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HAEMORRHAGIC FEVER VIRUS WITH RENAL SYNDROME IN SMALL RODENTS IN CZECHOSLOVAKIA

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Summary. — The antigen of haemorrhagic fever with renal syndrome (HFRS) was detected in the lungs of the following free-living small rodents trapped in different localities in Eastern Slovakia: *Clethrionomys glareolus* (2 positive samples of 7), *Apodemus flavicollis* (1 sample of 24) and *Apodemus agrarius* (7 positive samples of 66). The virus was first identified by indirect fluorescent antibody (FA) staining using human convalescent serum from a case of epidemic nephropathy (NE) of Scandinavia. The lung suspensions selected according to positive immunofluorescence were inoculated by intramuscular (i.m.) route into suckling rats; the antigen prepared from the lungs of these rats by sucrose-acetone extraction reacted with the prototype human serum in complement-fixation (CF) reaction. The results of the latter assay were in good agreement with those of the indirect FA staining.

Key words: haemorrhagic fever with renal syndrome; epidemic nephropathy of Scandinavia; free-living rodents; FA method; microcomplement-fixation reaction; Korean haemorrhagic fever; Hantaan virus

Introduction

In 1954 three fatal cases called nephroso-nephritis have been reported in Eastern Slovakia. The clinical symptoms of the disease were: high fever, rash, melaena, haematuria, albuminuria, oliguria and uraemia. Morphologically, the typical picture of nephroso-nephritis was found (Plank *et al.*, 1961). The aetiologic agent has not been detected as yet by virus isolation attempts in Czechoslovakia, though in other countries some small rodents, e.g. *Apodemus agrarius* in Korea (Lee *et al.*, 1978) and *Clethrionomys glareolus* in Europe (Brummer-Korvenkontio *et al.*, 1980; Gavrilovskaya *et al.*, 1983), were proved the natural hosts of HFRS. Therefore, we investigated free-living small rodents trapped in Eastern Slovakia as the possible reservoirs of the HFRS

virus. In addition to the FA staining, selected lung suspensions were inoculated into newborn rats. The antigen prepared from the lung tissue of these animals was examined by CF microassay as described by Casals (1967).

Materials and Methods

Small rodents. Free-living small rodents (*Clethrionomys glareolus*, *Apodemus flavicollis*, *Apodemus agrarius* and *Microtus arvalis*) were captured alive into the traps of Swedish type. In the laboratory, the animals were autopsied and the lung specimens were collected.

Prototype antisera. Human convalescent serum from a case of epidemic nephropathy (NE) of Scandinavia kindly provided by Dr. Brummer-Korvenkontio, University of Helsinki, Finland, was used for both FA staining and CF reaction. In addition, immune serum to Korean haemorrhagic fever (KHF) was used in the CF microassay only. The latter was kindly supplied by Dr. H. W. Lee from Collaborating Centre of KHF, Seoul, South Korea.

FA method. At least 20 cryostatic sections were prepared from 2 or more different levels of the lungs from each of 124 free-living rodents. The sections were fixed in acetone and stored at −20 °C. At staining, they were overlaid either with two dilutions (1 : 10, 1 : 20) of the prototype immune serum or control serum (diluted 1 : 10), respectively, and incubated for 30 min at 37 °C. After washing in phosphate buffered saline, the sections were stained with Sw-A-Hu conjugate (SEVAC, Prague) diluted 1 : 5. A series of sections was stained after 10 min washing in glycine buffer pH 2.9.

Complement-fixation (CF) antigen was prepared by sucrose-acetone extraction (Clarke and Casals, 1958) from the lungs of suckling rats infected i.m. with the 10% lung suspensions of free-living rodents selected according to the results of FA staining. The suckling rats were autopsied 14 days post-infection.

CF microassay was made on microplates with U-shaped wells (Dynatech). Veronal buffer (0.025 ml) was added into the wells No. 2 to No. 8, while to the first well was given 0.05 ml of inactivated immune serum. Using a microtitrator the serum was transfered to further wells. Into each well 0.025 ml (2 units) complement was added. Finally, the 0.025 ml of the lung antigen extract in different dilutions was added. Serum controls contained 0.025 ml each of immune serum, veronal buffer and complement (2, 1, 0.5 and 0.25 units in individual wells). Antigen controls consisted of 0.025 ml antigen, veronal buffer and complement (corresponding to 2, 1, 0.5 and 0.25 units). The solvent control consisted of 0.05 ml veronal buffer and 0.025 ml complement.

The microtitration trays were incubated for 18 hr at 4 °C, then 0.05 ml of haemolytic system (preheated for 30 min at 37 °C) was added to each well, sealed with a tape and incubated for 60 min at 37 °C (occasional shaking by 15 min intervals). After 60 min cooling at 4 °C, the trays were red using a convertible mirror.

Results

Detection of HFRS antigen by indirect FA technique

In the years 1982 and 1983, 124 lung specimens from free-living small rodents collected in Eastern Slovakia (Domica, Tarnava and Ruská Poruba localities) were searched for evindence of a virus serologically related to HFRS. By indirect FA method the antigen of HFRS was detected in the lungs of the following rodents: *Clethrionomys glareolus*, *Apodemus agrarius* an *Apodemus flavicollis* (Table 1). Two samples were positive from *Cl. glareolus*, 6 samples from *A. agrarius* and 1 sample from *A. flavicollis*. The highest proportion (28.5%) of infected *Cl. glareolus* species was trapped in the vicinity of the Domica cave. The specificity of granular immunofluorescence seen in the cells of alveolar lining and alveolar septa (Figs 1, 2) of the lungs of small

Table 1. Detection of specific immun fluorescence (IF) in the lungs of small rodents trapped in Eastern Slovakia

Species	Locality	No. of examined species	No. of positive by IF	% positive
Microtus arvalis	Domica	2	0	0
Clethrionomys glareolus	Domica	7	2*	28.5
Apodemus flavicollis	Domica	7	0	0
Apodemus agrarius	Tarnava	26	3*	11.5
Apodemus flavicollis	Tarnava	24	0	0
Apodemus agrarius	Ruská Poruba	40	4**	10
Apodemus flavicollis	Ruská Poruba	17	1*	5.8
Microtus arvalis	Ruská Poruba	1	0	0

* Specific immunofluorescence was confirmed by CF reaction (see Table 2).
** In 1 case slight positive IF was not confirmed by CF reaction.

rodents was checked in parallel sections by means of control human serum (Fig. 3). Pretreatment of the sections with acid buffer enhanced the brightness of the fluorescence. Lung specimens in which a single focus of fluorescence was found in semiserial sections were scored as probably positive (+), while those, which were repeatedly positive at several section levels, were scored as definitely positive (++, compare Table 2).

Microcomplement-fixation reaction with HFRS antigen prepared from the lungs of rats inoculated with mouse lung samples selected by immunofluorescence

In order to confirm the presence of HFRS antigen in the lungs of free-living small rodents by means of an additional assay, a CF microtest has been de-

Table 2. Comparison of the CF titres in the serum to NE of Scandinavia and in the serum to KHF with the HFRS antigens extracted from the lungs of small rodents

Strain No./isolated from	CF titres with NE antiserum	CF titres with KHF antiserum	IF
5/ *Clethrionomys glareolus*	32	0	++
9/ *Clethrionomys glareolus*	32	0	+
32/ *Apodemus agrarius*	8	0	+
37/ *Apodemus agrarius*	8	0	+
39/ *Apodemus agrarius*	8	0	++
82/ *Apodemus agrarius*	16	0	+
95/ *Apodemus agrarius*	8	0	+
112/ *Apodemus flavicollis*	8	0	++
117/ *Apodemus agrarius*	16	8	++
Control antigen	0	0	0

IF = Immunofluorescence (scoring explained in the Results).

veloped. The rest of the lungs, which had been used for sectioning, was homogenized and the 10% suspensions were inoculated by i.m. route into suckling rats. After 14 days, the lungs of the rats were removed under sterile conditions and the antigens were prepared by sucrose-acetone extraction. They were used in different dilutions for CF reaction with 2 sera, namely with the serum to NE of Scandinavia and/or with the serum to KHF. As seen in Table 2, in 9 out of 10 samples the results were in good agreement with those obtained by FA staining. The HFRS antigens from *Cl. glareolus*, *A. agrarius* and *A. flavicollis* reacted in sufficient titres with the serum against NE but only one antigen cross-reacted with the antiserum to KHF.

Discussion

The aetiologic agent of KHF (Hantaan virus, the prototype strain of KHF) has been isolated from patients suffering of HFRS by Lee *et al.* (1978). Later it was shown that the natural host of the agent in Korea was the field mouse *Apodemus agrarius*. Reservoir animals never showed symptoms of disease itself (Lee *et al.*, 1981), although the lung tissue of individuals captured in rural endemic areas may be possitive when stained with convalescent sera in FA test. The agent called Hantaan virus has been propagated in A-549 cells derived from human lung carcinoma (French *et al.*, 1981) as well as in Vero E6-cells. The Hantaan virus is a small single stranded RNA virus preliminary classified as bunyavirus (reviewed by Lee, 1982).

In the meantime it was demonstrated that sera from patients with clinical diagnosis of NE in Sweden (Svedmyr *et al.*, 1979) and in Finland (Brummer-Korvenkontio *et al.*, 1980) showed cross-reactivity with the KHF antigen. The NE antigen could be found by immunofluorescense in the lungs of *Cl. glareolus* trapped in endemic areas in Finland (Brummer-Korvenkontio *et al.*, 1980) and in the U.S.S.R. (Gavrilovskaya *et al.*, 1983).

In 1967 an outbreak of HFRS occurred in Bosna-Hercegovina. From July to September 114 people became ill in the Sarajevo region. The diagnosis of HFRS was based on the clinical course, epidemiological data and on post-mortem findings in 2 autopsied patients. The outbreak was preceeded by such an increased density of *A. flavicollis* population which had not been observed for more than 20 years before (Gaon *et al.*, 1968). The epidemic ended in September along with the decrease of population of small rodents. Recently it has been confirmed that the sera of some patients from that epidemic reacted with both, the NE and KHF antigens (Gajdusek *et al.*, 1982).

In Czechoslovakia, HFRS was diagnosed according to the clinical signs and necropsy findings (Plank *et al.*, 1961). The agent, however, was not isolated. It was of interest, therefore, to study the presence of HFRS antigen in the lungs of *Cl. glareolus*, as well as of *Apodemus* species. The free-living rodents were trapped in areas which the clinical cases of HFRS were coming from. By FA method a few definitely positive lung samples were found, while others were scored as probably positive (frequency ranged from 5.8—28.5%). In order to confirm the diagnosis in a further serologic assay, a simple CF micro-

test has been elaborated using lung extracts of rats previously inoculated with the mouse lung suspensions selected according to positive FA staining.

The sensitivity of rats to the HFRS virus has been confirmed previously (Lee *et al.*, 1982). HFRS virus was identified as the cause of 3 cases of acute renal failure in the staff handling laboratory rats in Belgium (Desmyter *et al.*, 1983). The transfer of HFRS virus to suckling rats seemed to be successful also in our experiments. It was of interest, however, that the lung antigens coming from rats inoculated with lung suspensions of *Cl. glareolus*, *A. flavicollis* and *A. agrarius* reacted predominantly with the serum to NE of Scandinavia and only in 1 case also with the serum to KHF virus. Attempts at isolation and further characterization of the virus from our material are in progress.

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Explanation to the Micrographs (Plate LV):

Fig. 1. Specific granular immunofluorescence in the alveolar cells of the lung (sample No. 112) from *Apodemus flavicollis* traped in Ruská Poruba. Serum of a patient with NE of Scandinavia was absorbed to liver powder and to rat lung suspension to remove possible nonspecific staining (magn. × 240).

Fig. 2. Detail of granular fluoresecence in the lining of interalveolar septa of the lung (sample No. 39) from *Apodemus agrarius* trapped in Tarnava (magn. × 360).

Fig. 3. The parallel section from the same lung sample as in Fig. 2 stained with control human serum (magn. × 160).