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Interferon production and sensitivity of Semliki Forest virus variants

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INTRODUCTION

Semliki Forest virus (SFV), a group A arbovirus, grows readily in calf kidney cells in tissue cultures, and has been found to form plaques on these cells under agar. However, SFV which has been grown only in the brains of mice (MB virus) forms plaques which are small and irregular. In an attempt to obtain a variant which might form better plaques, MB virus was passaged serially 10 times in calf kidney cells, but the resultant virus (termed CK) was not more satisfactory. It was however found that the MB and CK strains of SFV formed plaques on L cells which differed significantly in size. In further studies, other differences in the properties of the two strains were noted. The results obtained are presented in this paper. In particular, the relative growth of the two viruses in calf cells, the relative sensitivity of the two strains to added calf interferon, and the relative amounts of interferon which they themselves produce when growing in calf kidney cells were investigated.

MATERIALS AND METHODS

Viruses

MB virus. Semliki Forest virus (SFV) was received from Dr J. S. Porterfield (Medical Research Council, Mill Hill) having had a total of nine passages in the brains of mice since the original isolation (Smithburn & Haddow, 1944). This was inoculated intracerebrally at 10^{-3} into 17–18 g. mice. Fifty-three hours later, their brains were harvested, and made up in 10 % (w/v) suspension in normal horse serum (previously inactivated at 56° C. for 30 min.). This was designated MB (mouse brain) virus. Samples were stored at −20° C.

CK virus. Ten serial passages of SFV were made in primary calf kidney cells, starting with MB virus. In each passage, feeding bottle cultures containing confluent sheets of approximately $10^{7\cdot2}$ cells and 20 ml. of medium were inoculated with $10^{2\cdot0}$–$10^{4\cdot5}$ plaque-forming units (pfu) of virus. After the cultures had been incubated for 24–30 hr., the medium was harvested, clarified by light centrifugation and used, appropriately diluted, as seed for the next passage of the series. Seeds were stored until required as a 1/10 dilution in inactivated horse serum at −20° C. The 24 hr. harvest from the tenth serial passage in calf kidney cells was designated CK virus. Samples were stored at −20° C.

Other viruses. Influenza A (1947) virus, strain Kunz, and Sendai virus were

obtained from Dr D. A. J. Tyrrell (Medical Research Council, Salisbury), and passaged in eggs. Infected allantoic fluid was stored at −20° C., diluted 1/10 in inactivated horse serum.

Tissue cultures

Primary tissue cultures of the kidney cortical tissue of 5 to 7-day-old calves were prepared by trypsinization using standard techniques. The liberated cells were suspended in a growth medium of Hanks's saline with 0·5% lactalbumin hydrolysate, 8% calf serum and 0·05% sodium bicarbonate at a concentration of $0{\cdot}075 \times 10^6$ living cell aggregates (Bishop, Smith & Beale, 1960) per ml. Test tubes, 4 oz. medical flat bottles and Pyrex feeding bottles were seeded with 1, 12 and 20 ml., respectively. Tubes were incubated in sloped racks for 2 days, and then on a roller drum. Medical flat bottles were incubated on a levelled surface. Feeding bottle cultures were rolled throughout. Medium changes were given as necessary. Cultures were used after 6 or 7 days, when confluent cell sheets had formed.

L cells were grown in tubes and medical flats, using medium 199 with 10% calf serum.

All calf serum was inactivated at 56° C. for 30 min. before use. Cultures were incubated at 37–38° C. throughout.

Plaque assays for SFV

Unless otherwise stated, infective virus was assayed on monolayers of L cells in medical flat bottles by a plaque technique. An overlay of medium 199 with 10% calf serum and 1% agar was used. Plaques were counted after 3 days, and titres calculated as plaque-forming units (pfu) per ml. of undiluted virus.

Serum neutralization experiments

Sera were inactivated and diluted appropriately in medium 199 with 2% calf serum and 0·15% bicarbonate. Samples were mixed with equal volumes of virus diluted to contain approximately 200 TCD 50/ml. and held at room temperature for 1 hr.; 1 ml. of each serum–virus mixture was then added to each of five tubes of L cells. These were incubated in sloped racks and observed daily for 5 days. Serum 50% cytopathic-inhibition titres were calculated by the method of Thompson (1947).

Production of calf interferon

Interferon was made from calf kidney cells inoculated with small amounts of influenza A, strain Kunz, essentially as described by Tyrrell (1959). After incubation for 48–60 hr., the tissue culture fluid was harvested, lightly centrifuged and treated at pH 2 for 4 hr. at room temperature in order to destroy the virus present.

Assay of interferon

Interferon titres were measured by a quantitative haemadsorption-inhibition technique to be described fully elsewhere using tube cultures of calf kidney cells and Sendai virus. The titre of an interferon sample was the reciprocal of the log

dilution at which the amount of virus growth was reduced to 50 % of the level in control tubes. Statistical analysis showed that, with the technique used, fourfold differences in the interferon content of two preparations could be discriminated with 99 % certainty, if three tubes were used to test each interferon dilution. By using six to nine tubes for each dilution, twofold differences could be distinguished.

Growth curve experiments

Calf kidney cell cultures in tubes, medical flat bottles or feeding bottles were inoculated with SFV diluted in medium 199 with 0·5 % lactalbumen hydrolysate, 1 % chick embryo extract and sodium bicarbonate at 0·09–0·14 % as appropriate. At the time of harvest, medium was collected from groups of three to five replicate cultures and pooled. Inactivated horse serum was added to give a final 20 % concentration, and samples were stored at −20° C. until titrations could be performed. Before assay for interferon, media from SFV-infected cultures were treated at pH 2 overnight at 4° C., neutralized to pH 7, and heated at 56° C. for 30 min., in order to destroy all infective virus present (R. F. Sellers, personal communication).

RESULTS

Plaque sizes with MB and CK viruses on L cells

On L cell monolayers under agar, MB virus formed plaques which ranged from pin-point in size to 3 mm. diameter at 48 hr. after overlaying, and increased to between 0·5 and 5 mm at the 4th day. On other monolayers tested in parallel, CK virus produced plaques which were only pin-point in size at 48 hr.; additional plaques often became visible on the 3rd day, and on the 4th day the plaque sizes ranged from pin-point to 2·5 mm. With both strains the plaque sizes varied considerably at all times of reading, but MB virus formed plaques which were definitely the larger.

Serological identification of CK virus

It was necessary to demonstrate that CK virus was a variant of SFV, and not a bovine virus picked up from one of the serial passages in calf cells. Cross-neutralization tests were therefore carried out. In two experiments, an antiserum from a rabbit immunized with MB virus had a geometric mean titre of 1/224 against MB virus, and 1/110 against CK virus. An antiserum to CK virus had a geometric mean titre of 1/105 against MB virus, and 1/107 against CK virus. These results may be analysed for evidence of antigenic similarity by the method of Archetti & Horsfall (1950). The ratios (r_1 and r_2) of the titres with heterologous virus and with homologous virus are calculated with each serum, and also the geometric mean (r) of these ratios. The value r gives in a single figure the extent of the antigenic difference between the two viruses, a value for $1/r$ of between 1/1 and 1/5 implying that the strains concerned are antigenically indistinguishable. Applying this analysis to the data above, values are obtained for r_1 of 105/107, for r_2 of 110/224, and for $1/r$ of 1·4. It is concluded that CK virus is in fact a SFV variant.

Growth curve studies with MB and CK viruses in calf kidney cells

(i) *Formation of extracellular virus.* Feeding bottle cultures of calf kidney cells were inoculated with 100 pfu (approximately 2×10^{-5} pfu added per cell) of either MB or CK virus. With each virus, medium was sampled from one group of six bottles at intervals during the next 72 hr. Some of the medium harvested at 42 hr. was incubated for a further 24 hr. *in vitro* at 37° C. to give the rate of thermal inactivation of the progeny viruses. It will be seen from Fig. 1 that, compared with MB virus, CK virus accumulated more rapidly in the medium and reached a higher final titre at an earlier time. Under the microscope, cell damage was seen earlier and ultimately became more extensive in the cultures infected with CK virus than in those infected with MB virus.

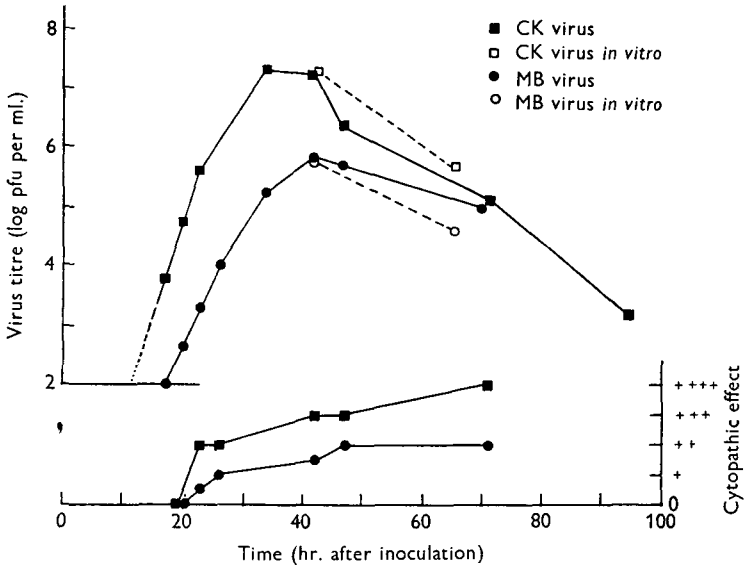

Fig. 1. Growth curves for MB and CK viruses in extracellular fluid of calf kidney cells. Inocula were 2×10^{-5} pfu per cell. Harvests at 42 hr. were incubated *in vitro* at 37° C. for 24 hr.

The rates of thermal inactivation *in vitro* of the medium harvested at 42 hr. were similar, at 0·053 and 0·057 log pfu per hr. for MB and CK viruses, respectively (Fig. 1). The level of infective virus in the medium of the cultures infected with CK virus declined from its peak at a comparable rate. In contrast, the rate of decline in the MB-infected cultures was definitely slower (0·029 log pfu per hr.) between 42 and 72 hr., suggesting that significant amounts of additional virus were being liberated from the cells.

In the same experiment, another group of six bottles infected with each virus was harvested at 24 hr., and again at 48 hr. and 72 hr. At each time, the medium was completely harvested, the cell sheets were washed once, and 20 ml. of fresh medium were added. These harvests contained the total amounts of infective virus liberated into the medium during the successive 24 hr. periods. These were found to be $10^{7\cdot4}$, $10^{6\cdot9}$ and $10^{5\cdot3}$ total pfu in the cultures infected with CK virus, and

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$10^{4\cdot7}$, $10^{6\cdot8}$ and $10^{5\cdot9}$ total pfu in those infected with MB virus. It was thus confirmed that after an inoculum of MB virus, virus was released from the cells more slowly and for a longer time than after a comparable inoculum of CK virus.

A final group of cultures received a 10,000 times larger inoculum of MB virus (0·2 pfu per cell). The virus in the medium rose to a peak level ten times higher than after the smaller inoculum of the same virus. This probably resulted from the operation of interferon mechanisms, as discussed below.

In a series of other growth curve experiments with relatively small inocula, it was again found that CK virus grew faster than MB virus, and reached a higher final titre (Table 1).

Table 1. *Rates of increase in and maximum titres of virus after equivalent inocula of MB and CK viruses*

Inoculum (pfu/cell)	Rates of increase in infective virus (log pfu/hr.) Virus		Maximum titre of infective virus (log pfu/ml.) Virus	
	MB	CK	MB	CK
2×10^{-5}	0·23	0·29	5·8	7·3
5×10^{-5}	0·30	0·40	n.d.*	n.d.*
2×10^{-3}	0·31	0·47	6·8	8·2
7×10^{-2}	0·47	0·55	6·7	7·4
$3{\cdot}8 \times 10^{-1}$	0·31	0·38	7·2	7·9
$7{\cdot}5 \times 10^{-1}$	0·30	0·48	7·2	8·2

These data from six growth curve experiments with calf kidney cells relate to extracellular infective virus.

* n.d. = not determined.

(ii) *Formation of extracellular virus in cells pre-treated with interferon.* A batch of calf kidney tubes was divided randomly into four groups, of which two were treated with a 1/10 dilution of interferon prepared from calf cells with Kunz virus. The remaining tubes were changed to medium without interferon and served as controls. On the following day, $10^{3\cdot1}$ pfu of CK virus were added to all tubes of one interferon-treated and one control group. The remaining interferon-treated and control tubes similarly received $10^{3\cdot1}$ pfu of MB virus. The tubes were incubated on a roller apparatus throughout this experiment. At various times between 1 and 96 hr. after inoculation of the seeds, medium was harvested and pooled from six tubes in each group. Results of assays are shown in Fig. 2.

The growth curves for infective virus in the medium of control tubes show the features described in the previous section. In this particular experiment, the differences between the two viruses were rather small. About 4 hr. after inoculation of MB virus, the amounts of infective virus in the medium of the control tubes increased rapidly. In the interferon-treated tubes, there was no increase until 15 hr. Thereafter infective virus increased slowly until about 60 hr., and reached a maximum level about 1000-fold lower than in the controls. The level then remained unchanged for at least a further 30–40 hr., during which virus must have been liberated from the cells at a rate sufficient to balance the amounts thermally inactivated.

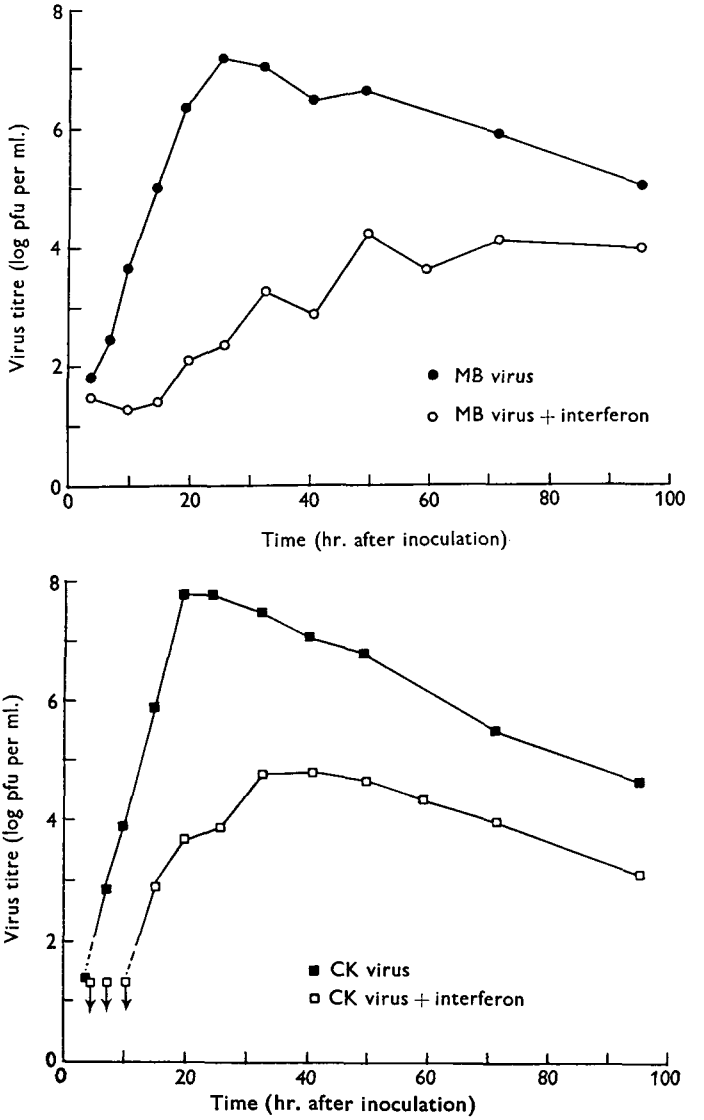

Fig. 2. Growth curves for MB and CK viruses in calf kidney cells which had been pre-treated with calf interferon, and in control cultures. Inocula of each virus were $10^{3 \cdot 1}$ pfu per culture.

The effects of added interferon on the growth of CK virus were similar but much less marked. Infective virus increased in the medium somewhat more slowly than in the controls, but the peak level was reached only 12 hr. later than in the controls. This level was again approximately 1000 times lower than in the corresponding controls, but it was five times greater than in the interferon-treated tubes infected with MB virus. From the shape of the growth curve, there was probably also some prolongation of the period of liberation of CK virus from the interferon-treated cells.

Similar results were obtained in a replicate experiment.

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(iii) *Formation of interferon by MB and CK viruses.* CK virus was added to groups of twenty medical flat bottle cultures of primary calf cells at a multiplicity, in terms of added virus, of 1·15 pfu per cell, and at 1/1000th of this level. Similarly MB virus was added to further bottles at 1·61 pfu per cell and at 1/1000th of this level. After incubation at 37° C. for 2 hr., the bottles were completely drained and washed, and fresh medium was added. From assays of the amounts of unadsorbed virus, it was calculated that 72 % of the larger inoculum of CK virus was taken up by the cells, and 38 % of that of MB virus. At various times up to 96 hr. after inoculation of virus, samples were taken from four or more bottles in each group, pooled and titrated for infective virus and interferon. Results from this experiment are shown in Fig. 3.

Considering first interferon production with the concentrated inocula, it will be seen that small amounts of interferon were first detected 10 hr. after inoculation of MB virus. After CK virus, interferon was not detected at this time, but thereafter the rates of formation were similar with the two viruses, and levels increased rapidly to reach the same maximum. After CK virus, the level of interferon declined after 48 hr. and became undetectable by 96 hr. In contrast there was little change over this period after MB virus. In other experiments, interferon formed after infection with CK virus consistently declined more rapidly than that formed after MB virus. Possibly the earlier and more extensive cell damage caused by CK virus leads to earlier release of enzymes which break down interferon.

After the dilute inoculum of CK virus, interferon was not detected in the tissue culture fluid until 25 hr. Thereafter it increased rather slowly to reach a peak which was the same as after the more concentrated inocula of either virus. In contrast, the dilute inoculum of MB virus led to a more rapid increase in interferon, and harvests at 36, 48, and 60 hr. after infection contained significantly more interferon than after any other inoculum in this experiment.

These differences between the two viruses in their relative production of and sensitivity to interferon may account for certain differences in the growth curves obtained after small inocula of the two viruses (Fig. 3). They may also account for differences in the curves after large and small inocula of MB virus. After the dilute inocula, the rates of increase in extracellular virus appear identical with the two viruses during the first 7 hr. Thereafter that of MB virus slowed relatively, and this virus grew to a lower maximum than CK virus. With the 1000 times larger inocula of the two viruses, the rates of growth and the maxima differed little. However, with MB virus the peak titre was more than twice that after the smaller inoculum. A tenfold increase in the peak virus titre after a 10,000 times greater inoculum of MB virus was described earlier. It seems likely that with dilute inocula, the cells infected initially produce not only infectious virus but also interferon. This will act on the cells which have not yet been infected and diminish their response to virus infection. In calf kidney cell cultures, such an effect will be more apparent with MB virus, which forms more interferon and is also more sensitive to its effects, than with CK virus. If on the other hand most or all of the cells are infected initially, interferon will be formed too late to influence virus growth. Under these conditions MB virus will grow at the same rate and to the same peak titre as CK virus.

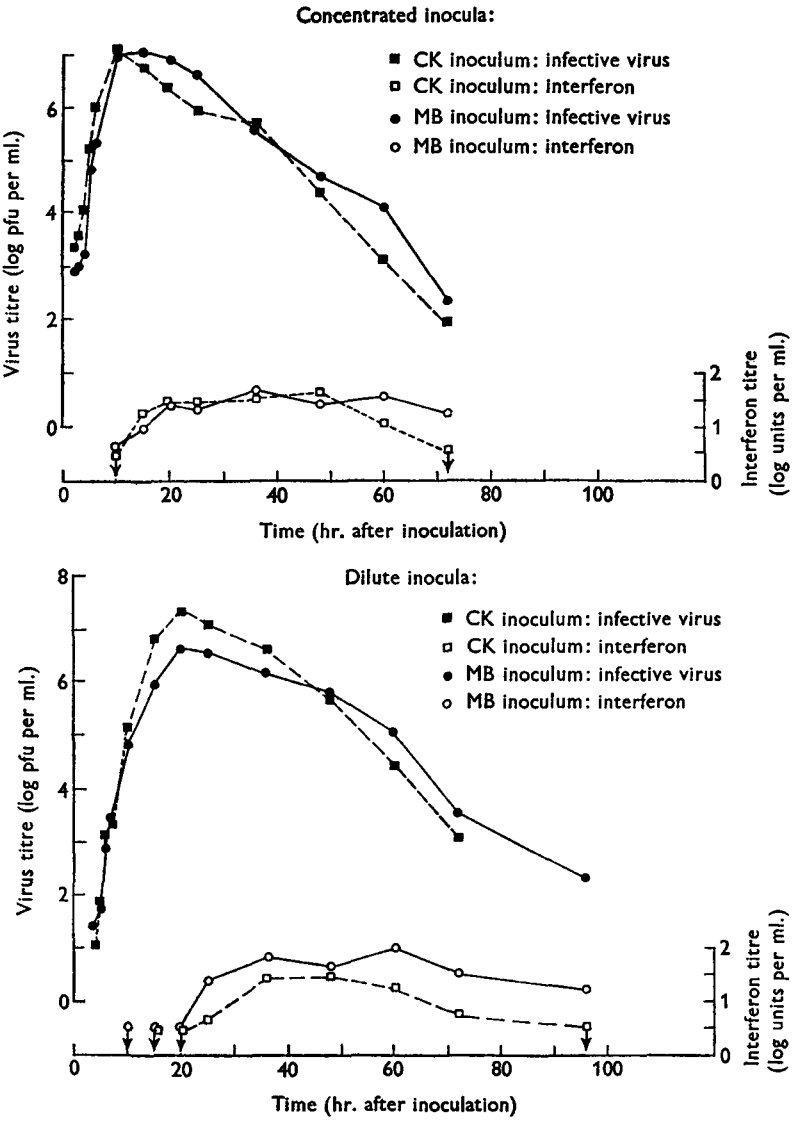

Fig. 3. MB virus was added to groups of calf cell cultures at 1·61 pfu per cell, and at $1{\cdot}61 \times 10^{-3}$ pfu per cell. CK virus was similarly added at 1·15 and $1{\cdot}15 \times 10^{-3}$ pfu per cell. After 2 hr., cultures were drained and washed and fresh medium was added. Samples of medium were taken from four or more bottles in each group at the times indicated.

In two comparable experiments, results were in general very like those shown in Fig. 3. The relative and absolute amounts of interferon formed after the different amounts of the two viruses varied somewhat from experiment to experiment. However, CK virus usually formed less and never more interferon than a comparable inoculum of MB virus. The differences between the experiments probably resulted from the use of particular batches of calf cells; when cells from several different kidneys were infected in parallel with the same inoculum of MB virus, the amounts of interferon formed after 48 hr. differed more than five-fold.

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Attempts to detect the presence of particles of CK type in MB virus

During the serial passages in calf cells, CK virus replaced MB virus as the predominant type, presumably because of selection under the conditions of passage. Attempts were made to isolate clones of large and small plaque-forming particles by picking plaques from L cell monolayers which had been inoculated with highly diluted MB virus under an agar overlay. The precautions suggested by Mosley & Enders (1961) were carefully observed to minimize the chances of contamination of the selected clones with other virus particles. In two experiments, no significant differences were found in the mean diameters of the plaques formed by progeny from the different clones. Thus the particular plaques selected may have differed in size at the time of picking due to physiological rather than genetic reasons. Alternatively, a high rate of mutation and back-mutation between particles forming plaques of the two sizes may occur on L cells.

These results suggest that particles of CK virus are present in MB virus in a proportion too low to detect without difficulty. Alternatively, they may have appeared as the result of chance mutation during the calf cell passages.

Growth of MB and CK viruses in other systems

Results of repeated assays of MB and of CK viruses in three different host cell systems are shown in Table 2. With both seeds, approximately the same figures were obtained for the amounts of infective virus per ml. using mice inoculated intracerebrally, and in plaque assays with L cells and in chick embryo fibroblasts. Thus particles infectious in the mouse brain were also infectious in L cells and in chick cells.

Table 2. *Titres of MB and CK viruses in three assay systems*

Assay		Virus			
		MB		CK	
Assay system	Units	No. of assays	Mean log titre	No. of assays	Mean log titre
L cells	log pfu/ml.	6	6·88	3	7·23
Chick embryo cells	log pfu/ml.	6	7·05	1	7·0
Mice infected intracerebrally*	log MLD50/ml.	2	7·0	2	6·93

* 17–18 g. mice inoculated with 0·03 ml. Titres are the 50% mouse lethal dose end-points, calculated by the method of Thompson (1947).

In a growth curve study, 18–20 g. mice were infected intracerebrally with 100 pfu of MB or CK virus. At intervals, mice were killed and the amounts of virus in 10% (w/v) brain extracts were measured. The mice infected with MB virus were moribund at 72 hr., at which time the virus content of the brains was $10^{8\cdot2}$ pfu per ml. (Fig. 4). In contrast, CK virus grew to a maximum titre of only $10^{6\cdot6}$ pfu per ml., and less than half of the mice inoculated were dead 8 days later. Thus CK virus grew less readily in the mouse brain than MB virus and was less virulent.

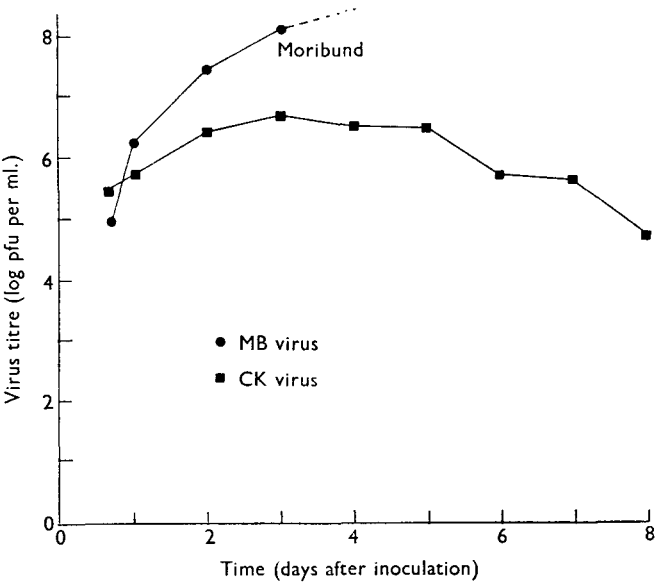

Fig. 4. Growth curves MB and CK viruses in the brains of mice. The inoculum of each virus was 100 pfu.

DISCUSSION

The two variants of SFV described in this report, MB, derived from mouse brain passages, and CK, from calf kidney cell passages, were first distinguished by the sizes of the plaques which they form on L cells under agar. In growth curve experiments with calf kidney cells, it was found that the MB variant was the more sensitive to the effects of added interferon. Also this variant usually stimulated production of more interferon, and never less interferon, than CK virus. It has already been pointed out that these differences probably account for the observed differences in the rates of growth of the two variants after small inocula, and for the differences in the rates of growth of the MB variant after large and small inocula.

Differences in interferon production and interferon sensitivity between strains of the same virus have been previously reported. De Maeyer (quoted by Enders, 1960) found that a monkey-virulent strain of measles virus produced 2–4 times less interferon in human cell cultures than an avirulent strain derived from it by egg passage. Sellers (1963) reported that attenuated strains of foot-and-mouth disease virus appear to produce more interferon, and to be more sensitive to its action, than cattle-virulent strains. Wagner *et al.* (1963) studied two variants of vesicular stomatitis virus which resembled the SFV variants described in this report in that they differed in plaque morphology as well as in rates of growth and in interferon sensitivity. No definite conclusions could be reached about their relative production of interferon.

The differences in interferon production and in interferon sensitivity between the two SFV variants may also be relevant to the phenomena of virus adaptation

and virus virulence. Compared with MB virus from which it was derived, CK virus grew poorly in the mouse brain and was less lethal. In calf cells, however, it grew faster, and it caused cell destruction sooner. Thus CK virus was less virulent than MB virus in the mouse brain, but more virulent in calf cells to which it had been adapted. The mechanisms behind these changes are of interest. It has been suggested that adaptation of a virus results from selection of variants capable of more rapid growth in the new host system (Burnet & Bull, 1943; Wang, 1948). Such variants may be present in the original inoculum as a small minority, or may arise during the serial passages as the result of chance mutations. Wang (1948) showed that mouse-adapted influenza virus grew considerably more rapidly, and to a 10 times higher titre, in the lungs of mice than an egg-passaged line of the same virus. These results are comparable to those found here with CK and MB viruses in calf cells.

A number of factors have been described which favour the growth of one variant of a virus rather than another. Such factors include the pH and bicarbonate content of the medium (Vogt, Dulbecco & Wenner, 1957; Mosley & Enders, 1962), and the temperature of incubation (Lwoff, 1959; Carp & Koprowski, 1962). The relative sensitivity of two virus variants to an inhibitor, such as a sulphated polysaccharide, may encourage the growth of one of them (Takemoto & Liebhaber, 1961). It is here suggested that another factor may be the relative production of, and sensitivity to, interferon of two variants.

In studies on viral interference in the allantois, Fazekas de St Groth, Isaacs & Edney (1952) inoculated eggs with heated influenza B virus, and challenged them after 24 hr. with influenza A virus. The total yield and the rate of growth of the challenge virus was markedly less than in controls. Similar results have been found with cells treated in tissue culture with interferon by Ho & Enders (1959), by Wagner (1961) and in the present study. The slower rate of growth and reduced final yields of challenge virus from cells previously treated with either interfering virus or interferon closely resemble the features described above for the growth of a virus in a new host system. This suggests that a common mechanism may be operating.

In support of this idea, it was found that when growing in calf cells, the unadapted MB variant usually stimulated production of more interferon than the adapted CK variant, and was also the more sensitive to the effects of added interferon. Such differences in interferon production and sensitivity could lead to overgrowth of MB virus during serial passages in calf cells by any particles of virus of the CK type present in the MB seed, or arising as the result of mutations. Thus while the evidence can only be regarded as circumstantial, these interferon mechanisms could play a part in, or even be mainly responsible for, the process whereby a virus adapts to a new host. In general, adapted viruses are more virulent in the host concerned. A correlation between increased virulence and decreased interferon production among virus variants has already been noted (Enders, 1960; Sellers, 1963).

Adaptation of SFV to calf cells was accompanied by loss of adaptation to growth in the mouse brain. The CK variant grew more slowly in the mouse brain, reached

a lower final titre and was less virulent than MB virus. It would be interesting to know whether the relative sensitivity to, and production of, interferon by the two strains in the mouse brain are, as expected, the converse of what obtains in calf cells.

SUMMARY

Semliki Forest virus was passaged 10 times in series in calf kidney cells, starting with virus passaged in the mouse brain (MB virus). A variant was obtained (termed CK virus). The two viruses were antigenically indistinguishable. When grown on L cells, CK virus formed smaller plaques than MB virus. In parallel growth curve studies in calf kidney cells, a small inoculum of CK virus grew more rapidly and to a higher final titre than a comparable inoculum of MB virus, and usually stimulated production of less interferon. Pre-treatment of cells with calf interferon reduced the growth of both viruses, but especially that of MB virus. The results are considered in relation to the phenomenon of virus adaptation. It is suggested that such differences between the two variants in their relative sensitivity to and production of interferon could have played a part in the emergence of CK virus as the predominant type during serial passages in calf cells.

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