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IMMUNOFLUORESCENT STUDIES OF THE REPLICATION OF RAT VIRUS (HER STRAIN) IN TISSUE CULTURE[1])

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Summary. — The replicative process during a single growth cycle of the HER strain of rat virus (RV) in rat embryo (RE), BHK-21, and L cell cultures was studied by immunofluorescent staining. In RE cells, after infection with a high input multiplicity, virus-specific antigen first became detectable at 5 hours, and was confined to the cytoplasm. By 8 hours, faint nuclear fluorescence appeared, thereafter increasing in intensity, with concomitant disappearance of cytoplasmic antigen. Nuclear localization of RV was followed by cell destruction as evidenced by retraction of cytoplasm, pyknosis and, after 20 hours, by a marked diminution of stainable antigen corresponding with the release of viral hemagglutinin. RV infection of BHK-21 and L cells under similar conditions also resulted in early production of cytoplasmic antigen, but cell destruction occurred in the absence of significant nuclear involvement or hemagglutinin formation.

Introduction

Rat virus is a member of the newly designated group of small DNA viruses, the "picodnaviruses" (Mayor and Melnick, 1966). Since its original isolation by Kilham and Olivier (1959), a number of reports have appeared describing rat agents which, although antigenically related to the prototype strain, possessed markedly different biologic properties (Lum and Schreiner, 1963; Payne *et al.*, 1964; Bergs and Scotti, 1967; ElDadah *et al.*, 1967).

One such agent recently isolated and under study in our laboratory (ElDadah *et al.*, 1967), appears to be unique among the known RV strains because of its ability to regularly produce fatal hemorrhagic encephalopathy in young rats (HER strain). As part of our initial studies of the HER agent, an investigation of its growth in several types of cell culture was conducted employing the fluorescent antibody (FA) technique to sequentially visualize the intracellular formation of virus-specific antigen.

The results, which form the basis of this paper, document an early step

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in the replication of the HER virus previously unreported in similar studies with other rodent picodnaviruses (Hampton, 1964; Mayor and Ito, 1968).

Materials and Methods

Virus. The original isolate of HER virus (ElDadah *et al.*, 1967) was serially passaged in suckling Wistar rat brain. A 10^{-2} dilution of 5th passage material was inoculated into a series of primary RE flask cultures. On the 5th day, when destruction of the monolayers was complete, the cultures were twice frozen and thawed, the fluids were pooled, clarified by light centrifugation and the resulting supernate constituted the stock virus used in these experiments. This preparation contained 10^{10} $TCID_{50}$ per ml as determined by titration in Wistar RE tube cultures.

Tissue cultures. Pregnant Wistar rats obtained from a commercial breeding colony free of RV (Microbiological Assoc., Inc., Walkersville, Maryland) served as a source of 15 day-old embryos used to prepare RE cultures. Plastic culture flasks, with a 75 cm^2 surface area (Falcon Plastics, Los Angeles, California) were seeded with approximately 3×10^7 washed, trypsin-dispersed RE cells suspended in 15.0 ml of Eagle's minimal essential medium (MEM) (Baltimore Biological Laboratories, Cockeysville, Maryland) containing 15% agamma calf serum (Grand Island Biological Co., Grand Island, New York), and 100 units of penicillin and 100 μg of streptomycin per ml. After incubation at 36° C in an atmosphere of air containing 5% CO_2, confluent cultures were either maintained on MEM containing 2% serum or used to prepare secondary coverslip cultures.

The continous hamster and mouse lines, BHK-21 and L-929 respectively, were obtained from the American Type Culture Collection (Rockville, Maryland) and grown or maintained on the same media used for RE cells.

Coverslip cultures were prepared by seeding of cells into 60×15 mm plastic culture plates (Falcon Plastics, Los Angeles, California) containing 11×22 mm glass coverslips.

Infection of cell cultures. The cell densities of the coverslip cultures varied with different experiments. The use of incomplete monolayers (15—20% coverage of surface area) permitted accurate counting of fluorescing cells during different stages of infection. Cultures were inoculated with HER virus at a multiplicity of approximately 100 $TCID_{50}$ per cell. Following a 2 hour adsorption period, free virus was removed by washing, and replaced with 3.0 ml of maintenance medium. Immediately following adsorption (2 hours) and thereafter at 3, 4, 5, 6, 7, 8, 9, 10, 12, 14, 20, 26, and 32 hours, 8 infected and 4 uninfected coverslips were collected, washed in saline, fixed in acetone, and stored at −20° C for subsequent FA and Giemsa staining. At the same intervals, fluids and cells from each of 2 companion cultures were harvested separately for hemagglutinin (HA) determinations. After removal of the fluid phase, cultures were washed and an equal volume of maintainance medium was added to each plate which was then twice frozen and thawed. Preparations were rendered free of cellular debris by centrifugation prior to testing for HA.

HA titrations were performed in plastic microtiter plates (Linbro Chemical Co., New Haven, Connecticut) using 0.4% guinea pig erythrocytes. Both antigen dilutions and erythrocytes were prepared in phosphate buffered saline (PBS) pH 7.6 containing 0.75% bovine plasma albumin (Armour Pharmaceutical Co., Kankakee, Illinois). HA titers were recorded as the reciprocal of the highest dilution causing agglutination of 50% of erythrocytes after 1 hour at room temperature.

Immunofluorescent staining. The ammonium sulphate-precipitated globulin fraction of HER virus hyperimmune antiserum prepared in Wistar rats was conjugated with fluorescein isothiocyanate (Baltimore Biological Laboratories, Cockeysville, Maryland) following the method of Marshall *et al.* (1958). Prior to use, the conjugate was mixed with a standard Evans blue counterstain solution (Cole *et al.*, 1970), and the mixture overlaid onto infected and control coverslip cultures. After staining 45 minutes at room temperature in a humid container, coverslips were rinsed in PBS, pH 7.6, and then in water. After drying, they were mounted with PBS, pH 8.0, containing 50% glycerol. The specificity of staining was verified by the blocking of fluorescence with unconjugated immune serum and by the absence of staining in uninfected cultures. A Zeiss standard fluorescence microscope equipped with an HBO-200 mercury vapor burner was used for examination of FA-stained preparations.

Results

Infection of RE cultures

Five hours after infection, fluorescent granules were observed scattered throughout the cytoplasm of 3% of the cells (Fig. 1, A). By 8 hours the number of cells containing cytoplasmic antigen increased progressively (Fig. 1, B and C), reaching 60%, and early fluorescence was first seen in the chromatin network of nuclei in 1—2% of cells (Fig. 1, D). At this early stage of nuclear fluorescence, the unstained nucleoli were readily visible.

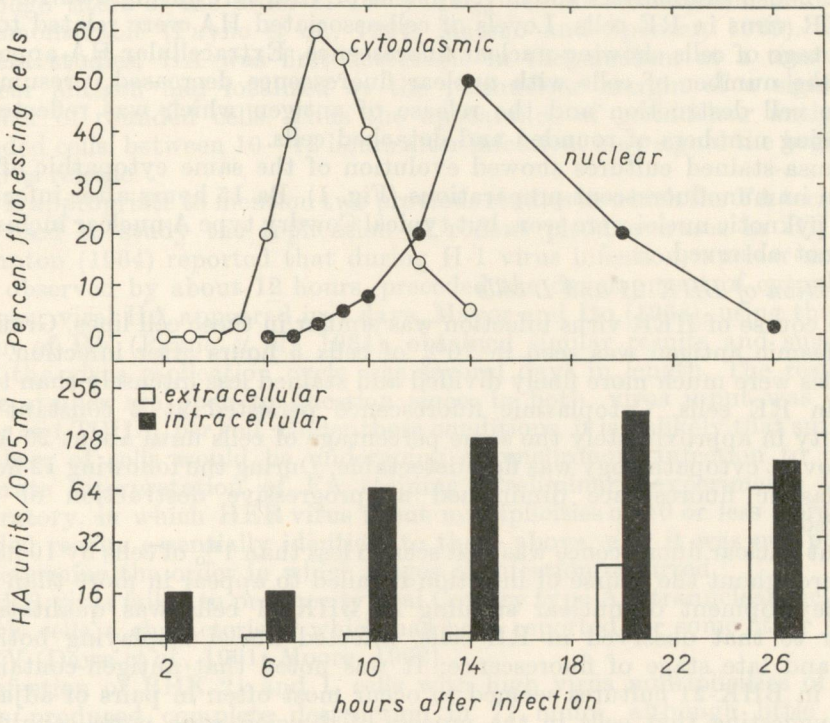

Fig. 2.

Rat virus (HER strain) infection in Wistar rat embryo cells. Development of fluorescent and hemagglutinating antigen following infection with an input multiplicity of 100 $TCID_{50}$ per cell

During the next 6 hours, intensely staining nuclear antigen appeared in an increasing number of cells (50% by 14 hours), eventually filling the nuclei and obliterating the nucleoli (Fig. 1, E). Associated with this late stage of nuclear fluorescence were signs of cytopathology evidenced by cytoplasmic retraction and cell rounding (Fig. 1, F). A small percentage (5%) of rounded cells were seen in which antigen was concentrated at the nuclear margin. Concomitant with the increase of cells containing nuclear antigen, there occurred a steady decrease in the number with cytoplasmic

antigen, the latter comprising only 5% by 14 hours. These changes in localization of fluorescence are graphed in Fig. 2.

Beginning at 14 hours, cytopathology and cell loss became increasingly evident. Thus, the number of cells with nuclear fluorescence declined to 20% at 20 hours and 2% by 26 hours. Concomitantly, there was an increase to 10% in the number of rounded cells with antigen concentrated at the nuclear margin. Also noted was the beginning of a second viral growth cycle between 14 and 18 hours, as evidenced by the appearance of fluorescence in the cytoplasm of previously uninfected cells.

Fig. 2 also summarizes the development of viral HA during a single cycle of HER virus in RE cells. Levels of cell-associated HA were related to the percentage of cells showing nuclear fluorescence. Extracellular HA appeared after the number of cells with nuclear fluorescence decreased, presumably due to cell destruction and the release of antigen which was reflected in increasing numbers of rounded and detached cells.

Giemsa-stained cultures showed evolution of the same cytopathic effects seen in immunofluorescent preparations (Fig. 1). Ba 15 hours after infection, many pyknotic nuclei were seen, but typical Cowdry type A nuclear inclusions were not observed.

Infection of BHK-21 and L cells

The course of HER virus infection was similar in these cell lines. Granular cytoplasmic antigen was seen in 70% of cells 5 hours after infection. The granules were much more finely divided and stained less intensely than those seen in RE cells. Cytoplasmic fluorescence persisted at a constant low intensity in approximately the same percentage of cells until about 20 hours when overt cytopathology was first detectable. During the following 12 hours, cytoplasmic fluorescence diminished as progressive destruction of cells occurred.

Faint nuclear fluorescence was first seen in less than 1% of cells by 10 hours, and throughout the course of infection it failed to appear in more than 5%. The development of nuclear staining in BHK-21 cells was qualitatively similar to that observed in RE cells, infected nuclei displaying both an early and late stage of fluorescence. It was noted that antigen-containing nuclei in BHK-21 cultures seemed to occur most often in pairs of adjacent cells suggesting that cells in the process of division were more capable of supporting productive viral infection. In contrast to RE and BHK-21 cells, nuclear antigen in L cells stained poorly and was difficult to visualize.

No viral HA levels, beyond those attributable to input virus, were detected either in BHK-21 or L cells.

Discussion

The appearance of HER viral antigen in the cytoplasm prior to its localization in the nucleus would not be unexpected for a DNA virus which is assembled in the nucleus (Bernhard *et al.*, 1963; Mayor and Jordan, 1966). Since cytoplasmic antigen was first detected in RE cells by the 5th hour after infection, transcription of viral DNA and translation of the resulting messenger RNA must have taken place before this time.

A gradual decrease of antigen in cytoplasm, coinciding with its continued increase in nuclei, occurred 8 hours after infection. Assuming the fluorescence in cytoplasm to be due to virus structural protein, this decrease may represent the migration of viral capsid precursors to the nucleus, for subsequent incorporation into mature virions.

Cells in transition from early to late stages of nuclear fluorescence were first observed between 8 and 10 hours, which provides an estimate of the duration of the replicative cycle of the HER virus.

Infectivity and HA activity of RV are associated with the intact virion, although titers of the order of 10^6 $TCID_{50}$ per ml are required before HA is demonstrable (Payne *et al.*, 1964; Matsuo and Spencer, 1969). In the present studies, HA was first detectable in the medium at a time when nuclear antigen had localized at the perinuclear margin of a significant number of rounded cells. Thus the appearance of perinuclear antigen in rounded cells, between 10—12 hours after infection, may signal the beginning of virus release.

It is appropriate to mention two previous studies in which the FA technique was used to study the replication of rodent picodnaviruses in RE cells. Hampton (1964) reported that during H-1 virus infection, nuclear antigen, first observed by about 12 hours, preceded the development of cytoplasmic antigen; viral HA appeared in 4 days. Mayor and Ito (1968), using the X-14 strain of RV (Payne *et al.*, 1964), obtained similar results and suggested that the virus replication cycle was several days in length. The results of these studies are open to question since, in both, virus input was of the order of 1 $TCID_{50}$ per cell. Under these conditions, it is unlikely that sufficient numbers of cells would be undergoing a synchronous infection to permit accurate interpretation of FA staining. Preliminary experiments in our laboratory, in which HER virus input multiplicities of 10 or less were used, yielded results essentially identical to those above, and it was not possible to determine the order in which stages of infection occurred.

HER virus failed to produce typical Cowdry type A intranuclear inclusions in RE cells, a characteristic which has been reported for some other strains of RV (Dawe *et al.*, 1961; Moore, 1962).

Infection of BHK-21 and L cells with high virus multiplicities of HER virus produced complete destruction in 72 hours, although little or no infectious virus was produced. Since less than 5% of these cells develop nuclear fluorescence, it appears that, in these cells, infection is cytocidal but largely non-productive.

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Explanation of Micrographs:

Fig. 1. Immunofluorescent staining of rat embryo cell cultures during different stages of infection with the HER strain of rat virus. All photographs. × 500.

A — Cell with cytoplasmic antigen 5 hours after infection.
B — Cytoplasmic antigen in cells 6 hours after infection.
C — Cells containing either cytoplasmic or early nuclear antigen 9 hours after infection.
D — Early nuclear fluorescence 9 hours after infection, with little residual cytoplasmic fluorescence.
E — Late nuclear fluorescence 10 hours after infection. Antigen fills the nuclei and cytoplasm is still intact.
F — Cells at different stages of antigen localization 12 hours after infection. Several rounded fluorescing cells are seen.