0·46	5·9 ± 0·72
	Mean	5·9 ± 0·2	5·8 ± 0·21	6·0 ± 0·23
III	T 3-1	0·7 ± 0·29	4·5 ± 0·25	4·5 ± 0·43
	T 3-2	0·04 ± 0·003	5·0 ± 0·5	4·4 ± 0·21
	T 3-3	0·2 ± 0·09	3·5 ± 0·33	3·3 ± 0·36
	Mean	0·3 ± 0·09	4·3 ± 0·24	4·1 ± 0·24

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SC virulence for mice. The difference between the IC LD 50 and SC LD 50 was more than 5·0 log.

A still more important marker for the differentiation of the tick-borne encephalitis virus into three different biological groups was provided by IC inoculation of monkeys (Table 4). Group I strains did not differ from 'typical' highly pathogenic strains and caused atrophic paralysis with an intense and regular viraemia lasting until death in all, in 7 days or less. Group II strains characteristically caused cerebellar symptoms in the infected monkeys (ataxia, in single cases an intention tremor and nystagmus). Some of the group II strains (T 2-4, T 2-5) also caused limb paralysis. Most monkeys infected with group II viruses also had a stable viraemia lasting 7–10 days. However, group II strains caused death of only about half of the infected animals, the rest recovering completely or with sequelae.

In monkeys infected with large amounts (6·8–8·4 log. LD 50) of group III strains no noticeable pathological changes were found in the nervous system. The only reaction was a short-term temperature rise. In many cases viraemia was absent.

Table 6. *Classification and basic properties of the various strains of tick-borne encephalitis virus isolated in West Siberia*

Pathogenicity group	Strain	Subcutaneous mouse LD 50	Intracerebral susceptibility of monkeys			Maximum titre on the chick-embryo cultures at 40°C.
			Clinical	Viraemia	Results	
I (Kemerovo)	K 1-1	7·5 ± 0·23	Paralysis	+	Death	5·8 ± 0·51
	K 1-2	5·1 ± 0·29	Paralysis	+	Death	5·7 ± 0·52
	K 1-3	6·6 ± 0·14	Paralysis	+	Death	6·2 ± 0·4
	N 1-1	7·6 ± 0·25	Paralysis	+	Death	5·9 ± 0·4
	Mean	6·6 ± 0·22	Paralysis	+	Death	5·9 ± 0·2
II (Tyumen)	T 2-1	5·3 ± 0·14	Ataxia	±	Death irreg.	3·5 ± 0·27
	T 2-2	6·8 ± 0·19	Ataxia	±	Death irreg.	2·6 ± 0·06
	T 2-3	5·2 ± 0·14	Ataxia	+	Death irreg.	3·1 ± 0·08
	T 2-4	6·1 ± 0·19	Ataxia paresis	+	Death irreg.	2·7 ± 0·46
	T 2-5	5·9 ± 0·17	Ataxia paresis	±	Death irreg.	3·1 ± 0·2
	Mean	5·8 ± 0·15	Ataxia	+	Death irreg.	2·9 ± 0·2
III (Tyumen)	T 3-1	2·2 ± 0·25	No illness	–	No illness	0·4 ± 0·29
	T 3-2	2·2 ± 0·27	No illness	–	No illness	0·04 ± 0·003
	T 3-3	2·3 ± 0·2	No illness	–	No illness	0·2 ± 0·09
	Mean	2·3 ± 0·16	No illness	–	No illness	0·3 ± 0·09

In CEF cultures (Table 5), all the group I strains grew at 40° C. more intensively (average titres 5·9 ± 0·2 log. LD 50/0·03 ml.) than the strains of group III. Group III strains differed essentially in that they either failed to multiply or grew very poorly (average titre 0·3 ± 0·09 log. LD 50/0·03 ml.) (Table 6). At 30° and 36° C. group I strains multiplied as actively as at 40° while group II strains multiplied better at 30° and 36° than at 40° C.

The correlation between neuro-virulence in mice and monkeys with the ability

of the same strains to grow at 40° C. indicates the importance of growth at this temperature as a genetic marker of virulence for susceptible animals. The neurovirulence marker of the TBE virus seemed to be related to the ability to multiply under unfavourable conditions of raised temperature.

SUMMARY

Investigations of the biological properties of tick-borne encephalitis viruses immediately after isolation from naturally infected *Ixodes persulcatus* from Western Siberia has revealed their different pathogenicity for monkeys and mice. Strains highly pathogenic for mice on extraneural inoculation and for rhesus monkeys on intracerebral inoculation have been isolated, but also strains with a low extraneural pathogenicity for mice and a complete non-pathogenicity or a mild pathogenicity for monkeys on intracerebral inoculation.

The viruses have been classified into three main groups by their virulence for mice and monkeys, and their ability to grow in tissue culture at 40° C. and the findings are summarized in Table 6. Group I included viruses highly virulent for mice by any route of inoculation, which caused fatal paralytic disease in monkeys, and which grew well in chick embryo culture at 40° C.

Group II strains also had a high virulence for mice, but in monkeys caused milder disease with a peculiar clinical course indicative of primary damage to the cerebellum. About half of the infected monkeys recovered completely or with mild sequelae (ataxia, pareses of the limbs). This group grew moderately well in chick embryo cultures at 40° C. Groups I and III differed essentially in their virulence for mice by subcutaneous inoculation, and virulence for monkeys decreased distinctly with transition from group I to group III. Group III strains showed a lower reproduction rate at 40° C. in chick embryo culture than group I. Groups I and II showed good correlation between extraneural pathogenicity for mice, pathogenicity for monkeys by IC inoculation, and growth rate in chick embryo cultures at 40° C.

Group I strains were isolated chiefly from foci with a high TBE morbidity and the strains of groups II and III in foci with sporadic cases. It is, however, highly probable that in any tick-borne encephalitis focus several variant strains of differing virulence exist.

Recent experience of vaccinating volunteers with naturally attenuated group III strains suggests that safe and effective live vaccines can be developed against tick-borne encephalitis. Vaccines against many other arbovirus diseases can probably also be derived from naturally occurring strains of virus isolated from arthropods in areas of low disease but high infection rate. This approach looks more promising than the usual approach of attempting to develop attenuated strains from highly virulent strains.

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