

VIRUS MENINGO-ENCEPHALITIS IN SLOVENIA

4. Isolation of the Virus from the Ticks "Ixodes ricinus" ^a

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SYNOPSIS

This article describes the steps taken to verify that the tick *Ixodes ricinus* is the main vector of Slovenian meningo-encephalitis.

In 1954, several unsuccessful attempts were made to isolate the virus from unengorged ticks collected at random, but in the following year this system was abandoned, and instead the search for specimens was carried out on the basis of evidence supplied by meningo-encephalitis patients with a tick-bite case history.

In June—the peak endemic period—140 unengorged ticks were collected from a part of a forest in central Slovenia where one of these patients had been working. The ticks were washed in alcohol, finely ground, and centrifuged, and the supernatant was inoculated intracerebrally into suckling mice, and intraperitoneally into adult mice and guinea-pigs. The infected suckling mice died between the fourteenth and eighteenth day after inoculation; a suspension of their brains was inoculated intracerebrally into a further batch of suckling and adult mice, which subsequently fell ill, showing signs in the central nervous system.

The virus strain obtained was subjected to neutralization and complement-fixation tests, in order to identify the infective agent.

Since 1946, a special form of meningitis of unknown origin has been observed in Slovenia, where occasional epidemic outbreaks have occurred. In 1953, during one such outbreak, a detailed study of 208 cases was undertaken. The epidemiological and clinical characteristics were analysed,^{1, 2} and a virus belonging to the group of arthropod-borne encephalitides was isolated.⁴ The agent was found to be indistinguishable from the agents called Russian spring-summer encephalitis virus, Czech encephalitis virus, Austrian encephalitis virus, or louping-ill viruses, which are believed at the present time to be strains of the same agent.³

The disease, which is endemic in the central part of Slovenia, is markedly seasonal, in that 95% of the cases occur during May-September. From

^a This work was sponsored by the Commission for Medical Research of the Secretary of State for Defence, Yugoslavia, and is the fourth of a series of studies (see references 1, 2 and 4).

the tick bites which were present in a very considerable number of cases, it could be seen that the tick *Ixodes ricinus* was the vector generally responsible for spreading the infection. The majority of the cases were found in rural districts, and only 9.1% occurred in town dwellers. Most of the patients were middle-aged, young children and old people being very little affected. No spread of infection from person to person was observed.

Epidemiological Approach

In 1954, several attempts were made to isolate the causative agent of meningo-encephalitis from the tick *Ixodes ricinus*, specimens of which were collected in woods near the villages where repeated cases had occurred. In order to collect the ticks, squares of linen or gauze, 50 cm × 50 cm, were drawn over the grass and low vegetation. Any ticks adhering to the fabric were dropped into special flasks, 3 cm in diameter, through a glass tube fixed in the centre of the cork stopper. The ticks were then identified and sorted in the laboratory.

In attempts to isolate the virus, only unengorged ticks were used, the specimens collected from animals being employed solely for entomological investigation.^b

During 1954, we tried six times to isolate the virus from the ticks thus collected. In these unsuccessful attempts about 5000 ticks were examined. In the following year, however, we stopped collecting ticks at random and, instead, selected typical cases of meningo-encephalitis, with an obvious tick-bite history, which had been admitted to the hospital in Ljubljana. On the basis of data collected from the patients, their families and the neighbourhood, we managed to establish the place and date of infection in each case. At the places in question, we collected all the ticks that we could find. In June, when infestation of the endemic area is at its height,^c we made two unsuccessful attempts to isolate the virus from 109 ticks collected in one place and 90 gathered from another source. However, a third attempt, which followed up evidence supplied by yet another patient (P. M.), proved to be successful.

On 24 June 1955, P. M., 31 years of age, a signalman employed at Verd railway station, was admitted to the Infectious Diseases Clinic in Ljubljana. Ever since 1946, repeated cases of meningo-encephalitis had been reported in Verd and neighbouring villages. P. M. fell ill for the first time on 6 June,

^b To prove that meningo-encephalitis was transmitted by *Ixodes ricinus* ticks, it was necessary to isolate the virus from unengorged specimens, since, on the one hand, the blood of an animal upon which the ticks had fed might have contained the virus, and, on the other, the virus present in the tick might have been neutralized by the antibodies in the blood of an immune animal, in which case the attempt at isolation would have had negative results.

In view of the fact that the ticks suck blood only once during each developmental stage, the isolation of the virus from unengorged ticks demonstrates that it is passed on from stage to stage. Most probably, as is the case in Russian spring-summer encephalitis and in the tick-borne rickettsial diseases, some infected female ticks transmit the virus through their eggs to the larvae of the next generation.

^c Kmet & Tovornik, unpublished observations

with malaise and fever that lasted 6 days. During the next 9 days there were no signs of illness, but on 22 June he developed typical symptoms of meningo-encephalitis. When tested with antigen prepared from the tick strain, the paired sera of the patient showed an eightfold increase of titre after 6 months.

P. M. had frequently worked in the woodlands situated close to the station and had often been bitten by ticks. The last occasion, before the onset of the illness, was on 27 or 28 May, in the evening of which day he found 4 ticks on his body.

On 5 July, we collected 140 ticks from that part of the forest where P. M. had been working. Of these, 18 were males, 16 were females and 108 were nymphs. All the ticks belonged to the species *Ixodes ricinus*.

Isolation and Identification of the Virus

After being washed in a 75% alcohol solution, the ticks were finely ground in a mortar. The material thus prepared was centrifuged for one hour at 3000 r.p.m. The clear supernatant was used to inoculate suckling mice by the intracerebral route, and adult mice and a pair of guinea-pigs by the intraperitoneal route.

The infected suckling mice became ill, displaying signs in the central nervous system, and died between the fourteenth and eighteenth day after inoculation. The adult mice and guinea-pigs showed no overt signs of illness during the same period. When inoculated with a further intracerebral passage of a pool of infected suckling-mouse brains, suckling and adult mice produced the same pattern of pathogenicity which had been observed in the first batch of infected suckling mice. The incubation period remained stable in all subsequent passages. The strain obtained, which was preserved in a 50% glycerol-distilled water solution, could be passed without difficulty through mice, both suckling and adult.

Neutralization and complement-fixation tests were used to identify the infective agent.

Neutralization tests on mice

Adult mice were inoculated intraperitoneally with 10^{-1} through 10^{-9} dilutions of brain suspension from the fourth suckling-mouse passage. In this way, the log titre of the virus was established as 7.3.

The chief object of this work being to demonstrate the existence of a relationship between our tick virus and other strains causing meningo-encephalitis in Slovenia, which had been isolated in our laboratory in 1954 and 1955, immune hamster sera prepared with our earlier strains and with a louping-ill strain isolated in Scotland were used to neutralize the new strain in mice.

TABLE I. CROSS-NEUTRALIZATION REACTIONS WITH VARIOUS ENCEPHALITIS SERA AND VIRUSES *

Immune hamster serum	Log of neutralized homologous virus	Tick virus	
		Log titre of virus	Log of virus neutralization by antiserum
Normal (control)	—	7.3	—
Slovenian virus	MF/54	2.1	5.2
	NF/55	1.5	5.8
	ZI/55	1.6	5.7
Louping-ill virus	6.9	1.6	5.7

* MF strain isolated from brain (for method of isolation and identification, see *Schweiz. Z. allg. Path. Bakt.*, 1956, 19, No. 2, p. 184); NF strain isolated from cerebrospinal fluid in the second stage of meningo-encephalitis; ZI strain isolated from brain.

Complement-fixation tests

A 20% brain suspension in saline from the fourth suckling-mouse passage was centrifuged for 2 hours at 4000 r.p.m. The complement-fixing antigens thus prepared were tested against tick-immune hamster serum and hamster serum immune to the Slovenian virus of meningo-encephalitis. As the complement-fixation tests on human beings had shown some cross reactions with lymphocytic chorio-meningitis antigens, an immune LCM guinea-pig serum was also included in the test. The results are shown in Table II.

TABLE II. CROSS-IMMUNITY COMPLEMENT-FIXATION TESTS WITH VARIOUS STRAINS OF VIRUS MENINGO-ENCEPHALITIS

Serum	Infected adult mouse			Infected suckling mouse			Normal mouse		Serum control
	1/2	1/4	1/8	1/2	1/4	1/8	1/2	1/4	
Normal hamster	0	0	0	0	0	0	0	0	0
Normal guinea-pig	1	1	0	0	0	0	0	0	0
Slovenia immune * (8 units)	4	3	1	4	4	4	0	0	0
Tick immune (8 units)	4	4	tr**	4	4	4	0	0	0
LCM immune guinea-pig (8 units)	2	1	0	2	tr**	0	0	0	0

* Serum prepared using MF strain

** tr = trace

Conclusion

The data discussed in this paper, combined with the epidemiological and virological evidence presented in the previous three articles of the series,^{1, 2, 4} demonstrate that the tick *Ixodes ricinus* is the main vector of meningo-encephalitis in Slovenia.

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RÉSUMÉ

Après des essais infructueux d'isolement du virus de la méningo-encéphalite de Slovénie à partir de tiques *Ixodes ricinus* récoltées au hasard, les auteurs ont abordé le problème par une voie nouvelle. Ils ont récolté des tiques dans des endroits où des malades avaient contracté l'infection, selon toute évidence à la suite de piqûres de tiques.

En 1955, 140 exemplaires d'*I. ricinus* ont été récoltés dans une forêt où avait fréquemment travaillé un employé, depuis lors atteint de méningo-encéphalite. Les tiques, lavées à l'alcool, ont été broyées; le broyat a été centrifugé et le liquide surnageant inoculé, par voie intra-cérébrale, à des souris. Ceux-ci moururent d'une atteinte du système nerveux central. Des passages successifs du matériel infectant provenant des cerveaux de ces souris ont provoqué la mort de souris jeunes et adultes. Des tests de neutralisation sur souris et de fixation du complément ont montré qu'il s'agissait bien du virus de la méningo-encéphalite. *I. ricinus* peut donc être considéré comme le vecteur principal de cette maladie en Slovénie.

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