Diversity of Lymphocytic Choriomeningitis Virus: Variation Due to Replication of the Virus in the Mouse

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SUMMARY

Depending on passage history, strain WE infectious LCM virus either damages L cells more or less severely or leaves them morphologically intact. Correspondingly, the plaques which are formed on L cell monolayers are of different appearance, ranging from intensely turbid to clear. Multiplication of LCM virus in certain mouse organs profoundly affects plaque characteristics. The brain, for instance, favours lytic variants while the spleen supports the replication of virus which forms turbid plaques. This statement holds if virus taken from organs of persistently infected mice or virus passaged from mouse to mouse is analysed and is true also if the initial preparation contains virus forming predominantly either clear or turbid plaques on L cell monolayers. Selection is not rapid and not absolute. It may take months of multiplication before a final state is reached, and even then the number of characteristic plaques is usually in great excess of the rest but never reaches 100%. Cloning procedures may alter the proportions, but with our experimental conditions no plaque has ever been isolated which would retain its characteristics upon passage. Differences of plaque type morphology were not reflected in differences of pathogenic properties, and both clear and turbid variants caused persistent infection if used to infect newborn mice and led to disease with signs of neurological involvement and death if inoculated intracerebrally into adult animals.

INTRODUCTION

Lymphocytic choriomeningitis (LCM) virus is said to leave the cell in which it multiplies virtually intact. While this statement is correct for virus which has established itself permanently in the mouse after neonatal or congenital infection, it cannot be accepted in its most general form. Thus, numerous observations attest to the virus’ ability to destroy cells grown in vitro, and especially permanent cell lines may be severely affected by LCM virus. Why, then, does one virus either kill or spare the host cell in which it multiplies, depending on whether the latter is growing in vitro under culture conditions or whether it is part of a tissue within an intact organism? This obvious question seemed to be answered when Hotchin, Kinch & Benson (1971) reported on the existence of virus variants with different cytolytic potentials. Only LCM virus, which was essentially non-cytopathic and gave

rise to turbid plaques, initiated persistent infection while the clear plaque type variant killed newborn mice. They also reported that clear and turbid plaque type variants preferentially multiplied, respectively, in brain and liver of the mouse which agreed with the older observation of Hotchin, Benson & Seamer (1962) that intracerebral or visceral passages of the virus affected its virulence for newborn mice, resulting in properties which were characterized as aggressive or docile, respectively. We have previously reported that in our hands three different LCM virus strains, passaged in either the brains or the visceral organs of mice, were all docile in the above sense (Lehmann-Grube, 1971), although Hotchin’s observation that such passages selected for lytic or non-lytic variants, respectively, was confirmed and extended as reported here. We then began studying the effects that multiplication of LCM virus in the mouse has on certain viral properties. Our results show that the passage history of an LCM virus profoundly affects its behaviour in vitro without changing its properties as they can be revealed by inoculation into adult or newborn mice. Presumably, LCM virus exists in a multitude of variants whose replication may be favoured under conditions as they occur both in nature and in the laboratory. All of these, however, seem to be able to initiate the most characteristic interaction between LCM virus and its natural host: persistent infection.

METHODS

Mice. Randomly bred albino mice, classified by the commercial source as specific pathogen-free, were used.

Cell cultures. L cells, clone 929 (Sanford, Earle & Likely, 1948–9) were employed after they had been freed of contaminating Mycoplasma by treatment with a hypotonic solution of antibiotics (Gori & Lee, 1964). Growth medium consisted of minimum essential medium (Eagle, 1959) supplemented with non-essential amino acids (Lockart & Eagle, 1959) and 5 % heated calf serum.

Virus. The WE strain of LCM virus (Rivers & Scott, 1936) was used. Most experiments were done with one of three virus preparations. (1) Standard Clear Plaque Type Virus was our common WE strain virus as it is being used for various experimental purposes in this laboratory. Of the plaques which develop on L cell monolayers more than 95 % are clear. (2) Standard virus as just described was cloned thrice. In spite of having been selected for clear plaque type morphology from individual infectious units, the resulting Cloned Clear Plaque Type Virus contained, nevertheless, 5 % turbid plaques; only 95 % were clear. For the preparation of pools, L cell monolayers were infected and virus was harvested from cells and media two days later. (3) The Turbid Plaque Type Virus as used in this study originated from L cells persistently infected with WE standard virus. An L cell carrier culture was established as has been described previously (Lehmann-Grube, 1967), and after the infected cells had been passaged 26 times, the virus was separated. Again, plaques were not of uniform appearance as to the desired morphology; only 97 % scored as turbid while 3 % had definitely to be classified as clear. In individual experiments the sources of virus forming predominantly either turbid or clear plaques on L cell monolayers were, respectively, blood from carrier mice more than one year after neonatal infection and homogenized brain from adult mice infected five days previously. In one instance Armstrong’s strain E-35o was employed.

Because of the great thermal lability of LCM virus, small samples were snap-frozen before storage at −70 °C and all manipulations were performed at ≤4 °C. For the same reason balanced salt solution (BSS) which was used to homogenize tissues and to dilute virus always contained 1 % heat-inactivated calf serum.
Variation of LCM virus

Titration of virus and classification of plaques. Vital infectivity was measured by means of a plaque assay which was performed as follows. L cells were dispersed with trypsin and $3 \times 10^4$ cells contained in 2 ml of growth medium were seeded into 35 mm plastic Petri dishes (Greiner, 7440 Nürtingen, Federal Republic of Germany). After incubation at 37 °C under 5 % CO₂ for not longer than 24 h the media were withdrawn and suitably diluted virus in 0.1 ml volumes of medium was allowed to adsorb for 30 min at 37 °C. Cells and virus were then covered with 2 ml/dish of growth medium which contained agarose at a concentration of 0.55 %. After four days of incubation, 1.5 ml of fresh medium without agarose was spread on top of the semi-solid overlay and incubation was continued for 24 h. On the fifth day, 1.5 ml of 10 % formaldehyde was added to each culture which was then left at room temperature for 1 h, thus fixing the cells and making them firmly adhere to the plastic surface. After all other contents of the dish had been discarded, the cells were stained with a 0.02 % solution of crystal violet in 10 % formaldehyde. This procedure led to well-delineated plaques which could easily be counted. It also allowed storage of experimental results without further precautions for later reference.

The same method permitted the ready assignment of individual plaques to one of the chosen categories, namely clear and turbid. In a few experiments the class turbid was further subdivided into light (semi-turbid) and intense. Classification of plaques was facilitated by including controls with virus of known characteristics in each assay. It should be mentioned that the above classification of plaques is but a crude representation of the actual variability observed. Thus, all degrees of turbidity were seen and plaques varied also as to size. However, for the sake of clarity further subdivision has not been attempted.

Cloning of virus. The progeny virus from individual infectious units was isolated as follows. L cell monolayers were infected and incubated under semi-solid medium as for plaque assay. On the fifth day, 1.5 ml of medium containing 0.55 % agarose and neutral red at a concentration of 1/5000 was spread on top of the older overlay and incubation at 37 °C was continued for approx. 9 h. The medium covering a well-separated plaque of desired morphology was aspirated with a Pasteur pipette and transferred to 1 ml of medium which was sonicated and used to infect further L cell cultures. These were harvested two days later either to be analysed as to viral plaque morphology or to be used for further plaque purification.

RESULTS

Plaque morphology after multiplication of virus in newborn mice

Mice less than 24 h old were infected intraperitoneally with 10³ p.f.u. of Cloned Clear Plaque Type Virus. At intervals of 24 h three mice from at least two litters were killed, snouts, skins, and guts were removed, and from the combined carcasses and BSS containing 1 % calf serum 10 %, homogenates (w/v) were prepared. Following centrifuging the supernatant fluids were frozen down at $-70 \, ^\circ \text{C}$ to be assayed altogether with regard to infectivity as well as plaque morphology. Unexpectedly, the latter parameter had not changed during the period of observation and therefore this experiment was repeated twice with similar results; these are illustrated in Fig. 1. While the concentration of infectious virus rose steeply, reaching a maximum on the seventh day, the proportion of turbid plaques remained low and did not significantly deviate from the proportion in the virus preparation used for infection.

As will be shown later, the spleen of a mouse favours turbid variants while the brain supports predominantly the multiplication of clear plaque type variants. An experiment resembling the one just described was performed. At intervals after neonatal infection of
newborn mice the virus contents of spleens and brains were analysed. Seven days after infection the spleen contained virus which formed plaques just like the infecting agent, which was the Cloned Clear Plaque Type Virus. On day 16 turbid plaques had risen to 30% in this organ and after 28 days spleen homogenates induced more than 70% turbid plaques. During this interval the plaque characteristics of the virus associated with the brain had not significantly changed.

### Plaque morphology after multiplication of virus in adult mice

Cloned Clear Plaque Type Virus or Turbid Plaque Type Virus was used for infecting mature mice either by the intracerebral or the intraperitoneal route of inoculation. Six days later the virus, which had accumulated in brains and spleens of seven individual mice, was assayed for infectivity and plaque morphology. The results of this experiment were clear-cut. They failed to reveal changes in the proportions of clear or turbid plaques, respectively, following the multiplication for six days in the adult mouse.

The next question to be answered was whether plaque morphology would be influenced by repeatedly passaging the virus in the tissues of adult mice. In a first experiment the effect of intracerebral passages on virus causing predominantly the formation of turbid plaques was studied. Turbid Plaque Type Virus passaged once in the brain of adult mice and blood from carrier mice were inoculated into the brains of adult mice, each animal receiving approx. 100 p.f.u. in 0.03 ml. Four or five days later the brains of five mice per group were homogenized and made 10% with BSS plus 1% calf serum. The homogenates were centrifuged and portions were stored at −70°C. For further intracerebral passages the virus was
Fig. 2. Effect of multiple passages in the mouse brain on plaque morphology of Turbid Plaque Type Virus (blank symbols) and blood from a neonatal carrier mouse more than one year old (shaded symbols). Circles and columns denote titres of infectious virus and percentages of clear plaques, respectively, in brain homogenates.

diluted again tenfold and five mice were inoculated as before. After the virus had been thus carried through the mouse brain 12 times, individual passages were assayed on L cell monolayers. The results in Fig. 2 show that intracerebral multiplication greatly favours variants forming clear plaque types. After nine passages the proportion of clear plaques which had been 10% or less initially had risen to 80% and two passages later the counts had reached 90%, which is the maximum attainable under these conditions.

In a further experiment the starting virus, which came from the brain of an acutely infected adult mouse, exhibited more than 95% clear plaque morphology on L cells. This virus was passaged in parallel 11 times in either the brains or the livers plus kidneys plus spleens of adult mice. The experimental conditions were similar to the ones just described and need not be described in detail. After conclusion of this double series, the proportion of clear plaques had not changed in the brain-passaged virus. In contrast, in virus which had been carried 11 times through internal organs of mice the proportion had dropped to 70%. Increase of turbid plaques to 30% was less than expected. A similar experiment was done with the Armstrong E-350 strain. Again, the starting material scored almost entirely as clear. However, after 11 passages in the visceral organs more than 90% of the plaques were turbid; less than 10% of the infectious units formed clear plaques.
It seemed important to know what kind of virus prevailed in carrier mice of long duration. Mice less than 24 h old were infected either with Cloned Clear Plaque Type Virus or with Turbid Plaque Type Virus. Four mice in each group were followed individually. They were bled from the retrobulbar venous plexus at intervals and all specimens were immediately assayed on L cell monolayers as to titre and plaque characteristics. The results of this experiment are presented in Fig. 3. Whereas the proportions of initially turbid plaques remained unchanged upon prolonged multiplication in one and the same mouse, clear plaques were slowly displaced. Thus, irrespective of the plaque characteristics of the inoculated virus, at the end of the observation period, i.e. six months after neonatal infection, the blood of carrier mice contained predominantly turbid plaque type variants.

As has been pointed out above, by classifying plaques as either lytic or turbid the actual findings are simplified. Turbid plaques are not of uniform appearance and range from light (semi-turbid) to intense. The question arose as to whether the proportions of subclasses within the class turbid underwent changes just as the relative proportions of lytic and turbid plaques did. Therefore, in several experiments turbid plaques were differentiated and plaques were counted as to whether they were light or intense. Plaques formed by the Turbid Plaque Type Virus from persistently infected L cells were almost entirely intensely turbid. When this virus was used to infect mice at birth, the proportion of turbid plaques did not change in these animals even after months (Fig. 3). However, there occurred a gradual shift from intense to light (data not shown) and when the carrier mice were bled longer than six months after neonatal infection, roughly one-third of the turbid plaques were of the light variety (Table 1). Conversely, if persistent infection was established with lytic virus, this was gradually replaced by virus forming turbid plaques which, again, could be classified partly as light and partly as intense (Table 1). Similar distributions were found when the blood of first and second generation congenital carrier mice was investigated (Table 2). Thus, the conclusion drawn above, namely that irrespective of plaque characteristics of the virus used
Table 1. *LCM* virus plaque morphology in the blood of carrier mice more than six months after neonatal infection with clear or turbid plaque type variants

<table>
<thead>
<tr>
<th>Plaque type inoculated</th>
<th>Age of mice (days)*</th>
<th>Total no. of plaques counted</th>
<th>Plaque morphology (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Clear</td>
</tr>
<tr>
<td>Clear</td>
<td>245</td>
<td>$1 \times 10^5$</td>
<td>1669</td>
</tr>
<tr>
<td>Standard</td>
<td></td>
<td>$(1\times 10^4-2\times 10^5)$†</td>
<td>(3-31)†</td>
</tr>
<tr>
<td>Clear</td>
<td>193</td>
<td>$6\times 10^4$</td>
<td>1518</td>
</tr>
<tr>
<td>Cloned</td>
<td></td>
<td>$(1\times 10^4-9\times 10^5)$</td>
<td>(2-38)</td>
</tr>
<tr>
<td>Turbid</td>
<td>193</td>
<td>$5\times 10^4$</td>
<td>1213</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$(7\times 10^3-1\times 10^4)$</td>
<td>(2-13)</td>
</tr>
</tbody>
</table>

* Ten mice in each group. † Variation between individual mice.

Table 2. *LCM* virus plaque morphology in the sera of neonatal and congenital carrier mice

<table>
<thead>
<tr>
<th>Carrier mice*</th>
<th>Plaque morphology (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Generation†</td>
<td>Clear</td>
</tr>
<tr>
<td>Approximate age (months)</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>6</td>
</tr>
<tr>
<td>2</td>
<td>12</td>
</tr>
</tbody>
</table>

* Pooled sera from at least 15 mice were assayed. † of neonatally infected; 1: first congenital generation; 2: second congenital generation.

to initiate persistent infection the eventual distribution of clear and turbid plaque variants was alike, can now be qualified by saying carrier mice harbour a variety of plaque type variants, the distribution of which is similar and independent of the virus used to establish persistent infection.

The possibility that different organs in one and the same carrier mouse favoured different variants was tested. Mice were made carriers by neonatal injection of either Standard Clear Plaque Type Virus or Cloned Clear Plaque Type Virus or Turbid Plaque Type Virus. At age 13 days or older two mice from each group were killed and homogenates from spleens and brains were assayed on L cells as to titre and plaque morphology. As is evident from the results in Table 3, in the brains of all animals the clear plaque variants prevailed. In contrast, virus from the spleens exhibited predominantly turbid plaque type characteristics.

Other properties of *LCM* virus after prolonged multiplication in the mouse

The obvious question arose whether changes of the virus, which so affected its interaction with the mouse cell in *vitro* that a drastically altered plaque morphology resulted, would be reflected in the effects the virus had on the intact organism. As markers we chose disease and death of adult mice following intracerebral inoculation of the virus and lethality of mice infected when less than 24 h of age. Irrespective of virus employed, intracerebral infection of adult mice led to signs of severe involvement of the central nervous system as they are characteristic of the acute *LCM* disease in these animals. As a rule, death was the ultimate consequence. Intervals between infection and death ranged from 5 to 9 days and...
Table 3. Plaque morphology of LCM virus in brains and spleens of carrier mice more than 6 months after their neonatal infection with clear or turbid plaque type variants

<table>
<thead>
<tr>
<th>Number</th>
<th>Age (days)</th>
<th>Plaque type inoculated</th>
<th>Brain Clear plaques (p.f.u./g)</th>
<th>Brain Clear plaques (%)</th>
<th>Spleen Clear plaques (p.f.u./g)</th>
<th>Spleen Clear plaques (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>184</td>
<td>Clear</td>
<td>2.0 × 10^5</td>
<td>83</td>
<td>7.9 × 10^6</td>
<td>22</td>
</tr>
<tr>
<td>2</td>
<td>184</td>
<td>Standard</td>
<td>7.9 × 10^6</td>
<td>90</td>
<td>7.9 × 10^6</td>
<td>13</td>
</tr>
<tr>
<td>3</td>
<td>131</td>
<td>Clear</td>
<td>2.7 × 10^6</td>
<td>86</td>
<td>2.2 × 10^6</td>
<td>10</td>
</tr>
<tr>
<td>4</td>
<td>134</td>
<td>Cloned</td>
<td>1.5 × 10^5</td>
<td>93</td>
<td>3.2 × 10^6</td>
<td>44</td>
</tr>
<tr>
<td>5</td>
<td>134</td>
<td>Turbid</td>
<td>1.4 × 10^5</td>
<td>75</td>
<td>6.2 × 10^6</td>
<td>5</td>
</tr>
<tr>
<td>6</td>
<td>131</td>
<td></td>
<td>3.5 × 10^6</td>
<td>84</td>
<td>1.1 × 10^6</td>
<td>8</td>
</tr>
</tbody>
</table>

were only slightly influenced by dose; they were not measurably influenced by the passage history of the virus. As is commonly observed with this strain of virus, a few mice always survive when high doses are inoculated. Provided comparisons were based on identical amounts of infectious virus, survival rates were equal even if plaque characteristics were not. When virus was inoculated into newborn mice, the majority survived and became carriers; again, the kind of virus did not influence the outcome and even after 10 or more intracerebral passages which resulted in virus exhibiting predominantly clear plaque characteristics, few mice inoculated when less than 24 h of age succumbed to the infection. This is the résumé of our observations as they have been recorded in a great number of experiments. In only one instance different results were obtained. Of 10 mice which had been given virus from a lightly turbid but extremely small plaque all survived, though they had been infected, which was proved by their later resistance to intracerebral challenge with standard virus. This observation, however, was an exceptional one and has not been analysed in detail.

In parallel with the work reported in this communication, we studied LCM viral interference in vitro and the role turbid plaques may play in the phenomenon. This extensive investigation will be published separately. It suffices to say that there was a mutual inhibition of virus replication and that c.p.e. caused by lytic virus were markedly interfered with by turbid variants.

After passaging in mice, the resulting clear or turbid variants were, in numerous instances, tested by neutralization tests as to their identity. There was always complete agreement between expected and observed; viruses were neutralized by high dilutions of an anti-LCM virus antiserum raised in the rabbit.

Discussion

As with other biological entities whose replication is governed by genetic mechanisms, viruses yield readily to selective pressure, and variants may emerge; LCM virus is no exception. Traub (1937) was the first to describe alterations of certain biological properties of LCM virus conditioned by its propagation in a new experimental host, and other workers have made similar observations since. More recently, Hotchin and his colleagues (1962) have stressed the great variability of LCM virus whose biological properties changed rapidly if multiplication was allowed to occur predominantly in selected organs of the mouse. They also found the plaque morphology, as it developed in cell cultures of several permanent cell lines, to be a useful in vitro marker. Thus, LCM virus passaged in the brains of mice formed predominantly clear plaques on BHK21 cells, caused neurological disease in adult
mice, and killed newborn animals, whereas the same virus passaged from liver to liver formed turbid plaques, killed adult mice with prolonged latent periods and uncharacteristic signs, and induced persistent infection in newborn mice (Hotchin et al. 1970). Though Hotchin did not give an explicit explanation, it seemed plausible to assume that a causal relationship existed between the cytolytic potential of the virus as exerted on cells grown in vitro and the death of newborn mice as well as the neurological disease in adult animals. This assumption was not borne out by our experimental findings. Virus causing the formation of lytic plaques as well as virus causing the formation of turbid plaques, which is the same as saying virus with high and low cytopathogenic potentials, both induced life-long carrier states when given to newborn mice. Also, death of adult mice following intracerebral infection was quite uniform and not at all influenced by passage history or plaque morphology.

Concerning the effect passage history has on plaque morphology, our data confirm and extend Hotchin's observations. Brain and visceral organs support the multiplication of clear and turbid plaque type variants, respectively. This was particularly convincingly illustrated in this study by showing that the brain of a carrier mouse harboured predominantly lytic virus, whereas the spleen of the same animal contained mostly infectious units which formed turbid plaques. We, too, failed in our attempts at deriving virus preparations exhibiting uniform plaque morphology. Prolonged multiplication in certain organs resulted in mixtures containing predominantly, but never exclusively, one variant. Of course, this was to be expected because contamination with virus from other organs via the blood stream was bound to occur. Contrary to expectation, however, when individual plaques were picked, the progeny did not score in a uniform fashion and even repeated cloning did not lead to a variant which bred true.

When trying to reconcile the obviously contradictory results concerning the biological properties of plaque variants as they were obtained by Hotchin and co-workers and by us, we must realize that plaque characterization as to clear and turbid is convenient but grossly simplifies the actual findings. In both laboratories a multitude of different plaques were observed with clear and turbid being the extremes of a continuous range. Thus, one variant may be entirely different from another, even though both are similar in appearance. Also, the experimental conditions were not entirely identical.

Though we conclude from our results that persistent infection can be established equally well with variants exhibiting either turbid or clear plaque morphology, the virus which finally persists was found to differ from either type. With the exception of monocytes, our strain of LCM virus does not multiply in cells belonging to the blood (F. Lehmann-Grube, unpublished data), and hence viraemia is caused by virus which spills from infected organs. Knowing that different tissues selectively support multiplication of different variants, it is not surprising to find that, irrespective of the type used to initiate infection, after a few months the blood of carrier mice contains mixtures of plaque variants which range from intensely turbid to clear. Undoubtedly, this merely reflects the relative production of variants in all the different tissues of the mouse. It takes months before an equilibrium is reached, which we interpret to mean that tissues of the mouse permit the multiplication of any variant and only favour certain types.

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