THE PATHOGENESIS OF EXPERIMENTAL INFECTIONS WITH ENCEPHALOMYOCARDITIS (EMC) VIRUS

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INFECTIONS produced by neurotrophic viruses in mice are often used in chemotherapeutic studies but little information is available in the literature about the pathogenesis of such infections. During work on the chemotherapy of encephalomyocarditis (EMC) virus infections the disease in mice and other animals has been studied and results are presented here.

EMC virus was originally isolated from a chimpanzee by Helwig and Schmidt (1945) and is closely related to Columbia–SK, Mengo and MM viruses (Dick, 1949; Warren, Smadel and Russ, 1949). These are now classified as strains of a single picornavirus. They are widely distributed and infect a variety of animals, including man occasionally. Melnick (1950) and Andrewes (1964) described their main characteristics but strains vary considerably according to their passage history. Since the work of Dick (1948), who described the viraemia and invasion of the nervous system by Mengo virus, little work on the disease in mice has been reported. McLaren and Sanders (1959) noted the greater susceptibility of very young mice to EMC, and Force and Stewart (1964) described changes in the neutrophil and lymphocyte counts in mice infected by Columbia-SK virus.

MATERIALS AND METHODS

EMC virus

The Helwig strain, which was supplied by Dr. F. K. Sanders, had had 54 mouse brain passages and had also been passaged by him in Krebs ascites tumour cells. It was used in a few tests here as similarly passaged stock material but usually as a mouse brain stock suspension. There was no difference in the behaviour of the 2 stocks *in vivo*. They were kept at −65° in 1 ml. amounts.

Animals

Albino mice from a commercial strain were used. They weighed 18–20 g. and the ages varied between 35 and 42 days. Other animals were used as indicated in the text.

Titrations of virus stocks and virus in mouse tissues

Serial 10-fold dilutions were made in nutrient broth and 0·2 ml. given i.p. to groups of mice; 3 mice per group were used for titrations of tissue suspensions and 5–10 for stock titrations (done several times during this work). Titres are expressed as the log. of the reciprocal of the dilution killing 50 per cent of the mice. This dilution was calculated by the Kärber method (1931) and refers to the i.p. route except where indicated. The i.c. route was used when greater sensitivity was required; 0·1 ml. was given under ether anaesthesia. Using the smaller groups of mice the estimations of individual titres were not very accurate but the error was small compared to the large increases in titres measured. Readings were made at several time intervals, and for various tissues, to follow the sequence of events.

For all relevant conclusions experiments were repeated at least once during the work. Blood was obtained by cardiac puncture immediately after death, using sodium citrate as an anti-coagulant. Tissue suspensions were prepared by grinding each whole organ with sand in a fixed volume of broth for each organ ; supernates were collected after centrifugation. Preparations were stored at $-20°$ until titrated. The use of convenient volumes of broth meant that the concentrations of original suspensions, as weight wet tissue in broth, varied, *i.e.* brain 21 per cent, heart 14 per cent, stomach plus small intestine 36 per cent, spleen 4 per cent, kidney 32 per cent, liver 33 per cent, lungs 14 per cent, hind leg 44 per cent. Correction factors were used to convert figures to the value per 0·2 g. original wet tissue. The average wet weights of the whole organs were : brain 0·42 g., heart 0·22 g., spleen 0·06 g., kidneys 0·64 g., liver 2·05 g., lungs 0·28 g., stomach plus intestine 2·2 g., hind leg 1·1 g. The amount of blood was taken as 1/12th the body weight. The carcass (less skin, feet, head, tail and organs) weighed 9·75 g.

Infecting doses used in pathogenicity experiments

Although stock virus suspensions were kept at $-65°$ titres varied slightly when titrated at different times. Therefore a " standard inoculum " was used which would be fatal to all the mice in all tests (i.p.) ; this was known because of many tests and contained 10–10^2 LD_{50}, according to titrations at different times. In other tests, when larger inocula were required, 10 or 100 times this dose was given, *i.e.* 10^2–10^3 and 10^3–10^4 LD_{50} respectively. If the virus was titrated during the test the actual value for that test is given.

RESULTS

Route of infection

EMC virus infects adult mice of the age and strain used in this work by the i.m., i.p., s.c., i.v., and i.c. routes. The LD_{50} values and average survival time (AST) are similar for the i.p., s.c. and i.v. routes but the i.c. route is ten times more sensitive (Table). The AST for the i.c. route is much less than for other routes. Death occurs when the virus titre in the brain reaches 10^5–10^7 LD_{50} per 0·2 g. wet tissue. If this dose is given i.c., most mice die within 1–2 days. Fig. 1 illustrates the percentage mortality and AST for log. dilutions of inocula by the i.p., and i.c. routes.

Suckling mice infected i.p. are more susceptible than adults infected either i.p. or i.c. and would be the most sensitive means of detecting EMC virus, even though the inocula are smaller (0·01 ml.).

Events after i.v., s.c. and i.m. infection

The i.v. route of infection was chosen to follow the disappearance of EMC virus from the blood and its early distribution in the body from 0–48 hr. Results are shown in Figs. 2, 3 and 4. After inoculation of 10^3–10^4 LD_{50} there was a rapid disappearance of virus from the blood and it was only just detectable for several hours. It was not detected in the liver and spleen at $5\frac{1}{2}$ hr. At about 12 hr., sometimes as early as 9 hr., virus levels rose in the blood and virus was also present in all the other organs tested. There was a steady rise of virus titre in the blood, in all organs, and in the carcass for the next 24–36 hr. and levels remained high until death.

Results for the s.c. route were similar (Figs. 5 and 6). Virus titres were low at 4 hr. and high in all organs tested at 24 hr. They remained high up to 4 days, when death occurred.

EMC virus grew well in the muscle of these mice. To demonstrate this, 0·1 ml. suspension (10^2–10^3 LD_{50}) was injected into the left hind leg muscle of a

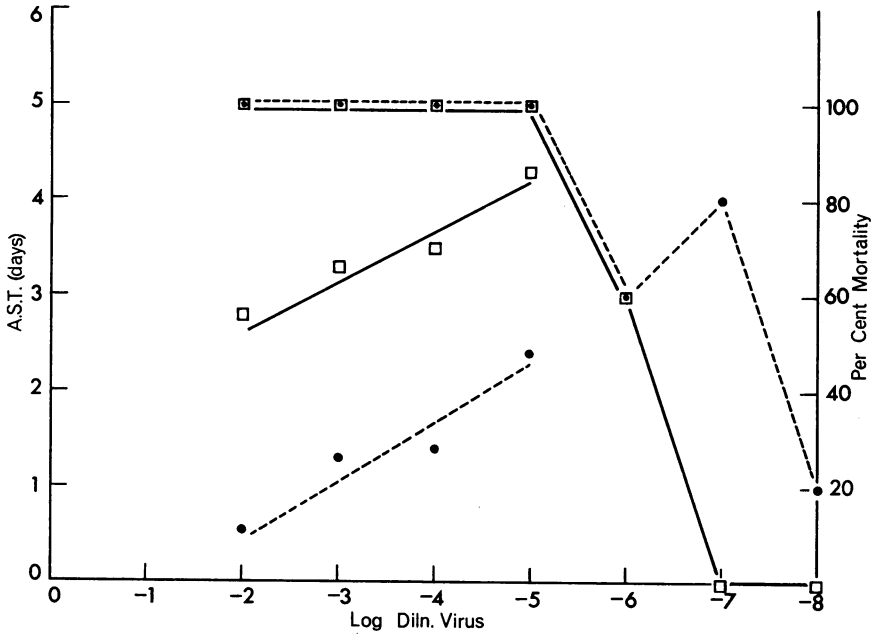

FIG. 1.—Percentage mortality and average survival time (AST) after i.p. and i.c. infection of mice by EMC virus.

● - - - - - - - - ● i.c.; □ ———— □ i.p.

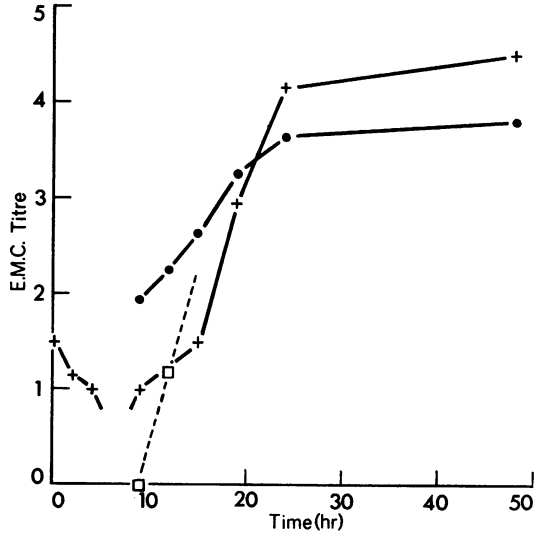

FIG. 2.—EMC virus titres per 0·2 g. wet tissue, expressed as log. reciprocal of LD_{50}, after i.v. infection by 10^3–10^4 LD_{50}.

\+ ———— + blood; ● ———— ● lungs; □ - - - - - - - - □ brain.

The blood levels between 8 and 12 hr. are irregular.

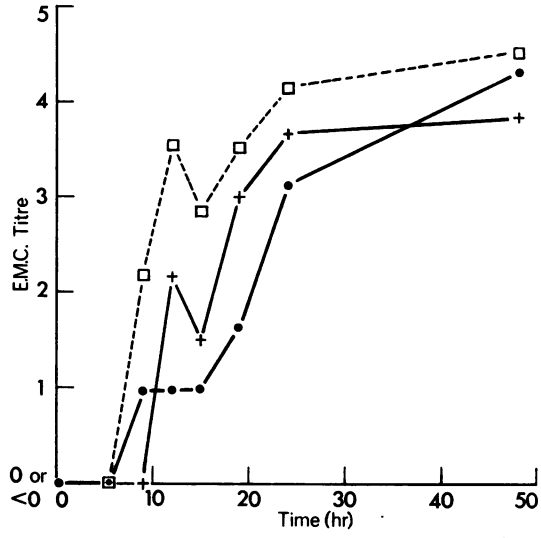

FIG. 3.—EMC virus titres per 0·2 g. wet tissue expressed as log. reciprocal of LD_{50}, after i.v. infection by 10^3–10^4 LD_{50}.

● ———— ● liver; □ - - - - - - - - □ spleen; + ———— + heart.

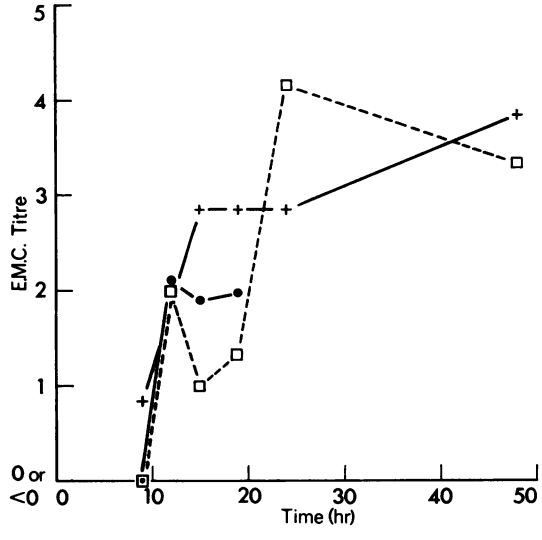

FIG. 4.—EMC virus titres per 0·2 g. wet tissue expressed as log. reciprocal of LD_{50}, after i.v. infection by 10^3–10^4 LD_{50}.

+ ———— + carcass; □ - - - - - - - - □ gut; ● ———— ● kidney.

TABLE.—*Titres* of Several EMC Virus Suspensions Titrated by Different Routes (Groups of 10 Mice, 10-fold Dilutions)*

	i.p.	s.c.	i.v.	i.c.
Suspension A	6·1	—	—	7·1
B	4·4	—	4·5	—
C	5·3	5·5	—	—

* Titres expressed as log. of reciprocal of 50 per cent end-point.

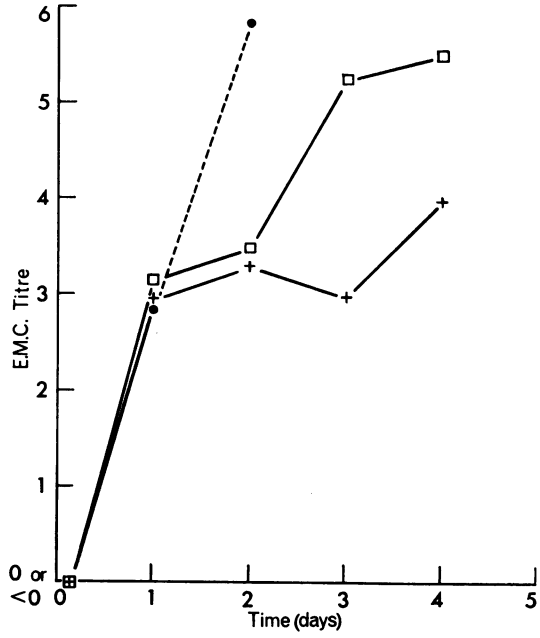

FIG. 5.—EMC virus titres per 0·2 g. wet tissue, expressed as log. reciprocal of LD_{50} after s.c. infection by 10^3–10^4 LD_{50}.

\+ ———— + liver; □ ———— □ heart; ● - - - - - - - - ● brain

(24 and 48 hr. readings only).

group of mice, two of which were killed at intervals. Virus levels for inoculated legs and also for the uninoculated legs and for the blood are shown in Fig. 7. After a rapid fall in titre, in the infected leg, the level was higher than the inoculum at 9 hr. Virus only appeared in the other leg when the level in the blood was increasing (16–20 hr.).

Events after i.p. infection

The i.p. route is commonly used in experimental chemotherapy. After 10–10^2 LD_{50} the virus was widely distributed in the body at 48 hr. In two experiments, each using pools of 5 mice, the titres per 0·2 g. wet tissue were serum 1·83, 1·49; spleen 1·86, 2·86; liver 0·97, 1·31; heart 2·83, 3·83; kidney 1·31, 1·31; brain 2·53, 0·86. Thus, the general picture at 48 hr. was similar to events after

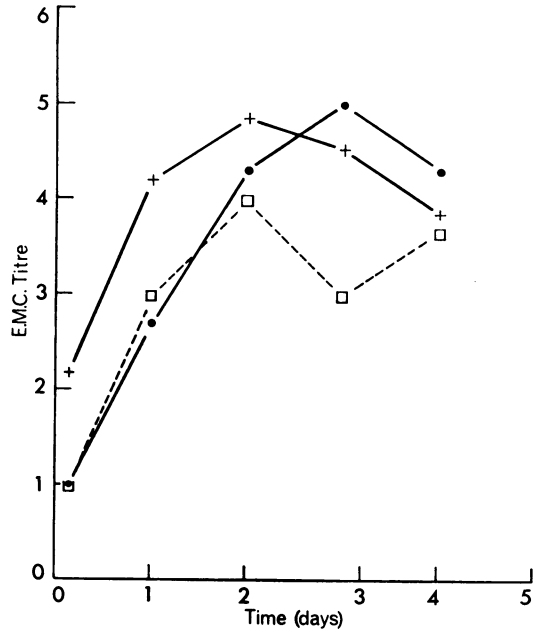

FIG. 6.—EMC virus titres per 0·2 g. wet tissue expressed as log. reciprocal of LD_{50} after s.c. infection by 10^3–10^4 LD_{50} virus.

● ———— ● gut ; + ———— + spleen ; □ - - - - - - - - □ kidney.

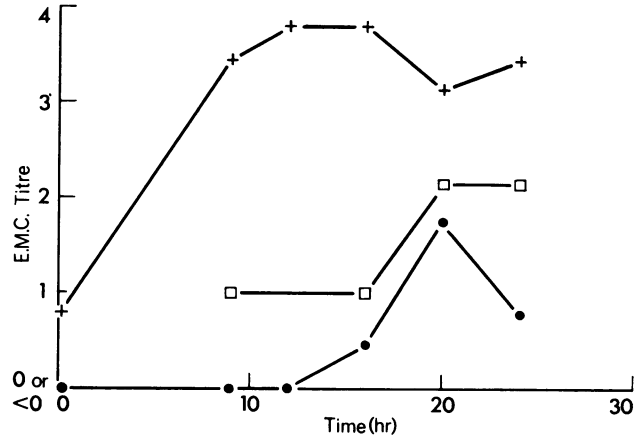

FIG. 7.—Growth of EMC virus in mouse calf muscle. Inoculum 10^2–10^3 LD_{50} in left leg titres per 0·2 g. wet tissue expressed as log, reciprocal of LD_{50}. Pools from 2 mice at each time interval.

+ ———— + left leg ; □ ———— □ blood ; ● ———— ● right leg (control uninoculated).

i.v. or s.c. infection but titres were lower since the inoculum for the i.p. tests was only 1/100 of that for the i.v. and s.c. tests.

The cells of the peritoneal cavity were investigated as a possible site of the primary multiplication of EMC. They were obtained immediately after death by washing out the peritoneal cavity with 1·5 ml. Hank's solution. About 10^6 cells can be obtained, mainly macrophages, lymphocytes and intermediate cells (Mims, 1964*a*). Total virus titres (pools of 2 mice at each time interval) fell slowly after inoculation with 10^2–10^3 or 10^3–10^4 LD_{50} virus. After 10–10^2 LD_{50} it was only just detected immediately after infection. After 10^3–10^4 LD_{50} the titres of washings were 3·5 at 0 hr., 3·5 at 1 hr., 1·83 at 4 hr. and 1·16 at 3 days.

More detailed experiments were then done on peritoneal washings, termed " macrophages " for convenience, to see if growth could be demonstrated.

Experiment I. Mice infected with 10–10^2 *and* 10^2–10^3 LD_{50} *i.p.*—Macrophages were collected from 2 pools, each of 3 mice, at daily intervals and titrated after freezing and thawing. Virus was detected at 0 hr. after 10–10^2 LD_{50} (1/3 mice died with the undiluted suspension) and again was just detected at 24 and 48 hr. (both groups). Of the 2 pools collected at 3 days, one pool had a titre of 1·0 and the other 2·83. Similar results were obtained after 10^2–10^3 LD_{50} virus, increased titres (again variable) only being demonstrated at 2–3 days when virus is widely distributed throughout the body after any route of infection.

Experiment II. Macrophages infected in vitro *and used to infect mice.*—Macrophages from 6 mice were collected and infected with EMC (final theoretical titre 1·9). The infected suspension was immediately given to 8 mice, two of which were killed at intervals and their macrophages titrated for virus content. There was no evidence of growth in 24 hr. At 0 hr. the titre of the inoculum in the absence of macrophages was 1·9 and in the presence of macrophages it was 1·83. Virus was detected only in the undiluted macrophage suspensions of mice killed at 2, 5, $7\frac{1}{2}$ and 24 hr., all mice surviving at 1/10th this dose.

In a similar experiment the inocula had a titre of 2·0 and macrophages at 24 hr. one of 1·79 ; groups of 7 or 10 mice were used in the titrations of pools of macrophages from 5 mice.

Thus there was no evidence of growth in peritoneal macrophages *in vivo*.

Entry of virus into the brain after parenteral infection

The time at which virus enters the brain is important in chemotherapy since after this time a drug can only be active if it passes the blood-brain barrier, unless it is given i.c. After 10^3–10^4 LD_{50} s.c., EMC virus was detected in the brain as early as 4 hr. after infection and regularly from 7 hr. onward. Concentrated normal brain suspension killed other mice when given i.c. so the less sensitive i.p. route was used for detection of the virus. After 10–10^2 LD_{50} i.p. the titre of brain tissue at 48 hr. varied but in one test was 2·53. Virus grew rapidly in the brain (Fig. 8) and, by extrapolation of the curve, virus must have been present in the brain by 12 hr., possibly earlier, allowing for the relative insensitivity of the assay.

The disease in other animals

10^2–10^3 LD_{50} EMC given i.p. did not infect either Wistar albino rats, aged 5 weeks, or albino guinea-pigs (commercial strain) aged 5–6 weeks. Three of each species were tested and no virus was isolated from the blood or brains, tested at 3 or 7 days. No anti-haemagglutinin was demonstrated in the sera after 7 days.

However, a species of jerboa, *Meriones libycus*, was highly susceptible, considerably more so than adult mice and at least as susceptible as suckling mice. About 10–10^2 LD_{50} (mouse) given i.p. or s.c. was rapidly fatal to adult jerboas, aged $2\frac{1}{2}$ months. The animals died within 3 days and the virus was isolated from brain and blood. The blood and brain titres from animals dying 3 days after s.c. infection were approximately : blood (2 animals) 4·82 and brain (1) 4·16. Results after the i.p. infection were similar and the liver titre was also high, approx. 3·5. Brain suspension from normal jerboas did not infect mice. A stock EMC suspension had a titre of 4·8 in mice (i.p.) and 7·0 in jerboas (i.p.) ; in jerboas s.c. it was 6·5.

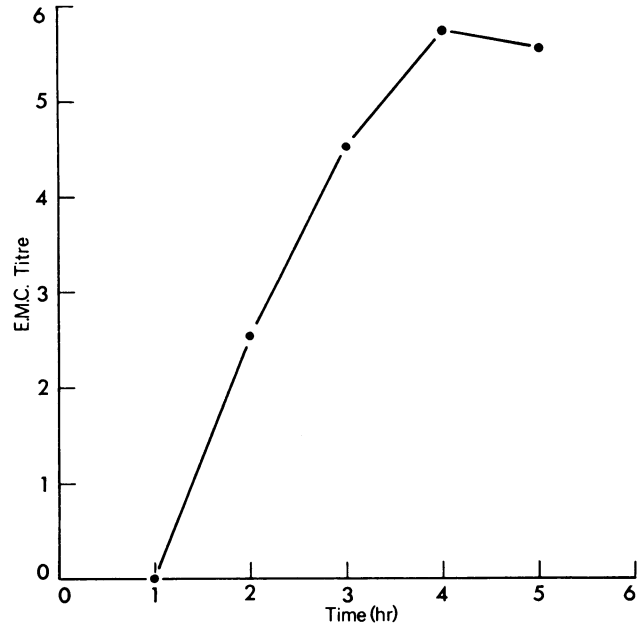

FIG. 8.—Growth of EMC virus in mouse brain. Inoculum 10–10^2 LD_{50} i.p. Pools of 5 brains at 2 and 3 days, 4 at 4 days (1/5 dead), and 2 at 5 days (3/5 dead).

DISCUSSION

Although EMC virus originally produced myocardial lesions in mice and other members of the group are capable of doing so, the rapidly lethal strain used in this work did not do so. Warren *et al.* (1949) stated that a rapidly lethal strain, obtained by repeated i.c. passage in mice, resulted in death before the heart lesions developed. However, the virus still grows well in muscle (Fig. 7) and muscle cells may be the site of primary multiplication. McLaren and Sanders (1959), using an inbred strain, found that only mice younger than 20 days gave great increases in EMC titre after i.m. infection. Mice over 30 days old did not do so but the reason was not an incapacity of the older cells to do so. The mice used in the present work were 35–42 days old and the muscle of these mice certainly supported the growth of EMC virus. More virus was recovered at 12–15 hr. than had been inoculated and considerably more than was recovered 15 min.

after infection. The rise in virus titre in the inoculated leg occurred by 9 hr., i.e. about the time usually found in single cycle growth experiments with EMC virus *in vitro*. The rise in blood level occurred only at 16–20 hr. and the virus appeared in the uninoculated leg at the same time.

The viraemia which occurred after i.v., i.p., s.c. or i.m. infection lasted until death and even low infective doses of this strain of EMC virus were rapidly fatal. Paralysed mice did not recover and those mice which did not develop an obvious infection (after a virus dose killing only a proportion of a group) were invariably found to be sensitive to a challenge infection. Antihaemagglutinin was not detected in survivors. However, towards the end of this work, a mutant which produced large plaques on L cells was supplied by Dr. E. A. Boulter. This strain, EMC/LP, did not kill adult mice so rapidly and many mice recovered, even after relatively large doses of virus i.p. Some mice developed only slight paralysis and others showed no obvious symptoms but the sera of all survivors tested had high anti-haemagglutinin titres 13 days after infection (1/320 to 16 u.). Anti-haemagglutin was not detected before 8 days.

No evidence was obtained for growth of EMC virus in the peritoneal macrophages and virus disappeared from the peritoneal cavity only slowly after i.p. infection. Mims (1964*b*) concluded from studies on tritiated thymidine that peritoneal macrophages in normal mice probably live up to a week before dividing, dying or moving elsewhere. Work discussed by Mims (1964*a*) and his own work on ectromelia virus (which grows in macrophages) indicate that particles can quickly leave the peritoneal cavity by some means. After growth, possibly in muscle cells, virus enters the brain and grows there rapidly until paralysis and death occurs. If large inocula are given, virus enters the brain before any multiplication can occur. It is still not clear how viruses enter the brain.

Virus is present in the kidney by about 9 hr. and is present in the urine. Hence infected faeces and litter are potential sources of infection but cross infection in cages does not occur, at least in short term experiments. As noted by McLaren and Sanders (1959) adult mice are not readily infected orally.

EMC virus has been isolated from several species of animals, and also from mosquitoes. Some strains originally produced experimental infections in many animals, including rats and guinea-pigs (references given by Andrewes, 1964). However, very variable findings have been reported with rats (Kilham, Mason and Davies, 1955), ranging from severe disease to inapparent infections. The strain used here did not grow in the 3 adult rats tested and was not recovered from the tissues, not did it infect guinea-pigs. However *Meriones libycus* was highly susceptible.

The EMC viruses seem particularly variable in behaviour and properties. Thus the virus from squirrels does not cause haemagglutination (Vizoso, Vizoso and Hay, 1964) and there are several plaque variants. The behaviour in different animals, especially rats, varies considerably according to the passage history of the strain, and possibly to its original host, as well as to the age of the animals used in the tests. In the early work strains were studied soon after isolation, *e.g.* from monkeys, but most strains now in use have been passed in mice.

Using EMC in mice as a model infection for chemotherapy studies, it should be possible to determine the mode of action of certain antiviral agents. Thus effects on the host defence mechanism could be determined by the rate of clearance of the virus from the peritoneal cavity. Possible prevention of entry into the

brain could be examined, if treatment began very soon after parenteral infection, and effects on multiplication in different organs readily determined. If another host is required *Meriones libycus* is particularly susceptible.

SUMMARY

The growth of a strain of encephalomyocarditis virus in mice, after infection by various routes, was followed by titration of virus in various tissues, with particular reference to the sequence of events in the early stages of infection. Growth was demonstrated in the muscle, but not in macrophages, *in vivo*. Viraemia lasted until death, which occurred after the rapid growth of virus in the brain.

This strain did not infect adult rats or guinea-pigs but adult jerboas (*Meriones libycus*) were even more susceptible than mice.

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