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In situ hybridization to detect Epstein-Barr virus DNA in oral tissues of HIV-infected patients

Thomas Löning[1], **Rolf-Peter Henke**[1], **Peter Reichart**[2], **and Jürgen Becker**[2]

[1] Institut für Pathologie der Universität Hamburg, Martinistrasse 52, D-2000 Hamburg 20, Federal Republic of Germany
[2] Abteilung für Zahnärztliche Chirurgie/Oralchirurgie Nord der Freien Universität Berlin, D-1000 Berlin

Summary. Thirty biopsies of oral mucosal lesions and normal oral mucosa were obtained from 26 HIV-seropositive individuals and studied for virus infections with Epstein-Barr virus-specific DNA probes (EBV). In situ DNA hybridization was carried out on frozen and formalin-fixed, paraffin-embedded tissues. Specifically bound biotinylated virus probes were detected with the streptavidin-gold-silver technique and visualized by standard and interference reflection microscopy. In 9/30 biopsies, EBV DNA was clearly demonstrated in the upper two thirds of oral epithelia. This finding corresponded to peculiar cytopathic effects including ground glass nuclei, basophilic nuclear inclusions, and ballooning of the cytoplasm, which were concentrated in the upper two or three layers of the stratum spinosum. Cytopathic effects together with the demonstration of EBV DNA were demonstrated in seven cases of tongue mucosa, and two cases derived from the gingiva. When comparing clinical and pathological findings with DNA detection rates, we saw 5/9 hairy leukoplakias associated with EBV infections. Four positive cases (two samples from the tongue, two gingival specimens) had not been regarded as hairy leukoplakia clinically. EBV infection of the oral epithelium occurred in male homosexuals (7 cases) and in male/female intravenous drug abusers (2 cases). Among the nine EBV-positive cases, 2 patients were asymptomatic, 4 patients were grouped into the ARC-, and 3 individuals into the AIDS-category. We conclude that HIV-seropositive patients are particularly prone to develop productive EBV infections in oral epithelia. This infection most frequently appears at the lateral border of the tongue, but may also occur at other sites of the oral cavity, and may already exist in a preclinical stage prior to the development of oral white lesions (hairy leukoplakia).

Key words: EBV infection – Oral epithelia – Hairy leukoplakia – In situ hybridization – AIDS

Offprint requests to: T. Löning

Introduction

Oral manifestations of HIV infection have been classified into five group of diseases: neoplasms, fungal infections, bacterial infections, viral infections, and lesions of unknown aetiology (Lozada et al. 1982; Lozada et al. 1983; Klein et al. 1984; Reichart et al. 1985; Schiødt and Pindborg 1987; Reichart et al. 1987). Recently, a peculiar white lesion was recognized to develop at the lateral or less frequently at the ventral tongue mucosa in 10–20% of HIV-seropositive individuals (Greenspan et al. 1984; Greenspan et al. 1985; Eversole et al. 1986; Greenspan et al. 1987). This lesion was called "hairy" leukoplakia (HL) because of the characteristic undulated appearance of the most superficial parakeratinized cell layers (Greenspan et al. 1984; Reichart et al. 1986). Initially, HL was regarded as particular epithelial reaction against the very common candida infections in these individuals. Since the lesions respond poorly to antimycotic treatments, however, and tend to disappear after application of virostatic drugs (acyclovir), members of the herpes virus group were suggested to be aetiologically involved. This assumption was soon substantiated by Greenspan and coauthors (1985), who detected EBV DNA and structural proteins in nearly all of their cases. These authors also reported on the presence of papillomaviruses in the same tissues. To our knowledge, the latter observation has not been confirmed using molecular virological techniques. In this study, we

present histological and cytological patterns of EBV-associated oral lesions including HL, and we relate these structural aberrations to the distribution of infected cells recognized by in situ hybridization with EBV DNA probes.

Material and methods

Thirty biopsies from 26 patients with HIV infection (HIV-infected: $n=3$; ARC: $n=7$; AIDS: $n=16$; see Table 1) were examined for the presence of EBV DNA. HIV-infection was diagnosed by ELISA and Western blot. The mean age was 37 years (22–83 years), the group of patients comprised 24 males and 2 females. All biopsies were removed under local anaesthesia except for six obtained post mortem. All tongue biopsies (14 samples from 13 individuals) were taken from the lateral part. The specimens from the gingiva propria (10 samples from 8 individuals) were obtained during tooth extractions from clinically normal oral mucosa. Palatal/buccal mucosa (6 cases) was received from patients some of whom suffered from Kaposi sarcomas (4 individuals).

Table 1. Clinical data and results of hybridization

No	Sex	Age	Risk group	Stage	HL	EBV-DNA
Tongue mucosa						
1.	m	35	homosexual	ARC	–	+
2.	m	30	homosexual	HIV	+/–	+
3.	m	40	homosexual	ARC	+/–	+
4.	m	29	i.v. drug ab.	HIV	+	+
5.	m	37	homosexual	AIDS	+	–
6.	m	37	homosexual	AIDS	+	–
7.	m	83	homosexual	AIDS	+	+
8.	f	25	i.v. drug ab.	ARC	+	+
Tongue mucosa (post mortem)						
9.	m	37	homosexual	AIDS	–	–
10.	m	44	homosexual	AIDS	–	+
11.	m	35	i.v. drug ab.	AIDS	–	–
12.	m	42	homosexual	AIDS	+	–
13.	m	28	homosexual	AIDS	+	–
14.	m	44	homosexual	AIDS	+	–
Palatal mucosa						
15.	(data as indicated under 14)				–	–
16.	m	46	homosexual	AIDS	–	–
17.	m	39	homosexual	AIDS	–	–
18.	m	32	homosexual	AIDS	–	–
19.	m	42	homosexual	AIDS	–	–
Buccal mucosa						
20.	m	45	homosexual	AIDS	–	–
Gingival mucosa						
21.	m	26	i.v. drug ab.	HIV	–	–
22.	m	22	homosexual	ARC	–	–
23.	m	31	homosexual	ARC	–	–
24.	f	35	ren.trpl.rec.	ARC	–	–
25.	m	45	homosexual	ARC	–	+
26.	m	26	homosexual	AIDS	–	+
27.	m	50	homosexual	AIDS	–	–
28.	m	34	homosexual	AIDS	–	–
29.	m	34	homosexual	AIDS	–	–
30.	m	34	homosexual	AIDS	–	–

m = male, f = female, i.v. drug ab. = intravenous drug abuser, ren.trpl.rec. = renal transplant recipient, HIV = human immunodeficiency virus-infected asymptomatic individual, for ARC and AIDS see CDC report (1986), HL = hairy leukoplakia, +/– incipient hairy leukoplakia

Routine histopathology (H & E, PAS) was conducted on either formalin-fixed, paraffin-embedded specimens or snap-frozen samples. The whole body of material was serially cut, and sections were transferred to aminoalkylsilane-treated glass slides (Rentrop et al. 1986; Henke et al. 1987b) in order to covalently fix the tissues to the slides and to avoid the otherwise inevitable floating of sections during the in situ hybridization protocol.

Hybridization was carried out as described elsewhere (Löning and Milde 1987; Henke et al. 1987a). Briefly, sections were covered with 20 µl of the hybridization cocktail containing 2 × SSC (1 × SSC: 0.15 M NaCl, 0.015 M sodium citrate, pH 7.2), 20% (v/v) deionized formamide, 10% (w/v) dextran sulphate, 0.1 mg/ml herring sperm DNA and 1 µg/ml biotinylated EBV DNA (Enzo, New York; prepared from the 3 kb BamHI "V" fragment cloned into the BamHI site of pBR322). After denaturation by heating to 90° C (for 10 min), hybridization was allowed to take place at 37° C overnight. Consecutively, sections were washed stringently in two changes of SSC buffer (1 × SSC, 45% formamide) at 37° C (corresponding to Tm −17° C), each for 10 min, and in further three changes of 2 × SSC at room temperature each for 5 min. Hybridized nucleic acids were detected with the immunogold-silver staining technique (Henke et al. 1987b). Sections were treated consecutively with 1. rabbit-anti-biotin-antibodies (Enzo, New York, USA), 2. biotinylated goat anti-rabbit-antiserum (Dianova, Hamburg, FRG), and 3. streptavidin-colloidal gold (5 nm)-conjugates (Janssen, Beerse, Belgium).

All reagents were diluted at 1:250 in Tris-HCl buffer (pH 7.5 for steps 1 and 2, pH 8.2 for step 3), and incubated for 1 h at 37° C. For the final silver development, a commercially distributed kit was used (Intense, Janssen, Beerse, Belgium). Sections were counterstained with haematoxylin and examined with standard and interference reflection microscopy (Verschueren 1985; Ambros et al. 1986).

Controls included: – the omission of the specific viral probe (EBV), the replacement of the EBV probe by biotinylated CMV (Enzo, New York, USA; insert sizes 17.2 kb and 25.2 kb, cloned into the BamHI site of pBR322) or HPV probes (HPV 6, 11, 13, 16, and 18 DNA harbouring plasmids were a generous gift of Drs. H. zur Hausen and L. Gissmann, German Cancer Research Center, Heidelberg, FRG) and the investigation of EBV harbouring cells and tissues (EBV positive peripheral blood cells from transplant recipients, nasopharyngeal carcinomas).

Results

The clinical findings are summarized in Table 1. HL was found in HIV-infected, asymptomatic individuals (2 cases), patients with ARC (2 cases), and with AIDS (5 cases). HL was observed exclusively at the tongue (7 patients). In further two patients, HL appeared clinically to be in an incipient stage. The residual four tongue biopsies were obtained from patients without any sign of HL. HL was seen in male homosexuals (7 patients) and in two male/female i.v. drug abusers.

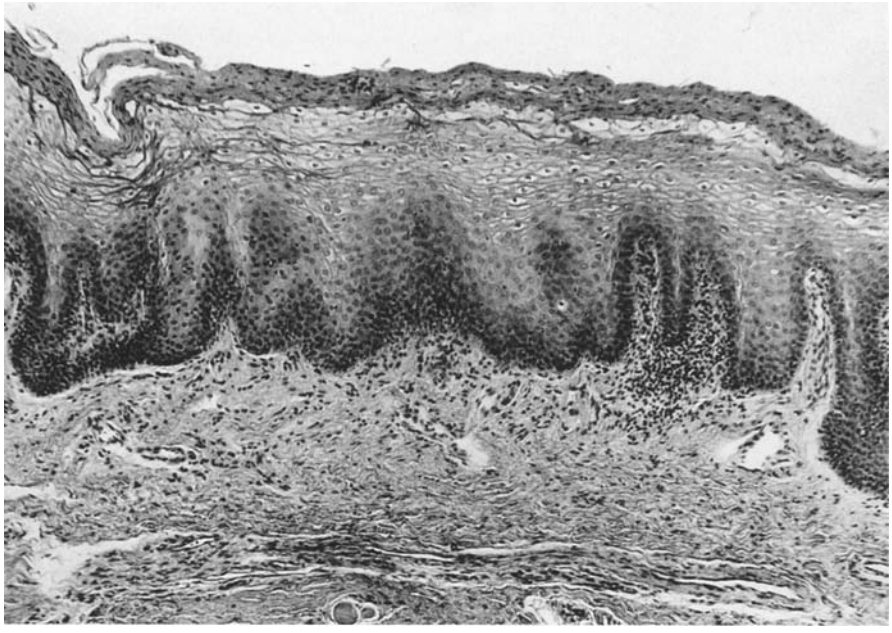

Fig. 1. Hairy leukoplakia (case No 8). Flat epithelium with parakeratotic surface. Note the broadening of the upper stratum spinosum. H & E, Mag. × 80

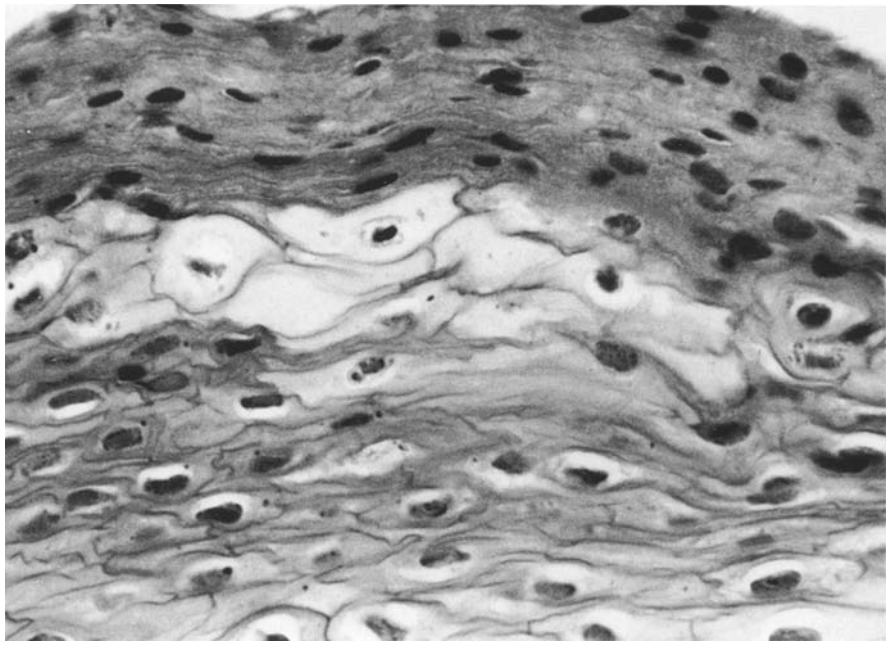

Fig. 2. Same case. Ballooning of keratinocytes beneath the parakeratotic surface. These cytoplasmic changes are associated with lysis/ fragmentation of nuclei. H & E, Mag. × 500

In this series of 30 incisional biopsies of patients with HIV infection, nine cases were observed to be positive for EBV (Table 1). Five of them were classified clinically as HL and were characteristically found at the lateral border of the tongue. Among the four additional cases positive for EBV, the lateral tongue mucosa and the gingival mucosa were involved each in two cases.

Independent of whether HL was diagnosed or not, EBV-positive oral epithelium was altered in a very particular manner. The basal cell layer was not strikingly enlarged and usually confined to a single row of keratinocytes. Above the basal cell layer an increasing degree of intracellular edema (ballooning) of spinous cells was noted (Figs. 1, 2). This feature was most striking at the top of the stratum spinosum just beneath the small zone of superficial keratosis, which, in most instances, consisted of 3–4 layers of parakeratinized flattened epithelia. Due to this spongy aspect of the most superficial spinous cells, the parakeratotic surface was often abruptly delineated from the stratum

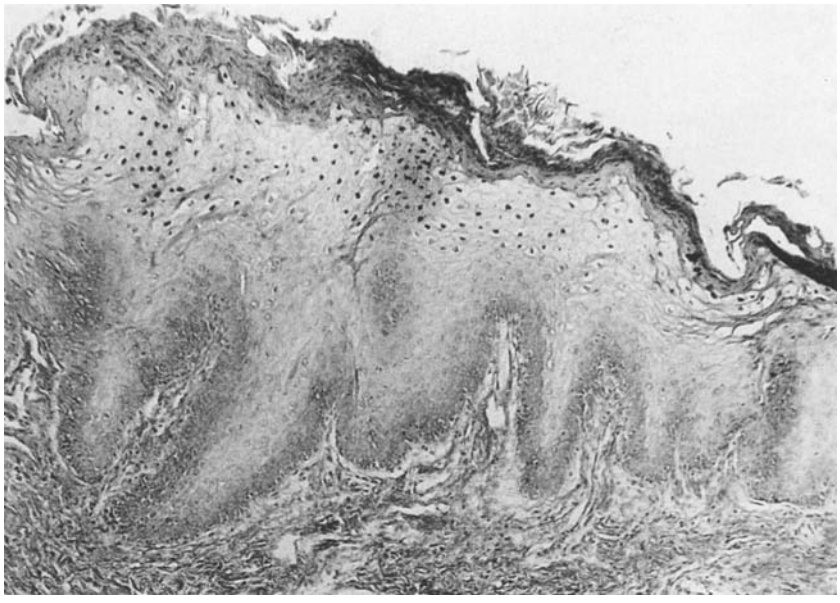

Fig. 3. In situ hybridization with EBV DNA probe (same case). Note the nuclear label starting in the upper part of the stratum spinosum. Immunogold-silver-detection. Mag. ×125

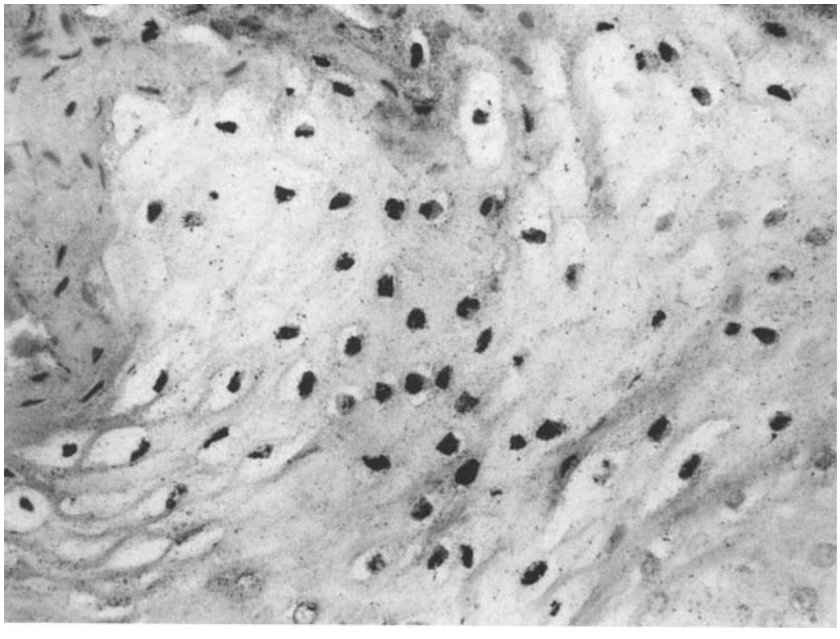

Fig. 4. Higher magnification. Intensive labelling of spinous cells with intracellular edema. In situ hybridization with EBV DNA probe. Immunogold-silver-detection. Mag. ×500

spinosum (Fig. 2). The enormous ballooning and consecutive enlargement of spinous cells caused a more or less pronounced corrugated appearance of the mucosal surface.

When looking at the ballooned keratinocytes in detail, it was evident, that nuclei lost their chromatin pattern and distinct borders with gradual changes toward the appearance of nuclear shadows. In addition, basophilic intranuclear inclusions occasionally occurred (Fig. 2). Sometimes, intracellular oedema was extensive enough, to give rise to vesiculations of the cytoplasm, and cells with this cytoplasmic changes were very similar to so-called koilocytes. Although slight in cases of HL, the architectural and cytological alterations described were seen in all EBV positive lesions. The cytoplasmic ballooning together with the ground-glass appearance of nuclei and nuclear inclusions represents the cytopathic effect of productive EBV infection, since in situ hybridization clearly showed a large number of hybridizing nuclei within the upper stratum spinosum (Fig. 3) and a particular relationship to the spongy keratinocytes (Fig. 4). Hybridization was always heavily concentrated

over the nuclei (Fig. 4), although minor signals were also recognized at cytoplasmic sites.

We looked carefully for positive cells outside the spinous cell compartment and for positive cells other than epithelial cells. Basal cells were never found to be unequivocally positive. Inflammatory mononuclear cells were always negative except for one case, which showed slight nuclear hybridization of perivascular infiltrates.

Positive results with CMV probes were not obtained. In addition, all EBV positive specimens were negative with the applied HPV probes 6, 11, 13, 16, 18. However, we observed 3 cases which were positive for HPV when hybridized with a mixture of 6, 11, 16, 18 under conditions of reduced stringency. In contrast, those cases were negative for EBV.

The four cases of HL negative for EBV DNA showed cytopathic effects consistent with EBV infection. In these cases (three of them being post mortem biopsies), poor tissue preservation and/or low copy numbers could have prevented successful hybridization.

Discussion

Early recognition of AIDS and its prodromal stages is of utmost importance for the patient and of particular relevance for public health (CDC 1985; CDC 1986). Despite of serological and molecular biological advances in the detection of HIV infection, diagnosis of the *disease* is still made on clinical grounds. In this regard, important clinical signs of AIDS or pre-AIDS (ARC, LAS) include oral manifestations in addition to the well acknowledged lymph node involvement and the development of skin tumours (Kaposi sarcomas) (L'Age-Stehr 1983; Helm et al. 1984; Hehlmann et al. 1985; Falk et al. 1986; Reichart et al. 1985; 1986). Besides recurrent oral herpetic and mycotic infections, peculiar white lesions were reported to occur in a high percentage of HIV infected individuals (mostly homosexual men) prior to the onset as well as in the course of the disease (Reichart et al. 1986; Greenspan et al. 1987). These lesions were called *hairy leukoplakia* because of their undulated white surface, and were regarded to be not only predictive of the development of the disease over short intervals of approximately 30 months, but also of progressive disease with a high rate of fatal opportunistic infections (e.g. pneumocystis carinii) (Kimmig et al. 1986; Greenspan et al. 1987).

Our findings strongly support the assumption that HL represents a characteristic spongy tissue reaction towards productive EBV infection, which was never recognized before probably, because EBV is never as actively replicated in "normal" epithelial cells as in these individuals (Sixbey et al. 1984; Greenspan et al. 1985). Morphology has more similarities with herpetic stomatitis than with any other virus infection. Both, intracellular oedema and ground-glass nuclei -typical cytopathic reactions of herpes simplex infections- are also characteristics of the EBV infection of oral epithelium.

In our understanding, leukoplakia is a misnomer of this oral lesion for two major reasons. From the clinical standpoint, only those white plaques of the oral mucosa should be called leukoplakia, which can not be related to any other disease (WHO 1978). The latter definition does not hold true for HL. From the viewpoint of histopathology, most oral leukoplakias represent hyperplastic conditions, which should not be confused with oedematous states (leukoedema) of the oral epithelium either at intercellular locations (spongiosis) or at intracellular sites (ballooning). Attempts to call these lesions oral condylomas (condyloma planus, Eversole et al. 1986) are not justified for the following reasons. None of our EBV positive cases were seen to be coinfected by HPV and the morphological picture of EBV-infected specimens differed from oral condylomas/papillomas in important aspects. Basal cell hyperplasia and koilocytosis were not present to a remarkable degree. In fact, koilocytosis-the cytopathic reaction of HPV infection and replication-(Löning and Milde 1987) is a phenomenon often confused with liquefaction degeneration caused by a number of extrinsic and intrinsic noxious agents.

True koilocytosis (e.g. in condylomas, cervical epithelial dysplasias, papillomas) starts to develop in the middle stratum spinosum, usually in non-inflamed epithelial compartments, and is characterized by often V-shaped (the V pointing to the rete ridges) fields of koilocytic cells, which typically contain a hyperchromatic distorted nucleus and a narrow perinuclear cytoplasmic halo (Löning and Milde 1987).

In our series of cases, HL was not regarded as an early sign of AIDS, neither could the lesion be evaluated as a prognostic label. In contrast to previous reports (Greenspan et al. 1984; Greenspan et al. 1985), EBV DNA production was not confined to tongue epithelium (2 positive cases of gingival mucosa) and, in addition, was even not restricted to clinically evident cases of HL (4 cases of clinically normal mucosa). From our findings, the questions emerge, at what frequency EBV

DNA infection occurs in oral keratinocytes either in a latent or productive form in non-selected populations compared with individuals with infectious mononucleosis and with immunocompromised patients.

In this context, DNA hybridization studies of throat washings are of particular interest. Sixbey et al. (1984) showed EBV DNA to be present in epithelial cells of 10/12 patients with infectious mononucleosis, and in four of these patients they showed by the detection of EBV specific RNA that keratinocytes harbour transcriptionally active EBV, indicating an activated (productive) viral state. In contrast, throat washings of healthy individuals including seronegative donors were usually negative for both EBV specific DNA and RNA except for one individual positive for EBV DNA, who could represent an asymptomatic carrier.

Salivary excretion of EBV is known to take place in patients with infectious mononucleosis, up to 20% of seropositive healthy individuals and more than 50% of patients under iatrogenic immunosuppression. Parotid duct epithelial cells harbour the EBV genome (Wolf et al. 1984). Oropharyngeal keratinocytes may represent another important reservoir of EB virus, which may persist in most individuals in a latent form. Viruses probably gain entrance into the upper squamous cells via functional EBV (C3d) receptors (Young et al. 1986). Those latent infections are probably hard to detect even with the most sensitive molecular virological assays. Greenspan and coauthors (1984, 1985, 1986, 1987) did not report on the presence of EBV DNA in the oral mucosa of individuals other than immunosuppressed HIV infected patients, and even in these individuals, positive results were only described for patients with HL, To our experience, in situ hybridization yields positive results only in those cells harbouring at least 100 copies of the nucleic acids in question. Thus, our demonstration of EBV DNA in gingival and tongue mucosa is certainly an expression of very active virus production (see also Greenspan et al. 1985) and corresponds to the development of cytopathic reactions in these cases. It is certainly interesting that cytopathic reactions with detection of EBV DNA were seen in cases in the absence of HL. However, we did not observe any case positive for EBV DNA, which lacked the characteristic cytopathic effects. In addition, in the individual case, hybridization was always confined to the described zones with pronounced intracellular edema.

We did not see these cytopathic effects before in oral lesions caused by other viruses (herpes simplex, cytomegalovirus, papillomaviruses). When considering our and other institution's interest and experience in oral pathology over the last twenty years, it is tempting to assume that hairy leukoplakia and the related EBV-exerted cytopathic effects really represent a hitherto unknown disease. In fact these lesions have never been reported to occur in congenital and acquired immunodeficiencies other than AIDS/ARC (Greenspan et al. 1987). Although initially regarded to be restricted to HIV-infected homosexual men, hairy leukoplakia was later recognized also in other risk groups (transfusion recipients, haemophiliacs, Greenspan et al. 1986). Our series of cases includes also two intravenous drug abuser (1 male, 1 female).

We have of course to study larger populations prior to claiming that the clinical and morphological spectrum of active EBV infection is being specific for HIV-infected individuals. At present, we conclude: – that EBV infects oral keratinocytes, and is active under certain circumstances, that EBV induces striking cytopathic effects in oral epithelia, which are certainly not restricted to tongue epithelium, that EBV-related cytopathic effects lead to clinically apparent lesions in some cases (so-called hairy leukoplakia), which morphologically still resemble a pronounced oedematous state of the oral epithelium rather than a true proliferative disease.

We do not know which mechanisms trigger the replication of EBV in oral epithelia. It is of course interesting, that cytopathic effects together with the demonstration of EBV DNA occurred in upper spinous cells, and seems to be under control of epithelial differentiation. In addition, extrinsic mechanisms have to be discussed (Birx et al. 1986), since previous studies showed a decrease in Langerhans cells in these lesions (Belsito et al. 1984; Daniels et al. 1986).

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