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LONGITUDINAL STUDY OF NATURAL FOCI OF CENTRAL EUROPEAN ENCEPHALITIS VIRUS IN WEST SLOVAKIA

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Summary. — A total of 2922 small terrestrial mammals of 12 species were collected in six localities of West Slovakia between 1981 and 1986. When examined for the prevalence of neutralizing antibodies (NA) to Central European encephalitis (CEE) virus we found that 14.6% had antibody. Nearly all (97%) of the 426 animals with antibody were *Clethrionomys glareolus*, the most abundant species (52.6% of mammals collected, 15.1% of those with antibody), *Apodemus flavicollis* (22.5% of mammals collected, 18.1% of those with antibody), *Apodemus sylvaticus* (14% of mammals collected, 8.5% of those with antibody), and *Microtus arvalis* (5.5% of mammals collected, 3.3% of those with antibody). In all locations studies the most abundant tick found on small mammals was *Ixodes ricinus* (larvae and nymphs). Less abundant, but present in all studied sites, were larvae and nymphs of *Dermacentor reticulatus* and *Haemaphysalis concinna*. Six strains of CEE virus were isolated from tissues of animals: four from *Clethryonomys glareolus* and one each from *Apodemus flavicollis* and *Sorex araneus*. Three of six isolates were from animals collected in February; none of the six had detectable neutralizing antibody to CEE virus. We discuss these observations with regard to possible mechanisms of persistence of CEE virus.

Key words: *Central European encephalitis virus*; *tick-borne encephalitis*; *ticks*; *terrestrial mammals*, *serologic survey*; *virus isolation*; *West Slovakia*

Introduction

The clinical entity tick-borne encephalitis (TBE) is caused by viruses belonging to the family Flaviviridae, genus *Flavivirus* (Westaway *et al.*, 1985). In the eastern Soviet Union the aetiologic agent of this disease is Russian Spring-Summer encephalitis virus, from the western Soviet Union to areas of Europe where TBE occurs the aetiologic agent is Central European encephalitis (CEE) virus (Calisher, 1988). Each year in these areas,

thousands of human infections occur, some with severe but most with mild to severe syndromes. Presence of virus is limited by the presence of abundant and competent tick vectors and competent vertebrate hosts. Disease foci occur in various naturally-occurring or man-made ecosystems. Numerous foci of CEE in Czechoslovakia are found in three geographically different regions: Herzynic, in Northern Moravia and Bohemia; Carpathian, formed by Carpathian Mountains and adjacent territories and covering most of Slovakia; and Pannonian, occupying most of the southern part of Slovakia (Grešíková and Calisher, 1988).

The study area included contiguous portions of Carpathian and Pannonian regions. These study sites were selected because CEE virus had been isolated from the brains of small mammals and NA to CEE virus were detected in serum samples from small mammals in the Danubian Lowland (Bárdoš, 1957). Also, NA were detected in serum samples from birds, game and pastured cattle in Záhorská Nížina lowland (Ernek *et al.*, 1975, 1977; Kožuch *et al.*, 1976) and haemagglutination-inhibiting (HI) antibodies to CEE virus were found in serum samples from humans (Grešíková *et al.*, 1973). Epidemiologic analyses had been consistent with these findings (Krajčír, 1972).

Six study sites situated in West Slovakia were selected for long term studies of CEE virus activity because antibodies to this virus have been found there previously and because they have ecologies typical of those in which CEE virus has been found. Two localities (Šaštínske Stráže and Kuklov) are situated in Záhorská Nížina (Carpathian region), one (Plavecký Mikuláš) is in the Little Carpathian mountains (Carpathian region), and three (Gabčíkovo, Jahodná, and Gbelce) in Danubian Lowland (Pannonian region).

Materials and Methods

Small terrestrial mammals were live-trapped in Swedish bridge metal traps, using oat flakes as bait. After transportation to the laboratory in Bratislava, blood for serological examination was taken from the orbital sinus using capillary pipettes and serum samples were separated from the clotted blood and stored at −20 °C. Sacrificed animals were necropsied and their organs (brains, lungs, livers and spleens) were collected aseptically. When infestation by ticks was observed, tick species were identified and the prevalence and intensity of infestation was calculated.

Serum samples were tested for the presence of NA by mixing equal 0.1 ml volumes of diluted serum and virus containing 200 Tissue Culture Infectious $Doses_{50}$ ($TCID_{50}$) of CEE virus (strain Hypr), incubating the mixtures at 37 °C for 1 hr and assaying for non-neutralized virus by inoculating PK (porcine kidney) cells (Kožuch and Mayer, 1975) with 0.1 ml of each mixture, such that the final serum dilution was 1 : 4 or greater and the final virus dose was 100 $TCID_{50}$.

Ten per cent suspensions of brains and lungs/organs pools were made in 2 ml of Minimal Essential Medium (MEM) containing 10% heat-inactivated newborn bovine serum. Suspensions were clarified by low-speed centrifugation (3,000 rev/min) for 15 min and the supernatant fluids were inoculated without further dilution. Each suspension was inoculated intracranially (i.c.) into five 1- to 4-day-old mice, 0.01 ml per mouse. Viruses were identified by a constant serum-virus dilution technique. Three-week-old albino mice inoculated intracranially with virus-serum mixtures were used as the indicator system. Hyperimmune goat serum prepared with Hypr strain of CEE virus was used for all identification tests; this serum has a log Neutralization Index of 4.0 (i.e. neutralizes 10 000 3-week-old mouse 50% lethal doses).

Virus isolates recovered from organ tissues were identified by virus neutralization tests using adult albino mice inoculated intracranially with mixtures of virus (tenfold dilutions) and hyperimmune goat serum (undiluted).

The two localities in Záhorská Nížina, Šaštínske Stráže, and Kuklov are situated Northeast and Northwest, respectively, of the town of Šaštín-Stráže. The former is an abandoned brickfield with underlying loess, brown earth, and growth of untended fruit trees, shrubs, and weeds. Kuklov lies in a depression within a pine (*Pinus nigra*) forest, with underlying sands blown on Neogene clays, brown earth, and a stand of alder (*Alnus glutinosa*) and isolated pines. Both localities have a warm, moderately dry climate with moderate winters (mean annual temperature 9.6 °C, total annual moisture 610 mm).

Plavecký Mikuláš is situated in a valley leading North from the Small Carpathian mountains. The underlying foundation is formed of Triassic limestone covered with brown earth and stands of typical Small Carpathian beech (*Fagus sylvatica*) trees. The climate is moderately warm and humid, with moderate winters (mean annual temperature 8.6 °C, total annual moisture 760 mm).

Gabčíkovo and Jahodná (Donube Lowland) are situated near communities of the same names, respectively, on recently formed, sandy clay sediments of the Danube River, with alluvial soils and mixed poplar-willow (*Populus* and *Salix* species) woods. Both localities lie on the banks of branches of the river. The climate is warm and dry, with moderate winter (mean annual temperature 9.7 °C, total moisture 570 mm). Gbelce, situated north of the community of the same name, is hilly land, with underlying Neogene, brown earth, and oak (*Quercus species*) stands. The climate is warm and dry, with moderate winter (mean annual temperature 10.4 °C, total annual moisture 566 mm).

Results

Neutralizing (N) antibodies

A total of 2922 small terrestrial mammals of 12 species were collected during 1981—1986; 14.6% of them had neutralizing antibody to CEE virus (Tables 1 and 2). Antibody prevalence in small mammals was as follows (in parenthesis are the lowest and highest rates in individual years): Šaštínske Stráže — 11.9% (6.6—18.1%), Kuklov — 15.6% (10.9—19.3%), Plavecký

Table 1. Neutralizing antibody to Central European encephalitis virus in terrestrial mammals, by location, West Slovakia, 1981—1986

Location	Number with antibody/ Number tested	Percent
Záhorská Nížina		
Šaštínske Stráže	77/648	11.9
Kuklov	63/404	15.6
Little Carpathians		
Plavecký Mikuláš	43/263	16.3
Danubian Lowland		
Gabčíkovo	125/768	16.3
Jahodná	80/569	14.1
Gbelce	38/270	14.1
Total	426/2922	14.6

Table 2. Neutralizing antibody to Central European encephalitis virus in terrestrial mammals by species, West Slovakia, 1981—1986

Species	Number with antibody/ Number tested	Percent
Apodemus flavicollis	130/717	18.1
Apodemus sylvaticus	36/408	8.8
Apodemus microps	0/9	0
Mus musculus	2/7	28.6
Micromys minutus	2/21	9.5
Clethrionomys glareolus	233/1538	15.1
Pitymys subterraneus	4/28	14.3
Microtus arvalis	14/161	8.7
Microtus oeconomus	0/2	
Glis glis	1/1	
Sorex araneus	4/29	13.8
Crocidura leucodon	0/1	
Total	426/2922	14.6

Mikuláš — 16.3% (0—23.0%), Gabčíkovo — 16.3% (4.1—23.9%), Jahodná — 14.1% (6.9—19.3%), and Gbelce — 14.1% (8.3—25.0%).

The most abundant rodent species were: *Apodemus flavicollis* (22.5% of 2 922 mammals), *A. sylvaticus* (14.0%), *Clethrionomys glareolus* (52.9%), and *Microtus arvalis* (5.5%). These four species represent 94.9% of all mammals collected. Nearly all (97.0%) of 426 animals with antibody to CEE virus were of the aforementioned species (30.5% of *A. flavicollis*, 8.5% of *A. sylvaticus*, 54.7% of *C. glareolus*, and 3.3% of *M. arvalis*). Prevalence of neutralizing antibody to CEE virus was 18.1% in *A. flavicollis* (range 9.1 to 19.6% in different years), 8.8% in *A. sylvaticus* (range from 5.5 to 23.8%), 15.1% in *C. glareolus* (range 10.8—20.8%), and 14.3% in *M. arvalis* (range 0—25.0%). For the two most abundant species. *A. flavicollis* and *C. glareo-*

Table 3. Prevalence of neutralizing antibody to Central European encephalitis virus in Apodemus flavicollis and Clethrionomys glareolus by three-month period, six West Slovakian study sites, 1981—1986[a]

Months	*Apodemus flavicollis*		*Clethrionomys glareolus*	
	Adult	Sabadult	Adult	Subadult
March-May	20.8	25.0	18.2	14.4
June-August	24.5	19.8	17.6	18.0
September-November	11.0	18.0	15.1	9.8
December-February	19.8	9.1	8.3	13.7

[a] Numbers signify percentages of animals with antibody to virus.

Table 4. Central European encephalitis virus isolates from mammals collected in Slovakia, 1981—1986

Isolate	Species	Locality	Month, year	Tissue
3571	*C. glareolus*	P. Mikuláš	February, 1981	brain
3573	*A. flavicollis*	P. Mikuláš	February, 1981	brain
3843	*C. glareolus*	P. Mikuláš	June, 1981	brain
3892	*C. glareolus*	Gabčíkovo	July, 1981	brain
4387	*C. glareolus*	Gbelce	February, 1982	lung-liver
5590	*S. araneus*	Jahodná	June, 1984	brain

lus antibody prevalence differed according to different seasons of the year and relative age of the animal. Nearly half (47.7%) of *A. flavicollis* with antibody to CEE virus (24.5% adults, 19.8% subadults and juveniles) and more than one third (36.8%) of *C. glareolus* with antibody to this virus (17.6% adults, 18.0% subadults and juveniles) were collected in the summer (Table 3). Titres of neutralizing antibodies ranged from 1 : 4 to 1 : 32.

Virus isolation

Six strains of CEE virus were isolated and reisolated, four from *C. glareolus*, and one each from *A. flavicollis*, and *Sorex araneus*; five were from brains and one was from a lung-spleen pool. Three from the six isolates were from animals collected in February (Table 4). None of these animals had neutralizing antibody when virus was isolated from them.

Table 5. Relationships between prevalence and intensity of tick infestations of small mammals, numbers of mammals captured, and antibody prevalence in these mammals

Index	Year of collection					
	1981	1982	1983	1984	1985	1986
Prevalence[a]	24.1	20.7	25.9	50.0	46.4	46.7
Intensity[b]	3.9	3.2	3.9	6.1	8.1	10.4
Trap rate[c]	32.3	13.1	39.4	22.3	20.1	28.6
A100[d]	30.4	8.7	39.8	68.0	75.5	138.9
Antibody prevalence	9.3	10.3	13.7	16.8	19.0	16.3

[a] Prevalence of infestation (percent of mammals with ticks).
[b] Intensity of infestation (mean number of ticks/infested mammal).
[c] Mean number of mammals trapped per 100 trap-nights.
[d] Mean rate of infestation on mammals trapped per 100 trap-nights (calculated by Prevalence × Intensity × Trap rate).

Tick infestation

In all localities the most abundant tick on small mammals was *Ixodes ricinus*, larvae and nymphs. Less abundant or rare, but present in every locality, were larvae and nymphs of *Dermacentor reticulatus* and *Haemaphysalis concinna*. From mammals at Plavecký Mikuláš, several nymphs and females of *Ixodes trianguliceps* were collected. The prevalence of infestation was 20.7—50.0%; intensity of infestation ranged from 3.2 to 10.4 ticks per animal. The mean numbers of ticks (of all species) on mammals collected in 100 traps during 1 night was 8.7—138.9 (Table 5).

Discussion

A longitudinal study of small, short-lived mammals, ticks, and viruses at six selected localities of West Slovakia confirmed the extensive distribution and persistence of CEE virus in this region. The mean prevalence of neutralizing antibodies in small mammals was 14.6%, differences during the years 1981—1986 being no more than 10% (9.3 to 19.0%); differences among localities were even smaller (11.9 to 16.3%). Similar results have been obtained by others studying nearby areas: in a short term study of different localities in Záhorská Nížina lowland, prevalence of neutralizing antibody to CEE virus in small mammals was 2.2—20.4% (Kožuch *et al.*, 1983); in Middle Považie (Central Slovakia), another focus of CEE virus activity, 15% of small mammals had neutralizing antibodies to this virus (Nosek *et al.*, 1982*a*); in areas surrounding villages in Tribeč mountains region antibody prevalence was 14.6% (Kožuch *et al.*, 1969); in a mountainous focus (Low Tatras) 10.7% of small rodents had neutralizing antibody to CEE virus (significantly, 18.2% of *A. flavicollis* nad antibody in this study) (Nosek *et al.*, 1982*b*); in district (northern Moravia) antibody prevalence was 15.8% (Kožuch *et al.*, 1976*b*); finally, and in northern Austria 13.3% of small mammals had neutralizing antibody to CEE virus (Kožuch *et al.*, 1969).

Ticks were not found on mammals collected in mid-winter, yet three isolates of CEE virus were obtained from animals collected at that time (1981—1982), suggesting the possibility that virus persists throughout the year. Previously, Ernek *et al.* (1963) isolated CEE virus from tissues of experimentally infected *C. glareolus* 21 and 28 days after infection. In this way, small mammals may serve as reservoirs for ticks feeding in the spring and subsequently amplifying the virus. Although CEE virus has not been isolated from the blood of small mammals collected in mid-winter, CEE virus isolated from tissues have lower pathogenicity (longer incubation periods in laboratory hosts) than those isolated from ticks collected in the spring (Kožuch and Labuda, unpublished observations). Whereas this apparent decreased pathogenicity of isolates from mammals may be due to lower titres than those developing in ticks, it may also be that hormonal or environmental effects in the spring allow virus to traverse biological barriers and initiate viraemias. None of the animals from which CEE virus was

isolated had antibody to that virus. During the years 1954—1975, 33 strains of CEE virus were isolated from tissues of small mammals throughout Slovakia, 12 of these were from *C. glareolus* (Nosek *et al.*, 1978).

In our study, the highest prevalence of neutralizing antibody to CEE virus was found in sera of *A. flavicollis* (18.1%). This species is preferred for feeding by larvae of *I. ricinus* ticks (Labuda *et al.*, 1989), the principial vector of CEE virus. *I. ricinus* is the most abundant tick species in all six localities, forming synusia with *D. reticulatus* and *H. concinna* ticks, as was shown for West Slovakia by Nosek and Krippel (1974). Antibody prevalence rates were higher in the period 1984—1986 (16.3—19.0%) than in 1981—1983 (9.3—13.7%). These rates correlated with higher tick infestation of small mammals (Table 5). Abundance of ticks on hosts (trapped in 100 traps in one night, which allows calculation of the abundance of both ticks and their hosts) seems to correspond with antibody prevalence in these small mammals. Our results emphasize the importance of the most abundant rodent species, *C. glareolus* and *A. flavicollis*, in the circulation of CEE virus in central Europe. Alternatively, the population density of insectivores was very low, therefore the relatively high antibody prevalence among these mammals (13.8%) can be disregarded in terms of their importance in the circulation of CEE virus in these localities. All foci of CEE virus in West Slovakia are of potential importance from the point of view of human infections, particularly in years when *I. ricinus* ticks are abundant and high populations of *C. glareolus* and *A. flavicollis* occur. Surveys of small rodents for the presence of neutralizing antibodies can be used to monitor the epidemic potential in a given focus so that human infections can be prevented.

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