Rate of Propagation of Japanese B Encephalitis Virus in Hamster Kidney Cell Cultures.* (24756)

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The usefulness of hamster kidney cell (HKC) cultures for detection and propagation of Japanese B encephalitis (JBE) virus has been established(1,2). The purpose of this report is to record the rate and pattern of viral yield and increase following inoculation of HKC cultures with known infective doses of virus.

Material and methods. The methods used for preparation and maintenance of HKC cultures for routine laboratory use have been described(2). Briefly reviewed, the methods used were essentially identical with those currently in use for processing of monkey kidney cell cultures with only minor modifications. The procedure employed for manipulating the labile JBE virus was unique in that the virus yield was harvested by addition of an equal volume of inactivated normal calf serum prior to shell freezing and storage at dry ice box temperatures. This practice permitted routine laboratory manipulations to be carried out without undue loss of titer. The Nakayama strain of JBE virus was used throughout our studies and the standard diluent for virus titrations consisted of maintenance medium fortified with 10% inactivated normal calf serum. All calf serum used was pretested for presence of JBE neutralizing antibodies and shown to have a neutralization index of less than 10. *Exp. A.* The 10th HKC passage pool of virus, which had a TCD_{50} titer of $10^{-6.0}$, was diluted in a 10-fold dilution series from 10^{-1} through 10^{-5}. Each of 72 replicate HKC culture tubes was then inoculated with 0.1 ml of the 10^{-3} dilution and each of an additional 72 replicate tubes received 0.1 ml of the 10^{-5} dilution. Therefore, the TCD_{50} used for these inoculations were approximately 1,000 and 10 respectively. At predetermined time intervals following inoculation, 6 tubes from each dosage level were harvested with the addition of an equal volume of normal calf serum. These shell-frozen and thawed harvests were then pooled and dispensed to glass-sealed ampules which were rapidly frozen in a dry ice alcohol bath and stored at dry ice box temperatures until thawed for titrations in HKC cultures approximately 2 weeks later. At time of titration, each sample of stored virus was melted at 37°C, diluted in a 10-fold dilution series and 0.1 ml of the appropriate dilutions inoculated into each of 3 replicate HKC culture tubes.

Results of these titrations plotted (Fig. 1) showed that following inoculation of 1,000 doses of virus, the fluids harvested 3 hours later still contained virus to a titer of $10^{-1.8}$/0.1 ml. Six hours after inoculation, virus content was still declining. Twenty-three hours following inoculation, the titer noted was $10^{-3.5}$ which then increased to a titer of $10^{-4.8}$ by the 32nd hour. The 46th, 52nd and 69th hour harvests showed further increases of infective content with titers of $10^{-5.8}$, $10^{-6.5}$ and $10^{-7.3}$ respectively. With the 10 TCD_{50} inoculum, the harvest stored immediately after inoculation failed to reveal the presence of virus. However, both the 1- and 4-hour harvest showed the presence of virus in 1 of 3 tubes inoculated with undiluted material. Virus increase was readily detected after 24 and 33 hours of incubation with titers of $10^{-2.1}$ and $10^{-2.8}$ respectively. The 47th, 53rd and 70th hour yields showed a further increase in titer from $10^{-4.5}$ to $10^{-5.5}$ to finally $10^{-6.3}$.

These data show form and pattern of virus replication to be essentially a straight line

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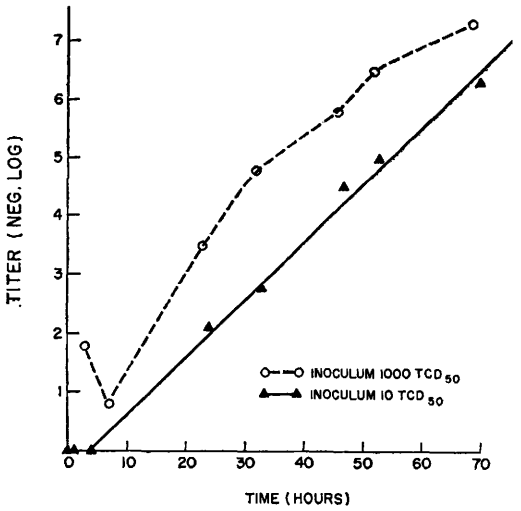

FIG. 1. Propagation of Japanese B encephalitis virus in hamster kidney cell cultures following inoculation with 10 and 1,000 tissue culture doses of virus.*

* Harvests collected 3, 6, 23, 32, 46, 52 and 69 hr after inoculation were titrated in 10-fold dilution series using 3 tubes of hamster kidney cell cultures per dilution for the 1,000 TCD_{50} inoculum. For the 10 TCD_{50} inoculum, harvests collected immediately after inoculation and after 1, 4, 24, 33, 47, 53, and 70 hr of incubation were titrated in a similar manner. Points plotted are TCD_{50} of the individual titrations. The data obtained for the 6 hr and 24 hr harvests are estimates only, since dilutions used for assay did not completely bracket the endpoint of infectivity.

logarithmic increase of infectivity after a brief latent period following inoculation. Although a slight decrease in rate of growth is suggested for the 1,000 TCD_{50} inoculum during the late time period, in neither instance was a definite plateau reached; therefore, it is not known whether the viral increment resulting from the smaller inoculum would have reached an ultimate titer equivalent to that attained by the larger dose. Over the period of observation, the 2 curves revealed slopes essentially identical but spaced approximately 1 log higher in the case of the larger inoculum.

Exp. B. For practical purposes of selecting the optimal day for harvest of tissue culture fluids following inoculation and to supplement the information gained with the 10th passage virus an additional experiment was designed to gain information regarding daily increase of infectivity by 24 hour time intervals, using the 25th passage level virus. The 25th passage pool of virus was estimated to have a mean TCD_{50} of $10^{-6.8}$ based on prior duplicate titrations carried out at ½ log intervals. As inoculum for this experiment, the stock virus was diluted to a concentration of $10^{-4.8}$. This dilution of virus in a volume of 0.1 ml, which represented approximately 100 TCD_{50}, was used to inoculate 52 replicate HKC culture tubes. Immediately following inoculation, 4 tubes were harvested in the usual manner. This harvest was designated as time zero. At 24 hour intervals thereafter, 6 replicate cultures were harvested and pooled in the same manner as in Exp. A. These pools of infective tissue culture fluids were stored at dry ice box temperatures for a period of approximately 2 weeks, at which time they were thawed and titrated in HKC cultures using 4 replicate tubes for each 10-fold dilution.

Following inoculation with 100 TCD_{50} of virus, the tubes responded in the usual way and showed minor evidence of cytopathic activity when examined 48 hours later. Morphologic changes at the end of 72 hours of incubation were recorded as 3+ prior to harvest of the 6 tubes, and from the 96th hour following inoculation onward, the cellular monolayers were graded as 4+ degeneration. The successive titers obtained, expressed as negative logarithms of infective titer, were: <1.0, 2.5, 5.8, 6.3, 6.5, 5.7, 4.4, <3.0, and <3.0 (Fig. 2). These results showed that viral increment as measured by infectivity reached its peak at the 72nd or 96th hour following inoculation, at a time when the cellular monolayer was first graded as 3 or 4+. The slope of increasing viral titers during the first 3 days appears to be somewhat steeper than the slope of decline as measured by titer changes at the end of the 5th and 6th days.

Exp. C. One final experiment was designed to gain a greater insight into the actual replication of virus by the infected HKC monolayer. The 25th passage virus again served as inoculum and the dilution used was prepared as described above for Exp. B. Each of 10 replicate tubes of HKC cultures received 0.1 ml of virus estimated to contain 100 TCD_{50}. Five of these tubes served as positive controls on virus activity and dis-

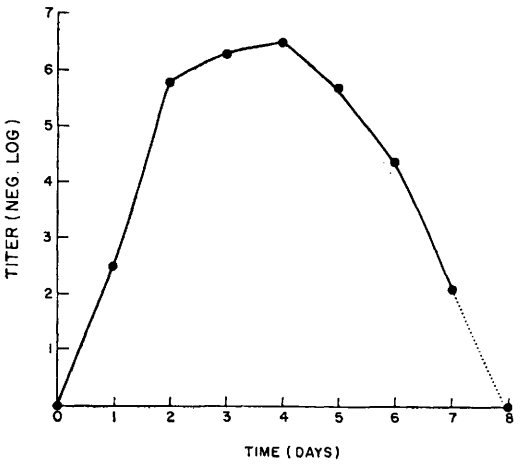

FIG. 2. Propagation of Japanese B encephalitis virus in hamster kidney cell cultures following inoculation with 100 tissue culture doses of virus.*

* Harvests were collected immediately after inoculation, and at 24 hr intervals thereafter for a period of 8 days. The stored fluids were titrated in 10-fold dilution series using 4 tubes of HKC for each dilution. The 0, 7, and 8 day titers are estimates of infective content since the dilutions used for assay did not completely bracket the endpoint of infectivity.

played 3+ degeneration 72 hours following inoculation, which progressed to complete, or 4+, at the end of 96 hours. Near the end of the 2nd day, beginning at the 45th hour after inoculation and continuing at hourly intervals to the 52nd hour, total fluids from the 5 test cultures were removed and pooled in a small flask. The monolayer was then washed one time with 1 ml of maintenance medium. One ml of fresh maintenance medium was added and the tubes returned to their stationary position in a 37° C incubator. The pool of tissue culture fluid obtained from the 5 cultures was stabilized by adding 5 ml of normal calf serum and then was dispensed to ampules which were shell-frozen prior to storage at dry ice box temperatures. Again near the end of the 3rd and 4th days the procedure was repeated. Therefore, hourly harvests were made on the 45th through the 52nd hour, similarly the 69th through the 76th hour and from the 93rd to the 100th hour following inoculation.

The accumulated dilution factor from the 45th hour to the 99th hour was of the order of 10^{-16} if one considers that a volume of 0.1 ml of fluid remained in the tube after pouring off the yield, adding and discarding the 1 ml wash and the addition of 1 ml of fresh maintenance media following each harvest (*i.e.*, 23 washings over a 3-day period each representing approximately a 10^{-2} dilution factor).

Approximately 3 weeks later, selected stored harvests were titrated for infectivity in HKC cultures using 10-fold dilutions and 3 tubes for each dilution. The results of these titrations are summarized in Table I.

As a further check on the validity of these results, the 69th and 70th hour yields (18 hours and 1 hour growth respectively) were retitrated using ½ log dilutions and 4 tubes per dilution. The TCD_{50} for the 18 hour period harvested at the 69th hour was $10^{-7.3}$ and for the 1 hour period harvested at the 70th hour was $10^{-6.6}$ (Table II).

Discussion. The first 2 experiments are in essential agreement, providing a reasonable approximation of the cumulated infective viral yield to be expected following inoculation of HKC cultures with from 10 to 1,000 TCD_{50} of at least partially HKC adapted JBE virus. The maximum titer is probably reached slightly more rapidly by the greater inoculum and there is a suggestion that the plateau is reached somewhat more rapidly by the 25th

TABLE I. Titers of Yields of Japanese B Encephalitis Virus in Hamster Kidney Cell Cultures Inoculated with 100 TCD_{50}* at 18 and 1 Hour Intervals during 2nd, 3rd and 4th Days after Inoculation.

Day after inoc.	Time of harvest (hr)	Titer† (neg. log)
2	45‡	5.3
	47§	4.8
	49§	5.3
	51§	5.5
3	69	7.8
	71	6.8
	73	6.5
	75	6.8
4	93	7.0
	95	5.5
	97	5.3
	99	4.8

* 0.1 ml of $10^{-0.8}$ dilution of 25th "rapid passage" virus.

† Titrations done in 1 log dilution series using 3 tubes per dilution from 10^{-4} through 10^{-9}.

‡ First harvest each day was of fluid placed in tube 18 hr previously.

§ All specimens after first of the day were fluids added just 1 hr prior to harvest.

TABLE II. Titers of Yields of Japanese B Encephalitis Virus in Hamster Kidney Cell Cultures Inoculated with 100 TCD_{50}* at 18 and 1 Hour Intervals at Beginning of 3rd Day after Inoculation.

Day	Time of harvest (hr)	Titer† (neg. log)
3	69‡	7.3
3	70§	6.6

* 0.1 ml of $10^{-6.5}$ dilution of 25th "rapid passage" virus.

† Titrations done in ½ log dilution series using 4 tubes per dilution from 10^{-5} through $10^{-8.5}$.

‡ Harvest was of fluid placed in tube 18 hr previously.

§ Harvest was of fluid placed in tube 1 hr previously.

passage virus than by the 10th, possibly the result of further adaptation.

From the first 2 experiments it is not possible to determine actual rate of virus growth or when replication ceases, for viral degradation is proceeding at an unknown rate and what is being measured is the difference between these two opposite forces.

Exp. C was designed to clarify this combination of replication and degradation, at least in part. It is evident from the combined results obtained that viral replication by the monolayer begins prior to the onset of marked cytopathogenic effect (CPE) and that virus continues to be released in the medium as long as cells remain in the monolayer. During the plateau phase shown in Exp. B, Fig. 2 (relatively constant level of active viral titers in tubes left undisturbed following inoculation) it is obvious that viral deterioration is progressing at a rate equal to that of viral release by the cells since during these same time periods (days 2 through 4) in Exp. C (Table I) the hourly harvests indicated a relatively constant rate of yield. After 120 hours following inoculation, when the monolayer had completely disappeared the rapid rate of logarithmic titer fall (Fig. 2) suggests that the released, degenerated cells were yielding no more virus and that the late portion of this slope (estimated in part) may represent the true rate of viral degeneration. Providing pH changes and other modifications of the medium due to cell growth have not greatly altered the rate of viral degeneration, it may be assumed that this goes on at a constant rate from time of release of virus from the first infected cells (less than 24 hours after inoculation) until all virus becomes inactive.

The infectivity titers attained in the harvested fluids are apparently largely the result of viral replication or release as it occurs during the previous hour (Tables I and II). The detectable accumulation of infective virus after overnight incubation does not exceed that of 1 hour growth by more than 1½ logs and in the more precise titration was only 0.7 logs. This fact emphasizes the lability of the Japanese B encephalitis virus at ordinary incubator temperatures in the usual tissue culture medium. Viral titers obtained by these hourly harvests beginning 24 hours following inoculation and during the next 48 to 72 hours are sufficiently high to suggest that this tissue culture system may provide an inexpensive source of potent virus-containing fluids which can be sufficiently concentrated by physical or chemical means to serve a number of purposes. The starting material need contain little protein or other extraneous materials as compared with mouse brain or chick embryo which has been used previously(3,4). Although not yet tested it appears reasonable to expect that during the phase of maximum viral release by the cells all serum may be omitted from the medium, since cell growth is no longer of importance. By the use of infected monolayers of HKC, which are releasing virus continuously, it should now be feasible to devise technics which would permit a continuous flow of fluid to be harvested directly into a container maintained at dry ice box temperatures during the period of optimal virus release. Such fluids would then provide an inexpensive starting material for subsequent concentration and purification procedures. This has obvious potentialities for an inactivated vaccine. It seems probable that studies similar to those which have been done on purification of poliovirus from infected tissue culture fluids(5) are now within the realm of possibility.

Preparations such as these, probably without concentration, and relatively devoid of inactive virus should have a real application in revealing the true neutralizing capacity of immune sera since it is probable that inactive virus is capable of combining with antibody

and thus altering the results of diagnostic neutralization tests. Certainly further work along these lines, comparing the results obtained with neutralization tests performed in the usual manner and with virus prepared from a 1 hour period of replication is indicated.

Finally, these active, hourly harvests of virus may provide a more delicate means for studying antigenic relationships between members of the arthropod-borne viruses, since animals immunized with active tissue culture virus may produce a more specific antibody than that now obtainable following immunization with virus suspensions containing not only large quantities of animal protein but also varying quantities of inactive virus. These actively replicating HKC cultures may also prove useful as new tools for study of the production of antigens for demonstrating complement-fixing and hemagglutinating activity of the virus.

Summary. Following inoculation of HKC cultures with JBE virus, virus content increases by geometric increment after a brief latent period and yields a maximum titer of virus usually of the order of 10^7 infective units per 0.1 ml of tissue culture fluid 72 to 96 hours following inoculation. Infectivity of the tissue culture fluids then decreases during the next several days as all of the cells of the monolayer are destroyed by the cytopathogenic activity of the virus. Further studies during the 69th to 75th hours following inoculation with JBE virus showed that the infected HKC monolayer is capable of releasing infective virus to a titer of the order of $10^{6.5}$ to $10^{6.8}$ per 0.1 ml at hourly intervals. The practical and theoretical implications of these findings for further studies have been discussed.

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Relation of Myxoma Deoxyribonucleic Acid (DNA) to Fibroma-Myxoma Virus Transformation. (24757)

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Transformation of fibroma into myxoma virus in tissue culture using myxoma virus inactivated by heat as transforming agent (TAM) has been described(1,2). Further findings indicate that TAM consists of myxoma virus particles which have lost infectivity due to denaturation of their outer protein coats. These particles are not affected by deoxyribonuclease (DNase)(3). Continuing investigations have aimed to remove outer coats in an effort to reveal an inner core of DNA. The present communication describes how urea treatment renders TAM susceptible to the destructive action of DNase, while not reducing its ability to transform. These findings suggest that DNA is essential to the Berry-Dedrick phenomenon(4).

Materials and methods. Recent modifications in procedures used in transformation experiments in tissue culture(1,2) may be outlined as follows: *Fibroma virus.* The live virus used was prepared from fibromas made into a 1:10 suspension with the medium used for tissue cultures. After clearing by repeated centrifugations at 3,000-4,000 rpm, the suspensions were stored at –70°C. *Preparation of TAM.* Myxoma virus was prepared as a 1:10 suspension of ground tumors in pH 7.2 phosphate-buffered saline containing 10% calf serum (inactivated) as well as penicillin