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STUDIES ON THE REPLICATION OF AFRICAN HORSE-SICKNESS VIRUS IN TWO DIFFERENT CELL LINE CULTURES (§)

By :

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Introduction

Previous studies on African horse-sickness (AHS) virus indicated that the virus is transmitted by blood sucking insects such as *Culicoides* (*Du Toit*, 1944) and mosquitoes (*Ozawa* and *Nakata*, 1965; *Ozawa* et al., 1966 b). However, many of properties of AHS virus are shown to be different from those of well-known arboviruses. For instances, AHS virus is resistant to deoxycholate and ether (*Howell*, 1962); it is labile in acid at pH 6.2 or lower (*Alexander*, 1935; *Ozawa* et al., 1965a); it has perhaps 92 capsomeres on a spherical body (*Polson* and *Deeks*, 1963).

Consequently, AHS virus has been classified as a member of a miscellaneous group of arboviruses.

Adaptation of all known 9 types of AHS virus to monkey kidney stable (MS) cell-line cultures (*Ozawa* and *Hazrati*, 1964) made it possible to use these cells for producing AHS tissue culture vaccines now widely used in Africa and the Middle East (*Ozawa* et al., 1965a; *Hazrati* and *Ozawa*, 1965). MS cells have also been used for serologic and virologic investigations of AHS virus (*Mirchamsy* and *Taslimi*, 1964; *Ozawa* et al., 1965b; *Hazrati* and *Ozawa*, 1965; *Ozawa*, 1966).

Susceptibility of green monkey kidney cells to AHS has been briefly reported previously (*Ozawa* and *Hazrati*, 1964). In the present experiment, a new African green monkey kidney cell-line designated as VERO was used* in comparison with MS cell-line which was originally derived from rhesus monkey kidney cells. Using these two cell-lines, effects of metabolic analogs, BUDR and IUDR, and antibiotics, actinomycin D (AD) and mitomycin C (MC), on the yield of AHS virus in these cell cultures were investigated with the object of determining the nucleic acid of the virus.

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* "Growth of AHSV in VERO" was reported at the International Conference on Equine Infectious Diseases, July 11-13, 1966, at Stresa, Italy.

Materials and Methods

Viruses. Type 9 vaccine strain of AHS virus designated as S 2 was isolated from the blood of an infected horse in Iran. The strain was adapted to MS cells after 102 intracerebral passages in mice. At least 5 passages were made in MS cells before using the virus in experiments with MS cells or before adapting it to VERO cells. The virus stocks were stored at 4° C.

Adenovirus type 5, strain adenoid 75, originally isolated by Dr. *R. J.Huebner* was obtained by the courtesy of Dr. *S. Uchida* of the National Institute of Health of Japan. After 5 passages in KB and several passages in HeLa cells, the virus was adapted to MS cells by 3 serial passages. The 4th passage was used in this experiment.

Identity of these viruses was confirmed by neutralization tests with specific antiserums in the manner described previously (*Ozawa* and *Hazrati*, 1964).

Cell lines. The MS and VERO cell lines were obtained from the National Institute of Health of Japan through the courtesy of Dr. *I. Tagaya*. The MS cell line was originally obtained from Dr. *Kanda Inoue* (*Kanda* and *Melnick*, 1959), and serial passages were made at the National Institute of Health in Tokyo. The 224th passage of the MS cell line was brought to Iran, and further passages were made before use.

The African green monkey kidney cell line (VERO) established by Dr. *Y. Yasumura* (*Yasumura* and *Kawakita*, 1963) was maintained at the National Institute of Health of Japan and the 152nd passage was brought to Iran.

Cell cultivation. Both MS and VERO cells were grown in nutrient medium (YLE) with 10% calf serum heated 30 minutes at 56° C. Medium YLE was prepared by adding to Earle's balanced salt solution 0.5% lactalbumin hydrolysate, 0.005% yeast extract, and 0.0015% phenol red. The maintenance medium contained all these constituents except for the reduced concentration (1.0% to 2.0%) of calf serum. All these media contained 100 units of penicillin and 100 μg of streptomycin per ml. The final pH was approximately 7.3.

These cell lines were subcultured approximately every 4 days. Cells dispersed with 0.25% trypsin were suspended in nutrient medium to make the final concentration approximately 7×10^4 cells/ml. Monolayers were usually formed within 3 day's incubation at 37° C.

Infection of cells. Monolayer cultures prepared in 4-ounce medical flat bottles were drained and inoculated with 2 ml. of virus either without inhibitors or with various concentrations of inhibitors. They were incubated at 37° C for specified adsorption periods. After washing 4 times with phosphatebuffered saline without magnesium and calcium ions (PBS-), 10 ml. of fresh maintenance medium with or without the test compounds was added to each bottle, and the cultures were incubated further at 37° C. At intervals specified in the text, 0.2 ml. of the fluid from each of 5 bottles treated in the same manner was harvested and pooled. After overnight storage at 4° C, all samples in small tubes were centrifuged at 1,000 r.p.m. for 5 minutes and the supernatant fluids were taken for titration.

Non-infected cultures of MS and VERO cells were held in maintenance medium with various concentrations of inhibitors for cell counting.

Titration of virus. Tenfold serial dilutions of the viruses were made with the maintenance medium. When monolayers of either MS or VERO cells had

just formed in 16 mm. × 150 mm. tubes, usually 2 to 3 days after seeding, the growth medium was discarded. Four tube cultures were inoculated with 0.1 ml. of each dilution and incubated I hour at 37° C before 1.5 ml. of the maintenance medium was added. The end points were read 10 days after infection of MS cell cultures and 12 days after infection of VERO cells. The infectivity titers were calculated by the method of *Reed* and *Muench* (1938) and expressed as $TCID_{50}$ per 0.1 ml.

Acridine orange staining. Monolayers on coverslips in Leighton tubes were washed twice with PBS-, and then fixed in acetone at room temperature for 10 minutes. They were air-dried, and stored at 4° C. These coverslips were placed for a few minutes in citrate buffer pH 3.6 to 3.8. They were then stained for 5 minutes with a 0.01% solution of acridine orange dissolved in the same citrate buffer. After washing in citrate buffer again, the stained coverslips were mounted in citrate buffer solution on a regular glass slide.

Fluorescent antibody staining. A neurotropic strain S 2 was inoculated intracerebrally into 5-week-old mice. The brains of infected mice were harvested at extremis, and a 10% brain suspension was made in PBS-. The emulsion was centrifuged at 2,000 r.p.m. for 15 minutes and 5 ml. of the supernatant was used as the inoculum for each injection. Hyperimmune horse serum was prepared by 10 consecutive injections of the virus supension. Six months after the first intravenous injection,9 intramuscular injections were given at weekly intervals. Two weeks after the last injection, the γ-globulin fraction was precipitated with cold half-saturated ammonium sulphate, conjugated with fluorescein isothiocyanate by the method of *Riggs* et al. (1958) and then passed through a 15 cm. column of DEAE cellulose (Brown 0.93 meq./g) to remove unconjugated isothiocyanate. After 3 days dialysis against PBS- (pH 7.2), the conjugate was adsorbed twice with acetone-extracted rabbit liver powder and centrifuged at 12,000 r.p.m. for 45 minutes. The supernatant fluid was freeze-dried and kept at —20° C.

The direct fluorescent antibody technique was employed to obtain immunofluorescence of infected cells. Control and infected cell monolayers on coverslips were washed twice with PBS-, fixed 10 minutes with cold acetone, and then washed again with PBS- after the acetone had evaporated. These coverslip cultures were stained overnight at 4° C with the conjugate. They then were rinsed in three changes of PBS- to remove excess conjugate and mounted in 10% glycerol in PBS.

Microscopic examination. All these preparations were examined with a Zeiss fluorescence microscope equipped with an Osram HBO-200 mercury arcvapor lamp, BG 38/2.5 and BG 12/4 transmitting filters, and Nos. 44 and 65 barrier filters.

Biochemical compounds. 5-Bromodeoxyuridine (BUDR) and 5-Iododeoxyuridine (IUDR) were purchased from Nutritional Biochemical Corporation, Ohio. Actinomycin D was originally supplied by the Merck Sharp and Dhome Research Laboratories. Mitomycin C was obtained from Sankyo K. K., Nihonbashi-Honcho, Tokyo.

Results

Single-passage Growth Patterns of AHS Virus

The development of AHS virus in MS and VERO cell cultures was compared by titrating extracellular virus in these cultures infected at high input multiplicity. MS cells were inoculated with the 8th passage of S2 virus at a multiplicity of approximately 18 per cell. VERO cells were infected with 3rd passage of the same virus in these cells at a multiplicity of approximately 30

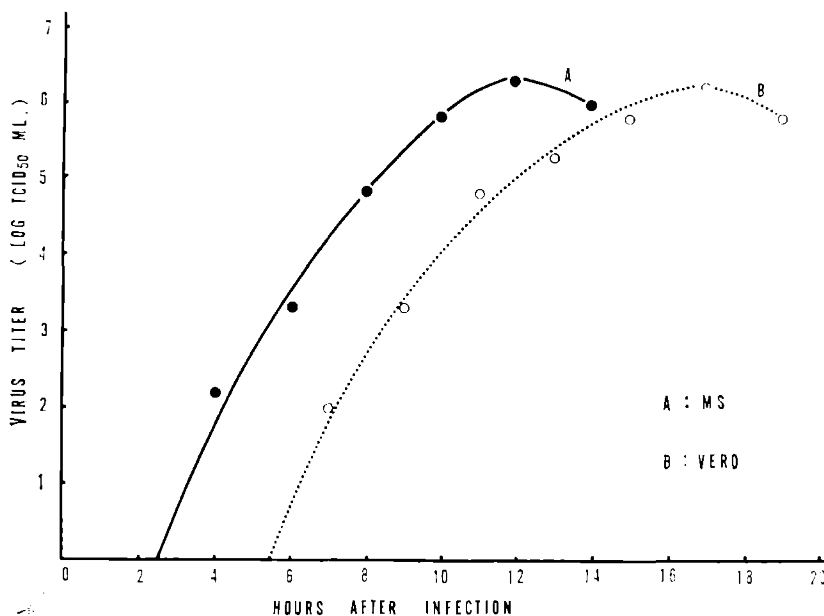

Fig. 1. Development of extracellular AHS virus (S2) in MS and VERO cell cultures infected at a high multiplicity (approximately 18/MS and 30/VERO cell).

per cell. Virus was allowed to be adsorbed for 2.5 hours on MS cells and 5 hours on VERO cells. These cultures were washed 4 times with PBS- before adding 10 ml. of fresh maintenance medium. Titers of virus harvested at various intervals are shown in Fig. 1. The growth curves were more or less parallel to each other and the maximum yield of the virus was almost the same.

Infected MS cells showing typical cytopathic changes, rounding and shrinkage, remain attached to the glass unless the bottle cultures were vigorously shaken. Infected VERO cells were irregular in shape and detached from the glass by two's and three's.

Acridine Orange Staining

When VERO cells were inoculated at a multiplicity of about 30, small

round bodies appeared in the cytoplasm 5 to 7 hours after infection. At first, the borders of the bodies were diffuse and the color was more or less the same as that of the cytoplasm. As the infection progressed, the number and size of the bodies increased. In general, these bodies were more concentrated around the nucleus. Approximately 10 to 14 hours after infection, they became fenestrated and the reddish orange color of the bodies increased in intensity (Fig. 3). The cytoplasm of the cells stained more intensely rust-red, and gradually disintegrated.

Such distinct cytopathic changes were not observed in the cytoplasm of infected MS cels. Although a few minute bodies were occasionally seen in the

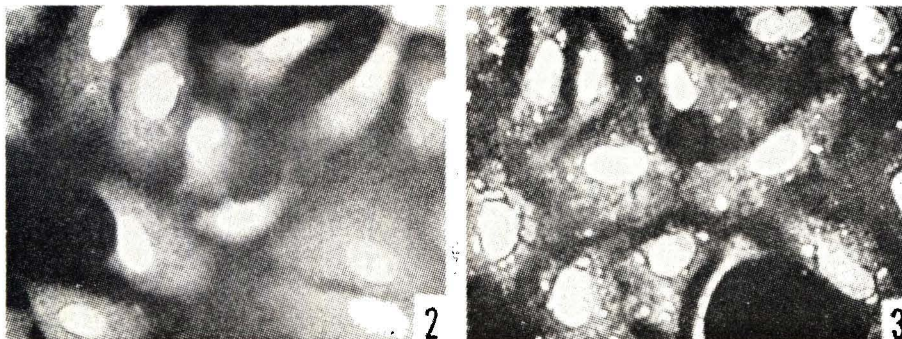

Fig. 2. Acridine orange staining of uninfected VERO cells. × 480.
Fig. 3. Acridine orange staining of VERO cells infected 12 hours previously with AHS (S2) virus at a high input multiplicity. Note cytoplasmic inclusion bodies of various size. × 480.

cytoplasm of MS cells, major changes appeared in the nuclei as described in previous reports (*Ozawa* et al., 1965b; *Ozawa* et al., 1966a).

Changes in the nucleus of infected VERO cells were not as marked as those in MS cells. In the nucleoli of infected MS and VERO cells the intensity of reddish color increased at early stages of infection. The color of the nucleus gradually changed from green to yellowish-green as infection progressed. Disintegration and margination of nucleoplasm were distinct in the infected pycnotic MS cells.

Fluorescent Antibody Staining

Appearance of antigen in the infected VERO cells followed the appearance of cytoplasmic inclusion bodies. However, fluorescent-tagged antibodies did not become fixed in the cytoplasm until the bodies were stained red by acridine orange. The antigenic bodies were usually concentrated around the nuclei of infected VERO cells. As infection progressed, the size and number of antigenic bodies increased (Fig. 5). The intensity of fluorescence of these bodies increased. At the late stage of infection, diffusion of antigen throughout the cytoplasm of infected cells was observed.

In infected MS cells, however, such characteristic changes in the cytoplasm were not observed. The appearance of small shining spots was observed in

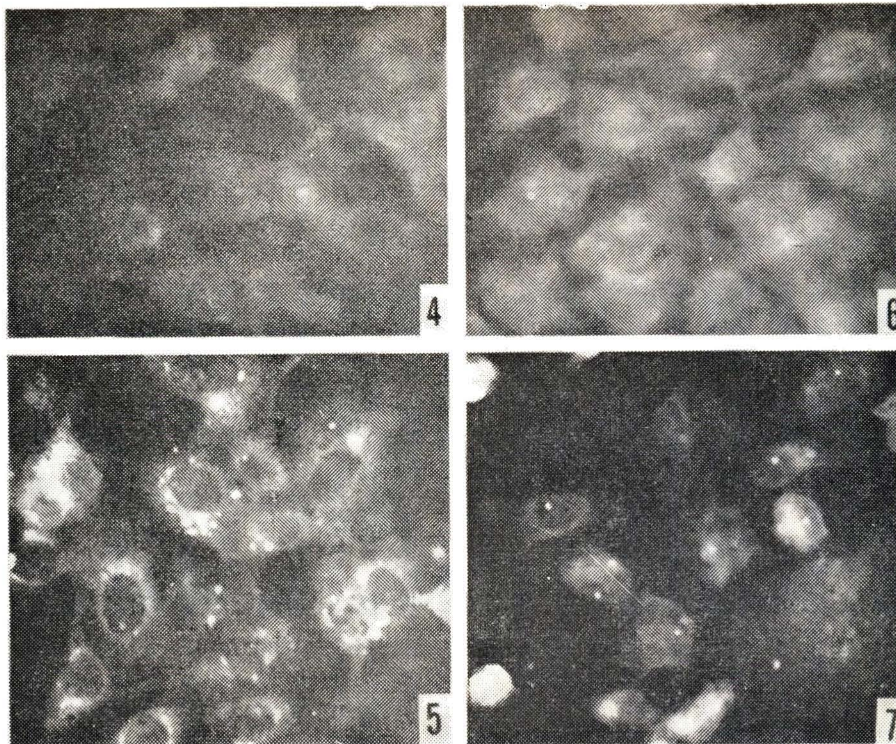

Fig. 4. Immunofluorescence study of uninfected VERO cells. × 480.

Fig. 5. Immunofluorescence study of VERO cells infected 10 hours previously with AHS virus at a high input multiplicity. Note appearance of antigenic bodies of various size around the nucle. × 480.

Fig. 6. Immunofluorescence study of uninfected MS cells. × 480.

Fig. 7. Immunofluorescence study of MS cells infected 8 hours previously with AHS virus at a high input multiplicity. Note one or two shining spots in each cell. × 480.

several preparation (Fig. 7). The shining spot was usually seen on or within the infected nucleus. However, in some cells, the spot was observed in the cytoplasm. In a few cells, the spots were seen both in the nucleus and cytoplasm. As infection progressed, the nuclei became pycnotic and the cytoplasm showing strong fluorescence covered the nuclei (Fig. 7).

Effect of BUDR and IUDR on the Growth of AHS Virus in MS and VERO Cell Cultures

In a preliminary exepriment with S2 virus in MS and VERO cell cultures infected at low input multiplicity it was noticed that maximum titers of the virus in the cultures with 50 μg. of BUDR or IUDR per ml. of medium were not always as high as those of infected cultures without these chemicals (Table 1).

To determine whether these chemicals significantly inhibit virus yield or not, single-passage growth patterns of S2 virus in MS and VERO cell cultures were compared with those of infected cultures containing 100 μg. of BUDR or

Table 1. Effect of BUDR and IUDR on the Yields of AHS (S2) Virus and Type 5 Adeno Virus in MS and VERO Cell Cultures

Virus and cells	Hours after infection	Virus titer (log $TCID_{50}$/0.1 ml.)		
		No drug	BUDR 50 μg.	IUDR 50 μg.
AHS in MS cells	8	2.3	2.1	1.7
	24	5.6	5.3	6.3
	32	5.6	5.6	5.5
	48	6.3	5.0	5.5
AHS in VERO cells	20	4.8	3.5	4.5
	42	5.7	6.7	5.3
	50	6.7	5.7	5.2
	66	5.7	5.5	6.0
Adeno in MS cells	24	4.3	3.0	3.3
	48	4.5	2.2	3.0
	72	4.5	1.2*	2.5**

* BUDR 50 μg. + Thymidine 100 μg. was 3.0.
** IUDR 50 μg. + Thymidine 100 μg. was 4.5.

IUDR per ml. of medium. As shown in Fig. 8, there was no significant decrease in the maximum yield of the virus in the MS cell cultures containing either BUDR or IUDR when infected at a high input multiplicity of approximately 18 $TCID_{50}$/cell. Similarly, there was no evidence that these chemicals inhibit the yields of the virus in VERO cell cultures infected at a low input multiplicity of 0.6 $TCID_{50}$/cell (Fig. 9).

MS cell cultures infected with type 5 adeno virus were used as controls. The maintenance medium with or without these inhibitors was added to each cultuer without washing away the inoculum. The inhibitory effect of these chemicals, which was reversed by adding thymidine, was evident (Table 1). A slight increase in the total number of MS or VERO cells per bottle was recorded when uninoculated cultures with these compounds were counted after 10 hours incubation. The increase, however, was much less than that in control cell cultures.

Effect of Mitomycin on the Yield of AHS Virus

Mitomycin C (MC) was added to MS and VERO cell cultures, infected at low multiplicities of 0.3 and 1.6 respectively, in concentrations ranging from 0 μg. to 9.0 μg. per ml. of maintenance medium. After 50 and 72 hours the cultures were harvested for titrating extracellular virus. As shown in Table 2, there was a slight decrease in virus titers in infected MS cell cultures at concentrations of 3.0 and 9.0 μg./ml. However, it was found that higher concentration

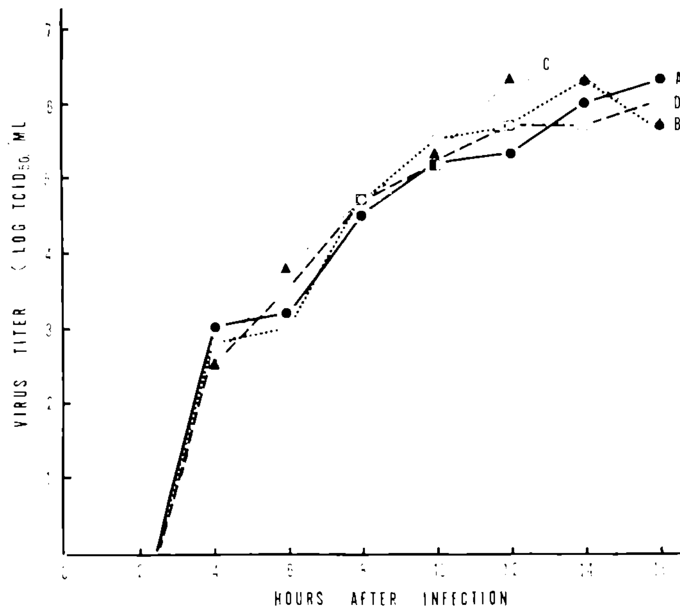

Fig. 8. Effect of BUDR, IUDR, and mitomycin C on the yield of extracellular MHS virus (S2) in MS cell cultures. A: control; B: BUDR 100 µg./ml.; C: IUDR 100 µg./ml.; D: Mitomycin C 6 µg./ml. Number of cells per bottle was 1.35×10^6 before infection; 2.00×10^6 with BUDR, 1.73×10^6 with IUDR, 1.33×10^6 with mitomycin, 2.39×10^6 without inhibitors (10 hours' exposure to these inhibitors at 37°C).

of MC was toxic for MS cells producing cytopathic effects. In VERO cultures, virus yield was not inhibited by addition of MC up to 9.0 μg./ml.

To determine the cause of this slight decrease in virus titers in MS cell cultures with higher concentrations of MC, a growth curve of S2 virus in MS cell cultures, in which 6.0 μg./ml. of MC was added to virus inoculum and maintenance medium, was compared with the growth patterns of the virus in control MS cell cultures. As shown in Fig. 8, there was no significant decrease in the yield of virus due to addition of MC when the cultures were infected at a high input multiplicity of approximately 18 $TCID_0$ /cell, although the toxic effect of MC on MS was evident from the results of cell counting. The number of cells per bottle in non-infected control cultures was 1.35×10^6 at the time of infection, 2.39×10^6 after 10 hours' incubation without MC and 1.33×10^6 after 10 hours incubation with 6 μg. of MC.

The growth of adenovirus in MS cell cultures was inhibited by MC at a 1 μg./ml., the titer decreasing to less than 1% of the control when titrated 72 hours after infection.

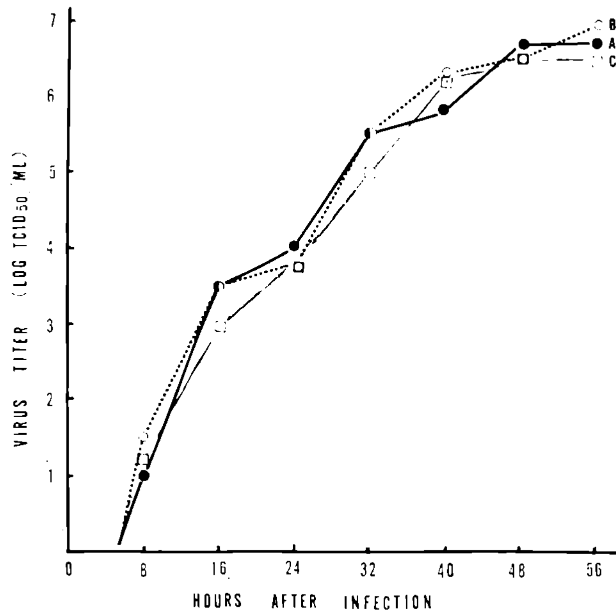

Fig. 9. Effect of BUDR and IUDR (100 μg./ml.) on the yield of extracellular AHS virus in VERO cell cultures infected at a low input multiplicity. A: control; B: BUDR; C: IUDR.

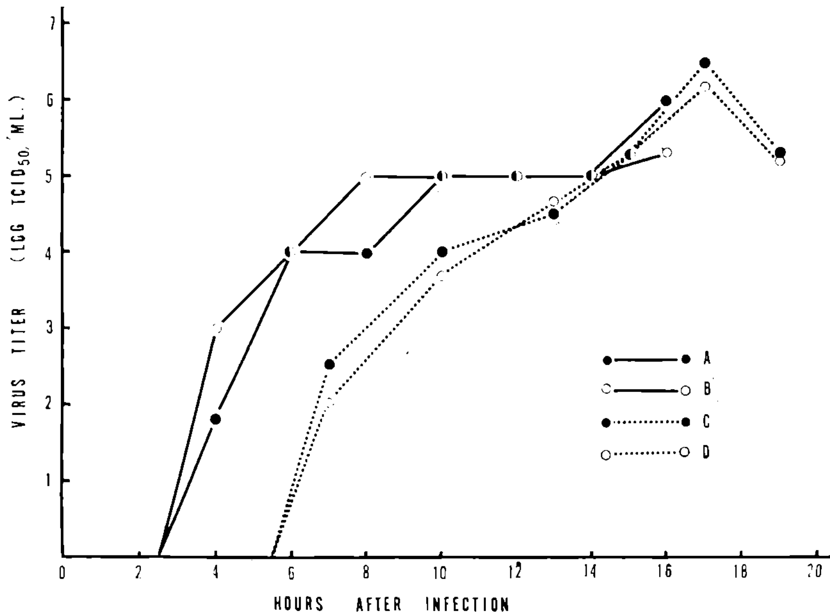

Fig. 10. Effect of actinomycin D on the yield of extracellular AHS (S2) virus in MS and VERO cell cultures infected at a high input multiplicity. A: Virus yield in MS cell cultures without actinomycin; B: MS cell cultures with actinomycin at 1.35 μg./ml.; C: VERO cell cultures without actinomycin; D: VERO cell cultures with actinomycin at 1.8 μg./ml.

Table 2. Effect of Various Concentrations of Mitomycin on the Yield of AHS Virus in MS and VERO Cell Cultures

Concentration of MC µg./ml.	S 2 virus titer (log $TCID_{50}$)	
	MS cells	VERO cells
0	5.3*	4.7**
0.1	4.5	5.3
0.3	5.0	4.7
1.0	4.5	5.5
3.0	3.7	5.0
9.0	3.5	5.2

* Virus was harvested 50 hours after infection.

** Virus was harvested 72 hours after infection.

In a preliminary experiment with actionmycin D (AD) at various concentrations, it was found that AD is toxic to these cell lines especially to MS cells. Most MS cells exposed to AD at concentrations between 0.45 µg. and 1.35 µg./ml. showed cytopathic changes and detached from the surface of the glass within 10 hours incubation at 37° C. Consequently, a marked decrease in virus yield was demonstrated in infected MS cell cultures with AD, when infected at a low input multiplicity of approximately 0.8 per cell. A maximum difference of 2 log in $TCID_{50}$ was recorded when virus was harvested 24 hours p.i. from MS cell cultures with AD at the concentration of 1.35 µg./ml.

To determine the direct effect of AD on the virus yield, growth patterns of the virus in MS and VERO cell cultures infected at high input multiplicity were compared with those of infected MS cell cultures with 1.35 µg. of AD, and of VERO cell cultures with 1.8 µg. of AD per ml. of virus inoculum and medium.

As shown in Fig. 10, the yield of S2 virus was not inhibited by AD at these concentrations. The input multiplicity was approximately 16 per MS cell and 30 per VERO cell. The number of cells per bottle was 1.12×10^6 MS and 7.15×10^5 VERO at the time of infection, 7.75×10^4 MS after 8 hours incubation with AD (control 2.27×10^6), and 1.11×10^6 VERO after 14 hours incubation with AD (control 1.14×60^6).

The growth of adenovirus was almost cmopletely inhibited by adding 0.1 µg./ml. of AD to infected MS cell cultures.

Discussion

MS cells available today are known to have properties in common with human cells, for instance, they have common antigens and the karyotype is similar to that of human cells. Apart from the arguments on the origin of MS cells, it is important to note that MS cells are the cells most susceptible to AHS virus so far reported, yielding virus of high titer and uniformly showing characteristic cytopathic changes.

MS and VERO cells were found to differ in several respects. VERO cells were larger and more resistant to inhibitors used in this study, and the cytopathic changes produced by AHS virus were different. In separate experiments using the plaque technique (*Hopkins* et al., 1966; *Ozawa* et al., in preparation), it was also found that the adsorption of AHS virus to MS and VERO cells increased linearly during the first 3 and 6 hours incubation at 37° C, respectively.

Although plaque assay methods of AHS virus with MS and VERO cells were being developed in our laboratory while this work was in progress, $TCID_{50}$,

was exclusively used in the present studies since titrations in tube cultures were easier and gave as high as or even higher titers than PFU titers.

It was previously found that freezing of AHS virus grown in tissue cultures resulted in a marked decrease in virus titers (*Ozawa* et al., 1965a), but that the virus was stable at 4° C. Since ultrasonic vibration was not done, only extracellular virus was titrated in the present experiment.

As mentioned above, VERO cells are usually larger than MS cells, the number of MS cells per bottle was almost twice as great as that of VERO cells. Consequently, the input multiplicity was usually higher in VERO cultures, but the speed of development of extracellular virus in VERO cell cultures was not greater than that in MS cell cultures. It also takes longer to produce the same size of plaque with AHS virus in VERO cell cultures (*Hopkins* et al., 1966, in preparation). However, the maximum titers of AHS virus in VERO cell cultures were almost the same as those of MS cell cultures or even higher in some cases. This indicates that the yields of extracellular virus per VERO cell is higher than that of an MS cell.

Before initiating the experiments with DNA inhibitors, it was ascertained by a series of preliminary experiments that these compounds have no effect on the adsorption of AHS virus to these cells by using plaque assay methods.

MS cells were shown to be very sensitive to DNA inhibitors especially to MC and AD at the concentrations used in the present experiment. This may be the main reason why in some experiments the yield of extracellular virus in MS cell cultures infected at a low input multiplicity appeared to be affected by the addition of these compounds.

Effects of DNA inhibitors on the production of interferon in these systems have not been investigated, since the presence of interferons has not been confirmed though interference phenomena between the serologically different AHS viruses have been demonstrated in MS cell cultures (*Ozawa*, 1966).

It is an important finding that VERO cells infected with AHS virus produced large cytoplasmic inclusion bodies containing RNA and antigens since this is one of the characteristics of similar animal viruses such as bluetongue virus (*Livingstone* and *Moore*, 1962) and reovirus (*Tournier* and *Plissier*, 1960).

Major cytopathic changes in MS cells infected with AHS virus appeared in the nuclei as described in the previous papers (*Ozawa* et al., 1965b; *Ozawa* et al., 1966a). Appearance of numerous particles in the vicinity of nucleloi of infected MS cells were observed in electron micrographs. In the cytoplasm of infected MS cells, dense bodies, vesicular bodies and numerous vacuoles were observed by electron micropgraphy. Because of these observations in infected MS cells and a slight decrease in virus yield in MS cell cultures containing DNA inhibitors when infected at a low input multiplicty, a possibility that AHS virus may have a DNA-moiety could not be disregarded prior to the present experiments.

Fluorescent-antibody fixing bodies often appeared in the vicinity of the nuclear membrane of infected VERO cells. In a separate experiment in progress, the uptake of H^3-uridine into infected MS and VERO cells was examined autoradiographically. The uridine uptake into the cytoplasm of infected MS or VERO cells in the presence or absence of AD was more or less the same relative to the control.

From these observations it may be presumed that the viral RNA is synthesized in the nuclei of VERO cells and the formation of virus particles takes place in the cytoplasm. However, replication of AHS virus in MS cells seems to take a little different steps as mentioned above.

Since FUDR has been reported to be inactive in some host cell systems especially in monkey cells (*Herrmann*, 1961), BUDR and IUDR were employed to investigate the inhibitory effects on AHS virus multiplication.

Two other DNA inhibitors, mitomycin C and actinomycin D, used in this experiment differ both in their mode of action and the extent of their action. Mitomycin C binds the two DNA strands convalently so that DNA replication and synthesis involving strand separation is inhibited. However, it presumably exerts no action on the template activity of DNA, such as its direction of messenger RNA synthesis (*Lyer* and *Szybalski*, 1963). Actimomycin D has been known to block the function of DNA and this causes a specific inhibition of DNA-dependent RNA synthesis. Therefore, actinomycin inhibits the growth of not only DNA viruses but also some of RNA viruses such as reovirus (*Gomatos* et al., 1962), influenza virus (*Barry* et al., 1962), and Rous sarcoma virus (*Temin*, 1963; *Bader*, 1964).

The fact that the yield of extracellular virus was not inhibited by these four compounds suggests that AHS virus contains an RNA moiety and that synthesis or function of DNA is not required for the replication of the virus.

Thus, the chemical requirements for AHS virus replication in infected MS and VERO cells may be the same, but the mode and site of virus replication may not be the same.

Summary

Single-passage growth patterns of African horse-sickness (AHS) virus in two different cell lines, MS and VERO, of monkey origin were compared by titrating extracellular virus in cultures infected at high multiplicity.

Both by fluorescent-antibody and acridine orange staining techniques, large antigenic bodies containing RNA were found in the cytoplasm of infected VERO cells. This was not so evident in infected MS cells.

Metabolic analogs, bromodeoxyuridine (BUDR) and iododeoxyuridine (IUDR) did not inhibit the yield of AHS virus either in MS or VERO cell cultures indicating that synthesis of new DNA is not required for replication of this virus. The yield of AHS virus was not inhibited by actinomycin D and mitomycin C, suggesting that synthesis or function of DNA is not directly required for the replication of the virus. Ms cells were more sensitive to the toxic effect of these chemicals than VERO cells.

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REFERENCES

Alexander, R. A.: Studies on the neurotropic virus of horsesickness. II. Some physical and chemical properties. Onderstepoort J. vet. Sci. **4**, 323—348 (1935).

Bader, J. P.: The role of deoxyribonucleic acid in the synthesis of Rous sarcoma virus. Virology **22**, 462—468 (1964).

Barry, R. D., D. R. Ives, and *J. G. Cruickshank:* Participation of deoxyribonucleic acid in the multiplication of influenza virus. Nature (Lond.) **194**, 1139—1140 (1962).

Du Toit, R. M.: The transmission of blue tongue and horse-sickness by *Culicoides.* Onderstepoort J. vet. Sci. Anim. Indust. **19**, 7—16 (1944).

Gomatos, P. J., I. Tamm, S. Dales, and *R. M. Franklin:* Reovirus type 3: physical characterization and interaction with L cells. Virology **17**, 441—454 (1962).

Hazrati, A., and *Y. Ozawa:* Serological studies of African horse-sickness virus with emphasis on neutralization test in tissue culture. Canad. J. comp. Med. **29**, 173—178 (1965).

Hazrati, A., and *Y. Ozawa:* Monovalent live-virus horse-sickness vaccine. Bull. Off. int. Epiz. **64**, 59—71 (1965).

Herrmann, E. C., Jr.: Plaque-inhibition test for detection of specific inhibitors of DNA containing viruses. Proc. Soc. exp. Biol. (N. Y.) **107**, 142—145 (1961).

Hopkins, I. G., A. Hazrati, and *Y. Ozawa:* Development of plaque techniques for titration and neutralization tests with African horse-sickness virus. Amer. J. vet. Res. **27**, 96—105 (1966).

Howell, P. G.: The isolation and identification of further antigenic types of African horse-sickness virus. Onderstepoort J. vet. Res. **29**, 139—149 (1962).

Iyer, V. N., and *W. Szybalski:* A molecular mechanism of mitomycin action: linking of complentary DNA strands. Proc. nat. Acad. Sci. (Wash.) **50**, 355—362 (1963).

Kanda, Y., and *L. M. Melnick: In vitro* differentiation of virulent and attenuated polioviruses by their growth characteristics on MS cells. J. exp. Med. **109**, 9—24 (1959).

Livingston, C. W. Jr., and *R.W. Moore:* Cytochemical changes of Bluetongue virus in tissue cultures. Amer. J. vet. Res. **23**, 701—710 (1962).

Mirchamsy, H., and *H. Taslimi:* Visualization of horse sickness virus by the fluorescent antibody technique. Immunology **7**, 213—216 (1964).

Ozawa, Y.: Interference between Afriacn horse-sickness viruses in tissue culture. Amer. J vet. Res. **27**, 106—109 (1966).

Ozawa, Y., and *A. Hazrati:* Growth of African horse-sickness virus in monkey kidney cell cultures. Amer. J. vet. Res. **25**, 501—511 (1964).

Ozawa, Y., A. Hazrati, and *N. Erol:* African horse-sickness live virus tissue culture vaccine. Amer. J. vet. Res. **26**, 154—168 (1965a).

Ozawa, Y., I. G. Hopkins, A. Hazrati, A. Mojitabai, and *P. Kaveh:* Cytology of monkey kidney cells infected with African horse-sickness virus. Nature (Lond.) **26**, 1321—1323 (1965 b).

Ozawa, Y., A. Mojitabai, I. Hopkins, A. Hazrati, and *P. Kaveh:* Sequential cellular changes produced by African horse-sickness virus in monkey kidney cells Amer. J. vet. Res. **27**, 558—565 (1966a).

Ozawa, Y., and *G. Nakata:* Experimental transmission of African horse-sickness by means of mosquitoes. Amer. J. vet. Res. **26**, 744—748 (1965).

Ozawa, Y., G. Nakata, F. Shad-del, and *S. Navai:* Transmission of African horse-sickness

by a species of mosquito, *Aëdes aegypty* Linnaeus, Amer. J. vet. Res. **27**, 695—699 (1966 b).

Polson, A., and *D. Deeks:* Electron microscopy of neurotropic African horse-sickness virus. J. Hyg. (Lond.) **61**, 149—153 (1963).

Reed, L. J., and *H. Muench:* Simple method of estimating 50% end points. Amer. J. Hyg. **27**, 493—497 (1938).

Riggs, J. L., R. J. Seiwald, J. H. Burckhalter, C. M. Downs, and *T. G. Metcalf:* Isothionate compounds as fluorescent labeling agents for immune serum. Amer. J. Path. **34**, 1081—1097 (1958).

Temin, H. M.: The effect of actinomycin D on growth of Rous sarcoma virus *in vitro*. Virology **20**, 577—582 (1963).

Tournier, P., and *M. Plissier:* Electron microscopic studies of cellular lesions produced *in vitro* by ECHO 10 (reovirus). C. R. Acad. Sci. (Paris) **250**, 630—632 (1960).

Yasumura, Y., and *Y. Kawakita:* Studies on SV 40 in tissue cultures. Nihonrinsho Japan) **21**, 1201 (1963).

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