Multiplication of Lymphocytic Choriomeningitis Virus in Cultivated Foetal Inbred Mouse Cells and in Neonatally Infected Inbred Carrier Mice

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SUMMARY

The replication of infectious LCM virus and its complement-fixing antigen was followed in cultivated foetal cells from colony-bred albino mice, foetal cells from CBA/Ca, C₃H/HeJ, C₅₇BL/6, and DBA/2 inbred mice, and L cells. Typical S-shaped growth curves were obtained in all instances and yields approached 10⁴ intracerebral mouse ID₅₀/cell. After the infection of newborn mice the multiplying virus rapidly reached a plateau of infectivity in tissues which remained thereafter. Ten weeks after neonatal infection the virus concentrations in blood and organs of ordinary albino mice and of CBA, C₃H, C₅₇BL/6, and DBA/2 inbred mice showed a wide range with highest levels in the kidneys and lowest in the blood.

The multiplication patterns and maximum yields of infectious virus and complement-fixing antigen were essentially identical in L cells and in foetal cells derived from five mouse strains; *mutatis mutandis* the same was true for persisting virus in the blood and organs of neonatal carrier mice.

INTRODUCTION

The interaction of lymphocytic choriomeningitis (LCM) virus with *Mus musculus* is regarded as an excellent model for the study of a variety of biological phenomena such as persistent virus infections, immunological tolerance, and pathological immune phenomena in virus diseases. Yet basic knowledge of the biological and physico-chemical properties of LCM virus is still limited. In this report, we describe the multiplication of the LCM virus in mice of inbred strains and in cell cultures derived from them. Differences between mouse strains were not observed.

METHODS

Mice. Inbred mice of the following strains were employed: C₅₇ (C₅₇BL/6J BOM) and DBA (DBA/2J/BOM) from Bomholtgård, Ry, Denmark; AKR and C₃H (C₃H/HeJ) from Jackson Laboratory, Bar Harbor, Maine; and CBA (CBA/Ca) from Animal Suppliers, London, U.K. Skin grafts between individuals of each strain were accepted permanently. The colony-bred albino mice (SCH) were from a local dealer.

Cell cultures. Growth medium for L cells consisted of minimum essential medium (MEM) (Eagle, 1959) supplemented with non-essential amino acids (Lockart & Eagle, 1959) and 5% heat-inactivated ordinary calf serum (CaS). Foetal mouse cells were grown with MEM

supplemented with 0.5 % lactalbumin hydrolysate (LaH) and 10 % CaS. The pH was maintained at 7.2 by means of NaHCO_3 , the concentration of which depended on the experimental conditions; at 37 °C and under 5 % CO_2 this was 0.14 %. All media contained 100 units of penicillin and 100 μg of streptomycin per ml.

Mouse L cells (NCTC clone 929), a permanent cell line of C3H mouse origin (Sanford, Earle & Likely, 1948–49), were routinely propagated in Roux bottles. Attempts to clear these cells of an infection by *Mycoplasma orale* were unsuccessful. However, this contaminant seemed not to harm the cells which showed a log-phase generation time of 13.5 h and a consistent cloning efficiency close to 100 %.

For the cultivation of foetal mouse cells, near-term foetuses were minced with scissors and cells were released by the action of 0.25 % trypsin. These were centrifuged and resuspended in growth medium, and 100 ml containing 100×10^6 single cells and small clumps were seeded into Roux bottles to be used for preparing cultures in Petri dishes after incubation at 37 °C for 2 days.

For cultural procedures, cells were counted after dispersal with trypsin. In conjunction with virus multiplication experiments, cells were counted as nuclei (Sanford *et al.* 1950–51). The number thus determined was approximately twice the number of intact cells actually present (F. Lehmann-Grube, unpublished results).

Virus. Most experiments were done with LCM virus, strain WE (Rivers & Scott, 1936) which had been provided by Professor W. Scheid, Cologne. It was propagated in L cells after two passages in L cells in this laboratory. Only in the experiment 'Replication of LCM virus in newborn mice' (see below) was a mouse-brain stock of virus used. Strain E-350 ('Armstrong') was obtained from the American Type Culture Collection. It was used after propagation in the mouse brain following intracerebral infection.

Strain CA 1371 was supplied by Dr W. P. Rowe, Bethesda, Md., as infected mouse brain. Its origin is obscure except that it came from the late Dr C. Armstrong and may be identical to strain E-350. Upon receipt, it was inoculated intracerebrally into SCH mice without passage in this laboratory, and a brain homogenate was used in these experiments. Appropriate tests showed that all three virus strains were free of the lactate dehydrogenase-elevating agent. Because of the great thermal lability of the LCM virus (Lehmann-Grube, 1968), care was exercised to prevent loss of infectivity due to thermal inactivation. Thus, if virus had to be stored it was snap-frozen in small samples in ampoules and kept at -70 °C, and virus diluents always contained 1 % heat-inactivated calf serum. Also, all manipulations involving virus were performed at 0 °C.

Virus assay. Quantal titrations were made with 5 assay units per 0.5 $\log_{10}$ dilution step. Usually, L cell tube cultures were used (Lehmann-Grube & Hesse, 1967). In addition, numerous titrations were performed in mice (Lehmann-Grube, 1964), and these parallel assays confirmed the previously established relationships between ID_{50} (L cell) and ID_{50} (mouse); the mouse was nine times more sensitive than the L cell tube culture for the WE strain of virus (Lehmann-Grube & Hesse, 1967), 27 times more sensitive for the E-350 strain of virus, and 16 times more sensitive for the CA 1371 strain of virus (F. Lehmann-Grube, unpublished observations). In order to permit comparison of the present results with those of other laboratories, the results from experiments performed *in vivo* are always given as ID_{50} (mouse), whereas results from cell culture experiments are expressed as ID_{50} (L cell). It should be emphasized that our assays in mice are based on infection and give higher values than assays based on death (LD_{50}); the difference is 0.44 $\log_{10}$ units for strain WE, 0.35 $\log_{10}$ units for strain E-350 (Lehmann-Grube, 1971), and 0.47 $\log_{10}$ units for strain CA 1371 (F. Lehmann-Grube, unpublished observations).

Complement fixation (CF) test. CF antigen (Ag) was measured by means of a micro-technique (Sever, 1962). Antiserum was prepared in rabbits which were immunized with WE strain virus propagated in primary rabbit kidney cells employing rabbit serum in growth and maintenance medium. The complement unit has been taken as the vol. of guinea pig serum required for lysis of 50 % of 3×10^6 sensitized sheep red cells after 60 min at 37 °C in a vol. of 0.125 ml. In the main test two complement units and antiserum in excess were employed. The Ag titre is defined as the reciprocal of that Ag sample dilution at which 50 % haemolysis occurs; this gives the Ag concentration in units per 0.025 ml.

Multiplication of LCM virus, strain WE, in mouse cells in vitro. L cell monolayers in Roux bottles were dispersed by means of trypsin, and 10^6 cells in 5 ml of growth medium consisting of MEM plus 0.5 % LaH and 10 % CaS were seeded into 5 cm plastic Petri dishes (C. A. Greiner u. Söhne, Nürtingen). After incubation for 48 h at 37 °C under 5 % CO₂ the media were exchanged against 0.5 ml of MEM without serum containing 10^3 ID₅₀ (L cell) of LCM virus. Adsorption at room temperature was terminated after 60 min by removing the inoculum and washing the cells once with balanced salt solution (BSS) (Hanks & Wallace, 1949). Five ml of pre-warmed (37 °C) virus multiplication medium consisting of MEM, non-essential amino acids (Lockart & Eagle, 1959), and 10 % CaS were then added (time 0), and incubation was at 37 °C in a humidified and gassed incubator. At intervals, three cultures were taken and immediately cooled to 0 °C. The cells from two cultures were scraped separately into the media, and cells plus media were treated with an ultrasonic drill (Mullard, U.K.) under conditions known to disrupt the cells but not to affect virus infectivity (Lehmann-Grube, 1968). The resulting homogenates containing virus were dispersed into ampoules and stored at -70 °C until titrated. The third culture was used for counting cells as nuclei at the time of harvest.

Secondary cultures of foetal mouse cells were prepared, infected, and harvested in the same manner.

Multiplication of LCM virus in newborn mice. SCH mice, less than 24 h old, were infected intraperitoneally with 10^3 ID₅₀ (mouse) LCM virus. Immediately after infection and then at 24 h intervals, three mice from at least two litters were killed. Snout, skin, and guts were removed, and from the combined carcasses a 10 % homogenate (w/v) was prepared with a mortar using as diluent BSS plus 1 % inactivated CaS. Samples were kept at -70 °C until titration.

Determination of virus concentration in blood and organs of carrier mice. Mice were infected intraperitoneally with 10^3 ID₅₀ (mouse) when less than 24 h old. At the age of 8 to 12 weeks they were killed. Blood was immediately diluted 10-fold with BSS, containing 10 units/ml of heparin. Kidneys, brain, and liver were ground individually in mortars with a little sand, and a 10 % (w/v) homogenate was made with BSS. Diluted blood and organ homogenates were dispersed by sonic treatment and centrifuged for 10 min at 4000 g. Supernatant fluids were titrated as soon as possible.

RESULTS

Experiments in vitro

Multiplication of LCM virus-infected mouse cells

During the log-phase of growth our uninfected L cells multiplied with doubling times of 13 to 14 h. After infection with virus of WE strain, multiplication was reduced though not abolished. The multiplication rates of normal foetal mouse cells have not been determined. However, these cells too did not stop increasing in number after infection.

Fig. 1. Multiplication of LCM virus, strain WE, in cultivated L cells and foetal cells from outbred SCH and inbred CBA, C3H, DBA, and C57 mice. Cultures were infected with 10^3 ID₅₀ (L cell), and after periods of incubation at 37 °C total yields of infectious virus and of complement-fixing antigen were determined in L cell tube cultures and on micro-assay plates, respectively. Titres are expressed as log₁₀ (ID₅₀ L cell) and log₁₀ antigen units per culture. Squares and circles denote results from separate experiments.

Morphological alterations of virus-infected cells were not apparent during the greater part of the observation period. Beyond the 60th h degenerative changes became visible which were seen but less pronounced in control cultures.

Production of infectious virus

Multiplication of infectious virus in mouse cells *in vitro* is depicted in Fig. 1. Little or no virus was demonstrable 2 h after infection. By 16 h progeny virus had appeared in considerable quantities, and for the next 20 h virus production was logarithmic. There was a plateau between the 42nd and 60th h and, towards the end of the observation period, the infectivities dropped again, presumably because of diminished replication combined with

Fig. 2. Yield of infectious LCM virus of strain WE from cultivated mouse cells. Cultures were infected with 10^8 ID₅₀ (L cell). After incubation at 37 °C cells and media were harvested for the determination of infectious virus in L tube cultures (see Fig. 1). At the same time the nuclei of parallel cultures were counted. Yields are expressed as ID₅₀ (L cell)/cell nucleus. Δ , L cells; $\blacksquare$, SCH; $\blacktriangle$, CBA; $\bullet$, C3H; *, DBA; $\odot$, C57.

Table 1. Yield/cell nucleus of infectious LCM virus of strain WE in mouse cell cultures during the plateau phase of virus growth

Source of foetal mouse cells	Time after infection (h)			Mean
	42	48	60	
	ID ₅₀ (L cell)/cell nucleus*			
SCH	229	200	347	259
C57	87	155	n.c.†	121
CBA	166	129	117	137
C3H	62	155	96	104
DBA	275	692	417	461
L	69	115	191	125

* Means of two cultures.

† Nuclei not counted.

thermal inactivation. A feature of the growth curves was their close similarity. This is also apparent in the comparison of virus yields per cell nucleus (Fig. 2). In order to define these similarities more quantitatively, the virus yields during the plateau phases were averaged for each cell type. Analysis of variance of these data (Table 1) revealed that the mean square within classes (mouse strains) exceeded that between means of classes, though not to a significant degree, and hence the maximal yields did not differ between L cells and SCH, C3H, CBA, DBA, or C57 foetal mouse cells.

Table 2. *Lethality among mice of different strains neonatally infected with LCM virus of strain WE*

Strain	Lethality*	
SCH	0/56,	0 %
C57	18/45,	40 %
CBA	80/105,	76 %
DBA	28/32,	88 %
C3H	58/65,	89 %
AKR	80/80,	100 %

* Number of mice dead between 6th and 25th day after intraperitoneal inoculation of 10^3 ID₅₀ (mouse) over number inoculated.

Fig. 3. Multiplication of LCM virus, strains E-350 ('Armstrong') and WE, in newborn SCH mice. Mice, less than 24 h old, were infected intraperitoneally with 10^3 ID₅₀ (mouse), and at intervals the virus concentrations of whole carcasses were determined. Infectious virus was assayed in L cell tube cultures and infectivities are expressed as ID₅₀ (mouse)/g. ●—●, E-350; ■- - ■, WE.

Strictly, a statistical treatment of the data in Table 1 is of little value because of uncertainty whether the experimental error is distributed linearly (as assumed to be the case) or logarithmically. Thus, if the data are converted to log values, analysis of variance leads to entirely different results; the mean square between classes then exceeds the mean square within classes by a factor of $F = 6.79$, and this difference is highly significant. However, the differences between the heights of the plateau phases for the mouse strains seen in Fig. 1, whether statistically significant or not, are not large enough to permit conclusions as to their biological or genetic relevance.

Table 3. Concentration of infectious LCM virus of strain WE in blood and organs of carrier mice 10 ± 2 weeks after neonatal infection

Organ	Strain of mice				
	SCH	C57	CBA	C3H	DBA
	Mean log ₁₀ ID ₅₀ (mouse)/1 ml blood or 1 g tissue ± single standard error*				
Blood	5.76 ± 0.11 (5)	5.86 ± 0.09 (3)	5.52 ± 0.15 (5)	5.87 ± 0.22 (5)	6.27 ± 0.15 (6)
Brain	7.29 ± 0.13 (5)	7.05 ± 0.18 (6)	7.33 ± 0.08 (6)	7.57 ± 0.14 (5)	7.66 ± 0.16 (4)
Liver	6.88 ± 0.17 (4)	7.52 ± 0.22 (7)	7.53 ± 0.34 (6)	7.17 ± 0.19 (5)	7.78 ± 0.18 (7)
Kidney	8.50 ± 0.19 (5)	8.38 ± 0.11 (7)	8.38 ± 0.08 (6)	8.68 ± 0.05 (4)	8.47 ± 0.10 (7)

* Number of mice in parentheses.

Table 4. Concentration of infectious LCM virus of strain CA 1371 in blood and organs of carrier C3H mice 10 ± 2 weeks after neonatal infection

Mouse	Blood	Brain	Liver	Kidney
	ID ₅₀ (mouse) per 1.0 ml blood or 1.0 g tissue			
1	5.90	6.88	6.64	7.92
2	6.31	7.21	7.04	7.84
3	5.46	6.91	8.27	8.14
4	5.71	6.65	7.21	8.54
5	n.d.	6.96	7.85	8.41
6	5.08	n.d.	n.d.*	n.d.
Mean and single standard error	5.69 ± 0.21	6.92 ± 0.09	7.40 ± 0.29	8.17 ± 0.14

* Not done.

Production of complement-fixing antigen (CF Ag)

The yields of CF Ag and infective virus are represented together in Fig. 1. As a rule, CF Ag passed the threshold of detection around the 30th h. It then increased and, again, there were no differences between the types of mouse cells investigated, either in rates of production or in maximum yields.

Experiments in vivo

Lethality in newborn mice after LCM virus infection

The general experience that newborn mice survive infection with the LCM virus irrespective of dose and route of inoculation was confirmed here with SCH mice; none died when 56 such animals were inoculated intraperitoneally with 10⁸ ID₅₀ (mouse) when less than one day old. Contrary to expectation, however, all inbred mice developed signs of severe disease after the 7th day of infection and most died (Table 2). Lethality was not reduced by increasing the virus dose or by using inbred litters nursed by SCH foster mothers.

Replication of LCM virus in newborn mice

Following the infection of newborn SCH albino mice, both the WE and E-350 strains of LCM virus grew rapidly, although strain E-350 was somewhat slower. By day 7 both viruses had reached maximum concentrations and these did not change during the rest of the 15 days of observation (Fig. 3).

Table 5. *Concentration of complement-fixing antigen in organs of LCM virus strain WE carrier mice 10 ± 2 weeks after neonatal infection*

Organ	Strain of mice				
	SCH	C57	CBA	C3H	DBA
	Mean log ₁₀ CF Ag units per 0.025 g ± single standard error*				
Brain	3.08 ± 0.09 (5)	3.18 ± 0.10 (4)	3.24 ± 0.06 (4)	2.94 ± 0.11 (4)	≥ 3.24 (± 0.12) (4)
Liver	2.55 ± 0.24 (4)	3.11 ± 0.15 (6)	3.28 ± 0.16 (4)	3.25 — (1)	3.19 ± 0.13 (7)
Kidney	≥ 3.32 (± 0.15) (5)	3.33 ± 0.06 (6)	3.36 ± 0.05 (6)	3.39 ± 0.08 (4)	≥ 3.52 (± 0.01) (7)

* Number of mice in parentheses.

Concentration of LCM virus in blood and organs of adult carrier mice

A synopsis of the results of virus titrations of blood and organ homogenates from adult mice infected neonatally is presented in Table 3. For a given organ the different mouse strains showed no significant differences in infectivity greater than that expected from sampling variation. In contrast, there were differences between organs, and the highest infectivities were observed for kidneys and the lowest for blood. We then investigated another virus strain and again the concentrations of infectious virus in blood and organs of C3H mice persistently infected with the CA 1371 strain of LCM virus were high and comparable to the concentrations of WE virus in C3H and other mouse strains (Table 4).

Concentration of complement-fixing antigen in the organs of carrier mice

Owing to high anticomplementary activities, the determination of CF Ag in the blood of carrier mice was not possible. The titres of the organ homogenates (Table 5) confirmed the findings with infectious virus and differences between mouse strains were not significant.

DISCUSSION

The laboratory model of LCM disease of the mouse is seen as an allergic phenomenon (Hotchin, 1962); direct effects of the infecting virus on the cell are assumed to be absent (Lehmann-Grube, 1971). However, when cells *in vitro* are infected, they are usually adversely affected to some extent, and under certain experimental conditions morphological alterations may be detected. In the present experiments frank cytolysis was not apparent, even at a time when virus infectivities were maximal and 90 % or more of the cells were filled with immunofluorescing antigen, but cell multiplication was slower and degeneration faster than for non-infected control cells.

Multiplication of the Traub strain of LCM virus in L cell suspension cultures was followed by Pedersen & Volkert (1966). Lehmann-Grube & Slenczka (1967) and Lehmann-Grube (1971) determined the behaviour of the WE strain in L cell monolayer cultures. Under these conditions the latent period did not vary with the input multiplicity; progeny virus appeared after 8 h and was largely cell-associated. Knowledge of the production of CF Ag in mouse cells *in vitro* is also fragmentary.

The present study indicates that the amount of infectious virus produced must be regarded as very high. Taking into account that the assay in L cell tube cultures underestimates the number of infectious units by at least a factor of 9, and that enumeration of cells on the basis of nuclei overestimates the actual cell number by a factor of about 2, each figure of

the last column in Table 1 must be increased 18-fold and hence, for instance in the case of cells from DBA strain foetal mice, approx. 8000 ID₅₀ of LCM virus of strain WE were produced per cell. A feature of our results is the similarity of virus production between L cells and foetal mouse cells originating from colony-bred albino mice or from inbred mice of strains CBA, C3H, DBA, and C57. Oldstone, Habel & Dixon (1969) reported differences of virus yields between foetal mouse cells *in vitro* from different inbred mouse strains.

The determination of the virus concentrations in blood and organs from carrier mice was difficult because of the high mortality after neonatal infection of inbred mice. While no SCH mice died during the period of observation, most inbred mice died after infection under the same conditions, and all AKR mice died. It should be pointed out that a few years ago Lehmann-Grube, then working in Marburg/Lahn, experienced no difficulty in establishing carrier mice of the same inbred strains, although even then more inbred mice died than did colony-bred albino mice. We know of no cause which satisfactorily explains the differences between mouse strains or the fluctuation within strains.

In contrast to cell cultures, in which the observation period was curtailed because of virus-induced and/or spontaneous degeneration of the cells, no such experimental limitation applied to the mouse. Having confirmed observations by others (Volkert & Hannover Larsen, 1965) that after neonatal infection a plateau of virus concentration is rapidly reached and maintained thereafter, the age of 8 to 12 weeks was chosen to determine the virus contents of blood and organs of SCH albino and four inbred strain carrier mice. Again no differences between mouse strains in respect of either infectious virus or CF Ag were found. This uniformity is in marked contrast to the results reported by Oldstone & Dixon (1968) who, employing the CA 1371 strain of LCM virus, found a gradient from high virus concentrations in SWR mice to very low concentrations in C3H/HeJ mice. However, even when we followed Oldstone & Dixon's protocol as closely as possible, using the same strain of virus and the same strain of mice, the concentrations of CA 1371 strain virus in the organs of neonatally infected C3H/HeJ mice were about 200-fold higher in blood and more than 10000-fold higher in kidneys than had been observed by them. Hoffsten & Dixon (1973) produced neonatal C3H and SWR carrier mice with the CA 1371 strain of LCM virus. At 2 to 10 months old the virus infectivities of sera ranged between 3.4 to 4.1 log₁₀ LD₅₀ (mouse) per 0.03 ml, which corresponds closely to the values determined here. Also there were no significant differences between the two mouse strains. Volkert & Hannover Larsen (1965) and Hannover Larsen (1973) worked with the Traub strain of LCM virus and found relatively high virus infectivities in C3H carrier mice.

The uniformity of inbred mouse strains in their ability to support LCM virus multiplication either *in vitro* or *in vivo* was also unexpected in view of the influence genetic factors are known to have on multiplication of other viruses (e.g. arboviruses, oncornaviruses) even in members of one species (Webster, & Clow, 1936; Webster & Johnson, 1941; Sabin, 1952; Vogt & Ishizaki, 1965; Hartley, Rowe & Huebner, 1970; Pincus, Hartley & Rowe, 1971). Of course, only few mouse strains were used and an extension may reveal differences which would increase our experimental tools for studying the relationship between LCM virus and the mouse.

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