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GROWTH OF AFRICAN HORSE-SICKNESS VIRUS IN MONKEY KIDNEY CELL CULTURES

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SUMMARY

Of various established cell lines susceptible to virus strains of African horse-sickness, monkey kidney, stable (MS) cells sustained the most distinct cytopathic changes, with both virulent and attenuated neurotropic strains of the virus.

After passage, the incubation period became shorter, and all the strains produced cytopathic effect in 2 to 3 days after inoculation.

A growth curve of Asian-type virus (S2) in MS cells was determined. There was an 8-hour latent period before virus production was demonstrable, and the maximum virus level ($10^{7.8}$ mice LD_{50} per ml.) was achieved in 48 hours.

Plaques were formed after 10 day's incubation. They ranged in size from 1 to 3 mm. in diameter.

The MS cells were considered to be the most suitable host for serologic studies, diagnostic techniques, vaccine production, and studies of the properties of the virus.

African horse-sickness, a highly fatal infectious disease of equine animals, is caused by a filterable virus (8). Because of its occurrence in the Middle

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East and India since 1959, we reconsidered its importance (15). The history and scientific knowledge of the disease have been summarized in recent publications(7,13).

As early as 1935, Alexander (1) demonstrated that the virus could be adapted to mice by intracerebral passage. Since then, mouse-adapted neurotropic strains of the virus have been widely used for vaccine production and studies of the disease.

Results of attempts to grow the virus in hosts other than horses and mice have not been consistent. Among those that were tried, chicken embryos seemed to have potential value for vaccine production, although results of the original work carried out by Alexander (2) were unsatisfactory.

Several workers have tried to find better hosts among various cell cultures but with little success; however, Mirchamsy and Taslimi (9) recently adapted an Asian strain of the virus to hamster kidney cells.

Using available established cell lines, we attempted to find cells suitable for multiplication of the virus, and for immunologic and virologic studies of the disease.

MATERIALS AND METHODS

Virus.—Seven antigenically different mouse-adapted neurotropic vaccine strains A501, OD, L, Vryheid, VH, 114, and Karen were obtained from the Onderstepoort Veterinary Institute. A few additional passages in mice were made, and the identity of these strains was confirmed by serologic examinations with homologous antiserums.

The 4th passage in suckling mice of an Iranian strain (10/60) (5) isolated from the blood of an affected horse was used as an Asian strain. The mouse-adapted Asian strain (S2) which was the 104th passage of the local strain was also employed as the neurotropic attenuated strain. All the viruses grown in tissue culture were stored at 4 C. throughout the experiment.

Cells.—Cell lines used were : MS (developed by Dr. Kanda "6"), normal human amnion (FL), epidermoid carcinoma of larynx (Hep-2), and normal green monkey kidney (GMK) *; baby hamster kidney (BHK-21) **; and bovine kidney (DBK).†

* Received from Dr. Tagaya, National Institute of Health, Kamiosaki, Shinagawaka-Ku, Tokyo, Japan.

** Received from Dr. Galloway, the Animal Virus Research Institute, Pirbright, Surrey, England.

† Received from Dr. Watanabe, National Institute of Animal Health, Kodaira, Kitatama, Tokyo, Japan.

Nutrient Mediums.—Nutrient medium (YLE) used for MS, GMK, FL, and Hep-2 was prepared by adding to Earl's solution(12) 0.5% lactalbumin hydrolysate, 0.005% yeast extract, 10.0% calf serum heated at 56 C. for 30 minutes, 100 units of penicillin per milliliter, 100 gamma of streptomycin per milliliter, and 0.0015% phonel red. The maintenance medium contained all these constituents except for the reduced concentration (1.0 to 2.0%) of calf serum. The final pH was approximately 7.3.

For DBK, Hanks' balanced salt solution(4) served as the base for the nutrient medium. The rest of the constituents were the same as for the other mediums.

Eagle's medium (Difco, dried) was the base for BHK-21 cell culture medium. The nutrient medium was prepared by adding 10% unheated calf serum, 5% tryptose phosphate broth (Difco), 100 units of penicillin per millilitre, and 100 gamma of streptomycin per milliliter. The final pH was adjusted to 7.0 by adding sodium bicarbonate solution.

Subcultivation.—The MS cell line was subcultured at least once a week, and all other cell lines were subcultured when complete monolayers were formed. The nutrient medium was removed when a monolayer was formed, and the cells were rinsed once with 0.25% trypsin in phosphatebuffered saline(3) (PBS) solution minus magnesium and calcium ions (PBS $^{-}$). The monolayer was then covered with the same trypsin solution and incubated at 37 C. for 5 to 10 minutes. An equal volume of cold nutrient medium was addeed to the cell suspension. After pipetting several times the cell suspension was centrifuged at 800 r.p.m. for 5 minutes. The sediment was resuspended in the appropriate nutrient medium, the volume of which was usually 3 times as much as the previous culture medium.

Inoculation.—Monolayers of cells were usually formed 5 days after subcultivation. The cell sheets prepared in bottles were washed once with PBS $^{-}$ and then were covered with appropriate virus dilutions. The inoculated cells were incubated at 37 C. for 30 minutes before fresh nutrient medium was added. For the 1st passage, infected mouse brain suspension in YLE (1 : 10 dilution) was employed after centrifugation at 3,000 r.p.m. for 10 minutes.

Subpassages were made whenever cytopathic effect (CPE) became widespread. If CPE was not observed, a blind passage was made on the 7th day, and the donor culture was kept undeer observation for another week.

Titration of Virus in Mice and in Monkey Kidney, Stable Cells.—Serail tenfold dilutions of virus were prepared by using the nutrient medium, YLE.

Titrations were made in 5 to 6-week-old mice by intracerebral injeciton of 5 mice per dilution with 0.05 ml. of inoculum. The mice were kept under

close observation for 2 weeks.

Titrations in MS cells were made in ordinary roller tube cultures prepared by seeding 1.5 ml. of cell suspension in the nutrient medium. After 5 to 7 days' incubation, the growth medium of prepared cell cultures was discarded.

Four tubes were inoculated with 0.1 ml. of each dilution and were incubated at 37 C. for 30 minutes before 1.5 ml. of the nutrient medium was added. The final reading of CPE was made 10 days after inoculation. Virus LD_{50} in mice and $TCID_{50}$ were calculated by the Reed and Muench (14) method.

All neutralization tests in cell cultures were carried out by using 100 to 1,000 $TCID_{50}$ units of virus and serial dilution of homologous anti-horse-sickness-virus rabbit sreum. Each virus-serum mixture was incubated at 37 C. for 1 hour and kept at 4 C. overnight before inoculation into roller tube cultures.

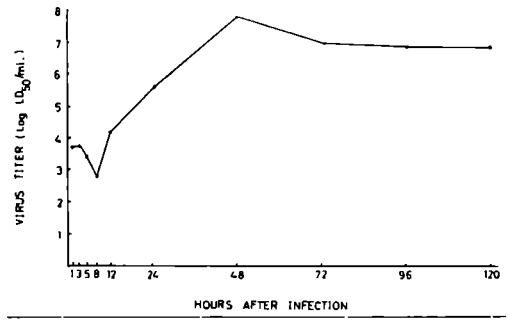

Fig. 1—Single-passage growth pattern of African horse-sickness virus (S2) in monkey kidney, stable cell cultures.

Plaque Assays in Monkey Kidney, Stable Cells.—The nutrient agar consisted of YLE containing 1.2% Difco bacto-agar washed 3 times, neutral red (1 :50,000) in place of phenol red, double concentration of sodium bicarbonate, and 5.0% calf serum.

The monolayer prepared in 2-oz. bottles was washed once with PBS,$^{-}$ and 0.2 ml. of each virus dilution was placed on the monolayer. The bottles were rocked 3 times during the 30-minute, 37-C. incubation period to distribute the inoculum over the entire surface of the cell sheets. Eight milliliters of the nutrient agar, kept at 43 C., was overlayed at the end of the incubation

period, and the bottles were returned to the incubator when the nutrient agar became solid. The agar was maintained on the upper side of each bottle.

Growth Curve of Virus in Cultures of Monkey Kidney, Stable Cells.—Monolayer cultures prepared in two 1-liter Roux bottles each having 190 sq. cm. of flat bottom, were washed once with PBS. Each monolayer culture was then inoculated with 1 ml. of S2-7 (the 7th passage of neurotropic Asian-type strain in MS cells) which contained 796,000 mouse LD_{50} of virus. The Roux bottles were incubated at 37 C. for 30 minutes, and then 100 ml. of warmed nutrient medium was added to each bottle. The bottles were immediately returned to the incubator.

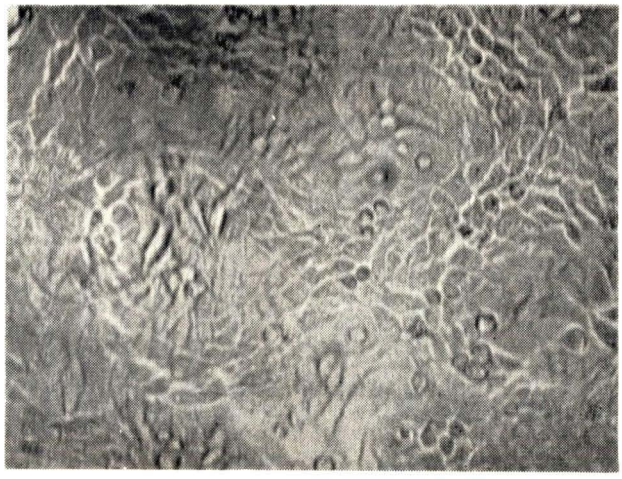

Fig. 2—Normal monkey kidney, stable cell culture. Unstained, x 300.

Samples (0.5 ml. for 1st 12 hr., then 0.2 ml.) were taken at intervals of 1, 3, 5, 8, 12, 24, 48, 72, 96, and 120 hours after infection. After each sample was taken, the same amount of fresh nutrient medium was added to each bottle. The samples taken from 2 bottles at the same time were pooled. Tenfold dilutions of the pooled sample were prepared in cold YLE and titrated in mice immediately.

Giemsa stain was employed for precise microscopic examination of infected MS cell cultures. Coverslip cultures of MS cells were infected with the 13th passage of S2 strain. The coverslips were taken at intervals of 12,

24, 48, and 72 hours. All the infected cell cultures and control cells were carefully washed 3 times with PBS. They were fixed in methanol and stained with Giemsa stain for 20 minutes.

Results

With the neurotropic Karen strain and the Asian strain (10/60), the susceptibility of each cell line was examined in a primary screening test. The MS cells produced distinct cytopathic changes 4 days after infection with the Karen strain and 7 days after infection with the Asian strain. The incubation period of both strains was markedly shorter after the 2nd passage.

After infection with the Asian and Karen strains, GMK cells had cytopathic changes. These changes were not as uniform as those in MS cell cultures. After granule formation within infected cells, the cells gradually shrank, became irregular in shape, and finally detacheed from the glass by two's and three's. The incubation period was shorter on the 2nd passage.

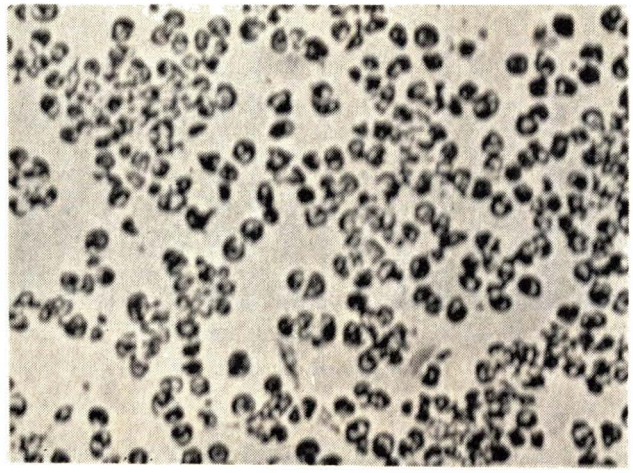

Fig. 3—Monkey kidney, stable cell culture infected with S2 strain of African horse-sickness virus. Unstained; x 300.

The BHK cells had cytopathic changes similar to those in GMK cells, but the incubation period was shorter.

The FL cells did not have definite cytopathic changes after the 1st passage with the Asian and Karen strains; however, a marked difference in

color change between the bottles of infected and noninfected FL cells was noticed. Blind passages were made up to the 5th passage, but the cells did not have definite cytopathic changes. The fluid of each passage did produce cytopathic changes in MS cells.

The Hep-2 cells also had a slight effect from the virus, but the changes were not definite even after passage.

Changes were not observed in DBK cells infected with the Asian and Karen strans of the virus.

Based on these results, further investigations were made on the relationship between African horse-sickness viruses and MS cells.

Cytopathic changes were observed in MS cell cultures at the 1st passage of all strains of horse-sickness virus used. The characteristic CPE was rounding of the infected cells (Fig. 3) similar to that produced in cells infected with polio viruses(10).

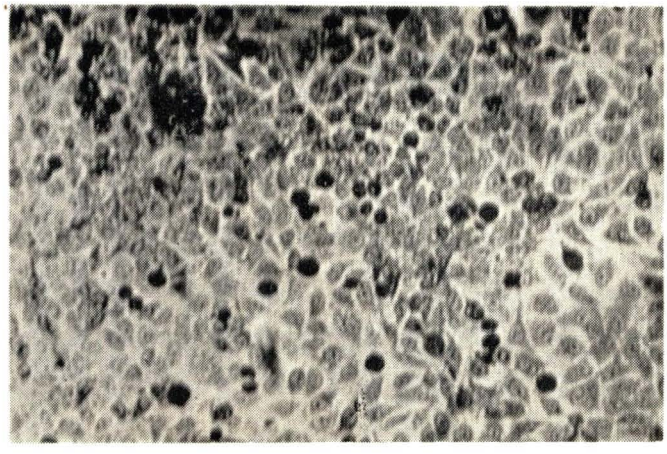

Fig. 4—Normal monkey kidney, stable cell culture. Giemsa stain; x 300.

Cytoplasmic inclusion bodies were not found in the infected cells. The nuclei of infected cells were, however, darkly stained with Giemsa stain (Fig. 5, 6).

The incubation periods were markedly shorter during the 2nd passage. After 3 passages, all the strains produced distinct CPE within 3 days after infection. After 10 passages, all the strains produced complete CPE within

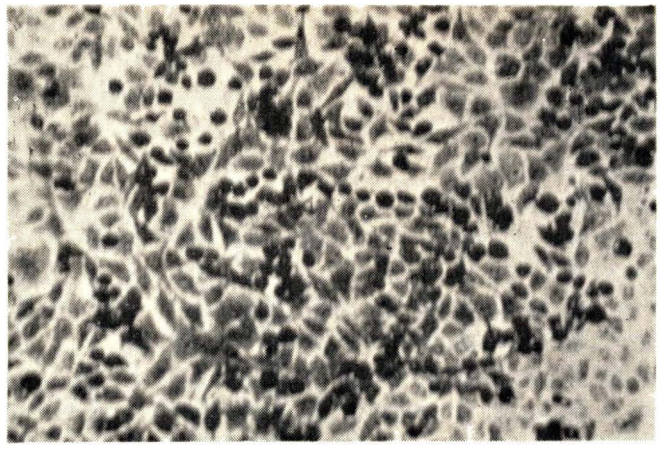

Fig. 5—Monkey kidney, stable cells infected with the 13th passage of S2 strain of African horse-sickness virus 24 hours previously. Giemsa stain; x 300.

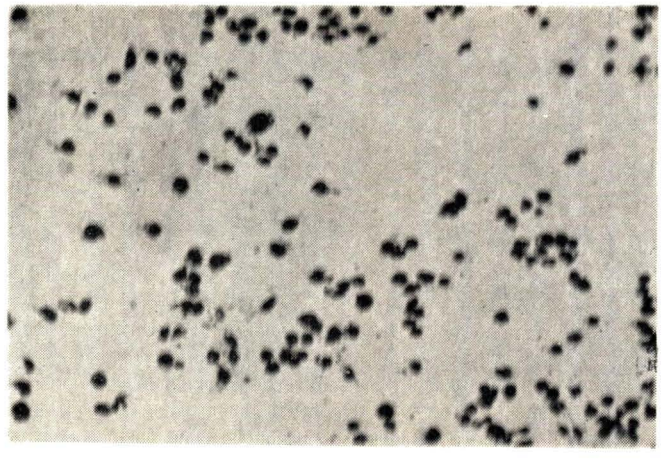

Fig. 6—Monkey kidney, stable cells infected with the 13th passage of S2 strain of African horse-sickness virus 48 hours previously. Giemsa stain; x 300.

2 days after infection. Differences were not observed in the types of CPE produced by different strains throughout the experiment. There were some difficulties in subculturing A501, OD, and VH strains of virus during the first few passages. It was observed that slightly longer incubation periods

were required to produce complete CPE if infected cells were maintained in plain YLE or 199 medium without calf serum.

Viruses harvested between 72 and 96 hours after infection were titrated in tissue culture tubes and in mice. All samples examined during the 1st 8 passages had titers between 10^6 and 10^7 $TCID_{50}$/ml. (Table 1).

A growth curve of S2 virus (8th passage) in MS cells was determined (Fig. 1). There was an 8-hour latent period before newly formed infective virus appeared in the medium. A maximum titer of $10^{7.8}$ mouse LD_{50} /ml. occured 48 hours after infection. At that time almost all the cells had cytopathic changes (Fig. 3). The affected cells gradually detached from the glass surface and, at the 72nd hour, a majority of the degenerated cells were floating in the fluid, and the titer had fallen to $10^{7.0}$ mouse LD_{50}/ml.

Death of affected mice first occurred approximately 3 days after infection among the mice injected with the highest concentration of virus. The incubation period of mice injected with lower concentrations of virus were longer, but most of them died within a week after injection.

TABLE 1—Infective Titers of African Horse-Sickness Virus Grown in Monkey Kidney, Stable Cells (Log $TCID_{50}$/ml.)

No. of passages	Strains of horse-sickness virus								
	A501	OD	L	Vryheid	VH	114	Karen	S2	Asian
2	....	....	....	....	...	...	6.5a*	6.3	6.5
3			....	....	....	....	6.0	....	6.5
4			6.3	6.6	....	6.7	..	.. .	....
5	....	....	....	....	....	....	....	6.5	....
6	....	6.5	....	....	....	....	7.0	....	....
7	. .	....	....	....	....	6.8	...	....	.
8	6.7	7.0	6.7	6.5	6.5	....	...	7.0b*	6.8c*

* The titers of the same samples in mice (log LD_{50}/ml.) were: a, 6.6; b, 7.0; and c, 5.3.

Plaque assay was made with 3 strains, the 8th passage of Vryheid, the 2nd passage of Karen, and 2nd passage of Asian strain (10/60). Plaques, first observed about 10 days after infection, were very small and faint but gradually increased in size and clarity until about the 16th day. They ranged in size from 1 to 3 mm. in diameter (Fig. 8). There were 1.9×10^7 plaque-forming units (PFU) per milliliter with Vryheid strain, 1.1×10^8 PFU/ml. with Asian strain, and 3.0×10^6 PFU/ml. with Karen strain. Simultaneous titrations in tube cultures were $10^{6.5}$ $TCID_{50}$/ml. with all 3 strains, and in adult mice were $10^{5.3}$ with the same Asian strain and $10^{6.6}$ with the Karen strain.

A marked difference was observed between the same virus fluid (prepared in YLE nutrient medium with 10% calf serum) stored at 4 and —20 C. The titer of the 2nd passage of Karen strain in MS cells stored at 4 C. was $10^{9.5}$ $TCID_{50}$/ml., whereas that of the same virus kept at—20 C. for 6 days was lower than 10^{3} $TCID_{50}$/ml., The 5th passage of St strain, which had the titer of $10^{6.5}$ $TCID_{50}$/ml., was stored at 4 and — 20 C. The titer of the virus kept at —20 C. dropped to 10^{3} $TCID_{50}$/ml. within a week, whereas that of the same virus stored at 4 C. for 2 months maintained the same titer, $10^{6.5}$.

The 5th passage of A501 and S2, 7th passage of OD and Vryheid, 8th passage of L and 114, 9th passage of Karen, 10th passage of Asian, and 11th

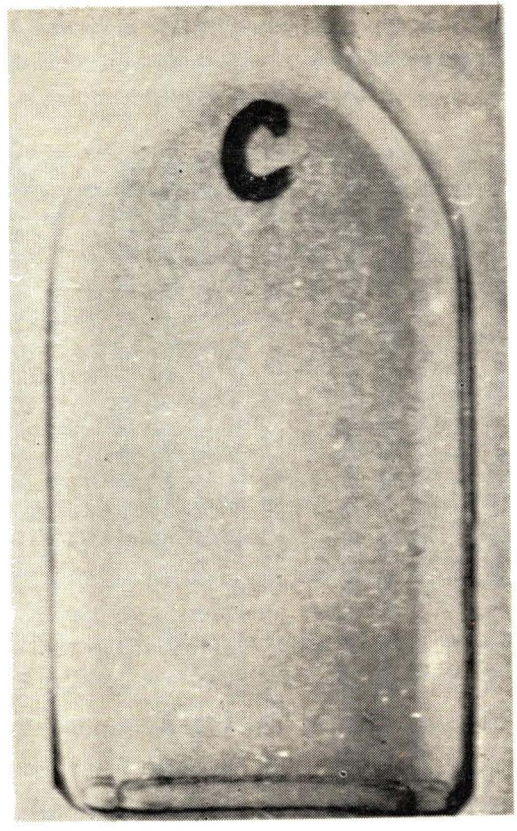

Fig. 7—Normal monkey kidney, stable cell culture in a plaque bottle.

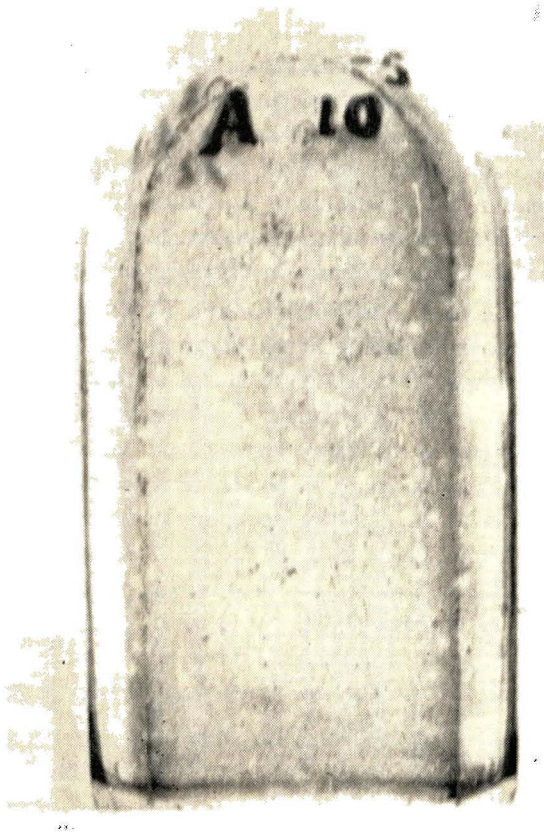

Fig. 8—Plaques on monkey kidney, stable cell culture 14 days after inoculation with 0.1 ml. of a 10^{-5} dilution of Asian strain of African horse-sickness virus.

passage of VH strain each were neutralized with specific antiserum prepared in rabbits. Growth of each virus in tube cultures was inhibited by specific antiserum, either undiluted or diluted tenfold.

Discussion

The MS cells were chosen for propagation of the virus because (*a*) all the strains of African horse-sickness virus easily became adapted to the cells and produced characteristic CPE from the first passage; (*b*) high titers were obtained and the incubation periods were shorter; and (*c*) MS cell cultures were easy to prepare, the cells were stable, and retained their halthy appear-

ance for a longer period. These characteristics are essential for serologic and plaque assays.

It appears that there is a distinct possibility of producing either monovalent or polyvalent tissue culture vaccine by using MS cell cultures, because all neurotropic vaccine strains of African horse-sickness virus were successfully adapted to MS cells yielding high titers of virus.

Infective titers in the growth curve were determined in mice because the S2 strain was initially a mouse-adapted neurotropic strain, and with mice it was easier to standardize conditions for titrating virus samples harvested at various intervals. The LD_{50} titer in mice and $TCID_{50}$ titer of 2 neurotropic strains of African horse-sickness virus grown in MS cells (during the first 8 passages) were almost the same. The titer of non-neurotropic Asian strain was, however, lower in mice. Alexander(1) demonstrated that the incubation periods of field strains of African horse-sickness virus were much longer during first few passages in young mice and that the mortality was lower.

It is previously observed that the incubation periods of neurotropic strains of African horse-sickness virus in mice vary. Karen strain has shortest incubation period of 2 to 3 days, and the other strains have a 3- to 4-day incubation period. After passage, all the strains including Asian strain of African horse-sickness virus produced complete CPE 2 to 3 days after inoculation, and titers of the virus in MS cells were slightly higher than usual titers in mice.

The $TCID_{50}$ titers (Table 1) were the titers of fluid harvested between the 3rd and 4th days after infection. It was found later that the maximum infective titeer was achieved on the 2nd day after infection. Therefore, the maximum infective titers of all these strains would be higher had they been harvested at the optimum time.

It is believed that the maximum titer ($10^{7.8}$ mouse LD_{50}/ml.) obtained with S2 strain after 48 hours' incubation in MS cells (Fig. 1) was the highest titer yet reported with African horse-sickness virus in hosts other than horses. The higher yield of virus obtained with MS cells may stimulate better antibody response in experimental animals used to produce antiserum and may be advantageous in diagnostic and serologic studies of African horse-sickness.

It was previously observed by us that the healthy appearance of MS cells was not affected by adding antiserums prepared in rabbits, guinea pigs, sheep, and horses to the cultures. This is important for neutralization tests in MS cell culturees.

According to Howell (5), there are 9 antigenic groups of African horse-sickness virus. The first 7 groups are represented by the following type

strains: group 1, A501; 2, OD; 3, L; 4, Vryheid; 5, VH; 6, 114; and 7, Karen. Strains of group 8 isolated in Africa were recently identified as a new antigenic group of virus, but none of them has been used as the neurotropic vaccine strain. All the strains, including the Asian (10/60) strain, isolated from the affected equine animals in the Middle East and Asia during epizootic seasons between 1959 and 1961 were identified as the virus belonging to group 9.

It appears that the subculturable MS cell line which was originally derived from a normal monkey kidney makes an excellent host for the vaccine production, serologic studies, diagnostic techniques, and studies of the properties of African horse-sickness virus, because of its ease of handling, its stability, and its ability to produce characteristic CPE yielding high titers of virus.

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SUMMARIO IN INTERLINGUA

Le Crescentia de Virus de African Morbo Equin in Cytoculturas de Ren Simian

De varie establite lineas de cellulas susceptibile pro lineas de virus de african morbo equin, stabile cellulas de ren simian (MS) suffreva le plus distincte alterationes cytopathic, tanto con virulente como etiam con attenuate lineas neurotropic del virus.

Post le passage, le periodo de incubation deveniva plus curte, e omne le lineas de virus produceva effectos cytopathic intra 2 a 3 dies post le inoculation.

In cellulas MS, un curva de crescentia de virus de typo asian (S2) esseva determinate. Il occurreva un periodo de latentia de 8 horas ante que le production de virus esseva demonstrabile, e le nivello maximal de virus ($10^{7.8}$ DL 50 murin per ml) esseva attingite in 48 horas.

Placas se formava post 10 dies de incubation. Lor diametros variava inter 1 e 3 mm.

Le cellulas MS esseva reguardate como le plus appropriate hospite pro studios serologic, technicas diagnostic, production de vaccino, e studios del pel proprietates del virus.