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PATHOGENESIS OF HAEMAGGLUTINATING ENCEPHALOMYELITIS VIRUS (HEV) IN MICE EXPERIMENTALLY INFECTED BY DIFFERENT ROUTES

By

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INTRODUCTION

Haemagglutinating encephalomyelitis virus (HEV), classified as a coronavirus, causes encephalomyelitis or so-called vomiting and wasting disease in suckling piglets. Some strains of HEV cause fatal encephalomyelitis characterized by squealing, vomiting, constipation, progressive paralysis and nervous signs in suckling piglets up to one week of age (Mitchell, 1963). Other strains have been shown to cause vomiting and wasting disease of piglets under 2 weeks of age, i.e. signs of gastrointestinal infection rather than encephalomyelitis (Alexander and Saunders, 1969; Cartwright, Lucas, Cavill, Gush and Blandford, 1969; Kershaw, 1969). On the other hand, Mengeling, Boothe and Ritchie (1972) have isolated HEV from tonsillar swabs of asymptomatic adult pigs.

Several coronaviruses are known to propagate in suckling mouse brain (Simpson and Groupč, 1959; McIntosh, Becker and Chanock, 1967; Bhatt, Percy and Jonas, 1972; Weiner, 1973; Horzinek, Osterhause, Wirahadiredja and der Kreek, 1978). HEV, however, replicates only in porcine kidney (PK) cell culture (Greig and Girard, 1963; Mengeling et al., 1972). Appel, Greig and Corner (1965) have reported that when some laboratory animals are inoculated orally with HEV-1 strain, they develop no clinical signs and virus cannot be re-isolated. So far as the authors are aware, the attempt to demonstrate the growth of HEV in laboratory animals has not been successful. In this report, we present some basic experiments elucidating the pathogenesis of HEV in mice, and the target cell for viral replication by immuno-fluorescent and electron-microscopic observations.

MATERIALS AND METHODS

Mice

Random-bred ddY mice were obtained from the Shizuoka Laboratory Animal Centre, Hamamatsu, Japan, and bred at our laboratory. Litters were weaned at 3 weeks of age and given a commercial diet and water ad libitum.

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Virus

The 67N strain of HEV used was supplied by Dr W. L. Mengeling, National Animal Disease Laboratory, Ames, Iowa, U.S.A., and was passaged 12 times in primary PK cell cultures and 10 times in suckling mouse brain. The suckling mouse-propagated strain, designated MB-67N strain, was used for further experiments.

Antiserum

Antiserum against the 67N strain of HEV was prepared in rabbits by intravenous injections of the virus propagated in PK cell cultures.

Mouse Inoculation

Inoculation of mice with the virus was made by the intracerebral (i.c.), intranasal (i.n.), intraperitoneal (i.p.) and subcutaneous (s.c.) routes. The inoculum was 0.02 ml for the i.c. and i.n. routes and 0.1 ml for the i.p. and s.c. routes. The mice were observed for clinical signs for 2 weeks after inoculation.

Infectivity Assay in Suckling Mice

For determination of infectivity titres, 10-fold dilutions of the virus or 10 per cent tissue suspensions were made in phosphate buffered saline (PBS, pH 7.2) containing antibiotics and clarified by centrifugation at 2000 rpm for 10 min. At each dilution 0.02 ml was inoculated by the i.c. route into 3-day-old suckling mice, using 4 mice per dilution. The inoculated mice were observed daily for 10 days. The LD_{50} of virus suspensions was calculated by the method of Reed and Muench (1938).

HA and HI Tests

Haemagglutinating activity (HA) and haemagglutination inhibition (HI) tests were performed by the method described by Hirai, Chang and Shimakura (1974).

Neutralization Test

A virus suspension of 0.5 ml (10^{2} LD_{50} per 0.02 ml) was mixed with an equal volume of serial 2-fold dilutions of serum heated at 56 °C for 30 min and incubated at room temperature for 60 min. Each virus-serum mixture was assayed in four 3-day-old mice by i.c. inoculation. The neutralizing antibody titre was expressed as the reciprocal of the highest serum dilution at which at least 2 of 4 mice survived.

Immuno-fluorescence Staining

Anti-HEV serum was collected from a rabbit inoculated intravenously with the 67N strain. Anti-rabbit IgG was prepared in guinea-pigs by intravenous injections of rabbit IgG, and conjugated with fluorescein isothiocyanate (FITC). The tissues were rapidly frozen in n-hexane chilled in dry ice and acetone, cut by cryostat to approximately 4 μm thickness and immersed in acetone at -20 °C for 20 min. Staining was by the indirect method at room temperature.

Electron Microscopy

The brains of suckling mice were fixed for 2 h in chilled 50 mM phosphate buffer (PB, pH 7.2) containing 2 per cent glutaraldehyde. Post-fixation was carried out in 1 per cent osmium tetroxide in 50 mM PB. Fixed specimens were rinsed with 200 mM PB containing 0.4 per cent sucrose, dehydrated in a graduated ethanol-water series and
propylene oxide and embedded in Epon 812. Sections were cut on a Porter Blum MT 2-B ultramicrotome, stained with uranyl acetate and lead citrate and examined with a Hitachi model HU-12 electron microscope at an accelerating voltage of 70 kV.

**RESULTS**

**Serial Passages in Suckling Mice**

The 67N strain of HEV propagated in PK cells was serially passaged in suckling mice by i.c. inoculation. On the first passage, 67N strain caused illness with an onset at 4 to 8 days post-inoculation (p.i.) and characterized by emaciation and weakness, followed by paralysis and death at 6 to 10 days. All 8 mice inoculated with 67N strain were affected. At the same time, 8 control mice inoculated with culture fluid of uninfected PK cells remained well. The subsequent passages were performed by i.c. inoculation with 10 per cent suspensions of pooled brain obtained from moribund or dead mice. In the course of serial passages, the incubation period decreased progressively, and death occurred consistently at 2 to 3 days p.i. Control material passaged in parallel with the infected passage series produced no clinical signs. In a 10 per cent suspension of the tenth passed brain, infectivity titres assayed in 3-day-old mice and PK cell cultures were $10^{7.4}$ LD$_{50}$ per 0.02 ml and $10^{4.0}$ TCID$_{50}$ per 0.2 ml, respectively. The adaptation of 67N to suckling mouse brain was repeated a second time. The 67N strain of HEV passaged 10 times in suckling mice was designated the MB-67N strain and was employed for further experiments.

**Haemagglutination Activity (HA)**

The MB–67N strain was tested for HA with erythrocytes of various species at room temperature. The virus agglutinated the erythrocytes of chicken, mouse and rat and their HA titres were 16 400, 8200 and 8200, respectively. Erythrocytes of guinea-pig, rabbit, dog, goat, sheep, calf, goose, turkey and human (O-type) failed to agglutinate under test conditions.

**Serological Tests**

The serological specificity of the MB–67N strain against anti-67N rabbit serum was examined by neutralization and HI tests. As shown in Table 1, the MB–67N and original 67N strains were neutralized by a 1 in 256 and a 1 in 512 dilution of anti-67N serum, respectively. The HI titre of anti-67N serum against the MB–67N was 160, compared with 1280 for the original 67N strain.

**Growth Curve of MB–67N in Mouse Brain**

Forty 3-day-old mice were inoculated i.c. with $10^3$ LD$_{50}$ of MB–67N. Three mice each were killed at 6- or 12-h intervals and a 10 per cent suspension of pooled brains was made in PBS for assays of infectivity and HA. Dead mice
TABLE 1

| Viruses                        | Antibody titres |
|--------------------------------|-----------------|
|                                | N       | HI       |
| Mouse-adapted MB-67N           | 256*    | 160†    |
| Original 67N                   | 512†    | 1280‡   |

Neutralizing (N) antibody titres assayed by 3-day-old mice (*) and PK cell cultures (†) expressed as the reciprocal of the highest dilution of serum that neutralized 100 LD₅₀ and 100 TCID₅₀ of the virus, respectively.

Haemagglutination inhibition (HI) antibody titres expressed as the reciprocal of the highest dilution of serum that inhibited 8 HA of the virus.

were excluded from the experiments. The infectivity titre increased logarithmically from 18 to 60 h p.i. A plateau of 10⁶⁸ to 10⁷² LD₅₀ per 0.02 ml was maintained between 60 and 96 h p.i. The HA titre reached 8200 at 60 h p.i. and remained at that value until termination of the experiment (Fig. 1).

Fig. 1. Virus growth curve in the brain of 3-day-old mice inoculated with MB-67N strain (10³ LD₅₀).

- - Inoculation titre; ▲ ▲ HA titre.
Relation of Mouse Age and Susceptibility

Further experiments on the pathogenesis of MB-67N were performed using various ages of mice and routes. Groups of 5 to 10 mice aged 3, 6, 9, 12, 16, 20 and 35 days were inoculated with a large dose (10^6 LD₅₀) of virus by the i.c., i.n., i.p. and s.c. routes. All mice inoculated by the i.c. route developed nervous signs and died regardless of age. Following i.n., i.p. and s.c. inoculation, suckling mice under 16 days old died with similar signs, whereas 20-day-old or older mice failed to show signs during the 14-day observation period (Table 2).

Since all mice inoculated by the i.c. route with a large dose of virus died regardless of age, 3- to 35-day-old mice were inoculated with 10-fold dilutions of virus suspension by the i.c. route and infectivity titres of the virus were calculated for each age group. Titres were 10^{7.4}, 10^{6.5}, 10^{5.3}, 10^{4.8}, 10^{4.3} and 10^{4.2} LD₅₀ per 0.02 ml in 3-, 6-, 9-, 12-, 16-, 20- and 35-day-old mice by i.c. inoculation, respectively. Similarly, comparative titration was performed in 3-day-old mice inoculated by the i.n., i.p. and s.c. routes. Titres in 3-day-old mice by i.n., i.p. and s.c. inoculation were 10^{6.9} LD₅₀ per 0.02 ml, 10^{3.2} and 10^{3.0} LD₅₀ per 0.1 ml, respectively (Table 3).

### Table 2

| Inoculation routes | Mouse age (days) |
|--------------------|------------------|
|                    | 3    | 6    | 9    | 12   | 16   | 20   | 35   |
| i.c.               | 10/10* | 8/8  | 8/8  | 7/7  | 8/8  | 6/6  | 9/9  |
| i.n.               | 8/8   | 8/8  | 5/5  | 5/5  | 6/6  | 0/5  | 0/6  |
| i.p.               | 10/10 | 8/8  | 5/5  | 5/5  | 6/6  | 0/5  | 0/6  |
| s.c.               | 10/10 | 8/8  | 5/5  | 5/5  | 5/5  | 0/5  | 0/6  |

Each mouse was inoculated with approximately 10^6 LD₅₀ of MB-67N and observed for 14 days after inoculation.

* Mortality (No. of dead mice per No. of inoculated mice).
  i.c. = intracerebral, i.n. = intranasal, i.p. = intraperitoneal and s.c. = subcutaneous.

### Table 3

| Mouse age (days) | Inoculation routes | Infectivity titre* |
|------------------|--------------------|--------------------|
| 3                | i.c.               | 7.4                |
| 3                | i.n.               | 2.9                |
| 3                | i.p.               | 3.2                |
| 3                | i.c.               | 3.0                |
| 6                | i.c.               | 6.5                |
| 9                | i.c.               | 5.3                |
| 12               | i.c.               | 4.0                |
| 16               | i.c.               | 4.3                |
| 20               | i.c.               | 4.3                |
| 35               | i.c.               | 4.2                |

* Infectivity titres expressed as log₁₀ LD₅₀ per 0.02 ml in i.c. and i.n. inoculation and as log₁₀ LD₅₀ per 0.1 ml in i.p. and s.c. inoculation.

Inoculation routes as in Table 2.
Viral Distribution in Mice

Groups of five 3- and 35-day-old mice were inoculated with $10^6 \text{LD}_{50}$ of MB-67N by different routes and killed when moribund or after 14 days' observation. Ten per cent suspensions of pooled organs were assayed in 3-day-old mice by i.c. inoculation. As shown in Table 4, the infectivity titres of brain suspensions from 3-day-old mice inoculated by the i.c., i.n., i.p. and s.c. routes were $10^{7.2}$, $10^{6.5}$, $10^{5.6}$ and $10^{3.6} \text{LD}_{50}$ per 0.02 ml, respectively. Low titres or no virus was re-isolated from organs other than lung following i.n. inoculation. In 35-day-old mice, the virus was re-isolated only from the brain after i.c. inoculation. Neither signs nor virus re-isolation were detected in other routes.

### Table 4

| Mouse age (days) | Inoculation routes | Infectivity titres* |
|------------------|--------------------|---------------------|
|                  | Brain | Lung | Liver | Spleen | Kidney | Intestine | Blood |
| 3                | i.c.  | 7.2  | –     | –     | –     | –         | –     |
|                  | i.n.  | 6.5  | 3.0   | –     | –     | –         | –     |
|                  | i.p.  | 5.6  | –     | –     | –     | –         | –     |
|                  | s.c.  | 3.6  | –     | –     | –     | –         | –     |
| 35               | i.c.  | 3.3  | –     | –     | –     | –         | –     |
|                  | i.n.  | –    | –     | –     | –     | –         | –     |
|                  | i.p.  | –    | –     | –     | –     | –         | –     |
|                  | s.c.  | –    | –     | –     | –     | –         | –     |

* Infectivity titres expressed as log_{10} \text{LD}_{50} per 0.02 ml of 10 per cent suspensions of pooled organs from each group of 5 mice.

† Virus detected only in undiluted 10 per cent suspension.

‡ These mice showed no clinical signs and were killed at the end for 14 days of observation.

Each mouse was inoculated with approximately $10^6 \text{LD}_{50}$ of MB-67N.

Inoculation routes as in Table 2.

In the final experiment, 3 each of 9-day-old mice inoculated with $10^{7.5} \text{LD}_{50}$ of the virus by the i.n. route were killed at 24 h, 48 h and 72 h and viral distribution was examined. Virus was detectable in the olfactory bulb, cerebrum and lung at 24 h p.i. and titres became higher in the olfactory bulb, cerebrum and cerebellum in the course of infection. Viral recovery from the olfactory bulb seemed to precede the appearance of virus in cerebrum and cerebellum. Similar titres of virus were detected in the lung for 72 h until the late stage of infection. In other organs, very low titres were detectable only at 72 h p.i. (Table 5).

Histological and Immuno-fluorescence Findings

Histological examination of the brain was carried out in the late stages of infection. Areas of focal necrosis were numerous in all parts of the cerebral cortex after all inoculation routes (Fig. 2). The lesions following i.c. and i.n. inoculation appeared to be more severe than those following i.p. and s.c.
HEV IN SUCKLING MICE

TABLE 5
VIRAL DISTRIBUTION AT DIFFERENT STAGES IN 9-DAY-OLD MICE INOCULATED WITH MB-67N STRAIN BY THE INTRANASAL ROUTE

| Time after inoculation (h) | Infectivity titres* |
|---------------------------|---------------------|
|                           | Olfactory bulb | Cerebrum | Cerebellum | Lung | Liver | Spleen | Kidney | Blood |
| 24                        | 2.0            | 2.0      | -         | 3.0  | -     | -      | -      | -     |
| 48                        | 6.3            | 4.5      | 4.0       | 2.5  | -     | -      | -      | -     |
| 72                        | 7.5            | 7.7      | 7.3       | 3.0  | ↑     | ↑      | ↑      | ↑     |

* Infectivity titres expressed as log_{10} LD_{50} per 0.02 ml of 10 per cent suspensions of pooled organs from each 3 mice.

† Virus detected only in undiluted 10 per cent suspension.

Each mouse was inoculated with approximately 10^{7.5} LD_{50} of the virus.

routes. The distribution of virus-specific fluorescence was closely correlated with that of the histological lesions (Fig. 3). A bright fluorescence was found in the cytoplasm of neurons and ependymal cells in sections at 48 h p.i.

Electron Microscopic Findings

Various features of the developmental process of the virus were found in brain sections from 3-day-old mice after i.c. inoculation. In the early stage, swelling of mitochondria and increase in endoplasmic reticulum were associated with accumulation of free ribosomes. Particles, presumably virions, approximately 90 nm in diameter, appeared individually or in short rows in the space between membranes of endoplasmic reticulum in some areas (Fig. 4). Similar particles were visible within electron-dense areas enclosed by a membrane or a double membrane (Fig. 5). In the next stage, the particles became more numerous. Amorphous tubular structures and a large number of vesicles containing virions were observed in the cytoplasmic space of the nerve cells (Fig. 6). The developmental process of individual particles appeared to be that of budding from intracytoplasmic membranes (Figs 7 to 9). In the late stage, vesicular or cellular walls were broken and the particles were frequently noted in the extracellular space. The mature particles had club-shaped projections and were 100 to 130 nm in diameter.

DISCUSSION

Several coronaviruses have been shown to grow in suckling mouse brain (Simpson and Groupé, 1959; McIntosh et al., 1967; Bhatt et al., 1972; Weiner, 1973; Horzinek et al., 1978), but HEV has not. HEV replicates only in porcine kidney cell cultures, because of its fastidious growth requirements. Appel et al. (1965) have reported that suckling mice inoculated orally with HEV-1 strain developed no clinical signs and that virus was not re-isolated from oral swabs.

We have demonstrated the growth of the 67N strain of HEV in suckling mouse brain following i.c. inoculation. The results of this study present confirmatory evidence that the MB-67N strain passaged in suckling mouse
Fig. 2. Focal necrosis in the cerebral cortex. 72 h p.i. by i.c. route. HE. Bar=50 μm.

Fig. 3. Viral antigen in cytoplasm of nerve cells in cerebral cortex. 48 h p.i. by i.c. route. Bar=10 μm.

Fig. 4. The particles, presumably immature virions, appeared individually or in short rows in the space between membranes of endoplasmic reticulum in the infected brain. 24 h p.i. by i.c. route. Bar=100 nm.

Fig. 5. A cluster of immature virions in an electron-dense area. 24 h p.i. by i.c. route. Bar=100 nm.
Fig. 6. Various stages of virus development in cytoplasm of nerve cells. Amorphous tubular structure (AT) and a large number of vesicles containing mature virions (V) are shown. Nucleus (N). 48 h p.i. by i.c. route. Bar = 0.5 μm.

Figs 7-9. Budding process of virions on vesicular wall. 60 h p.i. by i.c. route. Bar = 100 nm. The earliest event appeared to be the segment of a crescent developing on vesicular wall (short arrow in Fig. 7). The crescent bulged further into the vesicle (Fig. 8). It bulged away from the cytoplasm into the vesicle, and developed a dense underlying layer on the cytoplasmic side. Club-shaped projections were already visible on the surface of the vesicular side of particles (Fig. 9). A mature virion has been released into the vesicles (long arrow in Fig. 7). The underlying layer remained on the vesicular wall.
brain is identical in serological and morphological properties with the cell-propagated 67N strain of HEV. Mouse hepatitis virus (MHV), a coronavirus, is common and causes persistent infection in laboratory mouse colonies (Fujiwara, Takenaka and Shumiya, 1976), but does not agglutinate erythrocytes of any species. The MB-67N strain could agglutinate the erythrocytes of chicken, mouse and rat, and react with antiserum against the original 67N strain of HEV. Thus, the MB-67N was confirmed to be identical to HEV.

HEV infection of pigs is known to occur as three different types: fatal encephalomyelitis in suckling piglets (Mitchell, 1963), vomiting and wasting disease in piglets under 2 weeks of age (Alexander and Saunders, 1969; Cartwright et al., 1969; Kershaw, 1969) and asymptomatic infection of adults (Mengeling et al., 1972). In the present study, mice inoculated with the MB-67N strain by the i.c. route were shown to succumb without age difference, although a smaller dose of virus was fatal for suckling mice only. Adults did not die after i.n., i.p. or s.c. inoculation, even with a large dose of the MB-67N strain. Death occurred in suckling mice under 16 days old by i.n., i.p. and s.c. inoculation. These findings indicate that the susceptibility of mice for the MB-67N strain was influenced by age and inoculation routes, and appears to resemble that of HEV infection in pigs.

In transmission studies on piglets, virus was re-isolated from tonsil, respiratory tract, brain and spinal cord in late stages but not in the early stages of infection (Appel et al., 1965; Cutlip and Mengeling, 1972; Mengeling and Cutlip, 1972). The present results of viral distribution revealed that a high titre of virus was re-isolated from the brain of diseased mice after i.c., i.n., i.p. and s.c. inoculation with the MB-67N strain in the late stages of infection. These findings indicate that the MB-67N strain replicates mainly in certain cells of the central nervous system (CNS). Also, the finding that the virus found in the olfactory bulb preceded the appearance of the virus in the cerebrum and the cerebellum in i.n. inoculated mice, suggests neural transmission through the olfactory nerve.

Recently, Andries, Pensaert and Callebaut (1978) and Andries and Pensaert (1980a, 1980b) have determined the preliminary sites of viral replication and the route of viral spread in oro-nasally infected piglets by the direct fluorescent antibody technique and viral isolation. They stated that nasal mucosa, tonsil, lung and small intestine served as primary sites of replication and the virus then progressed via peripheral nerves to the CNS. The trigeminal nerve, vagal nerve and intestinal plexuses were also proposed as pathways to the CNS (Andries and Pensaert, 1981). Our findings in mice support their concepts in pigs. Furthermore, the olfactory nerve may also serve as one of the pathways to the CNS in i.n. inoculated mice. The relation between age-dependent susceptibility and maturation of the nervous system, regarded as the pathway of viral spread, will be an interesting subject for future research.

Because of the high fatality rate after a rapid course of infection, it was possible to examine the histological changes of the disease over a short period only. These findings revealed the characteristic lesions consisting of necrosis in almost all areas of the brain inoculated by the i.c. route. Similar necrosis of the
brain was observed after i.n. inoculation, but the reaction was slightly milder in i.p. and s.c. routes. McIntosh et al. (1967) reported that the predominant histological finding in mouse brain affected with human coronavirus (HCV) was round cell infiltration of the meninges and perivascular spaces of the cerebral cortex. In mice inoculated with sialodacryoadenitis virus (SDAV) of rats, diffuse and focal neuronal degeneration with minimal inflammatory cell response were found by Bhatt et al. (1972). The findings with the MB–67N strain are considered to be more severe than the findings cited above.

The morphogenesis of the virions in the infected brain was essentially in accord with that in PK cell culture (Mengeling et al., 1972). In addition, the amorphous tubular structures found in infected cells with HCV (McIntosh et al., 1967; Oshiro, Scieble and Lennette, 1971) and MHV (David-Ferreira and Manaker, 1965) were also observed in this study. The relationship of these structures to the developmental process of virus particles has not been elucidated. The findings by immuno-fluorescence and electron microscopy revealed the localization of large numbers of virus particles in the nerve cells. Thus, the nerve cell is considered to serve as a main target of virus replication. In this study, fatal infection of suckling mice caused by HEV was shown and a few similarities between infections of mice and pigs were also represented. Mice infected with the MB–67N strain will be utilized as an experimental model for HEV infection in pigs.

SUMMARY

Three-day-old suckling mice inoculated intracerebrally (i.c.) with the 67N strain of haemagglutinating encephalomyelitis virus (HEV) showed nervous signs and died. The virus was passaged 10 times in suckling mice and was designated the MB–67N strain. The pathogenesis of MB–67N was studied with various ages of mice and inoculation routes. All mice inoculated i.c. with a large dose of virus died regardless of age, although a smaller dose caused fatal infection only in suckling mice. By intranasal, intraperitoneal and subcutaneous inoculation, the virus also killed suckling mice under 16 days old, but not older mice, even with a large dose. The susceptibility of mice for the MB–67N strain was influenced by age and inoculation routes. High titres of virus were re-isolated from the brain of diseased mice after inoculation by any route, but not from other organs. Histologically, numerous areas of severe focal necrosis were produced in the cerebral cortex. Specific immuno-fluorescence and numerous viral particles were found in the cytoplasm of nerve cells by immuno-fluorescence staining and electron microscopy. These findings indicate that the MB–67N propagates mainly in the central nervous system and nerve cells serve as a main target of virus replication.

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