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Ultrastructural and immunohistochemical findings in oral hairy leukoplakia*

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Summary. Three cases of HL from the lateral border of the tongue of male homosexual AIDS patients were investigated by thin section electron microscopy. Keratinocytes contained condensed chromatin in their pyknotic nuclei and a few organelles in the oedematous cytoplasm. Chromatin was in close association to the nuclear membrane and showed a punched-out appearance. Particles typical of the herpes virus group were abundant in the upper two thirds of the epithelium in all three cases. Virus particles were seen frequently in the nuclei of the ballooned keratinocytes, but rarely in cells containing Candida albicans. Viral nucleocapsids were observed budding at the inner nuclear membrane, thereby acquiring the prospective viral envelope. Complete, enveloped virions were found in the endoplasmic reticulum and in the extracellular space. These virions were identified immunohistochemically as Epstein-Barr virus (EBV) using two monoclonal antibodies directed against EBV capsid and membrane antigen, respectively. Candida albicans was observed in the stratum corneum and in the upper layer of the stratum spinosum. Special cytoplasmic tubular structures arranged in parallel bundles were found in koilocytotic cells in addition to characteristic membrane structures composed of undulating convoluted membranes. Epithelial basement membranes were always intact.

Key words: Hairy leukoplakia – Electron microscopy – Epstein-Barr virus – Candida albicans

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Introduction

Oral hairy leukoplakia (HL), a new clinical entity, was first described by Greenspan et al. (1984) as pathognomonic for the HIV-infection. HL is characterized by whitish plaques at the lateral border of the tongue which cannot be rubbed off. The lesion, mainly observed in HIV-infected homosexual males, was also reported in heterosexual HIV-infected individuals, haemophiliacs, a transfusion recipient (Greenspan et al. 1986; Rindum et al. 1987), and intra-venous (iv) drug abusers (Reichart et al. 1986, 1987). A survival analysis showed that the probability of developing AIDS in patients with HL was 40% during 16 months and 83% during 31 months (Greenspan et al. 1987).

Light microscopic investigations (Greenspan et al. 1984; Greenspan et al. 1985; Eversole et al. 1986) indicated, that the lesion is characterized by parakeratosis, acanthosis, koilocytosis and lack of inflammation in the subepithelial tissue. Using electron microscopy (EM) particles of the herpes virus group were found in HL (Greenspan et al. 1984, 1985; Belton and Eversole 1986; Konrad 1986). Immunofluorescence studies demonstrated Epstein-Barr virus (EBV) antigen, and a nucleic acid-hybridization procedure revealed EBV viral DNA in a high copy number (Greenspan et al. 1985; Löning et al. 1987).

Purpose of the present study was to describe ultrastructural features of HL and to identify the virion particles using monoclonal antibodies against EBV core and membrane antigens.

Materials and methods

From July 1984 to July 1987 among 195 HIV seropositive individuals a total of 61 patients (male $n=59$, female $n=2$; homo-/

Fig. 1. HL on the lateral border of the tongue with whitish tightly adhering plaques

bisexual $n=55$, i.v. drug abusers $n=5$, blood transfusion recipient $n=1$) with the clinical diagnosis of HL have been observed. The average age of HL patients was 34.2 years. At the time of serodiagnosis four HIV positive individuals were clinically symptomless, 18 patients showed ARC-, and 39 patients AIDS symptoms. For the present study biopsies of HL were obtained from 3 male homosexual patients (age 39, 46 and 83 years), showing different AIDS manifestations (multiple Kaposi sarcoma: $n=1$, opportunistic infections: $n=2$). All three patients died within 2.8 months after biopsy. Biopsies were taken from the lateral margin of the tongue (Fig. 1) under local anaesthesia (Ultracain DS^R). Prior to biopsy all three patients had been treated for two weeks with topical (Mikonazole), two patients additionally with systemic antimycotic therapy (Ketokonazole).

Tissues were divided and one part was fixed in 2.5% glutaraldehyde in PBS for 3 hours. After washing with PBS specimens were postfixed in 1% OsO_4 for 1 h at 4° C, and afterwards treated for 1 h at room temperature within 1% uranyl acetate. After dehydration in a graded series of ethanol, specimens were infiltrated by 3 changes of propylene oxide and embedded in Epon 812 following routine techniques (Gelderblom et al. 1974). Semithin sections (0.5–1 μm in thickness of each specimen were cut, stained with toluidine blue, and evaluated light microscopically for pathognomonic changes. After finding suspective areas in light microscopy, ultrathin sections (40–60 nm) were cut. These were mounted on bare grids, poststained with lead citrate, stabilized with carbon and examined using a Zeiss EM 10A at 60 kV.

The other part of the biopsy was fixed in dimethylsuberimidate (DMS, Hassel and Hand, 1974) for 2 h, washed in PBS and infiltrated stepwise with 5%, 10%, 1.2 M and 2.3 M sucrose in PBS. Biopsies were deep frozen in liquid nitrogen. Semithin cryosections of 0.5 μm were cut using the Reichert FC4 cryo attachment (Reichert AG, Wien). Sections were incubated with monoclonal antibodies directed against EBV capsid (VCA) and membrane antigen (MA) (dilution 1:1000 in PBS). Immunobinding was visualized using a modification of the alkaline-phosphatase-mouse-anti-alkaline-phosphatase technique (APAAP; Becker et al. 1987). Controls included the use of second and third step antibodies, as well as normal tissues of HIV seronegative persons.

Results

Common features in all cases were hyperkeratosis, parakeratosis and acanthosis of epithelium. Above the basal cell layer an increased degree of intracellular oedema of spinous cells was noted. The ballooned keratinocytes often lost their chromatin pattern and sometimes basophilic intranuclear inclusions were observed. Candida albicans was found within epithelial cells of the superficial layers. Infiltration by inflammatory cells was not apparent, neither in the lamina propria of the mucosa nor in the epithelium.

In all cases, ballooned keratinocytes were seen in clusters within the spinous layer of the epithelium on electronmicroscopy. These cells were increased in size and contained only few organelles, including degenerated mitochondria and abundant intermediate sized keratin fibrils. Foci of condensed chromatin with a punched-out appearance were found in pyknotic nuclei mainly in close association with the nuclear membrane (Fig. 2). Candida albicans was frequently observed in the stratum corneum as well as in the upper layers of the stratum spinosum (Fig. 3).

Virus particles of the herpes virus group were present in the upper spinous layer mainly in and around koilocytotic cells, and in the intercellular space of flattened cells of the superficial layers

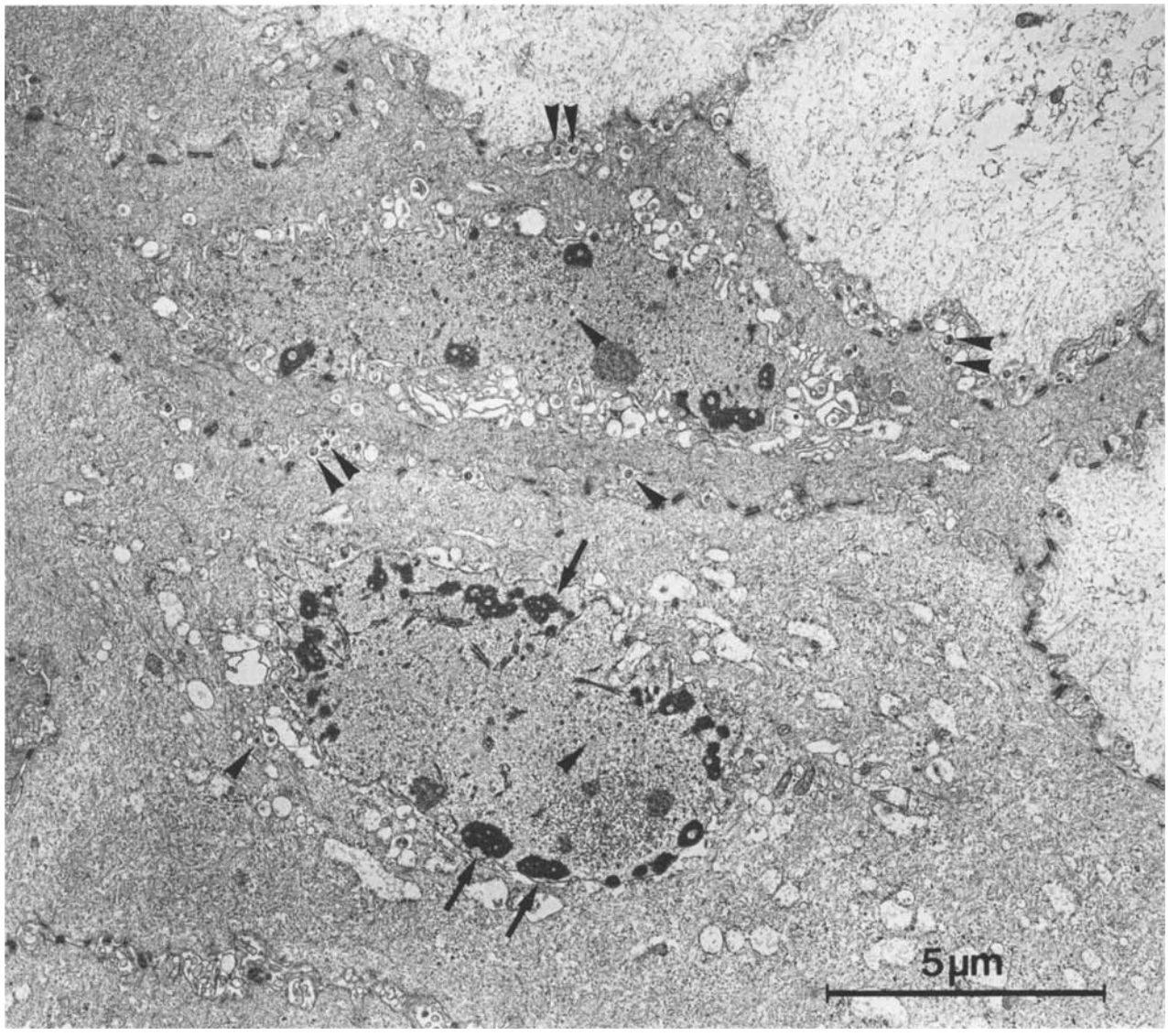

Fig. 2. In the stratum spinosum, two koilocytotic cells reveal degenerated mitochondria and dilated endoplasmic reticulum. Clumps of condensed chromatin, sometimes with a punched-out appearance (*arrows*), are located close to the nuclear membrane. EBV particles are detectable in the nucleus as well as in the cytoplasm of such cells, but are also seen in extracellular spaces (*arrowheads*). (EM ×8000)

(Figs. 2 and 4). Virus was also observed occasionally in cells infected by Candida albicans. The nuclei of infected cells contained varying amounts of viral nucleocapsids with a diameter of 100 nm (Fig. 4); while some capsids already contained the electron-dense toroidal desoxyribonucleoprotein portion typical for herpes viruses, others represented empty protein capsids. Occasionally, the egress of herpes virus capsids from the nuclei was observed (Fig. 5a). During egress the nucleocapsid acquires its future viral envelope mostly from the inner nuclear membrane in a budding process. The membrane was thickened in regions of nucleocapsid envelopment (Fig. 5a). Virus particles were also found in the cytoplasm, either free as nucleocapsids or as complete enveloped particles contained within the endoplasmic reticulum (Fig. 5b). Virions were finally released in abundant numbers into the extracellular space (Fig. 5c). Enveloped, mature herpes type virions within the cytoplasm and in the extracellular space showed identical diameters of 150 nm.

Two characteristic structures occurred within the cytoplasm of ballooned keratinocytes infected

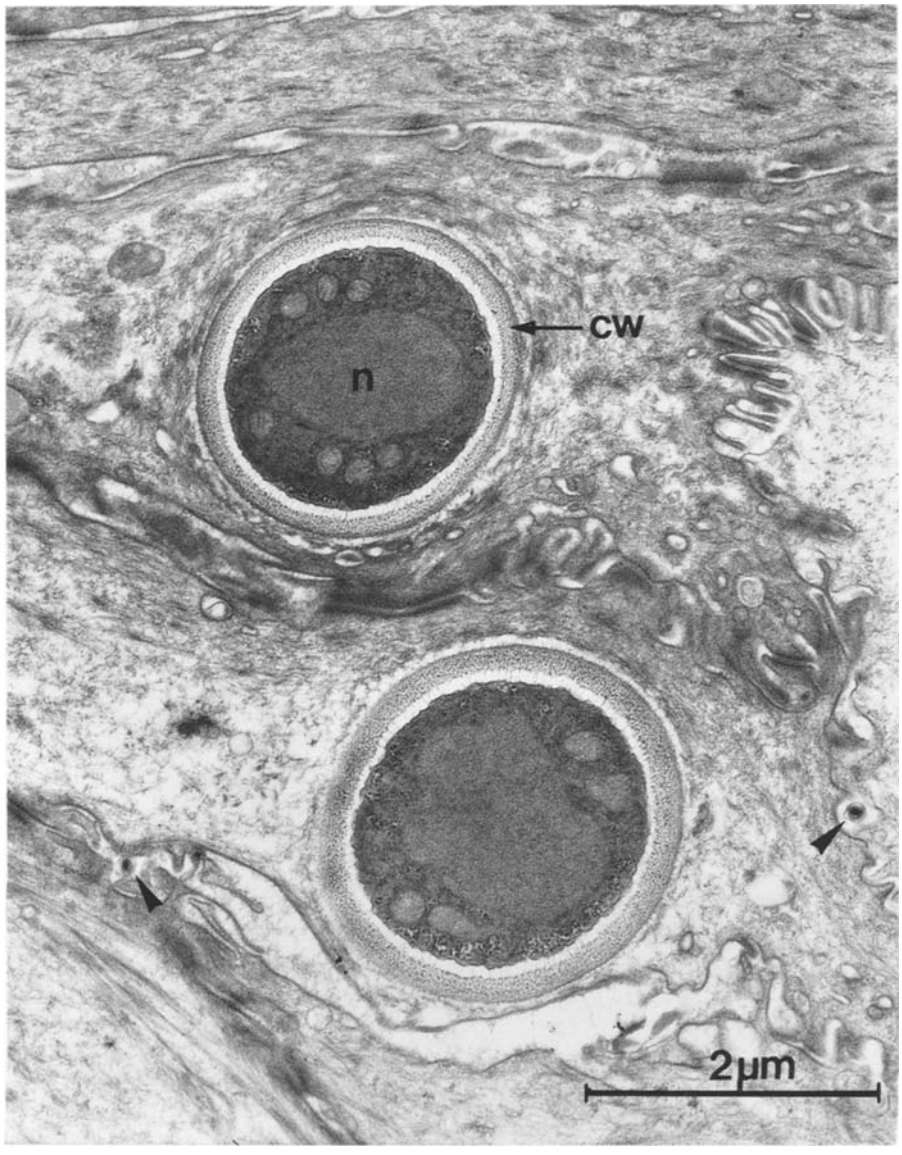

Fig. 3. Candida albicans in the cytoplasm of cells of the stratum corneum, showing an electron-lucent space (due to specimen shrinkage) adjacent to the cell wall (*CW*). Inside the hyphae mitochondria and nuclei are clearly seen. There is no clearance of Candida albicans by cellular immune mechanism. Virus particles are also present in the extracellular spaces (*arrowhead*). (EM ×19000)

by the virus. One consisted of tubules, 35 nm in diameter, arranged in parallel bundles about 1 µm in length (Fig. 6). Another inclusion body was composed of highly undulating, convoluted membranes. These were observed close to the nuclei of koilocytotic cells consisting of two, partly fused lipid bilayers. The inner fused part of this structure was electron dense and 25 nm in width (Fig. 7).

The subepithelial basement membrane did not reveal any irregularities. Langerhans cells were not observed within the HL lesions.

A specific immunostaining was noted in the upper two thirds of oral epithelia using semithin cryosections and EBV-specific monoclonal antibodies. Antibodies against EBV capsid (VCA) and membrane antigens (MA) revealed a completely different staining pattern (Fig. 8). While VCA was predominant in and around the nuclei of keratinocytes, MA was observed in high concentration in the interepithelial spaces in the same tissue areas, where EBV VCA was observed in consecutive sections.

Discussion

HL, an exclusively orally occuring lesion, was reported to be an early indicator of HIV-associated immunosuppression (Eversole et al. 1986; Silvermann et al. 1986). The occurrence of HL in differ-

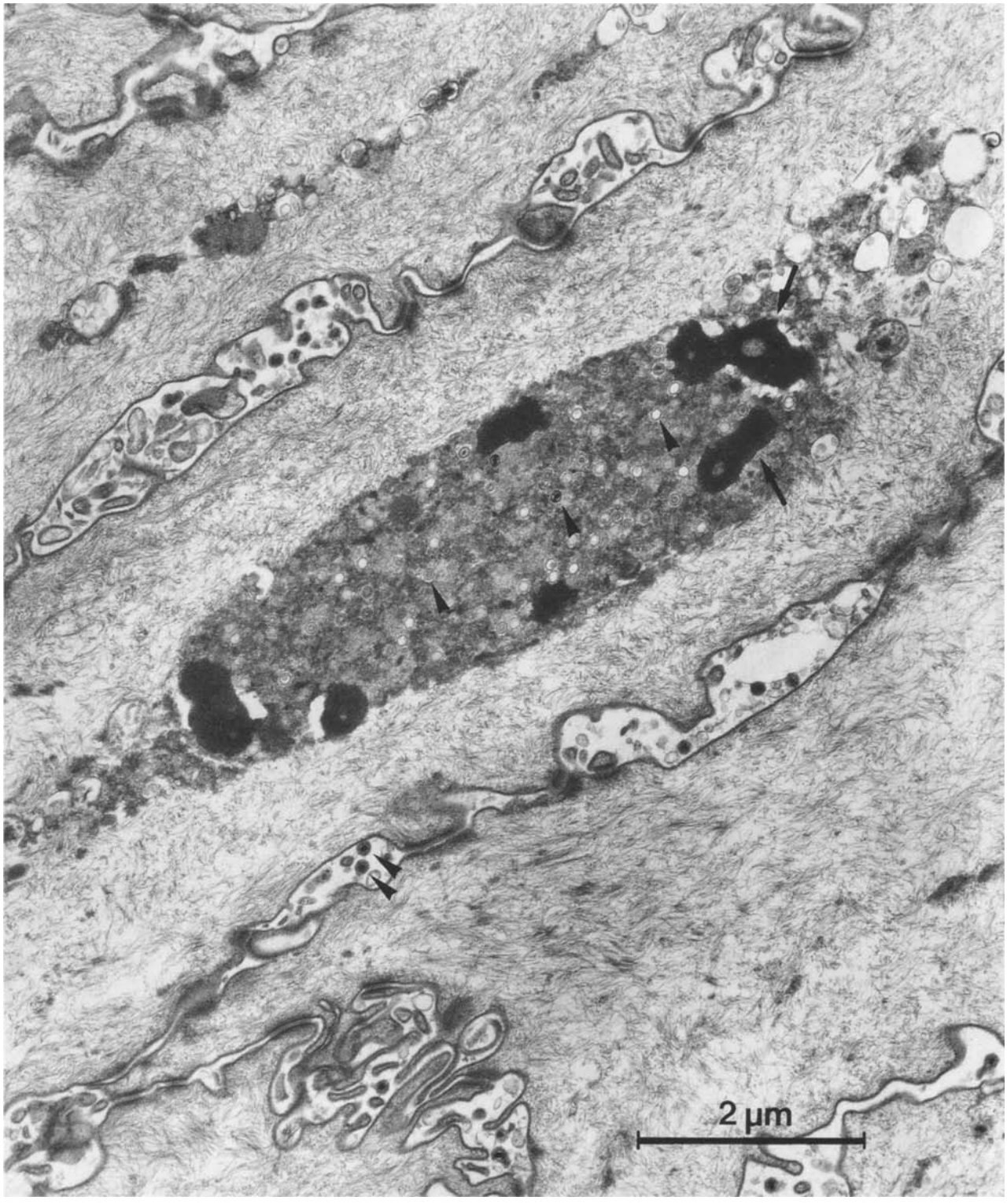

Fig. 4. EBV particles are present in the nuclei and the extracellular spaces of the flattened epithelium (*arrowheads*). In the nucleoplasm EBV capsids in different stages of assembly are densely packed. Note disappearance of nuclear membranes and the punched-out appearance of the condensed chromatin (*arrows*) of the koilocytotic cell. (EM ×19200)

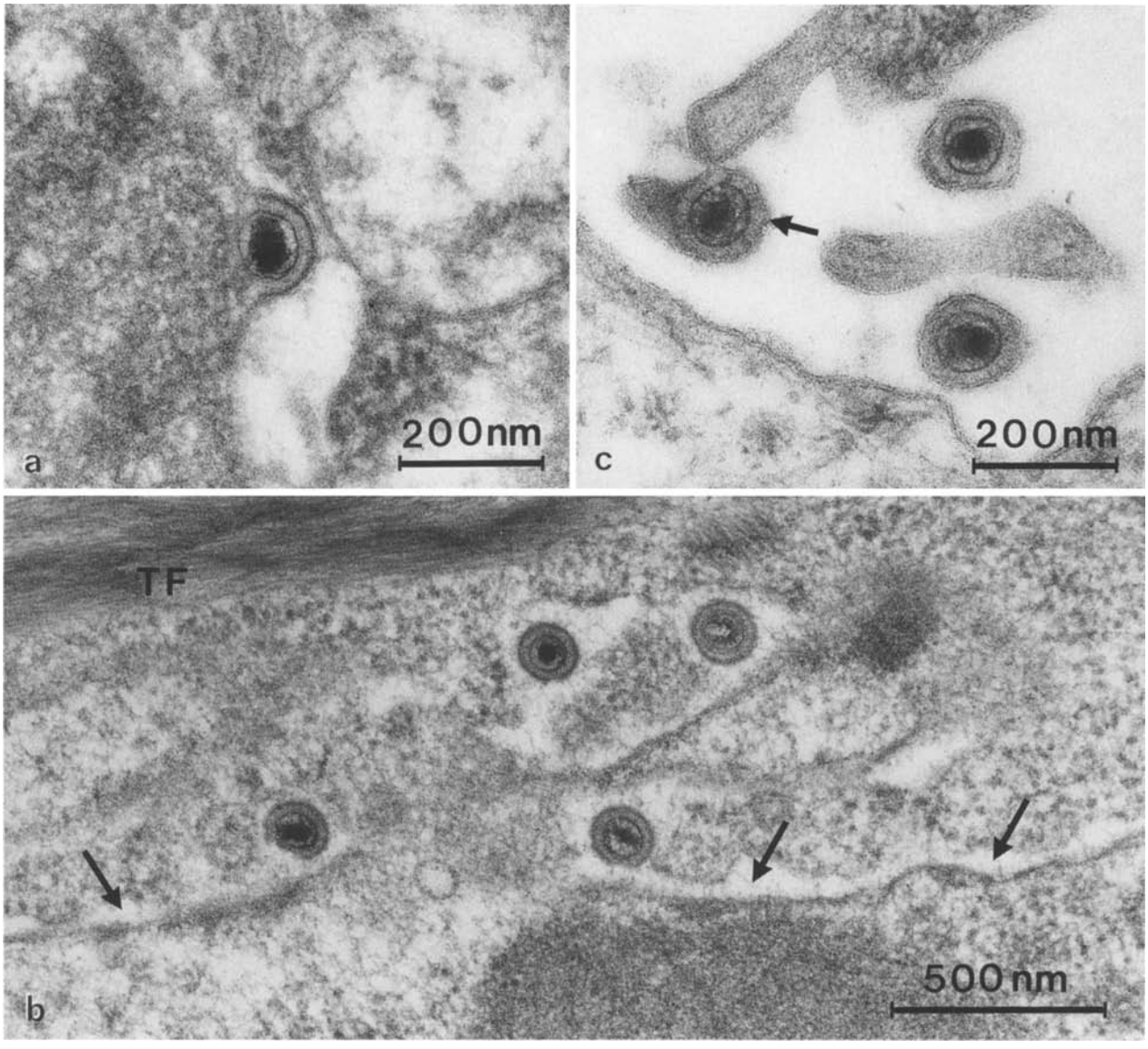

Fig. 5a. An EBV capsid acquiring its envelope by budding through the inner nuclear membrane, which is thickened in regions of nucleocapsid envelopment. (EM ×100000). **b** Enveloped, mature virions after egress from the nucleus. The lower particles are still in the perinuclear space (*arrows*), while the two upper virions are contained in the dilated endoplasmic reticulum. Abundant tonofilaments (*TF*) are seen in these cells. (EM ×60000). **c** Complete herpes type particles in the extracellular space of the upper spinous layer. The marked virion shows an extrusion of its envelope. (EM ×100000)

ent risk groups, as observed in the present study, has well been documented in the literature (Greenspan et al. 1984, 1986; Reichart et al. 1987; Rindum et al. 1987).

The aetiology of HL has long been supposed to be virus-related and in most investigations a herpes type virus was detected (Greenspan et al. 1984, 1985; Konrad et al. 1986). Furthermore, in some of these studies papillomavirus-like particles were observed in addition to the herpes type virus (Greenspan et al. 1984, 1985). While Greenspan et al. (1984, 1985) reported double infections in HL by EBV (using in situ hybridization) and HPV (EM, immunohistochemistry), we have not been able to detect papilloma virus in HL on the EM level in this investigation. In a further in situ hybridization study of seven HL all specimens were negative with the applied HPV type 6, 11, 13, 16, 18 probes while five of these specimens were EBV positive (Löning et al. 1987).

From the observation that papilloma virus-like particles were present in all 25 cases of HL at the

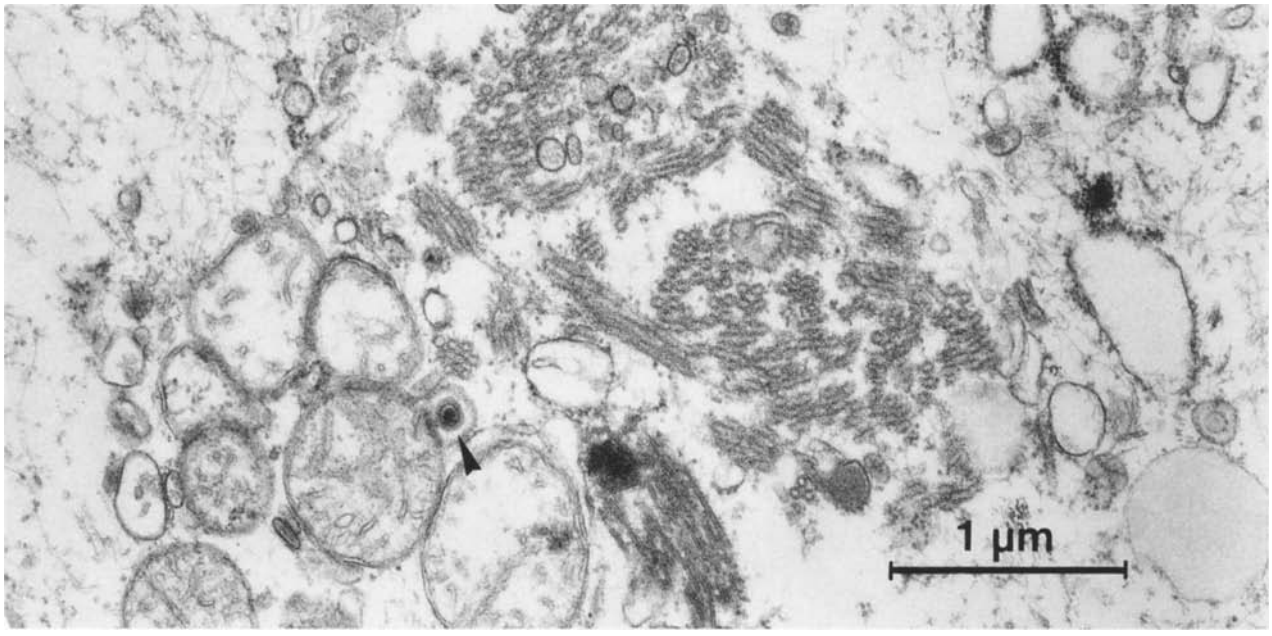

Fig. 6. Cytoplasmic structures consisting of tubules arranged in parallel bundles present in the cytoplasm of a koilocytotic virus producing cell. With different angles cross- and longitudinal profiles of the tubules become visible. Occasionally, a complete virion is found within an endoplasmic channel (*arrowhead*). (EM ×31250)

EM level Greenspan et al. (1985) considered whether the coexistence of EBV and HPV in HL is fortuitous. Since HPV was not demonstrated in our three cases or in a further 7 cases (Löning et al. 1987) our findings do not support the concept of simultaneous infection of HL. However, negative findings regarding HPV do not rule out a possible involvement of a second virus. In different tissues from patients with ARC or AIDS intracytoplasmic inclusions have been described, appearing as vesicular rosettes, test tube and ring-shaped forms (TRF), or tubuloreticular structures (TRS) (Onerheim et al. 1984; Kuntz et al. 1987). Within the cytoplasm of EBV producing cells, we observed only one type of parallel tubular structures (Fig. 6), which resembled in both size and appearance the inclusions described by Belton and Eversole (1986). The origin and nature of these aggregates is as yet unknown. While the occurrence of these parallelly arranged, intracytoplasmic tubular structures has only been described in HL, TRF inclusions have also been found in association with different immunological and neurodegenerative diseases as well as in viral infections. These membrane derived inclusions appeared to be associated with the production of alpha- and beta-interferon, but not of gamma-interferon (Onerheim et al. 1984). The occurrence of these aggregates may be a morphological indicator of interferon production, induced by viral infection, and may reflect the self-perpetuating immunological dysfunction. In addition, undulating membrane structures were observed within the cytoplasm of koilocytotic cells (Fig. 7), consisting of partly fused membranes similar to the above mentioned TRF/TRS. It is probable that these membrane structures are indeed precursors of TRF/TRS.

We did not observe Langerhans cells nor other signs of cellular immune reaction in the epithelium. Clearance of Candida albicans by cellular immune mechanisms was not found in our study nor in other reports (Belton and Eversole 1986). This is probably due to defective monocyte migratory function in AIDS-patients and gives reason to expect further functional disturbances in antigen processing and presentation by monocytes and Langerhans cells (Daniels et al. 1987; Becker et al. 1987).

Regarding the aetiology of HL, our EM and immunohistochemical findings corroborate the close association of EBV with this lesion. From clinical observations the virus seems to be involved in the pathogenesis of HL, since during treatment with high doses of acyclovir (4 × 800 mg/day during 14 days) clinical regression of the lesion has been found (Friedman-Kien 1986). The involvement of EBV in HL may be important for the further progress of the underlying HIV disease, be-

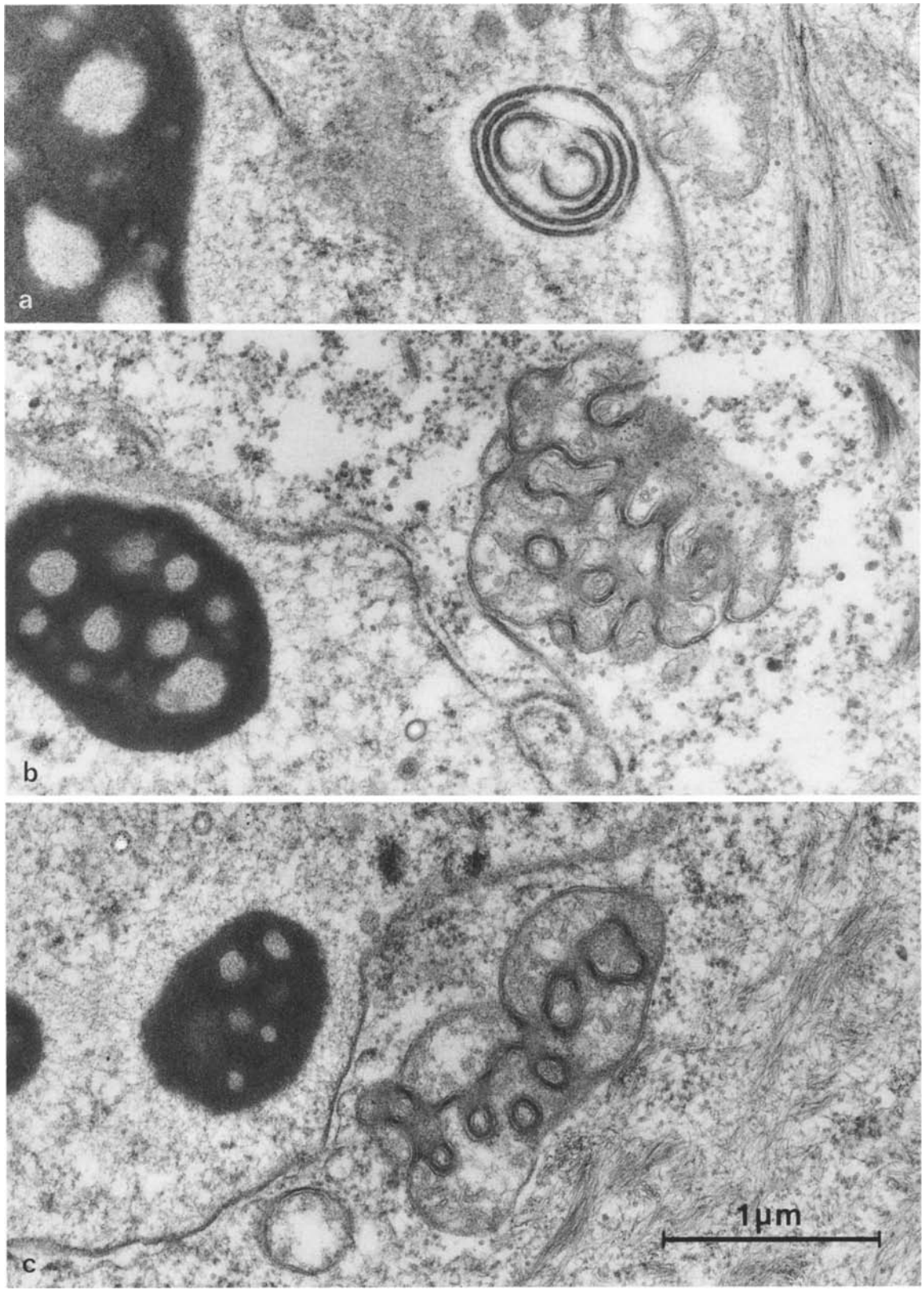

Fig. 7a–c. Features of cytoplasmic membrane differentiation occuring in cells of the stratum spinosum which are engaged in virus production. It appears that they consist of two, closely opposed and partly fused lipid bilayers, representing intermediate stages of "test tube like" structures. (EM ×40000)

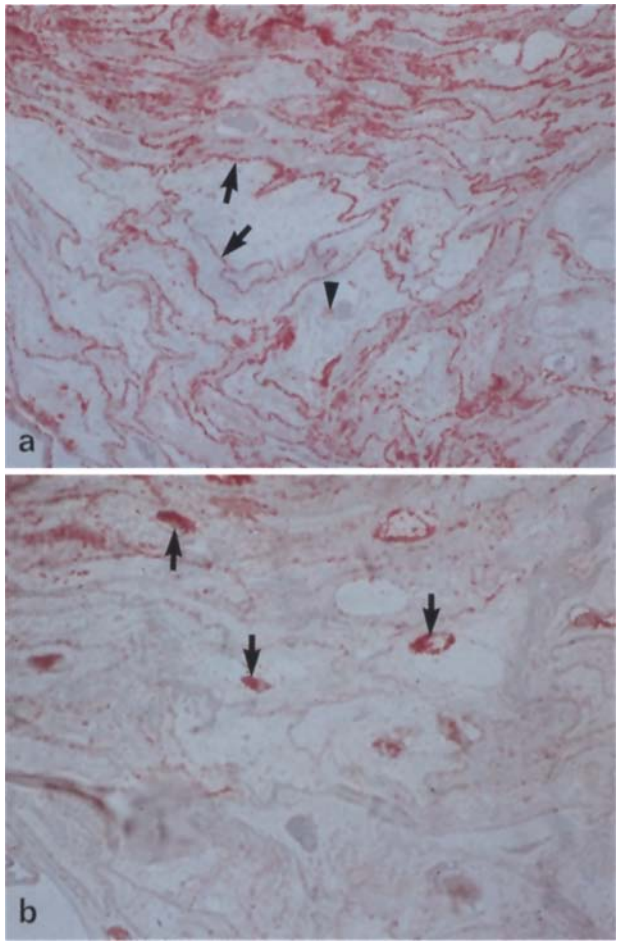

Fig. 8a, b. Semithin cryosections (0,5 μm): staining for EBV membrane antigen (MA; **a**) and EBV viral capsid antigen (VCA; **b**). Intense MA-staining is predominantly seen in the intercellular spaces (*arrows*; **a**). Additionally, a faint perinuclear distribution is observed (*arrowhead*). In contrast, VCA is detected predominantly within the nuclei of epithelial cells of the upper stratum spinosum (*arrows*; **b**), revealing a spotty distribution. (APAAP ×450)

cause EBV is known to be capable of transforming B lymphocytes so that they can be infected by HIV (Montagnier et al. 1984). The recruitment of such transformed B-cells in addition to the CD4+T-helper lymphocytes, monocytes/macrophages and Langerhans cells may encance HIV production considerably. This then directly leads to a higher level of infected cells engaged in HIV replication, and concomitantly to a continuously increasing loss of CD4+-lymphocytes by direct cytotoxicity of HIV and/or by the manifold HIV induced immunopathogenic mechanisms (Gelderblom et al. 1985; Klatzmann and Gluckman 1986).

Finally, the masses of EBV producing cells in HL are indicative of a severe immunodeficiency observed in these patients.

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