STUDIES ON THE INCREASE OF VACCINE VIRUS IN CULTURED HUMAN CELLS BY MEANS OF THE FLUORESCENT ANTIBODY TECHNIQUE*, ‡

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PLATE 37

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A number of different techniques have been used to study the development of vaccine virus infection. Himmelweit (1) examined the infected living chorioallantoic membrane of the chick embryo *in situ* with the oil immersion lens, using annular oblique, incident illumination. He described the increase in the number of bodies and the size of the infected area in the cell as a "shoal of fish in an ever extending pond." Merling (2) using the darkfield microscope described a similar type of increase for the virus in rabbit corneal cells. Bland and Robinow (3) cultivated rabbit corneal epithelial cells *in vitro*, and in the infected, Giemsa-stained cells demonstrated cytoplasmic bodies which increased in size with time. And, recently Gaylord *et al.* (4), using the electron microscope, have shown various stages in the development of the virus in cells of the chorioallantoic membrane of the chick embryo.

An excellent system was available to the present authors for the study of vaccine virus development in human cells. A fluorescent antibody system had been worked out for this viral agent (5) and it was well established that this technique was highly specific and gave good cytologic resolution (6–8). Furthermore, the strain of cells at hand were of an unusually large size with an extensive cytoplasmic area. This paper reports the findings obtained by the use of this system.

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Materials and Methods

Virus.—In the following experiments a dermotropic strain of vaccine virus derived from calf lymph was used which had had eight to ten passages on the chorioallantoic membrane of the chick embryo.

Cells.—The cells were originally isolated in tissue culture in 1952 by Fjelde (9) in roller tube plasma-clot cultures of a human epidermoid carcinoma primary in the larynx, which had been previously passed twice in x-irradiated rats by Toolan (10). The roller tube cultures were adapted to massive cultivation on a glass surface by one of the present authors. Cells of the strain grow rapidly in sheets directly on glass and are similar in appearance to Gey's HeLa strain (11). Vaccine virus multiplies very readily in them, and completely destroys the culture in 48 hours.

Cultivation of the Cells.—The feeding mixture was composed of 40 to 50 per cent human serum and 50 per cent Hanks's balanced salt solution adjusted to pH 7.6 with 2.8 per cent sodium bicarbonate, plus 100 units of penicillin and 100 μg. of streptomycin per ml. The cells were grown directly on glass in 250 ml. serum dilution bottles, starting with approximately 100,000 cells per ml. and a volume of 6 ml. per bottle. The fluid was changed at 3 day intervals and the culture transferred at approximately 10 day intervals. This was done by washing the growth from the side of the bottle with fresh medium and dispersing the clumps of cells by pipetting. Half the volume was used to start another culture bottle or to set up slide cultures for infection. The remaining cells were retained in the bottle as a "mother" culture.

Infection of the Cells.—The cells, dispersed as above, were allowed to settle onto small coverslips (10 × 10 mm.) in tubes having one flattened side. After the cells had multiplied to form sheets in 24 to 48 hours, the growth medium was removed and the cells washed twice with balanced salt solution. After 2 to 3 hours' incubation at 35°C., to allow attachment of the virus, 0.1 ml. of chick embryo extract was added per tube to furnish nutrient materials to the cells; otherwise the controls did not retain their normal structure. The tubes were then returned to the incubator for a suitable period.

Fixation.—The coverslips were removed and allowed to dry in the air at room temperature for ½ hour. Fixation was then carried out in acetone at room temperature for 15 minutes. The coverslips were then air-dried for 15 minutes before staining with fluorescent antibody.

Antiserum.—Hyperimmune sera were prepared in rabbits by repeated vaccination in the skin, followed by one to three intravenous injections of infective elementary body suspensions (12) over a period of 1 to 2 months. The virus used was derived from calf lymph and carried in rabbits through four to seven passages according to the method of Craigie and Wishart (12).

Florescein-Labelled Antiserum.—The antiserum was concentrated by precipitation with half saturated ammonium sulfate and conjugated with fluorescein isocyanate by the method of Coons and Kaplan (13). 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. to remove non-specific staining (13).

Staining.—After the cells had been fixed in acetone and dried, the coverslips were flooded with the fluorescein-labelled antiserum for 45 minutes. During this time the coverslips were covered with a Petri dish containing moist cotton to prevent evaporation. After staining, the slides were washed in two changes of buffered saline (0.8 per cent NaCl containing 0.01 M phosphate, pH 7.0) for a total of 10 minutes and mounted in buffered (pH 7.0) glycerol.

Controls.—Uninfected cells were stained and examined and in no instance showed staining. The specificity of the staining was demonstrated by the fact that pretreatment of the cells with homologous immune serum inhibited staining; normal rabbit serum did not inhibit the staining.

Fluorescence Microscopy and Photography.—The arc lamp used was of 13 amperes D.C. capacity and the $CuSO_4$ cell carried a Corning 5840 filter of ½ standard thickness. The micro-

scope was equipped with a standard darkfield condenser and a Wratten No. 2B (Eastman) filter in the ocular (7). The photomicrographs were taken with a 35 mm. film holder (visicam) on super XX film (Eastman).

RESULTS

Uninfected cells stained with hematoxylin and eosin and photographed in a bright field are shown in Fig. 1 for comparison with the darkfield photographs. The number of cells that stained with the fluorescent antiserum in the inoculated cultures was proportional to the amount of virus added, and the staining was usually confined to the cytoplasm of the cells and was mostly of a very granular nature. At an early period following infection (9 hours) the amount of antigenic material in the cytoplasm was very small and occurred as a few small granules in close contact with the nuclear membrane. This is illustrated by the cell in Fig. 2. In the cultures examined at 16 to 24 hours after infection, cells were observed in which progressively increasing amounts of particulate antigen were present in the cytoplasm and in close contact with the nuclear membrane, as illustrated in Figs. 3 to 5. In some instances the antigen was present in a sharply delimited area close to the nucleus, as in Figs. 6 to 9; furthermore, in some cells several areas of this type were seen. This observation is illustrated in Fig. 10. The cell in this figure appears to have three nuclei and there are four areas containing antigen about the upper nuclei. The size of the particles of antigen varied considerably as can be seen by comparing Figs. 11 and 12. The former shows a cell containing very large granules of antigen and the latter shows a dividing cell having numerous very fine particles of it. As the concentration of the antigen increased in the cytoplasm the staining appeared less particulate and finally became almost homogeneous (Figs. 13 to 15). Fig. 15 represents a dividing cell containing a high concentration of antigen. Nuclear staining was observed frequently in cells having large amounts of antigen and this can be seen in Fig. 15.

Cells which contained large amounts of antigen were observed to have cytoplasmic projections which contained antigen, mostly at their extremities and Fig. 16 shows a cell with these protuberances. These were not seen in the control preparations. In some instances protoplasmic connections between an infected cell containing antigen and a normal cell were observed; and cases were seen in which the antigen appeared to have extended into the second cell, as in Fig. 17. In this figure the antigen appears to have extended into the upper cell. Free antigenic debris was seen frequently in cultures infected for 24 hours. This observation is illustrated by the left section of Fig. 18.

DISCUSSION

The exclusive cytoplasmic occurrence of the particles of vaccine virus that has been observed in this study has been reported by numerous investigators

(16). In most instances the particulate cytoplasmic staining of the present work was due to groups or clumps of viral particles; but, in some cells individual elementary bodies were probably visible (see Fig. 12). The early perinuclear location of these particles has been reported previously by Goodpasture *et al.* (14) and recently by Gaylord *et al.* (4) who made use of the electron microscope. The cells which contained only a few particles of antigen in their cytoplasm apparently were supporting early stages of development of the virus, since they were found at the earliest postinfection periods. The high concentrations of antigen found at later periods following infection evidently resulted from the increase of the few original particles. The cells which were observed in late stages of division containing large amounts of viral antigen (Figs. 12 and 15) could indicate that the infection did not interfere with mitotic division; they could also indicate that division was halted at the point illustrated by the figures, while the infection progressed.

The change in the character of the staining from particulate to homogeneous in the late stages of development of the agent was due either to a very solid packing of the particles or to the presence of soluble antigen. Both occurrences may have been responsible for this type of staining. The presumptive soluble antigen may have diffused through the nuclear membrane and resulted in the specific staining that was observed in the nucleus; for this nuclear staining can scarcely have been caused by viral particles since it was always homogeneous in nature. A similar phenomenon has been observed with influenza virus by Liu (15).

The antigens were observed in situations which suggest that there are three mechanisms of spread of the virus to uninfected cells: (*a*) the cytoplasmic projections seen in the late stages of development could function to release virus, since they contained antigen in their extremities. This type of cytoplasmic projection of the cell in vaccine virus infection has been observed in electron microscope studies by Bang in chorioallantoic membrane cells (17), and in Giemsa-stained tissue culture preparations of rabbit corneal cells by Robinow (18). Both workers reported viral material in the projections. (*b*) the virus may be transmitted to new cells *via* cytoplasmic connections. (*c*) the infection may terminate in a disruption of the cell (since vaccine virus is cytopathogenic for these cells) and in a release of the agent, which is then free to infect new cells.

SUMMARY

The specific detection of the antigens of vaccine virus in cultured human cells by means of fluorescent antibody is reported. The antigenic material occurred in the cytoplasm in progressively increasing amounts and in the early stages was in close contact with the nuclear membrane. The antigens were present in a finely particulate form, either as foci or loosely spread out, up to

late stages of infection, at which time the staining assumed a homogeneous form due either to close packing of the particles or to soluble antigen, or both. In late stages, homogeneous collections of antigen were observed in the nucleus. They may have resulted from diffusion of soluble antigen through the nuclear membrane. The antigens were observed in situations which suggest a new method of spread from one to another of these cells, namely by transmission through cytoplasmic connections.

BIBLIOGRAPHY

1. Himmelweit, F., *Brit. J. Exp. Path.*, 1938, **19**, 108.
2. Merling, K. B. E., *J. Path. and Bact.*, 1940, **50**, 279.
3. Bland, J. O. W., and Robinow, C. P., *J. Path. and Bact.*, 1939, **48**, 381.
4. Gaylord, W. H., Melnick, J. L., and Bunting, H., *Proc. Soc. Exp. Biol. and Med.*, 1953, **80**, 24.
5. Watson, B. K., data to be published.
6. Kaplan, M. H., Coons, A. H., and Deane, H. W., *J. Exp. Med.*, 1950, **91**, 15.
7. Coons, A. H., Snyder, J. C., Cheever, F. S., and Murray, E. S., *J. Exp. Med.*, 1950, **91**, 31.
8. Coffin, D. L., Coons, A. H., and Cabasso, V. J., *J. Exp. Med.*, 1953, **98**, 13.
9. Fjelde, A., unpublished experiments.
10. Toolan, H. W., *Cancer Research*, 1954, **14**, 660.
11. Sherer, W. F., Syverton, J. T., and Gey, G. O., *J. Exp. Med.*, 1953, **97**, 695.
12. Craigie, J., and Wishart, F. O., *Brit. J. Exp. Path.*, 1934, **15**, 390.
13. Coons, A. H., and Kaplan, M. H., *J. Exp. Med.*, 1950, **91**, 1.
14. Goodpasture, E. W., Woodruff, A. M., and Buddinogh, G. V., *Am. J. Path.*, 1932, **8**, 271.
15. Liu, C., *J. Exp. Med.*, 1955, **101**, 677.
16. Van Rooyen, C. F., and Rhodes, A. J., Virus Diseases of Man, New York, Thomas Nelson & Sons, 1948.
17. Bang, F. B., *Bull. Johns Hopkins Hosp.*, 1950, **87**, 511.
18. Robinow, C. F., *J. Gen. Microbiol.*, 1950, **4**, 242.

EXPLANATION OF PLATE 37

FIG. 1. Uninfected cells of the epidermoid carcinoma; shown for orientation purposes, fixed in acetone, stained with hematoxylin and eosin and photographed in a bright field. × 400.

FIG. 2. An infected cell stained with fluorescent antibody and photographed in a dark field in the fluorescence microscope. Four small groups of viral antigen occur very close to the nucleus (the oval dark area). The largest are aggregations but the two smaller ones may be elementary bodies. A very early stage. × 700.

FIG. 3. Two cells at the upper left, both showing small aggregates of antigenic particles near the nuclear area. × 700.

FIG. 4. A cell, at the upper left, containing a somewhat larger number of particulate antigen bordering the nucleus. × 700.

FIG. 5. Many large aggregates of antigen in the cytoplasm, and several stained areas in the nucleus. × 700.

FIG. 6. A cell showing localized particles of antigen. × 700.

FIG. 7. A small, sharply delimited area of granular antigen is present in the cytoplasm. × 700.

FIG. 8. A large, local accumulation of particulate antigen is present just above the nucleus. × 700.

FIG. 9. At the upper left a cell shows nuclear staining and has a local accumulation of particles of antigen above its nucleus. × 700.

FIG. 10. A cell showing at least three nuclei, the uppermost surrounded by four distinct accumulations of antigen. × 700.

FIG. 11. A cell containing very large clumps of antigen which are grouped together below the nucleus. × 1000.

FIG. 12. A cell in last stage of division, showing a large amount of very finely particulate antigen in the cytoplasm. The smallest may be the elementary bodies of the virus. Areas are also stained in the dividing nucleus. × 700.

FIG. 13. An advanced stage of infection in which the cytoplasm shows a large area of homogeneous antigen and also some fine antigenic particles. × 700.

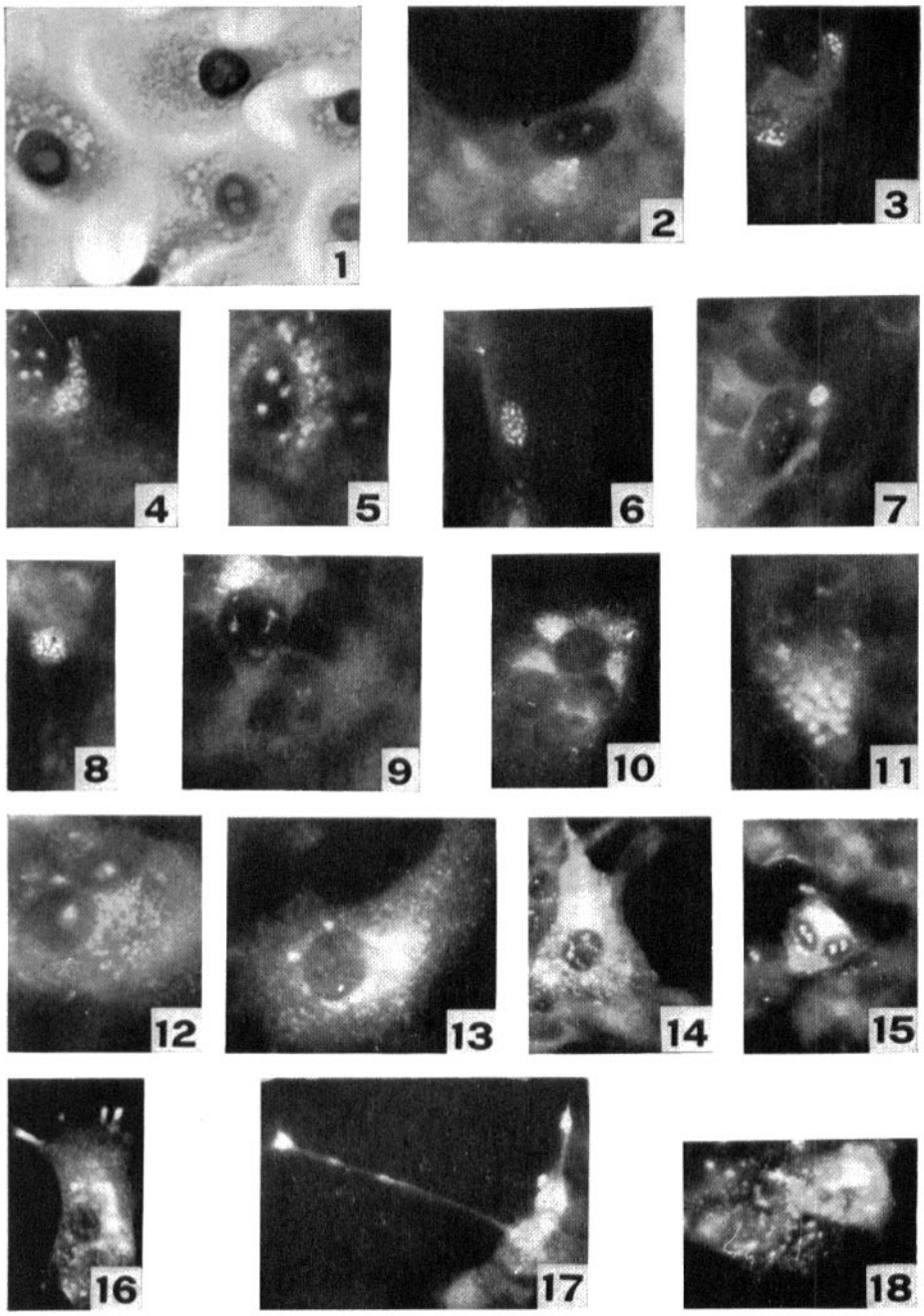
FIG. 14. The cytoplasm contains a large amount of homogeneous antigen as well as particulate antigen. A late stage of infection. × 340.

FIG. 15. A cell, obviously at a very late stage of infection, containing a great quantity of homogeneous antigen. The nucleus is in a late stage of mitotic division. × 340.

FIG. 16. An advanced stage of infection of a cell. It has four cytoplasmic protrusions which contain antigen at their extremities. × 280.

FIG. 17. Thin cytoplasmic process containing viral antigen between two infected cells. The antigen was apparently transferred from the lower to the upper cell. × 280.

FIG. 18. To the right a cell containing a heavy concentration of antigen. Antigenic debris to the left from a disrupted cell. × 280.



(Noyes and Watson: Vaccine virus in cultured human cells)