Isolation of Haemorrhagic Fever with Renal Syndrome Virus from Leukocytes of Rats and Virus Replication in Cultures of Rat and Human Macrophages

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SUMMARY

Newborn rats were inoculated intraperitoneally with haemorrhagic fever with renal syndrome (HFRS)-related virus (B-1 strain), and virus isolation from their various organs was attempted between 1 and 25 weeks after inoculation. Virus could be isolated repeatedly from lung, brain, spleen and kidney and also from peripheral blood. When virus isolation was carried out on fractionated peripheral blood cells, virus was associated mainly with the macrophage fraction and to a lesser extent with granulocytes. Virus replicated well in peritoneal exudate cells of normal rats and it grew in the adherent mononuclear cells from normal human peripheral blood. These data suggest that macrophages, permissive for HFRS-related virus replication, might contribute to the spread of viral infection in vivo.

INTRODUCTION

Haemorrhagic fever with renal syndrome (HFRS) has been reported in Scandinavia (Myhrman, 1951; Laedvinta, 1971), the Soviet Union (Casals et al., 1966) and the north of China (Ishii et al., 1942), and has been well known since 1951 as Korean haemorrhagic fever because of epidemics among United Nations troops in Korea (Smadel, 1953). Recently, there have been several reports of similar diseases in other areas (Cohen et al., 1981; Desmyter et al., 1983; Mery et al., 1983).

A viral agent, Hantaan virus (HV), was first isolated from the lungs of Apodemus agrarius coreae, a rodent reservoir, and it was proved that HV was the aetiological agent of Korean haemorrhagic fever by serological tests by Lee et al. (1978). Thereafter, HFRS-related viruses, which were antigenically related to HV, were isolated in various countries from wild and laboratory rodents (Lee et al., 1982a, b; Kitamura et al., 1983; Song et al., 1983; Niklasson & LeDuc, 1984; Yanagihara et al., 1984). In Japan more than 120 cases of this disease were reported between 1970 and 1984 from many medical institutions (Kawamata, 1983). We have made an independent isolation of a Hantaan-related virus (B-1 strain) from a tumour removed from a laboratory rat kept by a patient who developed clinical HFRS (Yamanishi et al., 1983). As a result of virological and pathological studies of animals infected with this virus, it was confirmed that systemic infection occurs in rats (Kurata et al., unpublished data). Recently, one of our colleagues, who had been involved in animal experiments, was accidentally bitten by a rat infected with the B-1 strain and typical HFRS developed in 2 weeks. Since the antibody titre in his serum increased, it is apparent that the B-1 strain can cause HFRS in man (unpublished data). Virus could be isolated from the blood of the patient, although it is presently
unclear in which leukocyte fraction HFRS virus resides. Interaction of HFRS-related virus with leukocytes may be important in relation to systemic infection and also in the pathogenesis of this disease. In this report, virus isolation from various rat organs including fractionated leukocytes is described and, in addition, virus replication in mononuclear cells from normal human peripheral blood and in peritoneal exudate cells from rats was investigated.

**METHODS**

*Cells.* Vero E6 cells were obtained from the American Type Culture Collection and cultured in growth medium containing a mixture of Medium 199 and Eagle's MEM supplemented with 5% foetal calf serum (FCS) or in maintenance medium which had the same composition but with 3% FCS.

*Virus and virus titration.* The B-1 strain was used. This strain was originally isolated in cell culture from a tumour specimen of a rat kept in a medical institution (Yamanishi et al., 1983), passaged 6 times in Vero E6 cells and prepared as stock virus. The virus titre was measured by a direct immunofluorescence (IF) method as follows. Vero E6 cells in Lab-Tek 8 chambers (Miles Laboratories) were inoculated with diluted virus solution and adsorbed for 1 h at 37 °C. The cultures were overlaid with the maintenance medium containing 1.3% methyl cellulose, and then kept for 7 days at 37 °C in a CO₂ incubator and fixed in a mixture of 90% acetone and 10% methanol for 15 min at 4 °C. IF tests were performed using clone 80 monoclonal antibody. The isolation and characterization of this monoclonal antibody has been reported previously (Yamanishi et al., 1984). Briefly, BALB/c mice were immunized with lysates of Hantaan virus (76-118 strain)-infected cells. Hybridomas were obtained from the cultures of SP/2 myeloma cells fused with spleen cells from immunized mice. Twenty clones were obtained and clone 80 reacted with a polypeptide of mol. wt. approx. 50000, presumably corresponding to the viral nucleocapsid protein. Antibody from ascites was purified by centrifugation followed by precipitation with ammonium sulphate, and labelled with fluorescein isothiocyanate (FITC). The number of foci was counted under the fluorescence microscope and the titre was expressed as the number of focus-forming units (f.f.u.) per ml.

*Animals.* Pregnant Fisher rats (F344/N strain), which were obtained from Shizuoka Laboratory Animal Center and kept in our Institute, were used. Three litters of newborn rats (total of 24 rats per experiment) were inoculated intraperitoneally (i.p.) with 0.05 ml of virus (8.5 × 10⁴ f.f.u./rat).

*Infection.* Virus isolation from brain, lung, spleen, kidney and peripheral blood of newborn rats was attempted at 1, 3, 5, 8, 11, 15, 20 and 25 weeks after inoculation. Each organ was not homogenized but minced, and put on to Vero E6 cells in plastic dishes. After 1 day of cultivation, cultures were washed to remove minced pieces of organs and then kept at 37 °C in a CO₂ incubator. Cells were trypsinized at 1 or 2 weeks after inoculation and seeded onto 12-well spot-slides. Subsequently, after overnight incubation, the cells were fixed with a mixture of 90% acetone and 10% methanol for 15 min at 4 °C and direct IF tests were performed as described above.

*Fractionation of rat or human peripheral leukocytes.* Human peripheral blood was obtained from healthy donors who had no evidence of HFRS and had no antibodies in their sera against the B-1 strain virus. Rat peripheral blood was collected by heart puncture at 5 weeks post-infection. Rat and human peripheral blood were collected in heparinized syringes and separated as described elsewhere (Kumagai et al., 1979).

Briefly, peripheral mononuclear cells were obtained by centrifugation on Lymphocyte Separation Medium (Litton Bionetics Laboratory Products, Kensington, Md., U.S.A.). After washing twice in RPMI 1640 medium by low-speed centrifugation, the cells were suspended in RPMI 1640 medium supplemented with 10% FCS. This suspension was placed in plastic dishes which had been coated with FCS by overnight incubation at 4 °C, then incubated for 2 h at 37 °C. Non-adherent cells were collected, washed and suspended in RPMI 1640 medium supplemented with 10% FCS adjusting the concentration of cells to about 1 × 10⁶ cells/ml. Non-adherent cells were used as the lymphocyte fraction. Adherent cells were detached from the plastic dishes by incubation at 4 °C in phosphate-buffered saline (PBS) containing 0-2% EDTA and 5% FCS, and then, after washing twice in RPMI 1640 medium, suspended in RPMI 1640 medium supplemented with 10% FCS so as to adjust the concentration of cells to about 1 × 10⁶ cells/ml. Adherent cells were used as the macrophage fraction.

For activation of lymphocytes, non-adherent cells were cultured with medium containing 0-015 mg/ml phytohaemagglutinin P (PHA-P) (Difco) for 3 days before virus inoculation, and the cells were cultured with RPMI 1640 medium containing 10% FCS and 20% Lymphocult-T (Biotest Diagnostics, F.R.G.) after inoculation. For activation of macrophages, adherent cells were cultured for 3 days with 0-001 mg/ml lipopolysaccharide (LPS) (Difco), which has an ability to stimulate macrophage function (Allison et al., 1973; Hotta & Hotta, 1982), and infected with virus.

Rat granulocytes from peripheral blood were obtained by additional centrifugation on 75% Percoll (Pharmacia). The purity of the granulocytes was assessed by Giemsa staining and was more than 95%.

*Peritoneal exudate cells (PEC).* Healthy Fisher rats aged 6 weeks were used for the collection of PEC. At 3 days after i.p. injection of thioglycollate, rats were sacrificed by heart puncture and PEC were obtained. The cells were suspended in RPMI 1640 medium supplemented with 10% FCS and cultured in plastic dishes which had been...
coated with rat plasma for 3 days. Non-adherent cells were discarded and adherent cells were used as PEC. The purity of the macrophages in PEC was tested as described elsewhere (Kumagai et al., 1979) and approached 100%.

**Virus infection to mononuclear cells from human peripheral blood and PEC from rats.** Lymphocyte and macrophage fractions of human peripheral blood, and PEC from rats were suspended in RPMI 1640 medium and cells were infected at multiplicities of 0-1 f.f.u./cell for human mononuclear cells and 0-5 f.f.u./cell for PEC of rats. After incubation for 1 h at 37 °C for adsorption, cells were washed twice with RPMI 1640 medium, resuspended in RPMI 1640 medium supplemented with 10% FCS and cultured in 24-well plastic plates in a CO2 incubator.

**Infectious centre assay.** Inoculated cells were harvested at the day indicated until 2 weeks after virus inoculation, and infectious centre assays were performed. Serially diluted cell suspensions were inoculated onto Vero E6 cells in Lab-Tek 8 chambers. After adsorption for 1 h at 37 °C, maintenance medium containing 1-3% methyl cellulose was overlaid. Direct IF tests were performed 7 days after infection as described above. At the same time, the number of living cells were counted by the exclusion test, using 0-2% (w/v) trypan blue dye.

**Detection of viral antigen in PEC infected with B-1 strain virus.** In order to determine the antigen-positive cells in PEC at various days after infection, adherent cells were treated with PBS containing 0.2% EDTA and 5% FCS to detach them from the plates, suspended in medium RPMI 1640 supplemented with 10% FCS and seeded onto 12-well spot-slides. PEC on slides were fixed and stained as described above, and the ratio of positive cells to total cells was determined by fluorescence microscopy.

**Antibody test by IF.** For antibody detection, cells infected with the B-1 strain were fixed and stained by the indirect IF test as described previously (Yamanishi et al., 1983).

**RESULTS**

**Virus isolation from newborn rats**

Newborn rats were inoculated i.p. with the B-1 strain. Virus isolation from brain, spleen, lung, kidney and peripheral blood was attempted as described in Methods. Rats became ill, with signs such as ruffled hair and hyper-excitability by 30 to 40 days after infection and some of them died (data not shown). Virus could be isolated from all samples repeatedly from 1 week to at least 25 weeks post-infection, and antibody titres measured by IF started to increase at 3 weeks and reached a maximum at 5 weeks post-infection (Table 1).

Next, rats infected with the B-1 strain were sacrificed at 5 weeks after infection and virus isolation was attempted from various fractions of rat peripheral blood. As shown in Table 2, virus could be isolated from the macrophage fraction (5-5 × 10^2 f.f.u./10^5 cells) and the granulocyte fraction (2-3 f.f.u./10^5 cells). Approximately 0-55% of the cells in the macrophage population were virus-infected, a proportion about 240 times higher than that of infected cells found in the granulocyte fraction. These data suggest that the virus would be highly associated with the macrophage fraction in vivo.

**Virus growth in PEC of rats**

Since the virus could be isolated mainly from macrophages of the peripheral blood of rats as described above, virus growth in PEC was tested. Cells were infected with the B-1 strain at a m.o.i. of 0-5 f.f.u./cell. Virus titres in supernatants of infected cells, the number of infectious centres and the number of cells having viral antigen were calculated from 1 day to 14 days after infection. As shown in Fig. 1, virus grew well in PEC. The maximum titres by infectious centre assay and in the supernatant were 2-7 × 10^4 f.f.u./10^5 cells and 4-9 × 10^4 f.f.u./ml respectively at 5 days after virus inoculation. The percentage of antigen-positive cells in PEC reached almost 100% at 5 days after inoculation. The fluorescence pattern in PEC is shown in Fig. 2.

**HFRS virus replication in human peripheral mononuclear cells**

Mononuclear cells from human peripheral blood were fractionated into non-adherent (consisting mainly of lymphocytes) and adherent cells (macrophages). A portion of the cell suspension was treated with PHA-P and Lymphocult-T for lymphocytes, or with LPS for macrophages, and infected with the B-1 strain at a m.o.i. of 0-1 f.f.u./cell. Virus titres in supernatants and infectious centres were measured on Vero E6 cells. Virus did not multiply appreciably in either non-activated or activated lymphocytes (Fig. 3a). In contrast, virus replication was observed in macrophage-rich adherent cells which were either unstimulated or LPS-stimulated (Fig. 3b). The maximum titre of virus was 7-1 f.f.u./10^5 cells by infectious centre
Table 1. Virus isolation from various organs and peripheral blood of rats inoculated with B-1 strain i.p.*

<table>
<thead>
<tr>
<th>Week after virus inoculation</th>
<th>Virus isolation from</th>
<th>Antibody titre (4⁺)†</th>
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<tr>
<td></td>
<td>Brain</td>
<td>Lung</td>
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<tr>
<td>1</td>
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* Minced organs and peripheral blood from two rats were put onto Vero E6 cells. Inoculated cells were tested by IF after 7 days or 14 days (passaged once) after inoculation. The positive rate was scored from − to +++; positive at 7 days after inoculation (+ ++), 14 days after inoculation (+).
† Antibody titres were measured by indirect IF as described in Methods.
‡ NT, Not tested.

Table 2. Virus isolation from various fractions of peripheral blood of infected newborn rats at 5 weeks after inoculation

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Virus titre (f.f.u./10^5 cells)</th>
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<tbody>
<tr>
<td>Lymphocytes</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Macrophages</td>
<td>5.5 x 10^2</td>
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<tr>
<td>Granulocytes</td>
<td>2.3</td>
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<tr>
<td>Erythrocytes</td>
<td>&lt;1</td>
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</table>

* Newborn rats were inoculated with HFRS virus B-1 strain within 24 h after birth. Virus isolation was performed at 5 weeks after inoculation. Fractions were separated as described in Methods.

Discussion

HFRS virus usually infects man by the air-borne route or by rodent bite, and systemic infection occurs. The major clinical manifestations are fever, prostration, proteinuria, haemorrhagic phenomenon and renal failure (Lee, 1982). When suckling mice were inoculated i.p. or intracerebrally with Hantaan virus, virus antigen was present in almost all organs including brain, spleen and lung (Kurata et al., 1983). In this paper, an HFRS-related virus (the B-1 strain), which was isolated from a laboratory rat, was inoculated into newborn rats instead of mice. Rats also manifested clinical symptoms such as ruffled hair and hyper-excitability as in the case of mice, and virus could be isolated from brain, spleen, lung, kidney and peripheral blood from 1 week to at least 25 weeks post-infection (Table 1). This evidence suggests that the infection in rats persisted in some organs for at least 25 weeks and moreover viraemia persisted for a long time in spite of the existence of high titred antibodies in the blood. It would be interesting to examine in which fraction of blood cells the virus exists persistently. As shown in Table 2, virus could be isolated from adherent cells (mainly macrophages) and virus growth in vitro in PEC of rats was considerable (Fig. 3b). These results suggest that HFRS virus will grow also in human macrophages in vitro.

When virus replication in human leukocytes was compared with that in rat macrophages from peritoneal exudates, limited replication was observed only in the macrophage fraction from
HFRS virus in rats

Fig. 1. Virus replication in PEC from rats. Cells were infected with the B-1 strain at an m.o.i. of 0.5 f.f.u./cell. After adsorption for 1 h at 37 °C, the monolayers were washed twice with RPMI 1640 medium, overlaid with maintenance medium and incubated at 37 °C as described in Methods. Cultures were harvested at the times indicated and virus titres were measured on Vero E6 cells. ●, Virus titre in supernatant (f.f.u./ml); ○, infectious centre assay (f.f.u./10^5 cells); △, numbers of antigen-positive cells (%).

Fig. 2. Immunofluorescence staining of PEC infected with the B-1 strain. Cells were infected with the B-1 strain, fixed 7 days after infection and stained by the direct IF method using monoclonal antibody clone 80 conjugated with FITC. (a) Infected cells; (b) uninfected cells. Note the defined granular dots in the cytoplasm.

Human peripheral blood and virus growth was not significant in the lymphocyte fraction (Fig. 3). It is known from several studies that mitogen-stimulated lymphocytes are permissive for the replication of herpes simplex virus (Nahmias et al., 1964; Westmoreland, 1978; Braun et al., 1984), poliovirus (Willems et al., 1969), measles virus (Joseph et al., 1975; Sullivan et al., 1975) and dengue virus (Theofilopoulos et al., 1976), and also that dengue virus could multiply in cultures of mouse peritoneal macrophages activated with bacterial LPS (Hotta & Hotta, 1982). In this paper, stimulated lymphocytes or macrophages were infected with the B-1 strain of HFRS virus. While virus growth in LPS-stimulated macrophages was enhanced, no viral replication was observed in PHA-stimulated lymphocytes (Fig. 3). These data suggest that virus replication is dependent on the state of cell differentiation.
Fig. 3. Growth curves of the B-1 strain in human mononuclear cells. (a) Lymphocytes were either stimulated with PHA-P (lower panel) or not (upper panel); (b) macrophages were either stimulated with LPS (lower panel) or not (upper panel) and infected with the B-1 strain and at each time point, samples of culture fluid or infected cells were harvested. The infectivity yield in fluid (○) (f.f.u./ml) and the numbers of infected cells (●) (f.f.u./10^5 cells) were determined as described in Methods.

It is evident that some viruses multiply in certain leukocyte fractions (Bang & Warwick, 1960; Goodman & Koprowski, 1962; Edelman & Wheelock, 1967; Wheelock & Edelman, 1969; Willems et al., 1969; Joseph et al., 1975; Sullivan et al., 1975; Rinaldo et al., 1978; Theofilopoulos et al., 1976; Turner & Ballard, 1976; Halstead et al., 1977; Levitt et al., 1979). These observations are interesting when we consider the mechanism responsible for systemic infection. Even though the macrophage might not be a primary target of HFRS virus, it is conceivable that systemic infection with HFRS virus could be caused by macrophages which might act as a carrier or propagator of the virus. It has been reported that viral antigen is present in endothelial cells lining blood vessels (Kurata et al., 1983). If the virus propagates in macrophages, infected macrophages could attach to the endothelial cells and consequently virus could be systemically spread to various organs. Moreover, it would also be possible for virus to pass the blood–brain barrier and to infect the brain. In fact, virus was isolated from the brain as early as the seventh day post-infection as shown in Table 1.

HFRS is a systemic disease and the antibody titres in sera of patients or infected animals were extremely high. Recently, the mechanism of prevention and recovery from viral diseases have been extensively studied with respect to both humoral immunity and cell-mediated immunity, including cytotoxic T cells, natural killer activity and delayed-type hypersensitivity (DTH). Macrophages have an important role in host defence mechanisms as phagocytic cells, as the effector cells in antibody-dependent cytotoxicity and in DTH, and also as the cells that produce interferon (Hirsch, 1984). If macrophages are incapacitated by viral infection, it would be expected that other defence mechanisms such as high titred antibodies and cytotoxic T cells will work cooperatively towards recovery from disease. The mechanisms for recovery from HFRS virus infection in animals are under investigation.

REFERENCES


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