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Isolation of Viruses of the Russian Spring-Summer Encephalitis-Louping Ill Group from Swedish Ticks and from a Human Case of Meningoencephalitis*

By

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Viruses belonging to the Russian Spring-Summer Encephalitis — Louping ill (RSSE-LI) group were recently found to be prevalent in Sweden (1, 2). Serological methods were used throughout these studies. The present paper concerns the isolation and identification of such viruses from ticks as well as from a human case of tick-borne meningoencephalitis.

Materials and methods

Specimens tested for the presence of virus

A. *Ticks.* Ticks were collected from the beginning of August through September 22, 1958, at nine farms situated about 60 miles northeast of Stockholm, i. e. within a region where tick-borne encephalitis is known to be endemic (1, 2). Freely creeping ticks were collected from the fur of cattle grazing on natural pastures. Altogether 898 starving adult ticks were thus obtained and processed. A preliminary classification of ca. 200 ticks suggested that they all belonged to the species *Ixodes ricinus*. Through the courtesy of Dr. *Harald N. Johnson*, Berkeley, Calif., U.S.A., five ticks were brought to Dr. *Glen M. Kohls*, Hamilton, Montana, U.S.A., who definitely classified them as adults of *Ixodes ricinus*.

In addition to the adult ticks mentioned 25 larval ticks and 35 nymphal ones were tested for virus. They were all obtained on September 22, 1958, from the bushes on the grazing fields of the farm Hallboda.

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The adult ticks were obtained as 23 separate pools. Like the two pools of larval and nymphal ticks these were stored separately in glass-sealed ampoules in a CO_2 ice cabinet before being processed.

B. *Human sera.* Virus isolations were attempted on the acute phase sera received from 26 Swedish patients suffering from acute CNS disease all of whom were known to have an at least fourfold rise of antibody titer in the convalescent serum as measured in the CF reaction with 2 units of an RSSE group antigen (HYPR strain). Only nine of the sera tested were negative in the CF test, however, thirteen having a titer of 2 and four a titer of 4. All specimens were collected during the summer and fall of 1958. They were stored at $-25°$ C without previous heat treatment.

C. *Cerebrospinal fluids.* Cerebrospinal fluids were obtained during the acute phase of the CNS illness of 14 patients.

All but three of these fluids originated from patients providing acute phase sera also tested. The fluids were stored at $-25°$ C.

Laboratory strains of viruses. The strain of Louping ill virus employed was kindly supplied by Prof. *N. Oker-Blom*, Helsinki, Finland, the HYPR strain of Central European encephalitis by Prof. *D. Blaškovič*, Bratislava, Czechoslovakia. Details about these strains are given in a previous paper (3).

Reference sera. Two immune sera against RSSE virus were generously supplied by Dr. *E. L. Buescher*, Washington, D. C., U.S.A. One was a guinea pig serum (B-693/1958) prepared by infection; accordingly it was supposed to be rather specific for viruses of the RSSE group. It was reported to have a CF titer of 32 to 64 against 4 units of RSSE antigen. The other specimen was a rabbit hyperimmune serum (3100/1955) which should neutralize more than 6 logs of RSSE virus in the intraperitoneal test in mice.

Preparation of immune sera. Immune serum against the strain HYPR was prepared by intravenous injection of 0.5 ml. of 10 per cent mouse brain material (from the 44th mouse passage) into guinea pigs weighing 300—400 g. Blood was drawn after 5 weeks (serum HYPR 1). A second inoculation (1 ml. intramuscularly) of a 10 per cent brain extract from the 45th mouse passage was given after 13 weeks. The animals were exsanguinated 9 days later (serum HYPR 2).

Sera were also prepared against two Swedish virus strains (see below), one isolated from nymphal ticks (strain T 1), the other from acute phase serum (strain 20536). The guinea pigs received two intramuscular doses at 4 weeks interval. The first dose comprised 0.5 ml. of a 10 per cent brain extract from the mice used for the first subpassage; next time 1 ml. of a similar extract from the second subpassage was given. The animals were exsanguinated 9 days after the last injection. Sera are referred to as serum T 1 and serum 20536, respectively.

A pool of convalescent sera from ten Swedish cases of tick-borne meningoencephalomyelitis was employed in some experiments.

All immune sera were stored at $-25°$ C following inactivation at 56° C for 30 min.

Tissue culture methods

Detroit- 6 cells grown in stationary tube cultures were used throughout. The preparation and maintenance of the cultures as well as the media used have been described in detail elsewhere (3).

Techniques used for isolation of viruses

Ticks were ground in a mortar with sand and diluent for about 30 minutes. The diluent consisted of equal parts of phosphate-buffered saline (PBS, 4) and inactivated (56° C for 30 min.) guinea pig serum; it contained also 500 I.U. penicillin, 500 μg streptomycin, 100 U. mycostatin and 60 μg neomycin sulfate per ml. Pools of about 30 ticks or less were made up to a final volume of 1.5 ml. Three larger pools, containing 131, 141 and 177 ticks, respectively, were each suspended in 5 ml. of the diluent. After centrifugation at 700 r. p. m. for 5 minutes part of the supernatants were immediately used for virus isolation attempts, the remaining fluid being stored in glass-sealed ampoules in the CO_2 ice cabinet.

Materials for virus isolation, i. e. tick extracts, sera or cerebrospinal fluids, were inoculated intracerebrally into albino mice as well as into tissue cultures in doses of 0.02 and 0.1 ml., respectively. Each specimen was tested in four tubes and five or six mice. The age of the animals varied for the separate experiments and will be specified below. In some of the experiments a single blind passage was performed in the same test system with undiluted tissue culture fluid and 10 per cent brain suspension, respectively.

During the period of the collection of ticks as well as during the isolation tests no animal work with laboratory strains of the RSSE-LI group was undertaken in the laboratory. With just a few exceptions, on days when the isolation experiments were not handled, no work with the laboratory strains was carried out in tissue culture either. As a further precaution to avoid laboratory pick-ups mice were, in isolation experiments, kept for 8—14 days only. At most one blind passage was performed (the conditions for virus isolations were thus not optimal); furthermore, all subpassages of materials from sick or moribund mice were postponed until the remaining original specimens had been tested.

Infectivity titrations

Titrations in Detroit- 6 cultures were performed with five tubes per tenfold dilution of virus. The cultures were observed for cytopathic changes for 10 to 11 days after inoculation. Titer values are calculated according to *Kärber* (5). Although an inoculum of 0.1 ml. was used the titer values are recalculated for 1.0 ml. Mouse titrations were performed with five 3 weeks old mice or six 2 days old ones per tenfold dilution of virus. The animals were injected intracerebrally with 0.02 ml. They were observed for 3 weeks. Titers are recalculated for 1.0 ml.

Neutralization tests

Neutralization tests in tissue culture were carried out with serial twofold dilutions of serum against ca. 100 TCD_{50} of virus. The virus dilutions were prepared in PBS containing 10 per cent filtered horse serum, 100 I. U. penicillin, 100 μg streptomycin and 100 U. mycostatin per ml. Equal parts of serum dilution and virus suspension were mixed and incubated at 37° C in a waterbath for one hour. Five cultures were used per dilution, each tube being inoculated with 0.1 ml. of the serum-virus mixture. The cultures were examined for cytopathic changes daily from the 4th through the 10th day. The medium was changed six days after inoculation. As a rule the cytopathic changes were obvious before the 7th day. The serum titer is recorded as the negative log of the initial dilution of serum (in the amount of 0.05 ml.) which

protected half of the cultures for virus degeneration. It was calculated according to *Kärber* (5).

Neutralization tests in mice were performed according to the constant serum dose-virus dilution method. The serum-virus mixtures were incubated at about 5° C overnight before 0.1 ml. doses were inoculated intraperitoneally into 5 mice (3 weeks of age) for each virus dilution. The simultaneous control titration of virus, intraperitoneally into 3 weeks mice, was diluted in PBS containing 50 per cent inactivated guinea pig serum.

The neutralization index, expressed as logarithm, represents the difference in MLD_{50} titer of the virus in the presence of immune serum as compared to normal serum.

Complement fixation tests

CF tests were carried out as box titrations according to the plate-technic of Fulton and Dumbell as described by *Svedmyr*, *Enders* and *Holloway* (6). Tissue culture antigens were produced in Detroit- 6 cultures containing a medium of bovine amniotic fluid with the addition of 10 per cent inactivated (56° C for 30 min.) guinea pig serum in order to avoid the appearance of anticomplementary activity (7). The treatment with β-propiolactone has been given in detail elsewhere (1, 3). Serum titers are given as the reciprocal of the highest initial dilution giving at least 50 per cent inhibition of hemolysis.

Results

Virus isolation experiments

A. *Human sera and cerebrospinal fluids.* None of the sera or cerebrospinal fluids caused cytopathic changes in Detroit 6 cultures during the observation period of 10 to 11 days.

Four of the serum specimens were tested in 2 weeks old mice, which remained healthy throughout the 2 weeks of observation. The remaining 22 sera as well as the 14 cerebrospinal fluids were all tested in 2 days old mice. All six mice inoculated with one of these sera, No. 20536, appeared seriously ill on the sixth day. At this time the brains were harvested for identification of the agent which below will be referred to as strain 20536*.

The patient from whom strain No. 20536 was isolated was a 15 years old boy. He fell ill on June 27, 1958, when on vacation in the northern part of the Stockholm archipelago. The disease ran a monophasic course, the predominant signs being meningitis and encephalitis. No preceding tick-bite is known. The patient was hospitalized on June 28. Blood specimens were drawn on June 29, July 2 and 8 and finally on August 20. Cerebrospinal fluid was drawn on June 29.

Virus was isolated from the first blood specimen obtained (no. 20536) but not from the cerebrospinal fluid of the same day. Remaining sera were not tested for virus as they had been inactivated at 56° C for 30 min. before being stored at −25° C. CF tests on the three sera first drawn gave negative results against HYPR antigen, whereas the convalescent serum had a titer of 64.

* Reisolation of this agent, in mice and in tissue culture, could not be carried out as the serum specimen had been used up.

No agent was recovered from any of the other sera or cerebrospinal fluids. For three of these groups of mice a blind passage was made on the 8th day into 4 days old mice, which all remained healthy during the 8 days of observation. All other groups of mice were kept for two weeks without subsequent blind passage; no signs of illness occurred in these cases.

B. *Tick suspensions.* Out of the 25 extracts of tick pools only the three larger groups of adult ticks and the group of nymphal ticks were virus-positive. These four pools were all inoculated into two weeks old mice. All mice of each group showed signs of either paralysis or general illness within 8 days. Those animals given the nymphal suspension had the shortest period of incubation, two mice being paralysed on the 6th day and the remaining 3 ones dead on the 7th day.

All these four tick pools originated from one single farm (Hallboda, about 60 miles northeast of Stockholm) but were collected during different periods of the summer and fall. The virus strains isolated are below referred to as strains T 1 (nymphal ticks), T 2, T 3 and T 4.

The remaining 21 pools of ticks were all negative; among them were two further pools from the farm just mentioned (the group of larval ticks and a group of 37 adult ticks). Five of the suspensions were tested in two weeks old mice. After 7 to 9 days blind passages were performed in 2 days old suckling mice which were observed during 8 to 11 days before the result was regarded as negative. The remaining 16 pools of ticks were inoculated into mice 2—4 days of age. After 8 days blind passages were made in mice of the same age. They were also kept for 8 days before being discarded as negative.

Detroit- 6 cultures were inoculated, in parallel with the mice, with 18 tick extracts later found to be negative in mice. The primary tubes were observed for 9—11 days. A slight but definite cytotoxic reaction was noted at the first examinations. The cultures regenerated, however, after the change of medium on the 6th day. A subpassage in tissue culture for 16 of the specimens was negative throughout the observation period of 9 to 11 days.

The four mouse-positive tick extracts were later titrated simultaneously in tissue culture as well as in 2 days mice and in 3 weeks mice. The mouse titers obtained for the different extracts varied between 2.4 and 4.5 per ml. (Table 1).

In tissue culture a certain toxic effect was noted, similar to that observed with the mouse-negative tick suspensions. After 7 to 11 days the entire titrations were, therefore, passed tube by tube. As again no significant cytopathic changes occurred a further passage was made with the pooled fluids from those tubes corresponding to the strongest original inoculum. The cells of this passage, i. e. the third tissue culture passage, showed some slight cytopathic changes on the 6th day. All

Table 1. Infectivity titers of tick extracts in mice 2 days and 3 weeks of age

Virus strain (tick extract)	Titer per ml. of tick extract		Period of lethality, days after inoculation	
	2 days mice	3 weeks mice	2 days mice	3 weeks mice
T 1	4.5*	4.2	6—8	7—10
T 2	≤3.9**	3.6	6—8**	8—12
	3.2	3.2	6—9	9—12
T 3	≤3.4**	3.4	7—9**	9—16
	3.2	2.4	7—9	9—11
T 4	4.0	4.4	7—9	8—11
	≤4.2**	4.0	6—10**	8—14

* Simultaneously performed titrations are recorded on the same line.

** Due to nonspecific death within 24 hours of the litter of the critical dilution the maximum titer only can be estimated (the nonspecific deaths being included when calculating the titer). The early nonspecific deaths are not recorded in the column giving the period of lethality.

suspensions were carried through altogether 10 passages in tissue culture, resulting in a slight increase in cytopathogenicity. The cytopathic effect remained, however, much less marked than the cytopathic effect caused by the laboratory strains LI und HYPR.

The comparative titrations in mice and in tissue culture were repeated with one of the tick-suspensions (T 4). This time, however, the tissue cultures of the original titration were all passed individually for 5 consecutive subpassages in tissue culture. Moreover, the presence of virus in the individual tubes of the last passage was checked by intracerebral inoculation into 3 weeks mice (2 mice per tube). Again slight cytopathic changes were at first noted in the third tissue culture passage and only in those cultures corresponding to the undiluted original inoculum and in 3 out of 5 of those corresponding to the 10^{-1} dilution. The specificity of this result was verified by the subsequent passages in tissue culture as well as by the final mouse passage.

The titration in tissue culture thus gave a titer of 2.1 per ml. of the tick suspension. On the other hand, the simultaneously performed titration in 3 weeks mice gave a titer of 4.0 per ml.

As the mouse-adapted laboratory strains, contrary to the newly isolated agents, showed a marked cytopathic effect already on their first passage in Detroit- 6 cells (3) brain extracts from the 6th mouse passage of all five new strains were tested for cytopathogenicity in tissue

culture. Cultures, inoculated with 10^5 to 10^6 MLD_{50} did, however, not show any signs of specific degeneration during the 7 days of observation. The second passages were also negative.

Typing of the newly isolated virus strains

The complement fixation reaction was used for preliminary identification of the newly isolated agents. Tissue culture fluids containing live virus as well as fluids inactivated with β-propiolactone were employed as antigens; a pool of sera from Swedish convalescents after tick-borne meningoencephalitis and various animal sera provided the immune sera. The data of Table 2 and 3 obviously suggest that the new agents belong to the RSSE-LI group.

All virus strains isolated were tested in mouse neutralization tests against immune sera to two of the new agents, T 1 and 20536, as well as against various control sera to laboratory strains of tick-borne viruses.

Table 2. Presumptive identification of isolated strains by CF tests against a pool of Swedish sera from convalescents after tick-borne encephalitis

Virus antigen*	Dilution of antigen	Titer of serum
20536	undil.	16
	1 : 2	64
	1 : 4	64
	1 : 8	16
T 1	undil.	64
	1 : 2	16
	1 : 4	<4
T 2	undil.	128
	1 : 2	64
	1 : 4	4
T 3	undil.	64
	1 : 2	64
	1 : 4	8
T 4	undil.	64
	1 : 2	16
	1 : 4	<4

* Active tissue culture fluids were employed as antigens. Strain 20536 was isolated in mice and carried for 3 passages in Detroit- 6 cells. Strains T 1—T 4 were isolated and carried for 10 passages in Detroit- 6 cells.

Table 3. Strains HYPR and 20536 tested in CF against various guinea pig immune sera

Virus antigen*	Dilution of antigen	Titer of serum			
		HYPR 2	20536	T 1	RSSE
HYPR	undil.	128	64	128	32
	1 : 2	64	128	128	16
	1 : 4	32	64	64	8
	1 : 8	8	8	4	4
	1 : 16	4	<4	4	<4
	1 : 32	<4	<4	<4	<4
20536	undil.	64	128	128	32
	1 : 2	64	128	128	32
	1 : 4	64	128	128	32
	1 : 8	32	8	32	8
	1 : 16	32	4	4	4
	1 : 32	<4	<4	<4	<4

* The HYPR antigen consisted of a pool of harvests from the 13th—17th passages in Detroit- 6 cells. The strain had previously undergone 44 mouse passages. Antigen 20536 was the same as that specified under Table 1. The HYPR antigen was killed by β-propiolactone treatment, the other antigen was used live.

As illustrated in Table 4 the new strains appear closely related to the HYPR strain of Central European tick-borne virus as well as to the LI and RSSE viruses.

For confirmation, neutralization tests in tissue culture were also performed. However, only the laboratory strains, LI and HYPR, were employed for the titrations since the new agents were considered not to give a cytopathic effect pronounced enough for a reliable neutralization

Table 4. Results of intraperitoneal neutralization tests in mice

Virus strain	Log neutralization index of antiserum				
	HYPR 1	HYPR 2	20536	T 1	RSSE-rabbit*
HYPR (44, 45)**	≧ 5.6	≧ 4.6	≧ 4.6	≧ 4.4	≧ 5.6
20536 (6)	≧ 6.2	≧ 5.4	≧ 5.4		≧ 6.2
T 1 (6).....				≧ 5.2	
T 2 (6).....			≧ 6.4		
T 3 (6).....			≧ 6.6		
T 4 (6).....			≧ 7.0		

* Serum diluted 1 : 2. Other sera used undiluted.

** Figures within parenthesis represent number of previous mouse passages.

test. As seen in Table 5 sera against the new agents neutralized the LI and HYPR strains to a similar extent as the other sera did. There is a

Table 5. Results of neutralization tests in tissue culture

Virus strain	Neg. log of serum dilution giving 50 per cent neutralization						
	HYPR 1	HYPR 2	20536	T 1	RSSE-rabbit	RSSE-guinea pig	TCD_{50} of virus
HYPR (mice 44, Detroit- 6-14)*	2.7	3.1	3.2	3.3	2.9	2.3	60—300
LI (mice unknown, HeLa 23, Detroit- 6-13)*	2.2	2.7	2.7	2.7	2.6	1.2	100—250

* Figures within parenthesis represent number of previous passages.

suggestion that they all gave slightly higher titers against the HYPR strain than against the LI strain. This is in accordance with our previous results (3).

Discussion

In a previous paper (3) it was suggested that cultures of Detroit- 6 cells may provide a system suitable for the isolation of viruses of the RSSE-LI group. In the present investigation four out of the five strains isolated by intracerebral inoculation into mice could also be isolated in Detroit- 6 cells. The mice were, however, sensitive to smaller doses of virus. The cytopathic effect of the isolated strains was, furthermore, not recognizable until in the third tissue culture passage and was even then only moderate. Harvests from the 3rd or 10th tissue culture passages did, however, contain enough CF antigen to permit a serological identification of the strains.

Mice, three weeks of age, were found to be as suitable as suckling mice for the isolation of the virus strains. The only difference noted was a somewhat longer incubation period in 3 weeks mice than in suckling mice.

The newly isolated strains could not be antigenically differentiated from the HYPR strain of Central European encephalitis virus, neither in tissue culture nor with the intraperitoneal mouse neutralization test. They were also found to be closely related to strains of RSSE and LI viruses.

Slonim has suggested (8) that the large number of mouse brain passages which the laboratory strains have usually undergone might have obscured antigenic differences originally present. It may be noted, therefore, that immune sera against two of the new strains, produced with materials

from the first and second subpassages in mice, gave as high neutralization titers in tissue culture against LI virus as did the immune serum to the old laboratory strain HYPR (44 to 45 mouse passages).

Summary

An account of the isolation of five Swedish virus strains belonging to the Russian Spring-Summer — Louping ill group is given. Four strains originated from ticks collected northeast of Stockholm, the fifth one from the acute phase serum of a case of meningoencephalitis. Four of the five strains recovered in mice could also be isolated in cultures of Detroit- 6 cells. A presumptive identification of the strains was performed by CF tests. The relationship of the strains to other viruses of the RSSE-LI group was shown in neutralization tests in mice and in tissue culture.

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