Nuclear Changes in Cells Infected with Parapoxviruses Stomatitis Papulosa and Orf: an in vivo and in vitro Ultrastructural Study

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SUMMARY

During ultrahistological investigations of naturally occurring cases of stomatitis papulosa in cattle and Orf in sheep, nuclear changes consisting of aggregations of double membrane-containing tubular structures (outer diam. 100 to 130 nm, inner diam. 50 to 65 nm) and filamentous material were observed. These changes could be reproduced in vitro after infection of bovine (BEL) and ovine (OEL) embryonic lung cell cultures with stomatitis papulosa virus and Orf virus isolates. Nuclear tubules were mostly associated with stomatitis papulosa, whereas filaments were regularly detected in Orf virus infections in vivo. Stomatitis papulosa virus also induced nuclear tubules in vitro in the two cell culture types employed, whereas tubular structures after Orf virus infection only developed in ovine embryonic lung cell cultures in addition to filamentous structures. Orf virus infection of BEL cell cultures induced the formation of filaments. Fluorescent antibody staining revealed parapoxvirus-specific antigens only in the cytoplasm of infected cells.

INTRODUCTION

Ultrastructural and biochemical studies of cells infected with poxviruses show in general that virus-induced alterations are mainly associated with the viroplasma zones in the cytoplasm. Since Dales (1973) investigated morphogenesis and maturation in the cytoplasm of poxvirus-infected cells using vaccinia virus as a model, speculations have arisen as to the involvement of the nucleus in these infections. Information on nuclear changes during the poxvirus replication cycle, however, is rare. Intranuclear inclusion bodies were observed light microscopically in cases of naturally occurring stomatitis papulosa (Griesemer & Cole, 1961). Furthermore, ultrastructural studies revealed nuclear morphological alterations in bovine stomatitis papulosa (Okada & Fujimato, 1975) as well as in Molluscum contagiosum (Dourmashkin & Bernhard, 1959; Middelkamp & Munger, 1964). In addition, fine filaments were seen in natural cases of swine pox (Reczko, 1962; Conroy & Meyer, 1971) and Junco pox virus infections (Beaver & Cheatham, 1963).

Recent biochemical studies have confirmed the requirement for the host cell nucleus in poxvirus replication (Hruby et al. 1979a, b). La Colla & Weissbach (1975) found that a significant fraction of vaccinia virus DNA synthesis takes place in the nucleus. Subsequently, Gafford & Randall (1976) detected fowlpox DNA in the nuclei of infected cells and recently this group presented evidence for a 36000 mol. wt. protein induced by fowlpox virus in the...
nucleus (Hardy et al. 1978). However, these results are still subject to controversy since poxvirus replication can take place under certain conditions at a reduced level in enucleated cells (Pennington & Follett, 1974). Furthermore, evidence has been presented recently that host nuclear transcription may not be required for vaccinia virus replication (Silver et al. 1979). Since the situation with respect to nuclear morphological changes is conflicting in poxvirus-infected cells, the nuclear involvement during replication of the parapoxviruses stomatitis papulosa and Orf in naturally occurring cases and in cell cultures was studied using ultra-thin section electron microscopy.

**METHODS**

*Histology.* Routine histology was carried out after fixation in 7% neutral formalin. Paraffin sections were stained with haematoxylin-eosin (HE) and periodic acid-Schiff reagent (PAS).

*Electron microscopy.* Tissues were either obtained from formalin-fixed material collected for histology during necropsy or taken from paraffin-embedded tissue blocks dating from previous years. After removal of paraffin with xylol (Bomhard & Pospischil, 1979) or removal of formalin, the tissues were washed for 4 h in 0.1 M-phosphate buffer, pH 7.2, containing 6.8% sucrose, post-fixed in 1% osmium tetroxide and stained with 0.25% aqueous uranyl acetate. Thin sections were prepared following dehydration in alcohol and embedding in Epon 812 as follows. Infected and control tissue cultures in 25 cm² Falcon plastic bottles were washed and fixed with 2% glutaraldehyde in 0.1 M-phosphate buffer containing sucrose for 15 min and again washed with buffer. Dehydration in alcohol was followed by embedding with Epon. Before total polymerization of the Epon the embedded cultures were removed from the flasks and cut into pieces.

*Virus assay from necropsy material.* Virus isolation was performed after preparation of 10% suspensions from tissues with lesions. The suspensions were centrifuged at 220 g for 30 min and supernates inoculated into secondary bovine and ovine embryonic lung cell cultures. Usually two to four blind passages were carried out with each sample.

*Cell cultures.* Bovine (BEL) and ovine (OEL) embryonic lung cell cultures were prepared according to standard methods (Mayr et al. 1974). Secondary cell cultures were used for all investigations. Culture media consisted of minimal essential medium with Earle's salt solution (EMEM) with the addition of 5% foetal calf serum (FCS) and antibiotics. For virus growth medium, FCS content was reduced to 2%.

*Virus strains.* Stomatitis papulosa virus strains V660 and V619 were passaged in BEL cell cultures after isolation from field cases. The strains were plaque purified three times, and the 12th tissue culture passage was used for all tests. Orf virus strain D1701 had been isolated in OEL cell cultures and passaged in BEL cell cultures. The 137th tissue culture passage of plaque purified material was used. Cells in 25 cm² Falcon plastic bottles were infected with approx. $10^6$ TCID₉₀ in 0.5 ml and the inoculum was allowed to adsorb for 60 min at room temperature. The cultures were washed and fed with 5 ml of EMEM, and then samples were taken for electron microscopy after 12, 16, 24, 36 and 48 h of incubation at 37 °C.

*Fluorescent antibody staining (FA).* BEL cell cultures were grown on coverslips in Leighton tubes and infected with about $10^6$ TCID₉₀ in 0.2 ml of either stomatitis papulosa or Orf virus. The inoculum was allowed to adsorb for 60 min at room temperature. Samples were taken at 6, 9, 12, 14, 18, 24, 36 and 48 h p.i. Harvested cultures were washed once with phosphate buffered saline (PBS) and fixed in acetone at room temperature for 10 min. Direct FA staining was carried out according to standard techniques using a FITC-con-
jugate prepared from convalescent serum of a sheep experimentally infected with Orf virus. The serum had an antibody titre to Orf virus of 1:64 as measured in neutralization tests described elsewhere (Wittek et al. 1980).

RESULTS

Case history

Stomatitis papulosa virus infection

Six animals aged between 3 weeks and 7 months with clinical signs of pneumonia or enteritis and lesions of stomatitis papulosa were investigated. Gross findings were mostly purulent or fibrinous gangrenous pneumonia, catarrhal enteritis and stomatitis papulosa in the oral mucosa and, in one case, also in the oesophagus and rumen. Histologically all lesions showed ballooning degeneration with cytoplasmic basophilic or eosinophilic inclusion bodies. No intranuclear changes could be observed. Virus isolation was attempted in four cases and resulted in the occurrence of cytopathic changes after one to three cell culture passages. All virus isolates were identified as parapoxviruses using neutralization tests and electron microscopy.

Orf virus infection

Eight animals aged from several days up to 12 months, with clinically diagnosed pneumonia and/or enteritis and lesions of papular or erosive stomatitis were investigated. Gross findings consisted mainly of catarrhal to haemorrhagic enteritis and fibrinous pneumonia. Papular or erosive stomatitis appeared around the nostrils, in the gingiva and, in one case, in the mucosa of the oesophagus. The histological changes in lambs with Orf
infection resembled those seen in stomatitis papulosa of cattle. However, cytoplasmic inclusion bodies were less frequent. Virus isolation was attempted in three cases and yielded one isolate. Morphology and neutralization tests showed that the isolate was a typical parapoxvirus.

**Electron microscopy of naturally occurring stomatitis papulosa**

The cells of the epithelial spinous layer were round to oval and attached to each other by many distinct desmosomes. The cytoplasm contained tonofilaments and only very few organelles, such as mitochondria, endoplasmic reticulum, Golgi apparatus and others. Sometimes polyhedral or round aggregates of different electron density were seen near the nucleus or at the periphery of the cells which were slightly larger in size than the nucleus. Mature virus particles were clearly visible in these cytoplasts. They displayed typical pox virus structures with a total length of up to 350 nm and a diam. of approx. 150 nm. Occasionally mature particles were spread all over the cytoplasm and also appeared extracellularly. Nuclei were round to oval and showed a clear hyperchromasia. Two different morphological alterations could be revealed in the nucleoplasm of infected cells. In five cases an accumulation of tubule-like structures (outer diam. 100 to 130 nm, inner diam. 50 to 65 nm) could be detected. Transverse sections reached a length of 250 nm. Some of these tubules were like horse-shoes (Fig. 1). The walls of these structures seem to consist of a double membrane. These tubules could only be detected in cells containing mature virus particles. In one case most of the central nuclear plasma was filled with bundles of filaments sometimes arranged like whirls (Fig. 2). No tubule-like structures could be found. Filamentous intranuclear structures did not appear in uninfected cells.
Nuclear changes in parapoxvirus-infected cells

Electron microscopy of naturally occurring Orf virus infections

The cytoplasm of the superficial stratified epithelial cells lacked most of the organelles usually found. Aggregates of mature and immature poxvirus particles comparable to those in stomatitis papulosa could be detected (Fig. 3). In many cells, mature particles, sometimes enclosed in a fine net of filaments, were spread all over the cytoplasm. Complete virions also appeared extracellularly.

Nuclei were mostly polymorphic. They showed signs of advanced destruction ranging from margination of chromatin to karyorrhexis. In infected cells which contained both mature and immature virus particles, hyperchromatic nuclei displayed a central, less electron-dense zone, filled with fine filaments arranged singly or in bundles (Fig. 3).

Electron microscopy of infected cell cultures

Bovine embryonic lung cells (BEL)

The first ultrastructural changes following stomatitis papulosa virus infection could be observed as viroplasmic factories in the cytoplasm. They contained immature particles formed of trilaminar structures at about 12 h.p.i. These particles were round with a diam. of 280 to 320 nm. The 'core' (diam. 200 nm) was surrounded by an electron-lucent space of 40 to 60 nm. Mature virions appeared at 16 h.p.i. and from then on mature and immature particles were present at the same time.

During virus replication in the cytoplasm, the nuclear chromatin started to condense near the nuclear membrane. At 16 h.p.i. nuclei of infected cells contained tubular structures resembling those in natural infections (Table 1, Fig. 4). Their occurrence was related to the appearance of complete virions which increased in number during the investigation period, sometimes filling the whole central part of the nucleus.
Table I. Formation of intranuclear tubules in BEL and OEL cell cultures infected with stomatitis papulosa and Orf virus at different times post infection*

<table>
<thead>
<tr>
<th>Time p.i. (h)</th>
<th>Stomatitis papulosa virus</th>
<th>Orf virus</th>
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<td>BEL</td>
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<td>48</td>
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* Tubule formation indicated by: —, none; +, little; ++, moderate; ++++, massive.

Fig. 4. Intranuclear tubules (arrow) appearing 16 h p.i. in BEL cells infected with stomatitis papulosa virus.

Orf virus replication in BEL cell cultures was accompanied by the morphological characteristics described for stomatitis papulosa virus. At 12 h p.i. infected cells already contained mature virions. From 36 h p.i. onwards, an accumulation of fine filamentous material appeared in the central part of the nucleus simultaneously with the margination of chromatin (Fig. 5). No relationship with time was observed for the formation of mature virions. At no time during the investigation period did nuclei of Orf virus-embryonic infected BEL cell cultures contain tubules.

Ovine foetal lung cell cultures (OEL)

Stomatitis papulosa virus replication in this cell type was comparable to that in BEL cell cultures. Complete virions were seen at 18 to 20 h p.i. At the same time nuclei of infected cells contained tubules similar to those seen in vivo and in BEL cell cultures. Again, the formation of tubules was related to the appearance of mature virions.

Morphological damage during replication of Orf virus strain 1701 in OEL cells was also comparable to that in BEL cell cultures. Complete virions were seen as early as 16 h p.i.
Nuclear changes in parapoxvirus-infected cells

Two different morphological alterations could be seen in the nucleoplasm of infected cells beginning around 36 h p.i.: (1) an accumulation of tubules comparable to those of stomatitis papulosa virus; (2) the formation of fine filaments as seen in Orf virus-infected BEL cell cultures. Both structures appeared at the same time after infection but did not show a dependence on the development of mature virus particles.

Fluorescent antibody staining of infected cells

Virus replication led to the appearance of c.p.e. in infected BEL and OEL cell cultures. With Orf virus D1701 c.p.e. began at around 12 h p.i. and progressed rapidly. By 48 h p.i. cells were mostly destroyed. First signs of c.p.e. were seen in stomatitis papulosa virus-infected cells at 16 h p.i. regardless of the strain used, and cell destruction reached 60 to 70% in both cell culture types at 48 h p.i. The first cells showing specific cytoplasmic fluorescence in Orf virus-infected cultures were detected at 10 h p.i. and increased gradually up to 20 h when about 70 to 80% of all cells were positive. A similar pattern was observed in stomatitis papulosa virus-infected cells although the first positive cells were seen at 14 h p.i. and the number of positive cells reached a maximum at 24 h.

The early fluorescence was granular; at later stages brightly fluorescing, inclusion-like patches were noticed in the cytoplasm. At no time after infection was nuclear fluorescence demonstrable in either cell type with any of the virus strains used.

DISCUSSION

Most of the results from ultrastructural investigations of parapoxvirus infections lack evidence for the morphological involvement of the nucleus during virus replication. In experimental infections of calves with stomatitis papulosa virus, Reczko (1957) demonstrated only cytoplasmic alterations. Similar changes were described by Knocke (1962) and
Kluge et al. (1972) after experimental infections of lambs with Orf virus. In addition, neither in cell cultures of bovine origin infected with stomatitis papulosa virus (Büttner et al. 1964) nor of bovine, simian or ovine origin infected with Orf virus (Knocke, 1962; Schulze & Schmidt, 1963; Büttner et al. 1964; Nagington et al. 1964; Kluge et al. 1972; Kim et al. 1977) were nuclear alterations detected. These results are in contrast to those described here and, in fact, there is only one report, published by Okada & Fujimato (1975), that describes tubular structures, in nuclei of infected cells in a naturally occurring case of stomatitis papulosa in cattle, which are similar to the nuclear tubules found in this study. Since, in five out of six cases, tubules could be demonstrated in stomatitis papulosa of cattle and, in six cases out of eight, filaments were demonstrated in Orf infection in sheep, it is believed that morphological changes in the nucleus of cells infected with these viruses under natural conditions are a regular occurrence. This statement is confirmed by the reproduction of the same nuclear alterations in bovine and ovine embryonic lung cell cultures in vitro with plaque purified parapoxvirus strains that were isolated from the natural cases under investigation.

At present there is no explanation of the apparent discrepancy with respect to the in vivo results of the other investigators. The different findings in vitro, however, could be due to the different cell culture types employed in the various studies. A dependence of nuclear changes upon the cell–virus system used has also been described in influenza virus infections (Saito et al. 1970). Our results indicate that the formation of nuclear tubules – at least with Orf–virus – appears to be dependent upon the cell type used for in vitro virus replication. Furthermore the lack of tubules in Orf virus-infected BEL cell cultures does not influence virus replication or maturation of infectious virus.

There may be some variation in the possible nuclear alterations since in Orf virus-infected BEL cultures, nuclear fine filaments were a regular finding, in contrast to stomatitis papulosa virus-infected BEL cells. These nuclear filaments are comparable to those seen in swine poxvirus (Reczko, 1962; Conroy & Meyer, 1971; Kim et al. 1977) and Junco poxvirus (Beaver & Cheatham, 1963) infections and could resemble part of the nuclear protein matrix (Wunderlich, 1978).

Function, origin and nature of the demonstrated tubules and filaments are, however, not known. They seem to be unrelated antigenically to parapoxvirions, as could be shown by fluorescent antibody studies with infected cell cultures using a hyperimmune serum produced in sheep against the whole virion. Therefore, the conclusion that these tubules and filaments are a by-product of virus replication would be feasible. It is well known that during infection with strictly cytoplasmic viruses, defined nuclear structures sometimes appear. They are composed of an overproduced component which, because it occurs in sufficient concentration, becomes self-assembled within the nucleus. Nuclear changes and structures have been reported, for instance, during the replication of influenza viruses (Anisimova et al. 1973) of parainfluenza-virus-3 (Reczko & Bögel, 1962) and possibly for other paramyxoviruses (Bachmann et al. 1979). A similar phenomenon may explain the current data. Further studies are required to show what role these morphological alterations play in the replication cycle of parapoxviruses.

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REFERENCES

Nuclear changes in parapoxvirus-infected cells


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