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Biophysical and Biochemical Characterization of Lymphocytic Choriomeningitis Virus

IV. Strain Differences

KENNETH P. CAMYRE AND CHARLES J. PFAU
Department of Microbiology, University of Massachusetts, Amherst, Massachusetts 01002

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Biological, biochemical, and biophysical properties of three lymphocytic choriomeningitis (LCM) virus strains were compared. The biological property examined was the concentration range of virus which would, when injected into neonates, cause a carrier state. The dosage range for the CA1371 and Traub strains was found to be as broad as the limits examined (5 to 100 LD_{50} units/mouse). The WCP strain, however, would only produce carriers within a 3 to 5 LD_{50} range. The biochemical properties examined were the growth rates in tissue culture and the effect of varying the input ratio of virus to cells. With identical input ratios, the Traub strain reached a peak titer 32 hr after infection. The CA1371 and WCP strain reached their peaks at the 40th hr. With a 10-fold decrease in the amount of CA1371 virus per cell, peak titer (as high as in the above experiments) was not obtained until 56 hr postinfection. The biophysical properties examined were stability in density gradients and inactivation rates at 4 C. In potassium tartrate gradients, full recovery of the CA1371 and WCP strain could be achieved. However, inactivation kinetics showed that only the CA1371 strain was much more stable than the Traub-LCM. The realization that marked differences in LCM strains exist is discussed in relation to certain inconsistencies in the literature.

It is apparent that most strains of lymphocytic choriomeningitis (LCM) virus have the following characteristics in common: (i) normal growth in the presence of halogenated deoxyuridines, (ii) ether sensitivity, (iii) ability to produce carrier-state or *pti* mice, (iv) inability to produce cytopathic effects (CPE) in a large number of tissue culture systems, (v) persistent infection of certain tissue cultures with high virus yields, and (vi) immunological relatedness. It is the purpose of this paper to show that various strains of LCM, while having the above similarities, may still be markedly different.

This study was initiated because of the finding that the CA1371 and WCP strains of LCM could be quantitatively recovered after density gradient centrifugation. Extensive studies on the behavior of the Traub strain of LCM in such gradients have already been reported by one of us (6). Subsequently, however, use of the technique for purification purposes was abandoned, since more than 90% of the input infectivity was lost under a wide variety of experimental conditions. In this communication, we show that it is the strain of virus used that determines the amount of recovery from density gradients. Differences between the above-mentioned strains are further illustrated by presentation of their growth curves in tissue culture, the kinetics of thermal inactivation, and the concentrations which can induce the carrier state in newborn mice.

MATERIALS AND METHODS

Virus assay techniques. The origin of the three strains of LCM was as follows. The WCP strain was that used in a previous study (7). The Traub strain used was a mouse brain suspension obtained from M. Volkert. The CA1371 strain (grown in African green monkey tissue culture) was obtained from R. E. Wilsnack. The virus was grown in L-cell monolayers, and the supernatant fluids were withdrawn when maximal infectivity was demonstrable. For all strains, this was usually 48 hr after infection. The WCP stocks used here were from the 17th through the 28th passages, the Traub stock was from the 4th and 5th passages, and the CA1371 was from the 3rd and 4th passages in L cells. The LD_{50} assays were carried out as previously reported (7). Swiss mice (12 to 14 g) from either Twin Oak Farms or Millerton Research Farm were used.

Virus inactivation. Survival of partially purified LCM at 4 C was determined by a previously used technique (7).

Carrier mice. Virus carriers were made by intra-

peritoneal injection of mice, between 8 and 24 hr after birth, with 0.03 ml of virus. The mice in these experiments were exclusively from Twin Oak Farms. The litters were watched, and deaths were recorded at 2-day intervals for 21 to 24 days. At this time, the survivor's blood was taken from the retrobulbar venous plexus and assayed for virus.

Production of non-serum-containing virus. So-called "non-serum-containing virus" stocks were obtained in the following way: 78-cm^2 prescription bottles were seeded with 1.5×10^7 L cells in 50 ml of Eagle's minimal essential medium (MEM) plus 10% calf serum. After 24 hr, the just-confluent monolayers were infected in the usual manner; i.e., the tissue culture fluid was poured off and replaced with 5 ml of virus suspended in growth medium. After 1 hr at 37 C, the virus was poured off, and the monolayers were washed three times with prewarmed phosphate-buffered saline (PBS) or MEM. The medium was then replaced to the original volume with Eagle's MEM *minus* calf serum. Controls consisted of monolayers treated in an identical manner except that MEM *plus* calf serum was the final replacement. The supernatant fluids were harvested 50 hr later. Both control and non-serum-containing stocks had identical titers.

Virus growth curves. L cells were grown and infected as outlined above for the production of non-serum-containing virus. After washing the monolayers with PBS and 5 min after the addition of MEM *plus* 10% calf serum, the medium was sampled. This was considered as the zero-time level of infectivity in the tissue culture fluid. Thereafter, at various intervals, samples were withdrawn and assayed immediately.

Density gradient techniques. These techniques were described in detail previously (6). In the present investigation, two previously unused types of gradients were included: Ficoll (Pharmacia, Uppsala, Sweden), and potassium citrate (Allied Chemical Corp., Morristown, N.J.). The former type was run for 15 hr and the latter for 2.5 hr. All gradients, except where noted, contained 6.25×10^{-4} M tris(hydroxymethyl)-aminomethane(Tris) base (7).

RESULTS

Density gradient experiments. Experiments to determine the stability of LCM in various density gradients were carried out by comparing the amount of virus initially in the gradient solution with that remaining after a specified time period in the presence or absence of an externally applied gravitational field. The results of major importance are presented in the first set of experiments in Table 1. In agreement with our previous observations (6), the Traub virus lost about 90% of its infectivity after centrifugation. The amount of recoverable virus could be increased somewhat by using Tris-buffered gradients (this result was also found several years ago in collaboration with I. R. Pedersen). With WCP virus, however, quantitative recovery (within the limits of the standard error of the assay) has always been noted. Full recovery was found with or without Tris addition, and also in potassium citrate gradients. Recovery of the CA1371 virus was comparable to recovery of the WCP strain.

TABLE 1. *Recovery of LCM virus after centrifugation through preformed potassium tartrate gradients*[a]

Strain	Gradient concn range (%)	Total infectivity[b] × 10^6		
		Input	Recovery after centrifugation	Noncentrifuged control
Traub[c]	45–20	48	4.8	32
Traub	45–20	48	15	48
WCP	45–20	48	32	48
CA1371	45–20	0.48	0.32	0.32
WCP	60–2	15	48	48
WCP[d]	60–2	48	4.8	32

[a] Each of three cellulose nitrate tubes for the Spinco SW39 rotor was filled with 4 ml of a preformed gradient. A 1-ml amount of virus-containing tissue culture fluid was added to each tube so that a sharp interface was formed. Two of the tubes were immediately inverted and mixed. One was then assayed and was considered as the amount of input virus. The other was held at 4 C. The third tube was centrifuged for 2.5 hr at 35,000 rev/min. It was inverted and mixed, and was assayed at the same time as the remaining noncentrifuged tube.

[b] The product of the LD_{50} per milliliter and the number of milliliters used in a defined experiment.

[c] Gradient not buffered with Tris.

[d] Non-serum-containing virus used.

The second experiment in Table 1 was run with WCP virus in steeper tartrate gradients. Again, recovery was quantitative when serum-containing virus stocks were used. Virus grown in the absence of serum, however, was quite labile. Ficoll and sucrose gradients were also used with serum-grown virus, but usually less than 1% of the input virus was recovered. Since the infectivity of most of the virus stocks used in the experiments presented in Table 1 was 1 to 2 log units higher than that obtained with the Traub virus in our previous studies (6), we wished to determine whether dilution of these stocks, prior to layering on top of the gradients, would affect final recovery. Thus, Traub and WCP virus were diluted 50-fold before centrifugation. The recovery of Traub virus was no less than in the undiluted controls (about 30% of the input). The results with similarly diluted WCP virus were less definitive. In three of five experiments, a 60% loss in infectivity of the diluted virus was encountered. As shown in Table 1, quantitative recovery of the CA1371

strain was found even though its titer was 2 log units lower than the other strains used.

Our previous work (6) on equilibrium sedimentation of Traub LCM in 60 to 2% potassium tartrate gradients revealed the presence of infectious virus in bands having densities of 1.24 and 1.15. The high-density band contained about 70% of the recoverable infectivity. The same type of experiment with WCP virus (with full recovery of input infectivity versus 10% in the above-mentioned experiments) indicated that infectivity was associated with bands having densities of 1.24, 1.20, and 1.17. These bands, in decreasing order of density, contained 63, 35, and 2% of the total infectivity of the gradient. When WCP virus was sedimented through 45 to 20% tartrate gradients, three bands were observed at densities 1.17, 1.15, and 1.12. The distribution of virus (in decreasing order of density) was almost identical to that observed in the 60 to 2% gradients.

To achieve a better separation of the virus bands, the concentrations of the starting potassium tartrate solutions were changed from 60 and 2% to 45 and 20%. As noted above, when WCP virus was sedimented through the latter type of gradient, most of the virus was recovered from the band having the highest density (1.17 g/ml). This density was markedly different (1.24 versus 1.17) from that of the band containing most of the infectious virus in the 60 to 2% gradient. When the 1.24 band was withdrawn and refractionated on a 45 to 20% gradient, 90% of the input infectivity was associated with the resulting single band having a density of 1.17. Why virus-containing material should band at different densities in supposedly equilibrium runs (15-hr centrifugations did not differ from the 2.5-hr runs) is not entirely clear to us. At the very least, these experiments point out to us the hazard of assigning absolute densities to virus recovered from tartrate gradients.

Synthesis of various LCM strains in L cells. Figure 1 shows the difference in growth rates of the three LCM strains. If one compares the three experiments, initiated with the same ratio of infectious virus to cells, it is obvious that Traub virus increased noticeably faster than the WCP and CA1371 strains. At 24 hr, when the Traub strain had reached 56% of its highest titer, the CA1371 and WCP strains had attained only 16 and 3.2% of their titers, respectively. At 32 hr, when the Traub strain had reached 100%, the CA1371 and WCP had reached 25 and 5.6%, respectively. Peak titers of both CA1371 and WCP strains were noted 40 hr postinfection. Cells infected with the high-titer stock of CA1371 reached peak infectivity 16 hr earlier than the culture initially exposed to low-titer stock.

Stability of LCM strains at 4 C. Figure 2 presents data on the inactivation of these three strains of LCM. The data on WCP-LCM closely parallel that already presented (7) on this strain, and also mimic the data on the Traub strain. It is clear that the CA1371 strain is the most stable of those tested.

Concentrations of LCM strains which will pro-

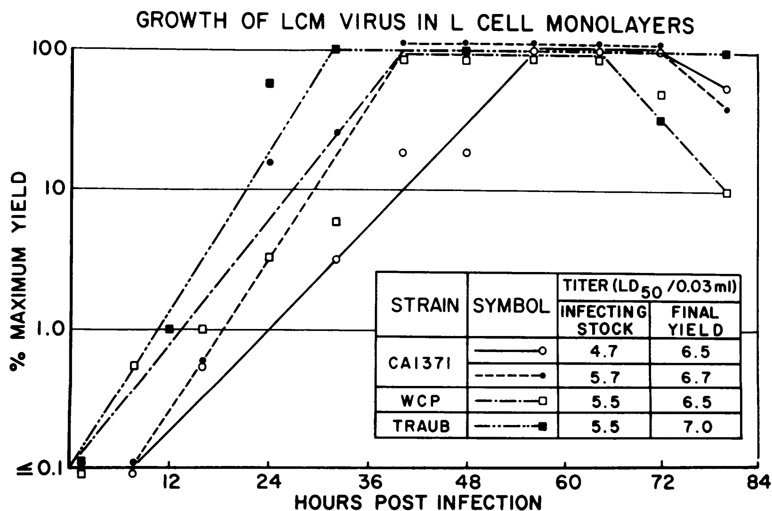

FIG. 1. *Growth curves of three strains of LCM in L cells. Confluent monolayers were exposed to 5 ml of the appropriate virus solution having, with one exception, almost identical titers. These infecting stocks were all 1:10 dilutions of original tissue culture harvests. After the adsorption period, the virus was removed by repeated washing. The cultures were then sampled and assayed at 8- or 12-hr intervals.*

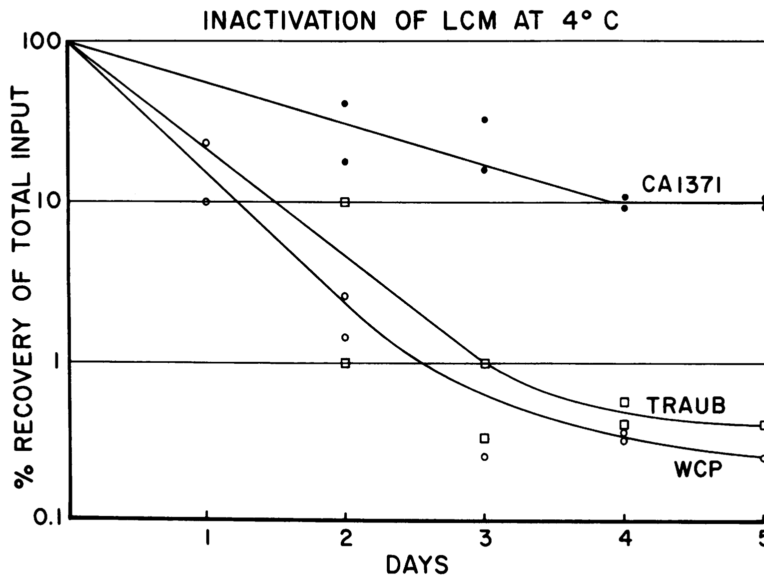

FIG. 2. *Stability of three strains of LCM at low temperature. LCM grown in tissue culture in the presence of serum (LD_{50}/0.03 ml between 6 and 6.5) was subjected to the usual low- and high-speed centrifugation cycle. The pelleted virus was resuspended to the original volume (usually with no loss in infectivity) with PBS, and was placed in tightly stoppered tubes at 4 C. The infectivity of the virus solutions was then determined at daily intervals.*

TABLE 2. *Induction of the carrier state with different strains of LCM*

Virus	LD_{50} per 0.03 ml per mouse[a]	Per cent dead within 21 days[b]	Survivors with blood titers <1.5LD_{50}/0.03 ml (%)
WCP	1	25	0
	2–3[c]	50	67
	4–5[c]	65	22
	10	90	0
	100	100	—
Traub	5	17	100
	10	12	86
	50	0	100
	100	0	100
CA1371	5	0	100
	50	0	100
	100	0	100

[a] The LD_{50} unit used in this table and throughout the paper refers to the amount of virus needed to kill 50% of adult (12 to 14 g) mice inoculated by the intracerebral route.

[b] Between 5 and 15% of the mice inoculated (in the above groups as well as saline-injected controls) died within the first 4 days. These were not included in the calculations, since death was probably due to mechanical effects. For each virus concentration given, 15 to 20 mice were used.

[c] Half of each litter was injected with one concentration or the other. There was enough variation in each stock of virus or each set of dilutions, or in both, so that reproducible results could only be obtained in the above manner.

duce virus carriers. Table 2 presents data indicating the levels of virus which, when injected into neonates, will result in carrier states. It is evident that a wide range of virus concentrations can be used to produce carriers with the Traub and CA1371 strains (no experiments were done beyond the stated virus concentrations). However, a very pronounced restriction can be seen with the WCP strain. In the latter case, if more than 2 to 5 LD_{50} units of virus were given, all mice died. If a virus dose just below that of the carrier-state level was injected, the mice became immune (as shown by challenge of the adult survivors with 100 LD_{50} units of the virus).

DISCUSSION

It is obvious from the data presented in Table 1 that certain strains of LCM are much more stable than others when subjected to density gradient centrifugation. Under almost identical conditions (type of gradient, time and speed of centrifugation, titer of serum-grown stocks) only the CA1371 and WCP stocks could be fully recovered after fractionation in potassium tartrate gradients. With the exception of potassium citrate, all other types of gradients led to significant decreases in infectivity. Furthermore, with the WCP strain, there may be a critical starting concentration of virus needed before full recovery (even in tartrate gradients) can be obtained.

As shown previously, WCP virus (7) loses about 0.5 log unit of infectivity per day at 4 C. This is in contrast to the CA1371 strain which

loses less than half that amount (Fig. 2). On the other hand, the inactivation kinetics of the Traub strain seem to be close to those of WCP-LCM. Thus, stability, on standing at 4 C, does not necessarily indicate whether a strain of virus will withstand density gradient centrifugation.

That the titer of the virus inoculum markedly influences the growth rate of LCM was first shown with the WE strain (1). This has also been confirmed for the Traub (5) and WE_3 strains (3) and in our experiments (Fig. 2) for the CA1371 strain. In each case, it has been found that increasing the input ratio of virus to cells decreased the time necessary for peak virus titers to be reached. The above type of argument cannot be used to explain the faster growth rate of the Traub versus the CA1371 and WCP strains, because the titers of the three infecting stocks were identical. Yet, the difference in growth rate might depend on the multiplicity of infection (strictly speaking, the ratio of *adsorbed* virus to cells) and on the adsorption kinetics. Determining the amount of reversible and irreversible adsorption of LCM is very difficult (M. P. McGinley, M.S. Thesis, Univ. of Massachusetts, Amberst, 1966), and as yet we have no reliable data to rule out or to substantiate the above proposal.

Three reports led to the experiments in Table 2. In 1960, Traub (10) showed that the pathogenicity of LCM, when injected into newborn mice, differed not only with the strain of virus used, but also with the mode of injection. This finding was confirmed by Lehmann-Grube in 1964 (2). In 1965, Volkert and Larsen (11) noted that the dose of LCM virus given to neonates influenced the establishment of the persistent tolerant state. These findings suggested that it would be of interest, with the three virus strains used in this study, to determine the concentration range that would induce the carrier state. From the data in Table 2, it is clear that there is a wide dosage range with the CA1371 and Traub strains. Virtually all the mice survived and were carriers. Furthermore, we have no reason to doubt that the range is wider than the limits we have chosen, i.e., 5 to 100 LD_{50}/mouse. Quite different results, however, were encountered with the WCP strain. This strain was extremely virulent. The virus concentration (2 to 3 LD_{50}/mouse) that would give a high percentage of carriers was very critical. The WCP strain was also extremely virulent for guinea pigs, 0.5 mouse LD_{50} units being the maximal amount of virus that could be given in the early part of an immunization program.

Knowledge of the literature led us to suspect that biological differences exist among the three LCM strains. It was also considered likely that physical and chemical dissimilarities exist, but we knew of no previous publications indicating this. The present communication dealing with these types of variations suggests that the discrepancies reported on the physiochemical nature of LCM might be due to strain differences. For example, using the CA1371 strain and fluorescent-antibody techniques, Wilsnack and Rowe (13) found viral antigen exclusively in the cytoplasm of infected cells. This observation seems also to apply to the WE (1) and Traub (5) strains. However, Mims (4), using the WE_3 strain, found antigen in both the cytoplasm and the nucleus. In preliminary studies, Pfau, Pedersen, and Volkert (8) found that synthesis of the Traub strain was not inhibited by actinomcyin D; this has been confirmed (L. Buck and C. J. Pfau, *unpublished data*). In contrast to this finding, Slenczka and Lehmann-Grube (9) reported that in most experiments there was no synthesis of LCM (presumably the WE_3 strain) in the presence of actinomycin D. Wainwright and Mims (12) have recently reported differences between various cell lines with regard to susceptibility to the WE_3 and Armstrong strains of LCM.

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