VISUALIZATION OF EGYPT 101 VIRUS IN THE MOUSE'S BRAIN AND IN CULTURED HUMAN CARCINOMA CELLS BY MEANS OF FLUORESCENT ANTIBODY*,‡

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Plates 38 and 39

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The antigens of a number of viruses have been visualized in the infected cells by the use of Coons's fluorescent antibody technique. Included are the antigens of the viruses of mumps (1), infectious canine hepatitis (2), influenza (3), varicella and herpes zoster (4), vaccinia (5), canine distemper (6), and primary atypical pneumonia (7).

This paper reports the first successful direct staining of the antigens of a neurotropic human virus—that of Egypt virus. (Poliomyelitis virus has been detected by means of the indirect staining method (8).) Egypt virus was isolated in 1950 by Melnick *et al.* from normal appearing children near Cairo, Egypt, and is closely related if not identical to the virus of West Nile fever and in lesser degree to Japanese B and St. Louis encephalitis viruses (9). This agent which is destructive to certain malignant cells has been used clinically in the treatment of advanced cancer (10).

Materials and Methods

Virus.—The 101 strain of Egypt virus which had been passed repeatedly in mice by the intracerebral route was used in these experiments. This virus was originally obtained from Dr. Joseph Melnick in the form of infected mouse brain.

Preparation of Antiserum.—Four week old Swiss-white mice infected by the cerebral route were killed in the terminal stages of the illness, 4 days after infection, by cervical dislocation. Ten infected brains were aseptically removed and ground in a mortar with the gradual addition of 25 ml. of 0.85 per cent NaCl solution. 5 pound male rabbits were then injected intraperitoneally with 10 ml. of a 1:2 dilution of this preparation. 4 weeks following

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the first injection the rabbits were given a second intraperitoneal injection of 5 ml. of the brain preparation which had been kept in sealed ampoules in a dry-ice cabinet. 2½ weeks after this second injection the rabbits were bled by cardiac puncture. The serum when tested by an intracerebral neutralization test in mice gave a protective level of approximately three logarithmic units.

Preparation of the Conjugate.—The pooled rabbit antiserum was concentrated by precipitation with half-saturated ammonium sulfate in the cold, washed with half-saturated ammonium sulfate, and dialyzed to remove ammonium sulfate and then conjugated with fluorescein isocyanate by the method of Coons and Kaplan (11). After dialysis in the cold to remove non-bound fluorescein, the conjugate was absorbed twice with 100 mg. of acetone-precipitated mouse liver powder per ml. of conjugate. This procedure removed the material responsible for non-specific staining (11).

Sectioning of the Mouse Brain.—The brains of mice moribund with infection were removed, frozen rapidly in tubes in a dry ice–alcohol mixture, and stored at −20°C. Sections of the frozen tissues were cut at 4 μ in a cryostat refrigerated at −20°C. by a modification of the method of Linderstrom-Lang and Mogensen as cited in reference 12. The sections, after drying at room temperature, were fixed in acetone for 15 minutes and dried in air for 15 minutes before staining.

Tissue Cultures.—The cell strain used in these experiments originated from a human epidermoid carcinoma primary in the larynx. It was carried for two generations in x-irradiated rats (13) and then serially in tissue culture since September, 1952 (14). It has been designated Human Epidermoid No. 2 (13). The cells are epithelial in character and grow rapidly in sheets on a glass surface. They have been used by the present author in a study of the multiplication of vaccine virus by means of the fluorescent antibody technique (5). Before infection with the Egypt virus, the cells, which had been grown on coverslips, were washed twice with Hanks's balanced salt solution to free them from the medium required for growth. This medium was composed of 50 per cent human serum and 50 per cent Hanks's balanced salt solution. After infection had progressed for a suitable period the cells were dried in air for 30 minutes, fixed in acetone for 15 minutes, and then dried again for 15 minutes in preparation for staining with fluorescent antibody.

Staining.—The brain sections or tissue culture coverslips were flooded with fluorescein-labelled antiserum and then covered with a Petri dish containing moist cotton to prevent evaporation. After staining for 45 minutes the preparations were washed for 10 minutes in cold buffered saline (0.85 per cent NaCl containing 0.01 M phosphate, pH 7.0) and mounted in glycerol buffered at pH 7.0. The preparations were examined under a fluorescence microscope according to the most recent modifications of Coons (2).

Controls.—Uninfected mouse brain sections and uninfected cultured human epidermoid carcinoma cells did not exhibit any staining when stained with the absorbed fluorescent antibody preparation and examined in the fluorescence microscope. Pretreatment of infected brain sections or infected cultured cells with rabbit antiserum against Egypt virus inhibited staining of the infected cells by fluorescent antiserum, whereas pretreatment with normal rabbit serum did not inhibit the staining.

RESULTS

Mouse Brain and Cord Sections.—Frozen sections of mouse brain, when stained with hematoxylin and eosin, exhibited poor cytologic detail. Therefore, a paraffin section of infected mouse brain stained with hematoxylin and eosin and photographed in a bright field is included as Fig. 1 for comparison with fluorescence photomicrographs. Sagittal sections and sections through

the midbrain, as well as cerebellar sections, when stained with fluoroescent antiserum, exhibited intense specific staining in every neuron both in the motor and sensory areas. The staining was strictly limited to the neurons and was not seen in the white matter. Sections of the spinal cord at both cervical and lumbar levels demonstrated intense staining of all the nerve cells in the substantia grisea including both the posterior and anterior columns. Figs. 2 to 4, which are increasing magnifications of comparable fields, illustrate the staining observed in the infected mouse brain sections. It can be seen that the specific antigenic material of the Egypt agent is confined exclusively to the cytoplasm of the neurons; also, that the staining tends to be irregular or stippled. This is well illustrated by the elongated cell near the center of Fig. 4.

Cultured Human Cells.—Fig. 5 shows uninfected human epidermoid carcinoma cells grown on small glass coverslips, fixed in acetone, stained with hematoxylin and eosin and photographed in a bright field for comparison with the darkfield photographs. In numerous experiments staining was not observed in the cultured human carcinoma cells inoculated with mouse brain-adapted stock of Egypt virus; however, upon repeated passage in this cell strain the virus acquired the ability to multiply and destroy the cells (15). The thirty-ninth passage virus[1] which was cytopathogenic for these cells was tested with the following results:—

Antigenic material specific to the Egypt virus was detected in these cells 24 hours after infection. The amount of antigen in the cell at this time was small and was strictly limited to the cytoplasm. At this stage of infection the antigen usually was localized about the nucleus as can be seen in Fig. 6 and in the cells at the upper right of Fig. 7. This figure also shows a cell with an unusually large amount of cytoplasm containing homogeneous antigen. Fig. 8 illustrates a more advanced infection and demonstrates the presence of antigen in the junctions of some of the cells. In the moderately advanced stages of infection the staining frequently had a sponge-like appearance as if the cell had been riddled with small holes, as is illustrated by the extremely large cell possessing two nuclei shown in Fig. 9. These dark areas that appear to be small holes in the cytoplasm were found to be lipid inclusions, since they were readily stained with Sudan black B. Cells in late stages of infection are shown in Fig. 10 which was taken 48 hours after infection at which time the cells contained the maximal amount of antigen. The entire cytoplasm of these cells is filled with homogeneous antigen, and it can also be seen in the cytoplasmic connections between some of the cells. No nuclear staining of any type has been observed in the cells infected with the Egypt agent.

The number of cells initially infected and containing antigens of the Egypt virus depended on the amount of virus that was added to the cultures. The area of infection remained well localized even after the agent was released from initially infected cells and the focal areas could be counted as "plaques." Counts were made at 48 hours after infection of the average number of "plaques" per low power field. In cultures that had been infected with a 10^{-3} dilution of the virus the number of stained focal areas averaged 3 per field with a magnification of 80, and cultures infected with a 10^{-4} dilution of the vi-

[1] Obtained through the courtesy of Dr. A. Moore.

rus averaged 0.5 per field with a magnification of 80. No infected cells were observed in cultures infected with a higher dilution of virus. Intracerebral mouse titrations gave an end point of 10^{-4}. These figures were obtained with a substrain of the human carcinoma cells which supported the multiplication of the virus to only a limited extent as compared with another substrain of these cells from a different "mother" flask which was used in all the other experiments reported above. The highly susceptible substrain was unfortunately lost owing to mold contamination, and no figures were obtained of the number of infected areas found after infection with different amounts of the viral agent. However, stained cells were regularly observed in cultures infected with a 10^{-5} dilution of virus.

DISCUSSION

The fact that all the neurons in both sensory and motor areas of the mouse's brain, as well as the cord, contained viral antigen is not surprising; for the mice from which the specimens were obtained were in a moribund condition just prior to death, and exhibited a flaccid paralysis with little evidence of life except for very shallow, slow breathing. The antigen of the Egypt agent was limited to the gray matter and to the cytoplasm of the neurons, and did not occur in the axons of the neurons.

In the human epidermoid carcinoma cells in culture it was noted that the antigen tended to remain localized about the nucleus in the early stages of infection. This has also been noted in vaccine virus infection of these cells, using the fluorescent antibody technique (5). However, in the case of vaccine virus the staining was particulate, whereas in the present case it was always homogeneous except for a stippled effect seen in some neurons. This difference in the distribution of the staining could be accounted for by the much smaller size of the viral particles, since the Egypt agent is probably identical with the West Nile virus (9), which has a diameter of 20 to 30 mμ.

The cells that contained antigenic material remained well localized around the original infected cell even after 48 hours. It would appear that a main mechanism of spread of this agent from cell to cell may be *via* their protoplasmic connections (see Fig. 10). Comparative titrations of the Egypt virus in cultures by means of fluorescent antiserum and by the intracerebral mouse technique show the former to be as much as ten times as sensitive, depending on the substrain of cells used.

SUMMARY

The antigens of the Egypt virus have been detected by means of the fluorescent antibody technique in the neurons of the brain and spinal cord, in both sensory and motor areas, of infected mice. The antigens have also been observed in infected human epidermoid carcinoma cells in culture. In both cases the

antigens occurred in the cytoplasm of the cells exclusively. In the early stages of infection the antigen was localized about the nucleus of the human cultured cells. In some cases the staining of the antigens produced a sponge-like effect, probably because of the numerous vacuoles of lipid material. The antigens were observed in the protoplasmic connections between cells and they probably served as a main route of spread of the agent in these human cell cultures. The number of cells initially infected and showing antigen was proportional to the amount of virus added, and titration with one substrain of cells proved much more sensitive than intracerebral titration in the mouse.

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EXPLANATION OF PLATES

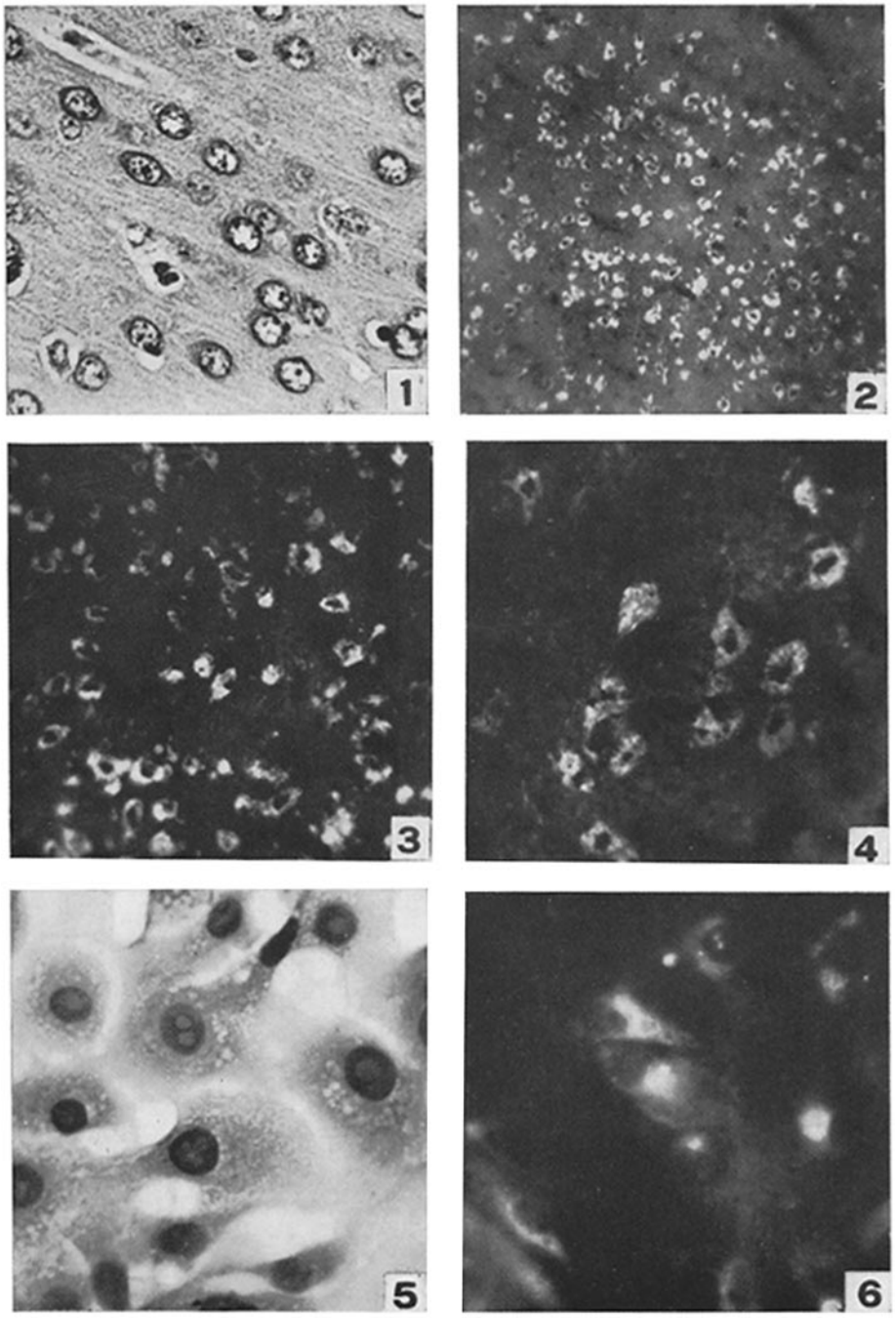
PLATE 38

FIG. 1. Section of cerebral cortex of infected mouse paraffin imbedded and stained with hematoxylin and eosin. × 400.

FIGS. 2, 3, and 4. Frozen sections of cerebral cortex of infected mouse stained with fluorescent antibody and photographed under the fluorescence microscope. Fig. 2 is a low power (× 140) view of numerous cells containing antigen. Fig. 3 is a higher power (× 280) of same field. Fig. 4 is a 540 magnification of a comparable field showing several elongated and one stellate cell (upper left). The staining gives a mottled effect to the central cell especially. The antigen lies wholly in the cytoplasm.

FIG. 5. Uninfected human epidermoid carcinoma cells in culture on glass, fixed in acetone, stained with hematoxylin and eosin and photographed in a bright field for comparison with the following fluorescent photomicrographs. × 390.

FIG. 6. Human epidermoid carcinoma cells infected 24 hours previously with Egypt virus and stained with fluorescent antibody. Cells contain a small amount of specific antigen in their cytoplasm, located close to the nucleus or partially surrounding it. × 540.



(Noyes: Visualization of Egypt virus)

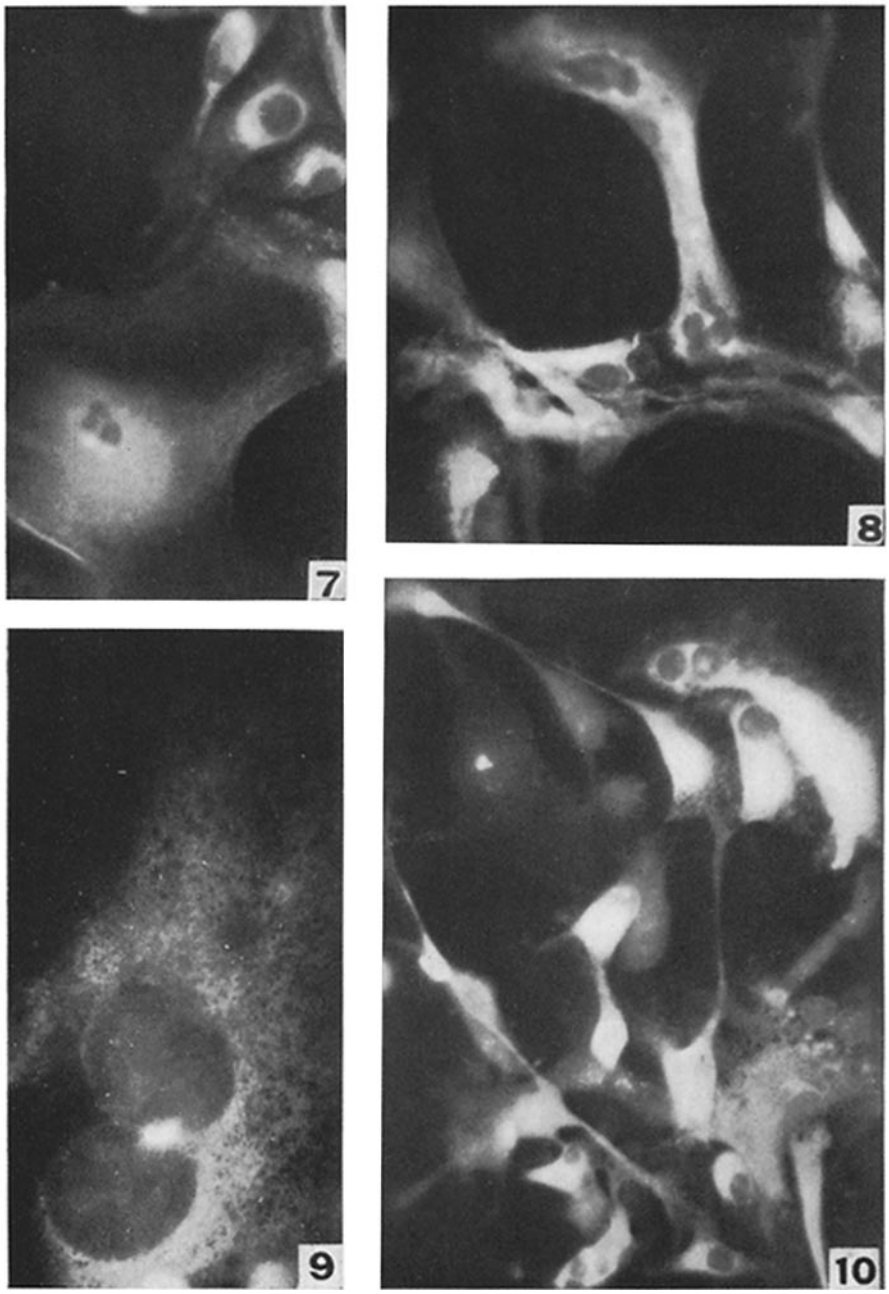
PLATE 39

FIG. 7. Slightly more advanced infection. The cells in the upper right have considerable quantities of antigen near the nucleus. The large cell at the bottom has cytoplasm containing almost homogeneous antigen in an amount that increases toward the nucleus. × 540.

FIG. 8. Antigen exists in quantity where the vertically placed cell is joined to the transverse one. This latter has several nuclei and antigen in concentrated form can be seen outlining them. × 540.

FIG. 9. An extremely large cell with two nuclei. The staining of the antigen in the cytoplasm gives it a sponge-like appearance owing to many scattered vacuoles. These can be stained with Sudan black. × 540.

FIG. 10. Cells in late stages of infection showing cytoplasm containing so much antigen that it appears homogeneous. It also exists in the thin cytoplasmic processes connecting the cells. × 280.



(Noyes: Visualization of Egypt virus)