Electron Microscope Study of Human NB and SMH Cells Infected with the Parvovirus, H-1: Involvement of the Nucleolus*

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SUMMARY

The sequence of changes in human NB and SMH cells that occurred after H-1 virus infection, was studied with the electron microscope. The earliest alterations in NB cells after infection were detected in the nucleolus, after 31 hr. The pars fibrosa appeared devoid of its formed elements and was occupied instead with mainly 'incomplete' H-1 virus. The pars granulosa was more diffuse than normal and its nucleolar granules more sharply defined. A few 'incomplete' and 'complete' virus particles were scattered about the nucleus at approximately the same time. The cytoplasm was intact. Shortly thereafter, margination of the nuclear chromatin occurred. The nucleoli became increasingly condensed and shrunken, and apparently formed a doughnut-shaped body with condensed walls containing empty virus. Eventually, most of the nucleolar elements disappeared as the nuclei filled with complete and incomplete virus particles as well as occasional crystals possibly of protein nature. As the nucleolus disappeared, the cytoplasm disintegrated except for a few scattered mitochondria and some recognizable areas of endoplasmic reticulum which contained complete virus particles in linear array. Seventy-seven hr after infection when the nucleus had also broken down, virus was found associated with the disintegrated nucleus, within fragments of endoplasmic reticulum, and attached to thickened and adjoining plasma membranes of the few remaining, apparently uninfected cells.

In SMH cells basically similar changes occurred except that the nucleoli appeared to fragment 31 hr after infection. Later, though most of the nucleolar sections disappeared, a few persisted and formed 'nucleolar inclusions' identical to those observed in NB cells. In contrast to the mixture of 'complete' and 'incomplete' virus observed in NB cells, the virus found in SMH cells was almost exclusively 'empty'. Virus seen outside the nucleus, either in NB or SMH cells, appeared complete.

INTRODUCTION

H-1 virus, one of the newly isolated parvovirus group (Int. Com. on Nomenclature, 1968) to which Kilham rat virus (RV) and the adeno-associated viruses (AAV) belong (Toolan, 1968), is a minute DNA animal virus which inhibits the oncogenic adeno-virus 12 both in vitro (Ledinko & Toolan, 1968) and in vivo (Toolan & Ledinko, 1968).
Such inhibition occurs even though the adenovirus acts as a ‘helper’ for H-1 in certain cell systems (Ledinko & Toolan, 1968; Ledinko, Hopkins & Toolan, 1969). H-1 virus also has the relatively rare ability to cross the hamster placenta and to infect and deform the developing embryo (Toolan, 1968). Recently it was learned that at least two of the parvoviruses, the minute virus of mice (Crawford et al. 1969) and RV (Robinson & Hetrick, 1969) have single-stranded DNA. H-1, which has been isolated from human tissues (Toolan, 1968), also appears to have single stranded DNA (M. Usategui-Gomez, H. W. Toolan, N. Ledinko, F. A. Al-Lami & S. Hopkins unpublished). The only previously reported single-stranded DNA agents have been bacterial phages.

Owing to the interesting findings associated with this agent, we have made an electron microscopic study of the host+virus relationship in cells infected with H-1 virus. Previously, only scattered and incomplete descriptions of such infections have been available (Bernhard, Kasten & Chaney, 1963; Portella, 1963) in all of which the H-1 was grown in rat-embryo cell cultures where another parvovirus, RV, may be latent. Since the NB (newborn human kidney) line (Shein & Enders, 1962) produces high yields of infective H-1, this was the cell of choice for study. These cells, however, were originally transformed by SV40, and although SV40 has never been observed by us in the NB cells nor in concentrated fractions of H-1 grown in NB cells, tests in Dr Enders’s laboratory (personal communication) have indicated that the SV40 genome is or may be present. Therefore, a second line of cells was infected with H-1 and examined with the electron microscope for comparison. The human ‘Salk monkey heart’ (SMH) was chosen for this purpose as these cells have been employed by us for plaque assay of H-1 (Ledinko, 1967). It was interesting that there was prominent involvement of the nucleolus in both cell types after the H-1 infection.

**METHODS**

**Cell cultures.** SV40-transformed newborn human kidney (NB) cell cultures (Shein & Enders, 1962) kindly supplied by Dr J. F. Enders, Children’s Hospital, Boston, were grown in bottles using Eagle’s minimal essential medium (MEM) containing 8% inactivated horse serum and 8% foetal calf serum with 2 x MEM amino acids, 2 x MEM vitamin concentrates and 0.025% lactalbumin hydrolysate (Nutritional Biochemicals, Cleveland, Ohio) added. The Salk ‘monkey heart’ (SMH) cells which have now been ascertained by various investigators to be of human origin (Brand & Syverton, 1962; Greene, Coriell & Charney, 1964) were originally obtained from Microbiological Associates, Bethesda, Maryland. These cells were grown in a medium similar to that used for the NB cells except that the only serum added was 10% foetal bovine and the amino acids and vitamins employed were in a concentration of 1 x.

**Virus growth and assay.** A stock of plaque-purified H-1 virus was prepared in NB cells by infecting bottle cultures containing approximately $6 \times 10^6$ cells with about $2 \times 10^4$ p.f.u. of virus in Eagle’s medium with 4% foetal calf serum added. After 7 days incubation at 37°C, the cultures were frozen and thawed three times to release virus. Cell debris was removed by centrifugation at 800 g for 15 min. and the virus-containing supernatant fluid was stored at $-20^\circ$. H-1 titres were determined using the plaque method of assay on SMH monolayer cell cultures as described previously (Ledinko, 1967).
Involvement of the nucleolus in human cells

Infection of cells. Confluent or nearly confluent NB or SMH monolayer cell cultures, containing about $2 \times 10^6$ cells in a 35 mm. plastic Petri dish, were washed twice with tris-buffered saline (Ledinko, 1967), after which 0.2 to 0.5 ml. of virus suspension was placed on the cell layer. A multiplicity between 10 and 15 was used in all experiments. (The multiplicity refers to adsorbed multiplicity as determined by detaching monolayers with EDTA and counting the cells in a haemocytometer.)

After 60 min. adsorption at 37°C the cell monolayers were washed three times with tris-buffered saline to remove unadsorbed virus. They were then covered with 2 ml. of Eagle's medium containing 5% foetal calf serum, and incubated at 37°C in a humidified mixture of 5% carbon dioxide in air. At various times, cultures were removed for growth studies or electron microscopy. For growth studies, cultures were frozen and thawed four times to release virus. Cell debris was removed by centrifugation at 800 g for 15 min. and the virus-containing supernatant fluid was frozen at $-20^\circ$.

Cell preparation for electron microscopy. At 24, 31, 45, 72 and 77 hr after infection, one to two infected control cultures from each set of NB and SMH cell cultures were removed for study. The cell monolayers were detached with a rubber policeman and centrifuged in their culture fluid at 900 g for 5 min. Supernatant fluids were withdrawn with gentle suction and the cell pellets then fixed in 3% glutaraldehyde in phosphate buffer (pH 7.4) for 1 hr, washed in several changes of phosphate buffer for 2 hr and post-fixed in 2% cold OsO$_4$ in the same buffer. They were subsequently washed briefly in distilled water, dehydrated in alcohol and embedded in Epon-812. Sections were cut on a Porter-Blum microtome, stained with uranyl acetate and lead citrate, and examined with a JEM-7 electron microscope.

RESULTS

Growth of H-1 Virus.

The eclipse period for H-1 in NB cell cultures was approximately 10 hr. The total (intra- and extracellular) infective titre subsequently increased until about 50 hr after infection, when approximately 80 p.f.u. were found per cell (Fig. 1). Extensive cytopathic changes were present when maturation was complete or almost complete. Growth of H-1 in SMH cells was also complete by 3 days after infection, but final yields of only 1 to 10 p.f.u./cell were obtained.

Electron microscopic examination of uninfected cells

Thick Epon sections of control cultures viewed with the light microscope showed that the typical NB cell was approximately 26 $\mu$m. in diameter, with a single lobular nucleus containing one or more prominent nucleoli about 1 $\mu$m. in diameter. Most of the chromatin was lightly stained or unstainable (euchromatin) (Fawcett, 1967). Stainable chromatin (heterochromatin) was located mainly at the nuclear periphery (Pl. 1). The nucleoli were distinct and round, and, as reported for other cell types (Fawcett, 1966; Bernhard & Granboulan, 1968), they were composed of a central portion, the *pars fibrosa*, surrounded by a more dense *pars granulosa* (Pl. 1 b). Extremely fine fibrils, obscured by a more or less homogeneous or finely granulated substance, formed the bulk of the *pars fibrosa*. The compact *pars granulosa* contained only one structure that could be recognized with certainty, namely, the nucleolar granules which were about 130 nm. in diameter; they were unevenly distributed throughout the region. The cytoplasm contained many vesicles and tubules of smooth-surfaced and,
to a lesser extent, rough-surfaced endoplasmic reticulum. Most of the ribosomes were freely disposed in the cytoplasm, either singly or in groups (polysomes). Lipid-like droplets were commonly seen.

Uninfected SMH cells were in many respects similar to the NB cells with three important differences. They were on the average smaller than NB cells (15 to 17 μm in diameter) with a single nucleus usually containing two large (2 to 2.2 μm in diameter) prominent nucleoli. The pars granulosa of the SMH nucleoli predominated over the pars fibrosa which was represented by a few small, ill-defined regions. The rough-surfaced endoplasmic reticulum (ER) was remarkably more voluminous in the cytoplasm of SMH cells than in NB cells.

![Graph showing the multiplication of H-1 in NB cell cultures](image)

Fig. 1. Multiplication of H-1 in NB cell cultures. The multiplicity of H-1 was 15. The plaque forming units represent the total (intra- and extracellular) units found per ml.

Observation of NB cells at relatively early stages of infection

Cells examined 8 or 24 hr after H-1 virus infection showed neither morphological changes nor detectable virus particles. At 31 hr after inoculation, however, about 50% of the cells were visibly infected. These cells showed some scattered complete and incomplete H-1 virus-like particles in their otherwise undisrupted nuclei. The first sign of alteration, however, was noted in the nucleoli of such cells (Pl. 2a). The pars fibrosa seemed to be completely devoid of any formed element and instead was occupied by numerous incomplete H-1 virus. The pars granulosa was more diffuse.
(a) Portion of an uninfected NB cell showing the lobular nucleus (N), and a prominent ring-shaped nucleolus (Nu). Most of the chromatin has a relatively low staining density or is unstainable (euchromatin). In the cytoplasm the endoplasmic reticulum is predominantly smooth-surfaced (ER); free and membrane bound ribosomes (unlabelled) and mitochondria (M) are present.

(b) High magnification of a nucleolus from a control NB cell, showing the pars granulosa surrounding the pars fibrosa (P). Arrows indicate two of the numerous nucleolar granules characteristic of the pars granulosa.

F. AL-LAMI, N. LEDINKO AND H. TOOLAN  
(Facing p. 488)
2(a) Portion of a nucleus with nucleolus (Nu) of a Nb cell 31 hr after H-1 virus infection. The nucleoplasm at this stage has a relatively normal appearance despite the presence of a few scattered complete (C), and incomplete (O), H-1 virus-like particles. In the nucleolus (Nu) however, the pars granulosa is less dense and its granules are subsequently easier to recognize than in control cells. Numerous particles of mostly incomplete H-1 virus can be seen in the otherwise depleted pars fibrosa (P).

(b) Portion of a nucleus (N) of Nb cell 45 hr after H-1 virus infection. Complete (C) and incomplete (O) virus particles can be seen in the depleted nucleoplasm. The nucleolus (Nu) has presumably condensed into a doughnut-shaped body filled with empty virus particles in the region of the depleted pars fibrosa (P).

F. AL-LAMI, N. LEDINKO AND H. TOOLAN
3(a) NB cell 45 hr after H-1 virus infection. The necrotic nucleus (N) is heavily populated with incomplete as well as complete H-1 virus (unlabelled). Near the margination and dense chromatin (Ch) at (A) there is an aggregation of regular granular components most of which seem to be surrounded by a light halo. At (B) a zone of dense irregular granules occurs interspersed with virus particles. Also shown is a portion of the considerably disrupted and broken down cytoplasm. A mitochondrion (M) and some vesicles of endoplasmic reticulum (ER) can still be recognized.

(b) Portion of a NB cell 72 hr after H-1 virus infection. The nuclear chromatin (Ch), which no longer has defined elements, is confined to a few peripheral areas but is no longer immediately adjacent to the nuclear membrane (NM). Numerous crystals (Cr) are present within the nucleus, H-1 particles, mostly complete, are associated with the crystal edges and are also scattered throughout the nucleus (arrows). Insert shows disrupted cytoplasmic region adjacent to nucleus (N) where complete virus particles are present in linear array within two fragmented cisternae of endoplasmic reticulum (ER).

F. AL-LAMI, N. LEDINKO AND H. TOOLAN
4(a) Portion of a NB cell nucleus (N) 72 hr after H-1 virus infection. A ‘nucleolar inclusion’ (Nu) contains ‘incomplete’ H-1 virus (unlabelled). A crystal (Cr) is present.

(b) NB cell 77 hr after H-1 virus infection with a portion of completely depleted nucleus (N) to the left. The cytoplasm on the right is represented chiefly by fragmented cisternae of endoplasmic reticulum within which are H-1 virus particles (arrows). In one region, an aggregate of H-1 particles is present (V); these tend to be arranged in regular array and associated with amorphous staining material. (M), a remnant of depleted mitochondrion.

F. AL-LAMI, N. LEDINKO AND H. TOOLAN
5(a) Nucleolar inclusion in SMH cell 31 hr after H-1 virus inoculation. Incomplete virus particles are evident within the inclusion as well as in the adjacent nucleoplasm (arrow).

(b) Nucleolar inclusion in SMH cell 45 hr after H-1 virus inoculation. The nucleoplasm is now filled with incomplete virus particles (unlabelled). There is a suggestion that some virus particles are being derived from the inner as well as the outer walls of the inclusion.
and its nucleolar granules easier to recognize than in the normal nucleolus. The cytoplasm of these cells appeared intact.

**NB cells 45 hr after infection**

At this stage over two-thirds of the cells appeared to be infected. Numerous incomplete and complete virus particles could be seen in the partially depleted nuclei (Pl. 2b, 3a). As in other virus-infected nuclei (Bedoya, Rabson & Grimley, 1968; Blalock, Rabin & Melnick, 1968; Dunn & Oogilvie, 1968; Jacob, 1968; Rabin, Jenson & Melnick, 1968) clumping, disaggregation and margination of chromatin were readily observed. Chromatin deformities (such as depicted at A and B; Pl. 3a) were in many respects similar to those displayed by lymphoma cells after infection with herpes simplex virus (Bedoya et al. 1968). High magnification of the intra-nuclear H-1 virus suggested ill-defined capsids for the complete virus particles.

Relatively few typical nucleoli such as those observed in control cells, were seen at this stage of infection. Instead, doughnut-shaped structures filled with incomplete virus particles were observed (Pl. 2b). These bodies appeared to be a progressive stage of the nucleoli described in 31 hr samples and we believe that they represented altered nucleoli. The walls of these ‘nucleolar inclusions’ (Bernhard & Granboulan, 1968) were compact and without definitive granules.

It should be noted that a few cells (about 8% of the total number) had depleted nuclei with marginated chromatin (not illustrated) but did not contain virus particles. Jacob (1968) described similar findings for a herpes virus. No nucleoli were discovered in such nuclei; suggesting a possible relationship between early disappearance of the nucleolus and an abortive virus infection.

At 45 hr after infection, the cytoplasm, including the plasma membranes and the mitochondria, was disrupted and partially broken down (Pl. 3a). Mitochondria often showed ill-defined cristae and sometimes dense fibrillar material in their matrix (Bedoya et al. 1968). Unaltered vesicles and cisternae of the endoplasmic reticulum could often still be identified in the cytoplasm.

**NB cells 72 hr after infection**

At this stage virtually all the cells showed nuclear alterations. All of the previously described stages were observed in various nuclei owing to apparent lack of synchrony in infection. At 72 hr after infection, nuclear peripheral chromatin often showed such degeneration that a gap occurred between the nuclear membrane and the chromatin; this region was usually occupied by complete H-1 virus particles (Pl. 3b). H-1 virus and, occasionally, crystals could be seen in the otherwise completely depleted nucleoplasm. Such crystals were possibly virus protein (Dunn & Oogilvie, 1968) or, alternatively, crystals of incomplete virus.

Incomplete virus particles seemed to be less numerous than they were in earlier stages and complete agents became predominant. This finding may be due to the association of ‘empty’ virus capsids within crystalline arrays. The nucleolar inclusions (Pl. 4a) were harder to find than in previous samples and the walls were somewhat dense when observed; otherwise they had the same morphological appearance as in the samples taken 45 hr after infection, including the presence of numerous incomplete virus particles within these inclusions. Nuclear membranes were often disrupted and
virus particles were seen for the first time in the severely damaged cytoplasm. Such virus, however, was observed only within the cisternae of the endoplasmic reticulum (Pl. 3b) and always looked complete.

**NB cells 77 hr after infection**

In this late stage of infection most nuclei were completely devoid of chromatin (Pl. 4b), and virus particles were found in or associated with cisternal fragments of endoplasmic reticulum which were almost the only recognizable component of cytoplasm remaining. The affinity of some viruses for membranous structures has been reported in many systems (Mattern, Takemoto & Daniel, 1966; Bedoya et al. 1968; Murphy et al. 1968). In a few areas, the virus appeared aggregated in paracrystalline array.

**SMH cells after H-1 virus infection**

No morphological alterations were seen in SMH cells 8 hr after infection. At 24 hr, however, detectable changes, as in the NB cells, were confined to the nuclei; the chromatin appeared less dense and the fibrillar components were more evident than in the control nuclei. Nucleoli were slightly swollen and their pars fibrosa somewhat depleted. However, no virus particles were detectable at this stage of infection.

After 31 hr of infection, about 80% of cells showed scattered incomplete viral particles in their depleted nuclei. Cytoplasmic contents were disrupted and mitochondria depleted (Pl. 5a). Again interesting changes were noted in the nucleoli which were dense and, unlike nucleoli of the NB cells, fragmented. Some of the fragments contained numerous incomplete virus particles and resembled the nucleolar inclusions seen in NB cells. The walls of these nucleolar inclusions were devoid of nucleolar granules while those nucleoli which still appeared normal had well defined granules. As many as three nucleolar inclusions (0.6 to 1 μm in diameter) were noted in one nucleus. Forty-five hr after infection and later, nucleolar fragments seemed to disappear except for these inclusions which, as in the NB cells, were seen even after 3 days of infection. Nuclei were more populated with incomplete virus particles than in earlier samples (Pl. 5b). The cytoplasm was severely disrupted and only a few complete virus particles were recognized with certainty, usually within fragments of remaining endoplasmic reticulum. In later stages of infection, the fate of the nuclei, nucleolar inclusions, and the cytoplasm was similar to that described for the NB cells.

It should be particularly noted that almost all the virus particles seen outside the nucleus in both NB and SMH cells were complete and, in general, associated with the endoplasmic reticulum or fragments thereof. In the case of the SMH cells, practically no complete virus was found within the nucleus.

**DISCUSSION**

While the nucleolus is the site of synthesis of ribosomal RNA precursors, the specific role of the fibrillar and granular components in RNA synthesis is not known. The organization and amount of these constituents, however, varies considerably both between various types of normal cells and following treatment by various chemical compounds as well as after virus infections (Bernhard & Granboulan, 1968). In our present study, a series of changes in nucleolar architecture was observed in NB or
Involvement of the nucleolus in human cells

SMH cell cultures after H-I infection. The earliest nucleolar alteration was a depletion of the fibrillar component. In the next stage, the granular region became more compact and the granular components less distinct. In later stages, a continuation of these processes resulted in the appearance of doughnut-like inclusion bodies. The functional significance of these morphological changes is not known.

A heavy concentration of incomplete virus particles was found within the nucleolus at the time when the earliest nucleolar changes were seen. Moreover, in late stages of infection in NB cells, almost the only site where incomplete virus particles (capsids) were found, was within the nucleolar inclusions. Some of these structures, as noted, were intact even 80 hr after infection. This apparently preferential site for accumulation of virus capsid-like material suggests that the nucleolus may play a specific and active role in H-I virus synthesis. It is noteworthy that nucleoli incorporate amino acids though it is not known whether they actually synthesize protein (Bernhard & Granboulan, 1968).

Nucleolar alterations, fragmentation and/or desegregation after virus infection (Stone, Shope & Moore, 1959; Almeida, Howatson & Williams, 1962; Sitori & Bosisio-Bestetti, 1967; Bedoya et al. 1968; Bernhard & Granboulan, 1968; Jacob, 1968; Noyes, 1968) or carcinogenic drugs (Bernhard & Granboulan, 1968; Flickinger, 1968; Reddy & Svoboda, 1968; Stenram & Willen, 1968) have been described in many systems. The functional significance of these structural changes in response to virus or drugs is not clear. In infected NB cells, though the nucleoli were shrunken and formed nucleolar inclusions filled with incomplete virus particles, they remained intact; on the other hand, nucleoli of SMH cells fragmented as early as 31 hr after virus inoculation. Some of these nucleolar fragments formed nucleolar inclusions similar to those seen in NB cells. That nucleoli of NB cells did not disintegrate could have been due to the presence of an SV40 [viral] genome, though it has been reported that SV40 caused nucleolar fragmentation in other cell cultures (Granboulan et al. 1963). It is also possible that, for the same reason, the yield of complete virus was much greater in NB than in SMH cells. Possibly the SV40 genome acted as a helper for H-I virus which in some systems is a defective agent. Ledinko & Toolan (1968), for example, reported that adenovirus is required as a helper for H-I virus in human embryonic lung cells.

The relationship between nucleolus, nucleus and cytoplasm has not as yet been completely elucidated. The rapid disintegration of the cytoplasm in H-I infected cells at about the time of the nucleolar disappearance suggests that changes in nucleolar function may have disturbed the cytoplasmic metabolism.

REFERENCES


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