

THE SIGNIFICANCE OF THE REGIONAL LYMPHATIC SYSTEM IN PENETRATION OF AN ATTENUATED STRAIN OF TICK-BORNE ENCEPHALITIS VIRUS INTO THE BLOOD OF EXPERIMENTALLY INFECTED ANIMALS

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Summary. — The passage of an attenuated strain of tick-borne encephalitis (TE) virus through the regional lymphatic system (RLS) and its influence on the virus level in blood were assayed in experiments on goats and mice. The results indicated that, after subcutaneous (sc) administration, the attenuated strain remained confined mainly to the RLS. As far as it penetrated into the blood, it was present only in a small amount detectable only after administration of large doses of virus.

Introduction

The effort to characterize the experimentally obtained attenuated strain of TE virus (Mayer, 1962a) led to experiments focussed to a more thorough investigation of its pathogenic properties. Mayer (1963, 1964) and Mayer *et al.* (1967) found that, as distinct from the virulent strain, the attenuated strain of TE virus, administered sc to experimental animals, did not usually cause viraemia in them, though it induced an antibody response. Considering that TE virus spreads after sc administration from the site of inoculation into the blood through the RLS, which is simultaneously the site of its primary multiplication (Málková and Fraňková, 1959; Málková, 1960, 1968), in this study we investigated the relationship of the attenuated strain to the RLS, in particular the influence of the passage through this system on the penetration of virus into the blood.

Materials and Methods

Virus and mode of infection. We worked with two clones of the Hypr strain of TE virus, namely the clone designated Hy-HK28“2” (Mayer, 1962a, 1966), attenuated for sc inoculated mice and intrathalamically inoculated monkeys *Macaca mulatta*, and the clone designated P III-E (Mayer, 1962b) which is significantly virulent for both mice and monkeys. We inoculated the virus suspensions sc into the hind limbs: above the interdigital spaces in goats, and into the paws in mice.

Experimental animals. The experiments were carried out on goats and laboratory mice. Mayer (1964) and Mayer *et al.* (1967) found no viraemia in these animals after inoculation with the attenuated strain. Adult goats, 2 to 3 years old, obtained from southwest Slovakia, from an

area which is known not to be a natural focus of TE, were used in the present experiments. No specific virus neutralizing antibodies against TE virus were found in their sera diluted 1:4. White mice weighing 18–19 g were obtained from the Děčín farm.

Collection of materials. In goats we examined virologically mainly the afferent and efferent lymph and the regional lymph nodes (RLN) — the popliteal nodes. The outlets for collection of lymph were prepared as follows: The goats were anaesthetized by Thiopental Spofa and injected 0.5 ml of a 4% Evans Blue solution into the interdigital spaces between the hoofs. The popliteal nodes of both hind limbs were dissected immediately thereafter. Into one limb a cannula of flexible nylon "OO" was introduced antidromically into one of the afferent vessels and the other vessels were ligated. In the other hind limb a cannula was similarly introduced into the larger efferent vessel and the remaining 1 to 2 vessels were also ligated. The cannulas' outlets were exteriorised and fixed on the skin to enable continual collection of the lymph. The continually outflowing lymph dropped into a test-tube fixed on the limb. The collection of the lymph for virological control lasted usually 5 minutes and at each interval the lymph was collected into a new sterile test-tube. The goats were injected 1 to 2 ml of 5000 units/ml of heparin Spofa intravenously during operation and 20 000 units (1 ml) of heparin Novo sc after operation.

In mice, the popliteal and paraaortic RLN, blood and spleen were assayed for virus. The removed organs were thoroughly washed, dried, weighed and homogenized in phosphate buffered saline containing 10% calf serum and 100 units/ml penicillin and 100 µg/ml streptomycin to obtain 10% suspensions. The latter were centrifuged at 2000 rev/min for 10 minutes and the supernatants diluted in the same medium. For each interval, materials from 6 mice were used.

Assay of virus. The lymph, blood, and the organ suspensions were inoculated intracerebrally (ic) in 0.01 ml doses into suckling mice 3 to 5 days old from the Děčín farm. We used a litter of 10 suckling mice for each dilution. The inoculated mice were observed for 14 days. The virus

Table 1. The attenuated strain of TE virus in the afferent and efferent lymph, RLN tissue and blood of goats

Material examined	Exp. No.	Hours after infection										
		1	2	3	4	5	6	8	10	24	48	
Afferent lymph	1	+	+	+	0	0	0	0	0	0	0	
	2	0	0	0	0	—	0	0	0	0	0	
	3	0	0	0	+	—	+	0	0	0	—	
Efferent lymph	1	0	0	0	0	—*	—	—	—	—	0	
	2	0	+	0	+	—	+	0	0	0	0	
	3	+	+	+	+	—	+	0	0	0	—	
Blood	1	—	—	—	—	—	—	—	—	—	0	
	2	—	—	—	—	—	—	—	—	—	0	
	3	—	—	—	—	—	—	—	—	0	—	
Lymph nodes	A**	1	—	—	—	—	—	—	—	—	0	
		2	—	—	—	—	—	—	—	—	0	
		3	—	—	—	—	—	—	—	0	—	
	B	1	—	—	—	—	—	—	—	—	—	+
		2	—	—	—	—	—	—	—	—	—	+
		3	—	—	—	—	—	—	—	—	+	—

+ = Virus detected ($< 0.5 \log LD_{50}$).

0 = No virus detected.

— = not done.

* Outlet obstructed.

** Lymph nodes at the afferent (A) and efferent (B) outlets.

titres were calculated according to Reed and Muench. The isolated virus was identified by means of specific immune serum and based on the determined character of their genetic markers (*sc, t, s*) (Mayer, 1966).

Results

Assay of the attenuated and the virulent strain of TE virus in the afferent and efferent lymph of the RLN of the goat

We examined the degree of penetration of the attenuated strain into the regional lymphatic pathways and the capacity of the nearest RLN — the popliteal node in our case — to take up virus, eventually in what extent the virus passed through the efferent pathways further into the RLS.

Table 2. The virulent strain of TE virus in the afferent and efferent lymph, RLN tissue and blood of goats

Material examined	Exp. No.	Hours after infection									
		1	2	3	4	5	6	8	10	24	48
Afferent lymph	1	+	+	0	0	0	0	0	0	0	—
	2	+	+	+	0	—	0	—	0	0	0
Efferent lymph	1	+	+	+	+	—	—	—	—	—	—
	2	+	0	0	0	—	0	—	0	0	0
Blood	1	—	—	—	—	—	—	—	—	0	—
	2	—	—	—	—	—	—	—	—	—	0
Lymph nodes	A	1	—	—	—	—	—	—	—	0	—
		2	—	—	—	—	—	—	—	—	0
	B	1	—	—	—	—	—	—	—	—	+
		2	—	—	—	—	—	—	—	—	+

For explanations see Table 1.

Three and two experiments were carried out with the attenuated and the virulent strain, respectively. One goat was used in each experiment.

The doses of attenuated virus in experiments 1, 2 and 3 were $10^{5.4}$, $10^{6.3}$, and $10^{6.7}$ ic mouse LD_{50} . They were administered in 0.1 ml amounts into each hind limb. The results summarized in Table 1 indicate that the virus penetrated into and persisted in the afferent and efferent lymphatic pathways of the RLN at the nearest intervals past infection (6 hours being the longest period). In the RLN at the site of the efferent outlet, where the afferent pathways were not ligated, it was possible to detect the virus still 48 hours p.i. The results showed a certain discrepancy which is not fully in correlation with the size of the dose and the spread of virus in the RLS.

In the control experiments with the virulent strain, the doses of $10^{5.1}$ (exp. 1) and $10^{6.1}$ (exp. 2) ic mouse LD_{50} were administered as above. As can be seen from Table 2 the virus, like in the foregoing experiment, was demonstrable in the afferent and efferent lymph of the RLN at the nearest intervals

Table 3. The attenuated strain of TE virus in RLN, blood and spleen of mice

Material examined	Exp. No.	Virus titres (log ic mouse LD ₅₀ /0.01 ml) at hours p.i.						
		1	3	6	12	24	48	72
Popliteal lymph nodes	1	> 2	0	1.5	0	> 1	1.5	—
	2	> 1	0	> 1	> 1	0	< 0.5 (?)*	—
Para-aortic lymph nodes	1	0	0	0	< 0.5	< 0.5	0	—
	2	0	0	0	0	0	< 0.5	—
Blood	1	0	0	0	0	0	0	< 0.5
	2	0	0	0	0	< 0.5	0	0
Spleen	1	—	—	—	—	—	0	1.0
	2	—	—	—	—	—	0	0

0 = No virus detected; — = not done.

* Mice were dying within the characteristic incubation period. Because of cannibalism, death specificity could not be checked.

p.i. At the end of the experiment (48 hours p.i.), it was similarly detected in the RLN at the site of the efferent outlet.

In view of the irregularity of findings and the relatively short uptake of virus, especially in the case of the virulent strain, immediately after the first experiment we examined the suitability of the blue used for staining of the lymphatic vessels. We tested the influence of 4% Evans Blue, 10% Direct Sky Blue and 10% Blue Patente on the virus titre. Direct Sky Blue was toxic for mice and thus could not be used. The two other dyes decreased the virus titre approx. by 1.5—2 log units. Since it was impossible to dissect the lymphatic vessels without use of these dyes, in further experiments 4% Evans Blue was administered in the least indispensable quantity only.

Passage of the attenuated strain in the RLS of mice from the site of inoculation into the blood

Two experiments were carried out. In experiment 1 and 2, doses of $10^{5.8}$ and $10^{3.8}$ ic mouse LD₅₀, respectively, were administered sc into both paws of the hind limbs in 0.01 ml amounts. The results, summarized in Table 3, indicate that the virus penetrated into the nearest RLN (popliteal) after both doses of virus; the difference between the doses was especially in the titre levels. In both cases the virus penetrated also into more distant RLN (para-aortic) and the difference after administration of the two doses appeared mainly in the earlier uptake of virus after the larger dose. The amount of virus detected in the para-aortic RLN was much smaller than that in the popliteal RLN which are located nearer to the site of inoculation. After the larger dose, the virus penetrated into the blood 72 hours p.i. in a small amount; this finding was confirmed by detection of virus also in the spleen. In case of the smaller dose, we found traces of virus at the interval of 24 hours p.i. in the blood, but we did not detect it in the spleen.

Discussion

The present results proved that the attenuated strain of TE virus penetrated into the RLS, spread in it and, in case of TE-susceptible animal such as the white mouse, was detected after sc administration into the paw also in the para-aortic RLN which are located in close vicinity of the thoracic duct, through which the lymph flows directly into the blood. After a larger dose administered to the mouse, a certain amount of virus penetrated also into the blood and spleen. During passage through the RLS into the blood, the detectable amount of virus gradually decreased, which proved that within the time of the experiment, i.e. within 72 hours p.i., no multiplication of virus occurred. A certain irregularity in the uptake of virus in mice is explained by the fact that another group of mice was always used for every interval.

In goats, the relatively short uptake of virus with low titres in the afferent and efferent lymph of the RLN is explained by a relatively small dose of virus administered to a large animal, in which the virus, especially the attenuated strain, did not multiply. A certain amount of virus was already taken up in the nearest RLN, which was proved by detection of virus in the popliteal RLN at the site of efferent outlet 48 hours p.i. The virus titre and the irregularity of uptake was further incontestably influenced by Evans Blue, though it evidently could have affected the virus in the animal in a substantially lower concentration than in the experiment *in vitro*. All these facts may offer an explanation why the results of the present experiments differed from those carried out on sheep (Málková, 1960), in which, after administration of a much larger dose of TE virus, the latter was demonstrated for a significantly longer period in the RLS in addition to viraemia. The negative finding of virus in the blood of goats reported by Mayer *et al.* (1967) may be explained by the fact that the subcutaneously administered virus remained confined to the RLS.

We conclude from the experiments on goats and mice that the attenuated strain of TE virus, after sc administration, remained confined mainly to the RLS in which it did not multiply. For these reasons only a small amount of virus passed into the blood, which was detectable only after inoculation with large doses of virus.

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