Replication characteristics and core size of intranuclear herpes simplex virus (HSV-1) in genital skin lesions: electronmicroscopy studies of a biopsy from a female patient

J. BODDINGIUS, H. DIJKMAN, W. van der MEIJDEN,* P. SCHMITZ,† T. van JOOST,* and E. STOLZ*

Department of Dermato-Venereology Research Laboratory, the Department of Dermato-Venereology of the Academic Hospital Dijkzigt, and †The Institute of Biostatistics, Medical Faculty, Erasmus University, P.O. Box 1738, 3000 DR Rotterdam, The Netherlands*

Summary. Herpes simplex virus (HSV) type 1 genital infection, leading to ulcerating lesions in a female patient, was studied by electronmicroscopy. Infection had probably been recent, through oro-genital contact with a cold sore on the husband's lip. Cell-culture typing and serological tests indicated that the patient currently had an HSV-1 secondary infection. Aspects studied in a skin biopsy from an ulcerating labium majus were epidermal cell types infected, stages in virus genesis, virus core diameter in intranuclear capsids and extracellular appearance of virus. Different stages in virus genesis, in virus envelope formation and in nuclear and cytoplasmic degeneration were observed in the few remaining, rounded and swollen, epidermal (?) spinosum cells. Their nuclei, some with marginated chromatin, harboured besides dense-cored or empty capsids, electron-dense blobs possibly representing clones of immature virus and falling apart into aggregates of small granules. In other nuclei, large clusters of dense-cored capsids, some distinctly hexagonal in shape, had accumulated in wide gaps in the nuclear membrane whereas remaining nuclear membrane portions were quadruple and often engaged in viral envelope formation. Partially enveloped capsids and naked dense-cored capsids were seen extracellularly indicating their survival outside cells. An occasional virion was present in dermal blood vessel lumina. Measurements of the electron-dense core (nucleoid) of intranuclear capsids in electronmicrographs showed that the HSV-1 core diameter differs very significantly from the core of intranuclear HSV-2 capsids, thus allowing a clear distinction by electronmicroscopy between the two HSV subtypes in plastic-embedded biopsies.

Introduction

Herpes simplex virus type 1 (HSV-1), a karyo-tropic dermato-tropic and facultatively neurotropic DNA virus, causes recurrent papular and vesicular eruptive lesions on epithelia at mucocutaneous borders and on the oral mucosa, whereby lips, oropharynx, eyes or the face are involved. Rarely, the virus causes encephalitis.

In the last decade, genital HSV-1 lesions have been reported. In females, the infection is considered to originate from oro-genital contact with partners who have oral HSV-1 lesions (Evrard, 1974; Whitney *et al.*, 1978), to be of unknown origin

but acquired after sexual intercourse (Kawana *et al.*, 1982; Kit *et al.*, 1983) or to occur in virgins whence diagnosed as progenital herpes (Ishiguro *et al.*, 1982). There are reports of genital HSV-1 in females and males (Kawana *et al.*, 1976; Smith *et al.*, 1976; Reeves *et al.*, 1981; Peutherer *et al.*, 1982), of the simultaneous presence of HSV-1 and HSV-2 in the human body (Maekawa *et al.*, 1981), and of the occurrence of sequential HSV-1 and HSV-2 infections in genital organs (Kit *et al.*, 1983). The rate of recurrent genital infections seems to be lower for HSV-1 than for HSV-2 (Reeves *et al.*, 1981). Type-specific drugs have been developed (DeClercq *et al.*, 1979, 1980).

There have been electron-optical investigations of HSV-1 and of its mutants in cell cultures (Epstein,

1962; Ciampor and Szanto, 1982; Dargan and Subak-Sharpe, 1983; Addison *et al.*, 1984) and of purified nucleocapsids by immuno-electronmicroscopy (Vernon *et al.*, 1981). There have been no electronmicroscopy studies on HSV-1 capsids and virions in biopsies of genital lesions.

In the ultrastructural work presented here, different microscopically-detectable intranuclear structures reminiscent of various stages in virus genesis were investigated as well as features of virus envelope formation, dimensions of the virus core, and the location of virus or virions extracellularly in the epidermis or dermis in acute, ulcerating, genital HSV-1 skin lesions in a female patient.

Patient and methods

Clinical details

A female patient (white European, 27 y, married, housewife) had suffered for 6 days from increasing vulval pain, particularly during micturition, washing and on touching. There was no previous history of genital disorders or of virus diseases other than verrucae plane on the left arm 5 y before. Recent treatment by her general practitioner had included miconazole ointment and oral doxycycline and metronidazole which had been unsuccessful. Examination revealed extensive ulcerations on the labia majora and minora (fig. 1), perineum and peri-anal skin. Inguinal lymph glands were enlarged and tender bilaterally.

A preliminary diagnosis of herpes genitalis was made, based on clinical manifestations, a probable source of recent infection (the patient's husband who did not suffer from genital disease but had a cold sore on his lip and had had oro-genital contact with his wife in the week before her lesions had appeared; when the patient was admitted, a crusted lesion still could be seen on the husband's lip), and dark-field examination of vulval smears and, later, vulval cultures and serological tests, results of which were negative for *Treponema pallidum*, gonococci and other sexually-transmitted organisms.

Samples for vulval HSV cultures, for the Tzanck test on a vulval ulcer, and blood for serological HSV tests were taken on the first day of admission. Vulval cultures revealed HSV-1 (Virological Laboratory, Municipal Health Department, Rotterdam). The Tzanck test showed atypical cells (but insufficient indications of malignancy), reactive changes (i.e., slight dysplasia due to inflammation) and a vaginal flora consisting predominantly of the virus. Serum sampled 6 days after the onset of symptoms (i.e., c. 8–13 days after genital infection with the virus, the incubation time of HSV-1 being 2–7 days) was tested. No specific IgM was found in the serum (Virological Laboratory, Municipal Health Department, Rotterdam). The final clinical diagnosis, therefore, was secondary HSV-1 infection. Previous (subclinical) genital infection from a cold sore of the husband is unlikely, and

Fig. 1. Ulcerating genital skin lesion of a 27-y-old female with a one-week history of HSV-1 infection probably contracted by oro-genital contact.

we surmise that primary infection may have occurred in childhood.

Under local anaesthesia, a skin biopsy was taken from an ulcerating lesion on a labium majus on the day of admission. The biopsy included clinically normal skin.

Electronmicroscopy

Within seconds of its excision, the skin biopsy was placed in a drop of GA/SPB fixative (see below) on a dental wax plate and divided with a fresh razor blade into four segments. After further fixation for 2 h in glutaraldehyde (GA) 3% in sucrose (5%) phosphate buffer (SPB), pH 7.25, on ice, the segments were rinsed in SPB overnight, postfixed for 2 h in OsO₄ 1% in SPB, dehydrated in graded alcohols (increasing with steps of 10%, 10 min each) contrasted in block for 45 min with uranylacetate (saturated solution in absolute alcohol) and transferred via epoxypropane to Araldite.

Semi-thin (1 µm) sections, stained with toluidin blue and basic fuchsin, were examined and photographed with a Leitz Ortholux microscope fitted with an Orthomat camera. Ultrathin sections, contrasted with uranylacetate

and Reynold's lead citrate, were examined with a Philips EM 300 electronmicroscope.

Measurements of capsid and core diameters were made on electronmicrographs taken at the highest feasible microscope magnification. For the calculation of exact micrograph magnifications, a reference grid was photographed (Fulham 1002 $\times$ shadow cast crossed lines carbon grating replica; 2160 lines/mm).

Results

Clinically normal skin. In the papillary dermis there was massive cellular infiltration. In the epidermis there were intracellular vacuoles and some infiltrating cells in the basal layer. Widened intercellular spaces with abounding desmosomal fragments and aggregates, possibly of ribosomes, were seen in the stratum spinosum. Neither capsids nor virions were seen.

Ulcerating skin. There was complete destruction of the normal epidermal architecture, with extensive necrosis. Only a few stratum corneum cells remained apically, and rounded, possibly spinosum, cells described in detail below were dispersed amongst abounding cellular debris that included thick curved bundles of tonofilaments (fig. 2a). Lymphocytes were amongst the many infiltrating cells.

The rounded and swollen cells mentioned above with ballooning nuclei (fig. 2 a, b, c) showed nuclear and cytoplasmic changes connected with virus infection and propagation.

By electronmicroscopy, marginated chromatin was seen in some of the nuclei with electron-dense round inclusions, aggregates of small granules, agglomerations of larger granules and dispersed virus capsids (fig. 3a). The disintegrating cytoplasm lacked tonofilaments. In other, larger, more distinctly ballooning nuclei, chromatin was not marginated and there were clustered and dispersed capsids. Nuclear membranes locally were reduplicated and occasionally involved in virus envelope formation, and a thick band of tonofilaments closely surrounded the nucleus (figs. 3b, 4, 5).

In more detail, the nuclear and cytoplasmic changes connected with virus propagation were as follows:

(1) Margination of chromatin (fig. 3a) in nuclei lacking clearly outlined or reduplicated membranes.

(2) Electron-dense round inclusions or blobs (figs 3a, b, 4a) measuring 0.35–0.65 micron in diameter in varying numbers and sometimes quite evenly spread, as if in "strategic positions", throughout the nucleoplasm (fig. 4a). On their surface, the inclusions often had a corona of small blebs and

nearby were rounded structures with a clear centre and a rim of radially arranged short arrays (fig. 3b) also seen in fully grown dense-cored virus capsids (figs. 4c, 5b). Some inclusions were falling apart into granular structures (fig. 3a, stage 2).

(3) Small granules 22–30 nm in diameter (fig. 3a, stage 3) besides small aggregates of larger granules 40 nm, in diameter (fig. 3a, stage 4), particularly in blob-containing nuclei with marginated chromatin.

(4) Virus capsids in the nucleoplasm with a round electron-dense core (figs 3b, 4c) and sometimes an electron-translucent centre (fig. 5b). The virus capsids were often hexagonal (fig. 4c). There were some empty vesicles of capsid size (figs 4c, 5b). Measurements of the diameter of round electron-dense cores in intranuclear capsids were made on photomicrographs of cores taken at the highest feasible electronmicroscope magnification. The frequency distribution of the diameters is illustrated in fig. 6. The average diameter of the HSV-1 core was 39.8 nm (SD σ = 3.5473; n = 100). The diameter of the virus capsid proper averaged 78 nm (SD σ = 5.5939; n = 100; frequency distribution shown in fig. 7).

(5) Nuclear membranes reduplicated in ballooning nuclei. Three phases of what we consider to be capsid passage through, and envelope formation by, the inner nuclear membrane were seen. (i) Nidation of the capsid in the inner double nuclear membrane. At the place of contact the membrane had become electron-dense and thickened and represented now the future inner coat of the virus envelope (fig. 4c, stage 1). A virus core is not detectable in the figure. (ii) Closure of the inner nuclear membrane above the enveloped capsid (fig. 4c, stage 2). (iii) The capsid "floated" in the space between the (double) nuclear membranes, the electron-dense inner coat was broadened, the virus core was very electron-dense (fig. 4c, stage 3). Fine structures radiated from the core and extended into the inner coat. Envelope formation not being a frequent phenomenon in our preparations, only a few partly enveloped virus particles were seen in the cytoplasm (fig. 4b). Naked dense-cored capsids or empty vesicles often embedded in tonofilaments were seen more often (fig. 4c).

(6) Wide gaps in the nuclear membrane and clusters of naked virus capsids (fig. 4a) barred off by a thick zone of tonofilaments from the cytoplasm. Rounded intact or flocculent portions of electron-dense blobs (above) were also present in or near these gaps (fig. 3b). When gaps were very wide, large clusters of naked capsids were seen "hanging" from the nucleus like bunches of grapes from a vine tree (fig. 5a).

Fig. 2. (a). Epidermis of ulcerating lesion on a labium majus. Note apically a few stratum corneum cells (*sc*) and, amongst numerous inflammatory cells, some dispersed rounded, possibly spinosum cells (*arrows*) which are enlarged in (b) and (c). Bar = 20 μ m. Semithin section; toluidin blue—basic fuchsin staining. (b) Two rounded cells (*thick arrows* 1,2) with translucent nuclei lined by a thick nuclear membrane. Note dark dots, also depicted by electronmicroscopy in figs. 3 and 4, in the nucleoplasm. Bar = 10 μ m. Semithin section. (c) High magnification of cell 1 (b). The translucent nucleus (*n*) is lined by a very thick (reduplicated) nuclear membrane (*arrow*). Bar = 3 μ m. Semithin section.

Fig. 3. (a) Virus propagation in nucleus with peripheral chromatin (*pc*). Possible stages in capsid formation: (1) electron-dense inclusion or blob (*bl*) possibly representing clones of immature virus; (2) falling apart of blobs; (3) segregation into individual 22–30-nm granules; (4) growth into 40-nm granules. Note mature naked dense-cored capsids (*d*). Bar = 0.2 μ m. (b) Electron-dense inclusion or blob (*bl*)—in the centre of the blob packed rounded structures; at the periphery a corona of small blebs (*dark arrow*); nearby, rounded faint structures (*open arrow*) possessing a radially striped outer zone. Note, at (*d*), a cluster of dense-cored virus capsids. Bar = 0.3 μ m.

Fig. 4. (a) Electron-translucent nucleus (*N*) harbouring electron-dense blobs (*bl*) and clusters of HSV-1 dense-cored and some empty capsids near or in gaps (*g*) in the nuclear membrane. Reduplicated nuclear membrane portions with capsid nidation and virus envelope formation are indicated by *arrows*. Note tonofilament bands (*t*) closely surrounding the nucleus. Bar = 0.5 μ m. (b) Partly enveloped virus in the cytoplasm (*arrows*) close to nucleus (*N*). Bar = 0.1 μ m. (c) Area $\longleftrightarrow$ in (a) shown in high magnification. Three possible stages (1, 2, 3) of virus envelope formation by the inner of the reduplicated nuclear membranes are depicted. Note hexagonal dense-cored capsid (*d*) and empty vesicles (*v*). Naked dense-cored capsids ($\leftarrow$) and empty vesicles are also present in perinuclear tonofilament bands. Bar = 0.1 μ m.

Fig. 5. (a) Clusters of dense-cored capsids (*d*) occurring in or protruding through nucleus (*N*), like bunches of grapes, into the cytoplasm. Note fragments of nuclear membrane (*arrow*) and ragged remainders of blobs. Bar = 0.5 μ m. (b) Area outlined to the lower right in (a), at higher electronmicroscope magnification showing clusters of HSV-1, generally with an electron-dense core (*d*), a few empty vesicles (*v*) and ragged remainders of blobs. Note the radially arranged zone around the core, the hexagonal shape of diameter of HSV-1 capsids located within nuclei of epidermal

Bar = 0.1 μ m.

Fig. 6. Frequency distribution (in nm) of the core (nucleoid) diameter of HSV-1 capsids located within nuclei of epidermal cells. Class width 2 nm. Mean value of the core diameter = 39.8 nm.

(7) Indistinctness of the cell membrane and its possible contribution to envelope formation. Extracellular virus particles, some possessing part of a virus envelope, were embedded in tonofilament bundles. More often, though, large clusters of naked capsids were found amongst cell debris.

(8) Absence of true virions in the ulcerating epidermis but virion-like inclusions intraluminally in dermal blood vessels (fig. 5c).

(9) Abnormal dermal nerves, particularly their myelinated fibres, without virions or capsids in them.

(10) Blood vessels in the dermis underlying the ulcerating epidermis showing characteristic features of inflammation (Boddington *et al.*, 1980), i.e., luminal and abluminal endothelial protrusions, fenestrations in or local disappearance of the endothelium with only the basal lamina remaining, and nuclear and cytoplasmic debris intraluminally.

Fig. 7. Frequency distribution (in nm) of the diameter of whole HSV-1 capsids in nuclei of epidermal cells. Class width 2 nm. Mean value of the capsid diameter = 78 nm.

Discussion

Studies on HSV infections are of importance because, in the last decade, possible associations have been revealed between HSV-1 infection and the occurrence of oral cancer (Shillitoe *et al.*, 1983, 1984; Hirsch *et al.*, 1984; Scully and Ward-Booth, 1984), brain tumours (Hadfield *et al.*, 1984), Kaposi's sarcoma (Dotz and Berman, 1983), myelogenous leukaemia or other haematological malignancies (Lam *et al.*, 1981), hairy cell leukaemia (Pozner *et al.*, 1978), Burkitt's lymphoma (Stewart *et al.*, 1965; Vernon *et al.*, 1981) and histiocytic lymphoma (Salomon *et al.*, 1982). Detection of virus antigen production can aid in monitoring mutations in cell metabolism (Campisi *et al.*, 1983; Mayman and Nishioka, 1985).

The only study of fresh biopsies of HSV lesions by light microscopy and electronmicroscopy concerned herpes oesophagitis (Bürrig *et al.*, 1984). Their illustrations and data on the virus core diameter (38 nm) indicate an HSV-1 infection. Genital HSV-1 lesions have not hitherto been investigated by electronmicroscopy.

Our present studies have shown by electronmicroscopy that the rounded epidermal cells with swollen translucent and slightly dotted nuclei seen by light microscopy were the main virus-containing cells in ulcerating genital skin. Two kinds of virus-containing nuclei were seen. We believe that nuclei possessing marginated chromatin, blobs and granules represent an earlier phase of virus propagation, subsequently "frozen" by necrotic processes, than do the ballooning nuclei with reduplicated membrane fragments in which virus propagation and envelope formation have proceeded further. The dots (above) in the nucleus were round electron-dense inclusions (0.35–0.65 μm) probably the same as the spherical bodies seen intranuclearly in wild HSV-1 infected tissue-culture cells by Dargan and Subak-Sharpe (1983) and held by them to be fragments of disorganised nucleolus; an explanation that does not fit the inclusions which we have seen. They initially were round and were quite uniformly distributed throughout the nucleus; they were located close to small 22–30 nm granules and seemed to loosen up into such granules, particularly in nuclei with marginated chromatin. Therefore, we believe that the blobs represent an early stage in the propagation of HSV-1 capsids, in the process of which some flocculent residual material remains and is extruded, together with some of the blobs, through wide gaps in the nuclear membrane into the cytoplasm or extracellularly.

The 22–30-nm granule aggregates, possibly coun-

terparts of 20-m μ (nm) aggregates described by Morgan *et al.* (1959), were found in the same area as the large electron-dense blobs (above). Because some of the virus capsids in the same nucleus had a 20-nm core, the 22–30-nm granules may represent a stage, in virus genesis, following that of the electron-dense blobs and preceding true virus capsid formation. Morgan *et al.* (1959) considered that the aggregates of granules represented templates for the sequential formation, at several foci, of virus. The 40-nm granules seen by us might represent another intermediate stage. If this is so, it suggests that before attaining a virus “membrane”, the core occasionally may increase further in size.

We suggest that virus escaped from the cell nucleus in the lesion examined, in two main ways. The first was by transmembranous passage through locally reduplicated nuclear membranes, with, in many cases, formation of part of the virus envelope from the inner nuclear membrane and by an increase in electron density, i.e., by maturation of the core (see also Toshima *et al.*, 1967). The core in this stage (fig. 4, stage 3) resembles that depicted by Nahmias and Roizman (1973, fig. 1). The presence of naked virus capsids between the nuclear membranes and in the perinuclear tonofilament bands indicates that naked capsids may gain access to the cytoplasm without much involving or disrupting the nuclear membrane. Such viruses remain enclosed in tonofilament bands after disintegration of the nucleus and most of the cytoplasm in infected cells. They are seen as naked or partly enveloped capsids in the extracellular bands.

The second was through wide gaps in the nuclear membrane. Clusters of virus may leave in this way, possibly because the thick tonofilament band closely surrounding the nucleus prevents gradual access of single virus particles to the cytoplasm. The clusters may correspond to the “dissolving viral crystals” seen by Morgan *et al.* (1959). The tonofilament alterations seen in infected cells may reflect cytoskeletal changes underlying cytopathic effects and aiding in virus dissemination (Krempien *et al.*, 1984).

Completion of virus envelope formation by nuclear or cell membrane (Toshima *et al.*, 1967) was not observed in our preparations. Possibly because of the absence of intact cell membranes, fully enveloped infective virions seemed no longer to be formed extensively during the phase of skin ulceration examined but, earlier, this may have been a feature, a pattern suggested by finding virions in the lumina of dermal blood vessels. Cell-culture studies have suggested that virus capsids

not surrounded by envelopes are unstable and are destroyed in the cytoplasm (Morgan *et al.*, 1959). However, naked capsids seen extranuclearly in the cytoplasm or extracellularly in the lesion examined by us seemed stable. According to Nahmias and Roizman (1973), a complete envelope is not necessary for infectivity.

Electronmicroscopy studies of the structure of HSV have been of untyped HSV in cell cultures (Morgan *et al.*, 1959; Epstein, 1962) and in smears (Smith and Coleman, 1983), HSV-1 purified nucleocapsids by immuno-electronmicroscopy to identify specific polypeptides (Vernon *et al.*, 1981), and HSV type-1 strains, including wild type and temperature-sensitive (ts) mutants, in infected cell cultures (Dargan and Subak-Sharpe, 1983; Addison *et al.*, 1984). Illustrations of intranuclear HSV-1 by Toshima *et al.* (1967), Dargan and Subak-Sharpe (1983) and Addison *et al.* (1984) are similar to pictures of intranuclear HSV in our work. This similarity in structure, and subtyping by cell culture, confirms the identity of our virus as HSV-1. In electronmicrographs by Toshima *et al.* (1967) of cell lines from lymphoma of the liver in patients with Burkitt's lymphoma, the intranuclear capsids are similar to the capsids seen in our biopsy, i.e., slightly hexagonal capsids with a clear centre (figs 4c and 5b).

Few data on the dimensions of the virus core and of the whole virus capsid of HSV-1 have been published. Epstein (1962) gave a core (nucleoid) diameter of 35 m μ (nm) for HSV (untyped) in osmium methacrylate preparations. Morgan *et al.* (1959) found that the core of virus capsids within one nucleus varied in size, shape and density; the core dimensions were in the range 20–40 m μ (nm), some scattered virus having the largest core with a

Fig. 8. Comparison of the core diameter of HSV-1 and HSV-2 intranuclear capsids in skin biopsies fixed and processed for plastic embedding by identical methods. Note that the HSV-1 core is significantly larger than the HSV-2 core.

central region of diminished density. This is in full agreement with the frequency distribution that we found for the HSV-1 core diameter (fig. 6).

For measurements made by us on electronmicrographs taken at the highest possible magnification, the mean value for the diameter of the intranuclear virus capsid was 78 nm and of the core 39.8 nm. In concurrent studies, the HSV-1 core diameter was found to differ very significantly ($p \leq 0.001$) from

the core diameter of intranuclear HSV-2 in identically fixed and embedded HSV-2 genital lesions (Boddingius *et al.*, unpublished observation). The present finding of a significant difference in core diameter between HSV-1 and HSV-2 (fig. 8), which we have already reported (Boddingius *et al.*, 1984), indicates that a clear distinction can be made between the two HSV subtypes by electronmicroscopy of plastic-embedded biopsies.

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