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VISUALIZATION OF HORSE SICKNESS VIRUS BY THE FLUORESCENT ANTIBODY TECHNIQUE

by

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Summary. Horse sickness virus was grown in tissue cultures of monkey kindey cells, and virus was detected by a direct fluorescent antibody technique. Virus was detected at 8 hours in or around the nucleus of cells, and at 24 and 48 hours after infection it was also seen in the cytoplasm.

INTRODUCTION

Horse sickness disease caused heavy losses of solipeds throughout the Middle East when the disease was first introduced by way of the Persian Gulf in 1960. Extensive work has been done in the Razi Institute of Iran in order to supply the Onderstepoort type of vaccine to Iran and neighbouring countries for vaccination of the equine population. Seven antigenically different types of virus adapted to mice were incorporated in a neurotropic polyvalent vaccine (Rafyi, 1961).

HSV was first adapted to hamster kidney cells by the authors (Mirchamsy and Taslimi, 1962, 1963). Characteristic cytologic changes were observed and the occurrence of an eclipse phase of 8 hours followed by an increase of released virus was noticed. The virus was then adapted by the authors to the hamster kidney cell line, BHK 21, received through the courtesy of Professor M. G. P. Stoker, Institute of Virology, University of Glasgow, and to HeLa and FL cells. Microplaques were seen in cultures of BHK 21 cells when the cultures were overlaid with a thin layer of Nobel agar (Mirchamsy and Taslimi, 1963). Recently Ozawa and Hazrati were able to adapt the virus to a monkey kidney cell line, MS, and palques were seen after 2-3 weeks (personal communication).

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* *Immunology*, 1964, 7, 213.

We have applied the direct fluorescent antibody technique to monkey kidney cells infected with HSV and have obtained evidence of the presence of virus at various periods after infection. Evidence is presented that this technique may be used for the visualization of HSV.

MATERIALS AND METHODS

Virus

Strain S28 was isolated in 1960 from blood collected in Khorram-Abad in Iran, from a horse showing symptoms of the acute cardiac form of horse sickness. The virus was passed four times in mice, then eight times in hamster kidney cells and ten times in MS cells. Strain S2 was also isolated in 1960 from a horse in Shiraz-Iran, showing symptoms of horse sickness. After sixty-five intracerebral passages the strain was neurotropic and could be used as a vaccine strain (Hazrati and Taslimi, 1962).

By cross neutralization tests in mice and in MS tissue culture we found that strain S2 was antigenically identical with strain S28. Strain S2 was found by Hazrati and Taslimi (1963) to be antigenically related to a vaccine strain of type 6 of HSV. By applying cross neutralization tests in mice and in MS tissue culture we have recently shown (unpublished data) that S28 and S2 were identical with another local strain, 10/60, also isolated in 1960 in Iran. On the other hand, strain 10/60 was found by Howell (1962) to belong to group 9 of HSV, but he also found some antigenic relation between the prototype strain of group 9, and group 6.

Cells

Line MS of monkey kidney cells was received through the kindness of Dr. Y. Ozawa of this Institute. The cells were kept in continuous passage, being transferred by resuspension with a mixture of trypsin and versene. The cells were grown on cover-slips in leighton tubes at 37° for 3-4 days. CSV 6 medium (Cooper, Wilson and Burt, 1959) was used with 15 per cent calf serum for growth and with 10 per cent calf serum for maintenance. The medium was brought to a final pH of 7.2 before use. One hundred units of penicillin, 100 gamma of streptomycin and 100 units of mycostatin per ml. were added.

Infection of Cells

The cell cultures were washed with phosphate buffered saline pH 7.2 and were inoculated with 0.1 ml. of a 10^{-1} dilution of virus, strain S28, in phosphate buffered saline. The undiluted virus contained $10^{6.5}$ mouse LD 50

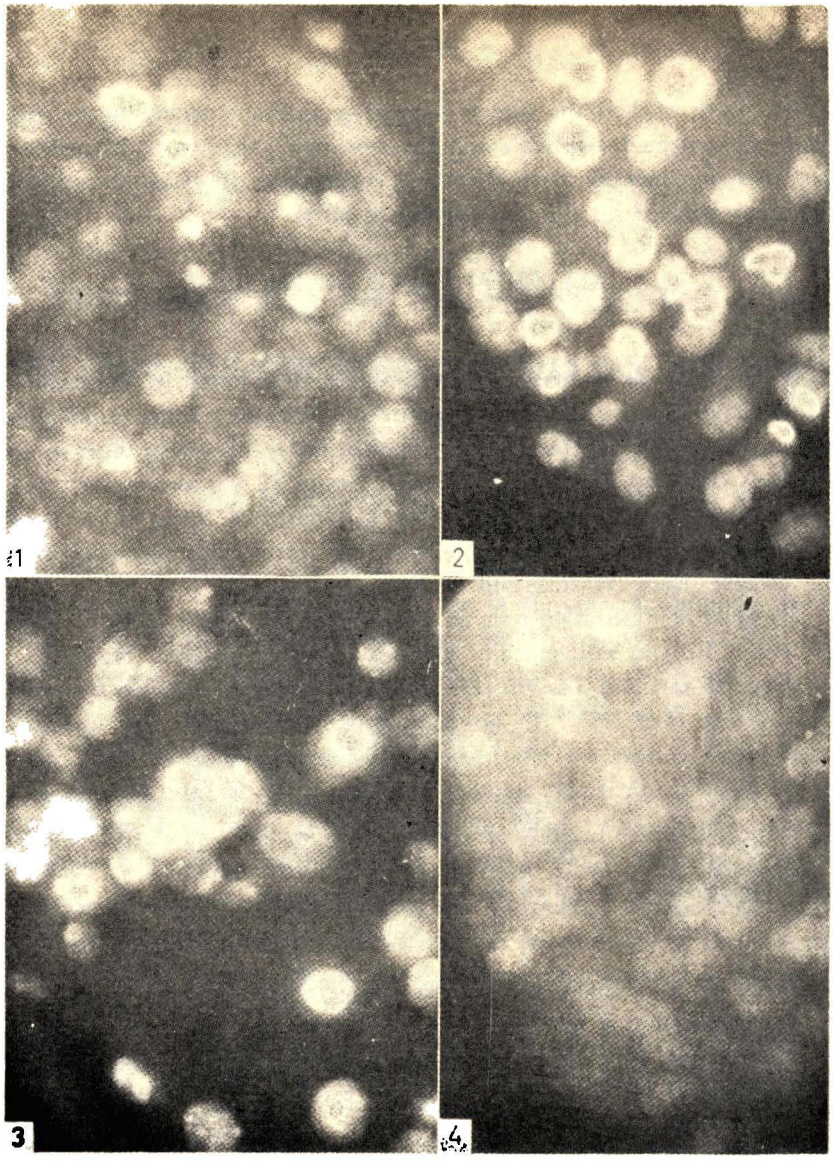

Fig. 1. MS cells, 8 hours after infection. The specific bright green fluorescence is in or closely surrounds the nucleus of a number of cells. At this stage fluorescent material was not seen within the cytoplasm.

Fig. 2. MS cells, 24 hours after infection. Diffuse or granular distribution of bright green material within the cytoplasm and the nucleus.

Fig. 3. MS cells, 48 hours after infection. The large cell in the centre shows a granular distribution of specific fluorescence within the cytoplasm. The nucleus shows less fluorescence.

Fig. 4. Uninfected MS cells. Bright green fluorescence is not apparent in these control cells.

per ml. Absorption time was fixed at 45 minutes after which 0.9 ml. of the maintenance medium was added to each tube. The cultures were incubated at 37°. Cover-slips were removed at various times after infection, fixed 4 hours in acetone at — 20°, dried and kept overnight at 4°.

Immune Serum

Strain S2 virus in a dose of 10^5 mouse LD 50 was injected intracerebrally into 3-weeks-old mice. Brains of mice which showed typical nervous symptoms, and which died under observation 4-7 days after injection, were immediately removed and quickly frozen. A 10 per cent suspension of the pooled brains, containing 10^6 -10^7 mouse LD 50 per 0.03 ml. was used as antigen for immunization. A total of 5 ml. of antigen in five doses over a 20 day period was given intravenously to rabbits. Ten days later the same course was repeated and the animals were totally bled 8 days after the last injection. Immune sera were prepared in sheep and horses by using the same routine but injecting higher doses of virus.

Conjugation of Serum

The immune serum was conjugated with fluorescein isothiocyanate by the method of Marshall, Eveland and Smith (1958). Five ml. of serum were diluted with 20 ml. of 0.125 m sodium chloride and 4 ml. of carbonate-bicarbonate buffer 0.5 M, pH 9.0 After the solution was cooled to 4°, 0.025 mg. fluorescein isothiocyanate was added for each milligram of protein. The mixture was stirred overnight with a magnetic stirrer at 4°. The conj gated serum was then dialysed through cellulose casing and against 0.01 M phosphate buffer solution, pH 7.0, until the dialysate was free of fluorescence under ultraviolet light. To eliminate the slight precipitate the dialysate was centrifuged at 4° for 20 minutes at 8000 rev./min. and then stored until used for staining.

Absorption of Serum

To prevent the non-specific uptake of fluorescein dye, the conjugated serum was absorbed with a suspension of MS cells. To 1 ml. of undiluted conjugate, 1 ml. of a suspension of cells having 10^6 cells per ml. was added. The mixture was shaken for 1 hour at 25°. The cells were then removed by centrifugation for 15 minutes at 2500 rev./min.

Staining Procedure

For staining, the cover-slips were flooded with conjugated serum, diluted 1 :10 with phosphate-buffered saline, and left for 25 minutes at room tem-

perature. They were then washed in four changes of phosphate-buffered sasline and mounted in buffered glycerol. A Zeiss fluorescence microscope, with an Osram HBO 200 W and a BG12 exciter and UG 5 (47/50) barrier filter was used.

RESULTS

Rabbit fluorescent antibody was specifically attached to horse sickness virus present in infected tissue culture of monkey kidney cells. A bright green fluorescence was observed in or closely surrounding nucleus of infected cells 8 hours after infection. This reaction was not seen at 2 or 5 hours after infection nor in uninfected control cells. The bright green material was seen more diffusely within the cytoplasm and within the nucleus 24 hours after infection; 48 hours after infection when cytopathic changes were almost complete and the cells were all rounded in unstained preparations, granular distribution of specific fluorescence was evident within the cytoplasm. At this stage the nucleus showed less fluorescence.

DISCUSSION

One of the major problems in the study of all antigen-antibody systems in tissue culture with fluorescent techniques is the non-specific uptake of fluorescein dye by cells.

Following Coons and Kaplan (1950) we treated the undiluted conjugated serum twice with acetone-dried mouse liver powder. This method was not effective when we used hamster kidney cells from either a primary or a continuous line culture. It was however of some value when MS cells were used. The treatment of conjugated serum with a suspension of a continuous culture line of monkey kidney cells proved to be effective when this cell line was also used in the fluorescence experiments.

In various experiments we used horse, sheep and rabbit immune sera. The index of neutralization of each of these sera was high, but in regard to specific fluorescence, rabbit serum was the serum of choice. With 1 :80 dilution of conjugated rabbit serum we got a bright green fluorescence without any disturbing background, whereas with the same dilution of sheep or horse serum no fluorescence was detectable. When 1 :10 or 1 :20 dilutions of horse or sheep immune sera were used the non-specific fluorescence obscured the specific fluorescence. The results described and illustrated in the present

paper (Figs. 1-4) were all obtained with rabbit serum.

The appearance of specific fluorescence 8 hours after infection confirmed our previous finding that there was an eclipse phase of 8 hours when the HSV was grown in primary cultures of hamster kidney cells. The appearance of specific fluorescence on the nucleus coincides with the elaboration of new virus, and the absence of fluorescence at certain times during the first 8 hours after infection suggested once more the existence of an eclipse phase of not more than 8 hours.

After 48 hours there was an almost complete cytopathis effect on the MS cells and a maximum yield of new virus; the fluorescent technique showed a large number of cells with diffuse or granular fluorescent material within the cytoplasm. At this stage nuclei seemed to be less fluorescent. These observations suggested that the virus of horse sickness was synthesized in the nucleus and that as new virus was released it passed through the cytoplasm.

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