Viral Antigen in Rat Embryo in Culture Infected with the H-1 Virus Isolated from Transplantable Human Tumors: Cytochemical Studies*

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SUMMARY

The infection of rat embryo cells with H-1 virus was followed, with Coon’s method used for staining with fluorescent antibodies. After a period of 12 hours the stain revealed a diffuse fluorescence that occupied the nucleus except for areas corresponding to the nucleoli. The stained nuclei increased in number during the subsequent 16, 20, and 24 hours corresponding to the log phase of viral growth. On the 2d day after inoculation, when the viral growth curve had reached a plateau, a homogeneous type of fluorescent antibody stain was present in nuclei. Some cells contained granular or diffuse stain in the cytoplasm. At this stage acridine stain showed nuclear structural changes and homogeneous nuclei of DNA-like material. Treatment with pepsin and nucleases indicated the presence in the homogeneous nuclei of a deoxyribonucleoprotein resistant to DNase. Phase microscopy also showed nuclear alteration. By the 3d day, when the viral antigen was present in most of the cells, inclusion bodies and cytopathic effect were present. These changes were followed by the release of hemagglutinating virus.

A group of viruses, characterized by their specific osteolytic action in hamsters, have recently been isolated from human tumors (H.Ep. #1 and H.Ep. #3) growing in rats (14, 16) and from rat tumors and rat leukemia (6). Viruses of this group, designated as H viruses (8), have also been found in tumors and other tissues of cancer patients and in human fetuses (15). The significance of these viruses in oncology is as yet not clear.

The H viruses can be grown in rat embryo tissue cultures, when they give rise to cytopathic effect (CPE) and hemagglutinin (7). This communication reports cytological and cytochemical changes which the H-1 virus, isolated from the H.Ep. #1 tumor, produces in the course of its growth in rat embryo tissue culture. These studies have included immunofluorescent procedures, phase contrast microscopy, and acridine orange staining of nuclease- and protease-treated preparations. The appearance of infective virus and hemagglutinin is correlated with the cytological changes. The H-3 and Kilham’s rat virus (RV) (6), which are immunologically distinct from the H-1 (7), have also been studied by phase contrast microscopy and acridine orange staining. The results are compared with those obtained with the H-1.

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MATERIALS AND METHODS

Cell cultures.—Embryos from 18-day, pregnant, Sprague-Dawley rats were removed aseptically. After being washed they were minced, covered with 0.25 per cent trypsin, and stirred in a magnetic stirrer at room temperature for 1 hour. The free cells were centrifuged, washed 3 times in Eagle’s medium (5), and seeded into milk dilution bottles. The medium was changed the next day, and 2 days later the monolayer cultures were ready to be trypsinized for preparing cultures for cytological studies or titrations.

Hemagglutination (HA) tests.— Cultures to be tested for hemagglutination were first diluted 1:10 in 0.01 M phosphate buffer, pH 7.4, in saline and successive two-fold dilutions were made. To each 0.5 ml. of dilution was added 0.5 ml. of 0.4 per cent washed guinea pig red blood cells. The HA titer was the highest viral dilution that produced complete hemagglutination. This dilution was also considered one hemagglutinating unit.

Viruses.—Pools of each virus were made by inoculating rat embryo (RE) monolayer cultures with seed virus and harvesting the virus when the HA titer had reached 320 or more. Fluids were frozen and stored at —20°C. in 1-ml. quantities. The medium used throughout was Eagle’s basic medium with 5 per cent human serum.

Tissue culture titrations.— Serial tenfold dilutions of virus in Eagle’s medium were added in 0.1-ml. quantities to
each of two tubes containing rat embryo cell monolayers. The tubes were inspected daily for cytopathic effect (CPE) which usually began in 2-3 days and proceeded to complete cellular destruction in 5-6 days. The end-point was taken after no further CPE was noted and was expressed as the dilution at which 50 per cent of the tubes showed CPE (TCID₅₀).

Preparation for cytological and cytochemical studies.—A suspension of approximately 5 × 10⁴ cells/ml of medium was added to Petri dishes containing coverslips (#H). After 3 days' incubation at 37°C. in a CO₂ incubator the medium was removed, the cells were washed, and the coverslips were covered with 0.1 ml. of Eagle's basic medium containing 10⁻⁴ TCID₅₀ of virus/ml. Adsorption was allowed to take place for 2 hours at 37°C. in the CO₂ incubator, with frequent tilting of the Petri dish. The cell layer was then washed 3 times, and Eagle's medium with 5 per cent human serum was added. The cells were further incubated at 37°C. in the CO₂ incubator, and the coverslips were removed and fixed at different time intervals. Simultaneously, aliquots of the supernatant medium were obtained for determination of hemagglutination (HA) titer and TCID₅₀.

For phase microscopy, duplicate cultures grown in Sykes-Moore (13) chambers were given inoculations of virus as described above. Photomicrographs were made with a Wratten B green filter and 34 × 4½ Ansco Versapan film. The Zeiss microscope was fitted with a 6-mm. condenser. Observations were made 2 hours after inoculation and daily thereafter. Simultaneously, the supernatant medium was collected, and its HA titer was determined.

Preparation of fluorescent antibody.—Fluorescent antibodies were made from the globulin fraction of hamster serum by Rigg's (10) modification of Coon's method (3). Hamster sera containing antibody against the H-1 virus were treated with sodium sulfate (12) to obtain the globulin. They were conjugated with fluorescein isothiocyanate by the method of Rinderknecht (11). One fluorescent antibody preparation inhibited hemagglutination of 16 units of the H-1 virus when diluted 1-640; the other, when diluted 1-3200. Neither inhibited hemagglutination by H-3 or RV. The solutions were stored at 4°C. when diluted 1-3200. Neither inhibited hemagglutination by H-3 or RV. The solutions were stored at 4°C. in an open dialyzing bag with weekly changes of dialyzing fluid (isotonic buffered salt solution).

The coverslips were fixed for 10 minutes with acetone at 0°C. When fixed for 50 minutes they lost their stainability. Air-dried preparations were also satisfactory, but localization of antigen was sharper in the acetone-fixed preparations. After fixation the coverslips were kept in the refrigerator for periods of over a month without losing their stainability. None of the fluorescent antibody preparations stained noninfected cells. The staining of infected cells was blocked by treating with the anti-H-1 globulin prior to staining. Control tests were done with fluorescein-labeled normal hamster globulin which did not stain infected or noninfected cells.

To stain, a suitable dilution of fluorescent antibody in buffered salt solution was put on the fixed cells, which were then kept in a humidified chamber for half an hour at room temperature. The stain was rinsed off with two changes of phosphate-buffered saline during 10 minutes, and the coverslips were mounted with 9 parts of glycerin to 1 part buffered salt solution. For fluorescence microscopy, a Zeiss standard microscope was used. A mercury lamp (Osram OB 0200) provided the ultraviolet light. Exciter filters were a combination of BG14, 3 mm. thick, and UG1, 3 mm. thick. To exclude the ultraviolet light from the eyepiece or the photographic film, the Wratten 2B and Corning 3389 filters were used. Black and white photomicrographs were taken on 3½ × 4½ Kodak Royal Ortho film. Super Anscochrome film daylight type, size 120, was used for color pictures, with the filters previously mentioned.

Acridine orange staining.—The technic described by Armstrong (1) was followed. Coverslips were fixed for 10 minutes in Carnoy’s fixative (9 parts ethanol and 1 part glacial acetic acid), washed 4 times with water, left in 0.1 M acetate buffer pH 5 for 10 minutes, and stained with 0.05 per cent solution of acridine orange for 10 minutes. They were washed 4 times with buffer and mounted wet.

The BG12 filter (3 mm. thick), BG14 (3 mm. thick) as an exciter filter, and the Corning 3486 (2 mm. thick) barrier filter were used. Black and white pictures were taken on Ansco Versaplan film and color pictures on Anscochrome daylight type.

Enzymatic removal of viral inclusions.—Cells grown on coverslips in Petri dishes were infected with the H-1 virus as previously described. When the abnormalities associated with virus infection (Fig. 12) were present, the coverslips were fixed in Carnoy’s fluid as described above, treated with the enzymes, and then stained with acridine orange. Controls were uninfected cells similarly treated. Deoxyribonuclease (DNase, once crystallized, Worthington Biochemical Corporation, Freehold, New Jersey) was employed in a solution containing 30 μmoles of magnesium chloride in 1 ml. of phosphate buffer, pH 7.4, 0.003 M. The smallest concentration of enzyme that completely removed DNA from uninfected cells was 20 μg/ml and was used in the experiments reported under “Results.” Whenever DNase was used the cytoplasm lost its brilliant red color and stained brown-green.

Crystalline ribonuclease (RNase, Worthington) was used at a concentration of 200 μg/ml of distilled water. The cells were incubated with DNase and RNase for 1 hour at 37°C. Pepein (1:60,000, crystalline, Sigma Chemical Co., St. Louis, Missouri) was used at a concentration of 200 μg/ml of 0.1 M acetate buffer pH 2.7 and incubated with the cells at room temperature for 2 hours.

RESULTS

Chart 1 shows the development of nuclear antigen, infectious virus as measured by tissue culture, and the hemagglutinin following infection of rat embryo cells with 10⁻⁴ TCID₅₀ of the H-1 virus. It combines the results of three separate experiments.

At the time of inoculation of the coverslips cells had formed an almost complete fibroblastic layer. Six hours later examination by phase microscopy showed an increased nuclear cytoplasmic ratio in a few isolated spindle-shaped cells with condensed nuclei. At the same time, viral antigen was detected by fluorescent antibody stain-
ing in the scarce cytoplasm and homogeneous nuclei of a few shrunken cells. Little or no infective virus was detected in the medium. After 12 hours, a few nuclei showed low-intensity fluorescent antibody stain of the strands and spheres following the pattern of the chromatin net (Fig. 1). Phase microscopy of the such cells showed that the one or two unstained areas in the nuclei corresponded to nucleoli. Unstained nuclear areas sometimes contained a stained granule. The antigen-containing nuclei increased in number during the subsequent 16–20 hours, and at 20 hours involved 20–30 per cent of the cell population (Fig. 7). Some nuclei were broken, and part of their content was visible in the cytoplasm. Uninfected control cells showed no stain (Fig. 9). At this time infective virus was being released into the medium (Chart 1), although no hemagglutinin was detected.

During this time phase observations and acridine staining revealed normal nuclei in most of the cells, the few exceptions showing changes in the distribution of the chromatin (Fig. 2).

On the 2d day after inoculation the nuclei stained with fluorescent antibody had a diversified appearance (Figs. 3, 11).

In some cells diffuse or granular fluorescent antibody stain (Fig. 4) appeared in the cytoplasm on the 2d day, with or without concomitant nuclear stain. At this time, in about 10 per cent of the cells, acridine staining showed nuclear changes consisting of margination of the chromatin and condensation around the nucleoli, thus leaving the rest of the nucleus empty or filled with a coarser chromatin net with increased stainability (Fig. 5). Especially outstanding were homogeneous nuclei filled with a yellow material of intense fluorescence, the same color as the chromosomes of mitotic cells (the normal nuclei stained green). The nuclear membrane was prominent. The red nucleoli were visualized with difficulty and often located adjacent to the nuclear membrane. Most often the cells with the homogeneous nuclei were spindle-shaped and poorly attached to the glass (Fig. 6). The nature of those nuclei was studied by treating them with enzymes before acridine staining. RNase removed the staining properties of the cytoplasm and nucleolus but not those of nuclear DNA or the homogeneous nuclei. Pepsin treatment did not alter staining properties of the controls or of the infected cells. DNase prevented the DNA stain of noninfected cells and of cells of the infected cultures with normal chromatin, but the homogeneous nuclei still stained. Treatment with pepsin followed by DNase removed not only the normal chromatin but also the DNA of the homogeneous nuclei.

Phase microscopy also revealed nuclear abnormalities in the 2d day after inoculation. This consisted of chromatin aggregation around the membrane and filling of the nucleus with homogeneous grey material (Figs. 7, 8).
Fig. 9.—FA staining of an uninoculated control culture. Notice the absence of specific staining. X 270.

Fig. 10.—FA staining of RE cells 24 hours after inoculation, showing the predominance of stained nuclei with unstained nucleoli. X 270.

Fig. 11.—FA staining of RE cells 2 days after inoculation. Homogeneous type of stain present in the nucleus. Many cells show cytoplasmic staining. X 280.

Fig. 12.—FA staining of RE cells 3 days after inoculation. Perinuclear and cytoplasmic stain predominates. One nucleus on the right shows a stained, shrunken body separated from the nuclear membrane. The cell sheet is disrupted. X 280.
Fig. 13.—Acridine staining, 2 days after inoculation with Kilham’s RV. Appearance of a nucleus with dense green fluorescence for DNA. Two nucleoli are centrally located. Vacuoles containing a DNA core are seen scattered through the nucleoplasm. × 770.

Fig. 14.—Phase observation of a cell 2 days after infection with Kilham’s RV showing a nucleus with grey material. × 770.

Fig. 15.—Acridine staining of a binucleated cell 4 days after infection. A core of dense chromatin is present in both nuclei. × 770.

Fig. 16.—Phase observation of a cell 24 hours after infection with Kilham’s RV showing fractionation of the nucleoli. × 770.

Fig. 17.—Acridine staining of a nucleus 24 hours after infection with Kilham’s RV showing small globular inclusions green for DNA and fractionation of the nucleoli. × 770.

Fig. 18.—Phase observation of cells 24 hours after injection with Kilham’s RV. Left nucleus, similar to Fig. 17, contains small grey globules, and larger and darker ones probably resulting from nucleolar fractionation. Nucleus on the right shows margination of chromatin.
On the 3d day after inoculation the viral antigen had a predominantly perinuclear and cytoplasmic location (Fig. 12). The number of nuclei carrying antigen decreased. Acridine stain showed nuclear inclusions of yellow material, separated from the nuclear membrane; their connective strands were visible. Some highly stained nuclei had unstained vacuoles, sometimes enclosing a granule of DNA-like material. Very rarely, DNA-like material was found in the cytoplasm.

Phase microscopy showed an increased number of dark nuclei. Infective virus and HA were present in the media. At this time, or on the 4th day, CPE was evident. The cell sheath was disrupted, and a large number of cells had pyknotic nuclei and vacuolated or granular cytoplasm. On the 7th day only a few cells, rounded or with long processes, remained on the coverslip. The production of infective virus and HA had decreased.

H-3 infected cells showed the same type of nuclear change as the H-1 infected cells, and in the same sequence. Kilham's RV infected cells also presented the same changes during the 1st, 2d, and 3d days (Figs. 13, 14). In addition, a new type of nucleus appeared on the 4th day. It consisted of a dense core stained green as DNA, centrally located and surrounded by a zone of normally stained chromatin (Fig. 15). Later, the chromatin around the central core became rarefied and clumped on the nuclear membrane. In some cells infected with RV phase microscopy showed early changes in the nucleoli leading to fractionation (Fig. 16). Some nuclei contained small globular inclusions of grey material which were less dense that the nucleoli (Fig. 18). These inclusions stained green for DNA with acridine staining (Fig. 17).

**DISCUSSION**

The acridine orange stain showed that the homogeneous nuclei produced after infection with the H-1 virus contained a deoxyribonucleoprotein which resisted pancreatic DNAse under conditions when DNA in nuclei of normal appearance was digested. The fluorescent antibody technic revealed that these nuclei also contained viral antigen. This, and previous findings (2, 9) of DNAse-resistant DNA and viral particles in inclusion bodies, suggest that the homogeneous nuclei could contain viral DNA protected from the action of DNAse by its protein coat (2).

The insusceptibility of the abnormal DNA to the action of DNAse and the presence of DNA in the cytoplasm of cells with unbroken nuclei has been previously correlated with viruses of DNA nature (9) and suggest that the H-1 virus could be of this type.

The cytoplastms of most of the cells contained viral antigen 2 days after infection and, in a very few, DNA. The cytoplasmic DNA could have been less easily detected than the antigen, owing to the relative abundance of RNA. H-3 and the Kilham's RV, related to the H-1 by some of its characteristics, are immunologically distinct from the H-1 and have been grouped as the H-3 type viruses. The H-3 virus produced the same kind of nuclear inclusions as the H-1. Kilham's RV produced a different type of nuclear inclusion from the H-1 or the H-3 viruses, characterized by a dense chromatin core. As the halo of chromatin surrounding the core became rarefied, the inclusions resembled those described by Dawe et al. (4), using May-Grünwald stain in rat embryo cells infected with Kilham's RV.

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**REFERENCES**

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