Immunocytochemical detection of herpes viruses in oral smears of HIV-infected patients


Cytologic smears (CS) were taken from the lateral border of the tongue of HIV-seropositive patients (HIV+) (n = 39) and of seronegative controls (HIV—) (n = 19) and examined by immunocytochemistry (APAAP) and in situ hybridization (ISH) (biotinylated DNA probes) for the presence of viral antigens/DNA of EBV and CMV. While none of the HIV controls showed positive results for EBV antigen, 61% (APAAP) resp. 79% (ISH) of oral epithelial cells in the group of HIV+ patients were EBV-positive. While all CS taken from areas with the clinical diagnosis of hairy leukoplakia (HL) were EBV positive (APAAP and/or ISH), the detection of EBV in CS from uninvolved oral mucosa seemed to be associated with the later development of HL. In the group of HIV+ patients the detection rate for CMV was about five times (APAAP) resp. three times (ISH) higher than in HIV— persons. This non-invasive technique seems to be a valuable tool to screen for viral antigens/genomes.

Material and methods

39 HIV-seropositive patients and 19 HIV-seronegative controls were included in this study. HIV seropositivity was stated by ELISA (DuPont) and confirmed by Western blot. Complete histories and examinations of the oral cavity were done initially and reevaluated after 8 months. The clinical status was diagnosed according to the definition of the Centers for Disease Control (CDC) (7). Progression of HIV-associated disease was defined as clinical deterioration of a patient, resulting in the change of CDC-group (7). CD4/CD8 ratios and absolute numbers of T-helper lymphocytes per μl blood were determined according to standard methods (8).

Cytologic smears (CS) were taken from the lateral border of the tongue using a metal spatula. After dissolving in phosphate buffered saline (PBS) and centrifugation (150 g/5 min) trypsin (0.2%) was added to the cell solution in order to separate the epithelial cells from adhering detritus and mucus. The cell suspension was repeatedly washed with PBS, centrifugated (100 g/5 min) and was filtered using a teflon filter. Afterwards the superfluous liquid was removed and the quantity of cells was controlled in a Neugebauer counting chamber (optimal density of cells: 80–100 cells/μl). After adding 10 μl acetone the cell suspension was immediately distributed onto poly-L-Lysine coated glass slides, air dried (10 min), fixed with acetone (5 min), air dried (10 min) and stored at −20°C. After fixation (5 min) and air drying (10 min) one slide/patient was counterstained with hemalum to control the quality of the cell preparation.

For detection of structural proteins of EBV (nuclear antigen: NA, virus capsid antigen: VCA, early antigen: EA) (dilution: 1:25; Dakopatts) monoclonal antibodies were applied using the APAAP technique (9). As pretreatment for this procedure slides were defrosted (30 min), fixed with acetone (10 min) and air dried (10 min).

EBV and CMV DNA probes were obtained from Enzo (Ortho Diagnostics). For in situ hybridization (ISH) slides were defrosted (30 min), air dried (30 min) and fixed with acetone (10 min). To avoid unspecific reactions with the streptavidin horseradish peroxidase complex resp. due to the presence of endogenous phosphatase slides were...
treated with 0.5% triton (10 min) resp. 
H₂O₂/methanol (5 min). After washing (aqua dest) and air drying (5 min) the 
probe reagent was applied and the slides 
were covered with acid-cleaned cover 
glasses. Denaturation was performed by 
placing the slides in a 92+2°C incubator 
for 4 min and then immediately placing 
the slides in a 37°C incubator for 20 
min to allow for hybridization. 

Following hybridization, the cover 
glasses were removed, the slides incubated with the post hybridization solu-
tion (1 mM phosphate buffer, 13 mM 
NaCl, 50% formamide) and repeatedly 
washed (10 mM PBS/5 mM EDTA). 
Hybridized biotinylated DNA probes 
were detected using an avidin-biotiny-
lated horseradish peroxidase system 
(10) with aminoethylcarbazole as chro-
mogen. Finally the cell preparations 
were lightly counterstained using Fast 
Green (4 min) and coverglassed with 
glycerin jelly. 

Using the APAAP technique the spec-
ificity of monoclonal antibodies was test-
ed on Raji cells (negative control for 
EBV) and EBV-infected Raji and 
P3HR1 cells (positive controls for EBV), 
on HEL cells (negative control for CMV) 
and CMV (strain AD169) infected HEL 
cells (positive control for CMV). For in-
situ hybridization the specificity of 
probes was tested on Ramos and P3HR1 
cells (negative control for EBV) and on 
Raji cells and EBV (strain B95-8) in-
fected Ramos and P3HR1 cells (positive 
control for EBV), on MRC-5 and HEL 
cells (negative control for CMV) and on 
CMV infected MRC-5 and HEL cells 
(positive control for CMV). 

To exclude unspecific reactions cyto-
logic slides, not incubated with the pri-
mary antibody respectively DNA 
probe, were included in all other test 
procedures. 

The slides were microscopically eval-
uated using a Leitz Orthoplan micro-
scope with a primary magnification of 
×250. All cells of each slide – in average 
about 1500 cells – were evaluated. The 
slides were scored as being either posi-
tive or negative. Positive cases with less 
than five positive cells/slide were classi-
ded as “ + ”, those with 5 to 10 positive 
cells as “ ++ ” and those with more 
than 10 positive cells as “ +++ ”. Posi-
tivity was also designated as nuclear, 
cytoplasmic or whole cells. Positive cells 
were characterized as cytomegalic, 
expanded or normal sized cells. The mean 
value and standard error of the mean of 
the number of positive cells/slide were 
determined. To decide whether there 

was a significant difference or correla-
tion between the immunocytochemical 
results gained from patients of different 
clinical groups a parameter free test (U-
test Mann-Whitney) was used. Spear-
mann’s correlation test was applied to 
determine relations between the immu-
noeytochemical results and the clinical 
and immunologic data. 

Results 

Clinical results 

Nineteen healthy HIV-control persons 
(average age: 33 years; men n = 9, 
women n = 10) were included in the 
study. HIV+ patients (men: n = 35, 
women: n = 4; average age: 34.9 yr) were 
in different risk groups for acquiring 
HIV-infection: homo- or bisexual males 
(28/39), i.e. drug users (9/39; men: n = 
6, women: n = 3), hemophilic (1/39) 
and heterosexual women (1/39). The 
patients were in different clinical stages 
of HIV-infection: 6 patients were 
asymptomatic, 12 patients showed ARC 
symptoms (CDCIII/IVA) and 21 pa-
tients suffered from AIDS manifesta-
tions (CDC IV B–E). 

During the observation time of 8 
months in 7 patients progression of 
HIV-associated disease was noted, 
while 11/21 patients with AIDS mani-
festations died. 

In all HIV+ patients the CD4/CD8 
ratio was decreased (average: 0.3; range: 
0.04–1.0), while in 60% the absolute 
number of CD4 cells was below 100/μl 
(average: 195/μl; range: 7–744/μl) 
(Table 1). All patients with a CD4/CD8 
ratio ≥ 0.1 and/or an absolute num-
ber of CD4 cells ≤ 100/μl showed clinical 

<table>
<thead>
<tr>
<th>CD4/CD8</th>
<th>HIV+</th>
<th>ASY</th>
<th>ARC</th>
<th>AIDS</th>
<th>HL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of patients</td>
<td>39</td>
<td>6</td>
<td>12</td>
<td>21</td>
<td>19</td>
</tr>
<tr>
<td>Average</td>
<td>0.3</td>
<td>0.7</td>
<td>0.4</td>
<td>0.1</td>
<td>0.3</td>
</tr>
<tr>
<td>Standard deviation</td>
<td>0.04</td>
<td>0.1</td>
<td>0.5</td>
<td>0.02</td>
<td>0.06</td>
</tr>
<tr>
<td>Median</td>
<td>0.3</td>
<td>0.7</td>
<td>0.4</td>
<td>0.1</td>
<td>0.2</td>
</tr>
<tr>
<td>Range</td>
<td>0.04–1.0</td>
<td>0.3–1.0</td>
<td>0.2–0.8</td>
<td>0.04–0.4</td>
<td>0.04–1</td>
</tr>
</tbody>
</table>

Table 1. Ratio of CD4/CD8 positive cells and absolute number of CD4 positive cells/μl (CD4 abs/μl) (peripheral blood). 

HIV+: HIV seropositive patients; ASY: Asymptomatic HIV+ patients; ARC: HIV+ patients with ARC symptoms; AIDS: HIV+ patients with AIDS symptoms; HL: HIV+ patients with hairy leukoplaikia (HL). 

Fig. 1. Positive reactions in EBV infected Raji cells were located within nucleus, occasionally within cytoplasm as well. APAAP × 200.
Herpes viruses in oral smears

Positive reactions were found for EBV capsid and/or membrane antigens (A). Flattened (B) or polygonal (C) keratinocytes positive for EBV revealed nuclear (D) and/or cytoplasmatic staining, but in addition cells morphologically not altered also stained positive (CS of HIV+ patient. A: APAAP, ×300. B-C: APAAP, ×400.

symptoms of AIDS manifestations. Between the clinical stage of HIV-associated disease and the CD4/CD8 ratio (Spearmann-test: P = 0.03) as well as the absolute number of CD4 positive cells (Spearmann-test: P = 0.03) significant correlations were found.

Within the oral cavity clinical manifestations of 
C. albicans infection (26/39), HSV1/2 associated lesions (5/39), Kaposi’s sarcoma (8/39) and non-Hodgkin’s lymphoma (1/39) were observed. In 19 patients (asymptomatic: n = 1; ARC: n = 7, AIDS: n = 11) hairy leukoplakia (HL) was clinically diagnosed at the lateral border of the tongue. During the observation time of 8 months three more patients developed this lesion. There was a significant correlation between the clinical finding of HL and the progression of HIV-associated disease (U-test Mann-Whitney; P = 0.015). No correlation was found between HL and the clinical stage of HIV-associated disease, serologic immunoparameters (CD4/CD8 ratio, absolute number of CD4 positive cells) or the presence of other oral lesions.

At the time of examination 15 patients received antiviral drugs: AZT (Retrovir) n = 10, pentosan polysulfates (HOE/BAY 946) n = 3, Gancyclovir (DHPG) n = 1; two patients were under treatment with Acyclovir (Zovirax), starting 2 days before examination. In eight of these patients the clinical symptom of HL was diagnosed; however, antiviral medication showed no effect on the clinical presentation of this lesion.

Immunocytochemical results:

EBV antigens – Using the APAAP technique Raji cells were negative for EBV, while positive reactions were found in EBV infected Raji cells (Fig. 1) and in P3HR1 cells. Positive reactions were mainly located within the nucleus, in the nucleus and cytoplasm and occasionally only within the cytoplasm.

Table 2. Number of cases positive for EBV nuclear (NA), capsid (VCA) and early antigens (EA) (APAAP) and for EBV DNA (in situ hybridization).

<table>
<thead>
<tr>
<th>EBV antigen</th>
<th>NA</th>
<th>VCA</th>
<th>EA</th>
<th>EBV antigen total</th>
<th>EBV DNA total</th>
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<tr>
<td>HIV+</td>
<td>18/39 (46%)</td>
<td>13/39 (33%)</td>
<td>3/39 (8%)</td>
<td>24/39 (61%)</td>
<td>31/39 (79%)</td>
</tr>
<tr>
<td>ASY</td>
<td>2/6 (33%)</td>
<td>3/6 (50%)</td>
<td>0/6</td>
<td>4/6 (55%)</td>
<td>4/6 (67%)</td>
</tr>
<tr>
<td>ARC</td>
<td>9/12 (75%)</td>
<td>3/12 (25%)</td>
<td>2/12 (16%)</td>
<td>9/12 (75%)</td>
<td>11/12 (92%)</td>
</tr>
<tr>
<td>AIDS</td>
<td>7/12 (33%)</td>
<td>7/12 (33%)</td>
<td>1/21 (5%)</td>
<td>11/21 (52%)</td>
<td>16/21 (76%)</td>
</tr>
<tr>
<td>HL</td>
<td>13/19 (68%)</td>
<td>9/19 (47%)</td>
<td>1/19 (5%)</td>
<td>16/19 (84%)</td>
<td>19/19 (100%)</td>
</tr>
<tr>
<td>HL*</td>
<td>16/22 (73%)</td>
<td>11/22 (50%)</td>
<td>3/22 (14%)</td>
<td>19/22 (86%)</td>
<td>22/22 (100%)</td>
</tr>
<tr>
<td>HL-<em>/HL</em></td>
<td>2/17 (12%)</td>
<td>3/17 (18%)</td>
<td>0/17</td>
<td>5/17 (29%)</td>
<td>9/17 (53%)</td>
</tr>
<tr>
<td>HIV-</td>
<td>0/19</td>
<td>0/19</td>
<td>0/19</td>
<td>0/19</td>
<td>3/19 (17%)</td>
</tr>
</tbody>
</table>

[Expression of more than one antigen/patient]

HIV+: HIV seropositive (HIV+) patients [n = 39]; ASY: Asymptomatic HIV+ patients [n = 6]; ARC: HIV+ patients with ARC symptoms [n = 12]; AIDS: HIV+ patients with AIDS symptoms [n = 21]; HL: HIV+ patients with hairy leukoplakia (HL) [n = 19]; HIV-: HIV seronegative persons [n = 19]; HL*: HIV+ patients with HL or later development of HL [n = 22]; HL-*/HL*-: HIV+ patients without HL or later development of HL [n = 17].
While all cytologic cell preparations (CS) from control persons were EBV negative, 24/39 (61%) CS of HIV+ patients were positive for EBV capsid and/or membrane antigens (APAAP) (Fig. 2A) (Table 2). Keratinocytes positive for EBV showed flattened (Fig. 2B) or polygonal morphology (Fig. 2C) with nuclear (Fig. 2D) and/or cytoplasmic staining (Figs. 2B, C), in addition some cells morphologically not altered stained also positive.

While no correlation was found between the number of patients with positive results and the clinical stage of HIV-associated disease (Table 3), the amount of positive cells/positive case correlated with HIV-associated clinical symptoms (Table 3) (Spearmann-test: $P=0.001$): in the group of asymptomatic patients all positive cases were scored as "++", while in the group of ARC and AIDS patients most positive cases showed more than 10 positive cells/slide (Table 3). In the group of patients with HL 16/19 patients (84%) revealed positive results for EBV-antigens. There were significant correlations between the clinical finding of HL and patients with positive results for EBV antigen (Spearmann-test: $P=0.001$). Additionally, correlations were found between the presence or development of HL and the expression of certain EBV antigens resp. the number of positive cells/case: all patients showing positive results for EA (ARC: $n=2$, AIDS: $n=1$) (Table 2) or positive results scored as "++" or "++++" for VCA (AIDS: $n=3$) and/or NA (ARC: $n=5$, AIDS: $n=5$)
showed the clinical symptom of HL (n = 16) or developed HL during an observation time of 8 months (n = 3) (Table 3). Compared with 61% positive cases (24/39) in the group of HIV+ patients and 86% (19/22) in the group of patients with presence or later development of HL only five of 17 patients without HL (29%) were EBV antigen positive (Table 2).

**EBV-DNA** - On Ramos cells and P3HR1 cells positive reactions were not observed, while positive staining was located within the nucleus and in the cytoplasm of Raji cells, in EBV infected Ramos and P3HR1 cells (Fig. 3A, B).

EBV-DNA was found within the nucleus and the cytoplasm of cells with intranuclear inclusions and occasionally also in cells morphologically not altered. CS of HIV+ patient, ISH × 300.

While HEL cells were negative for CMV structural antigens, CMV infected HEL cells showed positive reactions mainly within nucleus and occasionally within cytoplasm as well. APAAP × 350, without counter staining.

There was a strong correlation between the occurrence of HL and positive results for EBV-DNA and high numbers of positive cells/positive CS (Spearmann-test: P = 0.0002): all CS scored as “++” or “+++” were taken from patients with HL (n = 7) or who later on developed HL (n = 1) (Table 3). All CS taken from areas with the clinical diagnosis of HL (19/19) and from clinically uninvolved areas in patients who developed HL (n = 3) were positive for EBV-DNA (Table 2).

**CMV antigen** - Positive staining for CMV structural antigens and for CMV DNA was found within the nucleus and the cytoplasm of cells with intranuclear inclusions and occasionally as well in cells morphologically not altered. While HEL cells were negative for CMV structural antigens, CMV infected HEL cells showed positive reactions mainly within the nucleus and the cytoplasm (Fig. 5).

Using the APAAP technique in the group of HIV+ patients 22/39 CS (56%) were positive for CMV, while 2/19 controls (11%) showed positive results as well (Table 4). Expression of viral structural antigens was observed within keratinocytes revealing enlarged nuclei (Fig. 6A) and perinuclear halo (Fig. 6B), mostly associated with cytoplasmic positive reactions.

While in the group of asymptomatic HIV+ patients and in HIV-seronegative controls less than 5 positive cells/case were found, positive CS of ARC and AIDS patients were mainly scored as “++” or “+++”. No correlation was found between the presence of HL and positive results for CMV antigen (Table 4).

**CMV DNA** - MRC-5 cells and HEL cells were negative for CMV DNA, while within the nucleus of CMV infected MRC-5 cells (Fig. 7) and HEL cells positive reactions were observed. By means of ISH 27/39 (69%) of HIV+ patients showed positive results for CMV DNA (Fig. 8), while in 4/19 (21%) HIV seronegative controls positive results with less than 5 positive cells/CS were found (Table 4). In the different clinical stages the number of patients with positive results for CMV DNA was similar. Compared with asymptomatic HIV+ patients ARC or AIDS patients showed an increased frequency of positive cases scored as...
Positive staining for CMV structural antigens was found within nucleus and/or cytoplasm of keratinocytes revealing as signs of viral infection enlarged nuclei (A) with perinuclear halo (arrow) (B). CS of HIV+ patient: A: APAAP, ×450. B: APAAP, ×300.

Fig. 6. Positive staining for CMV structural antigens was found within nucleus and/or cytoplasm of keratinocytes revealing as signs of viral infection enlarged nuclei (A) with perinuclear halo (arrow) (B). CS of HIV+ patient: A: APAAP, ×450. B: APAAP, ×300.

“++” or “+++” (Table 4). Positive results for antigens/DNA of both viruses were not correlated with the medication of antiviral drugs (n = 15 patients). However, one AIDS patient under medication with Gancyclovir (DGHP) (duration: 4 months prior sampling) was negative for EBV and CMV in both techniques, while 2 patients receiving Acyclovir (Zovirax) for 2 days before sampling revealed positive results for viral antigens/DNA.

Discussion

The pathogenesis of AIDS may be influenced by several cofactors (11, 12). Herpes viruses may be of special importance, because they are ubiquitous, have a particularly high prevalence in certain high-risk populations and cause increased morbidity and mortality in patients with AIDS. Herpes viruses are characterized by three biologically and clinically distinct features: latency after initial infection and initial replication, reactivation during alterations of the T-cell mediated immunity, and potential transformation of the host cell to a malignant state (13). Virus persistence and latent infection may occur due to different mechanisms: viruses may continue to replicate very slowly, eventually producing disease due to cytolytic infection. They may also cause chronic cytopathic infection with continuous viral production that does not cause disease, such as persistent asymptomatic shedding of CMV in urine or EBV and HSV in saliva. Virus replication and subclinical shedding may also be intermittent without producing lesions (14). In each of these cases the infection is subclinical either because dying host cells are replaced rapidly in the tissues or because the host has little or no inflammatory response (13-15).

There is ample evidence of EBV infection in patients with AIDS (16, 17); titers of EBV-related antibodies as well as numbers of EBV-infected cells in the peripheral blood of HIV-infected patients are higher than those in HIV-seronegative healthy persons (17). While human B lymphocytes are the usual target, EBV can also infect and replicate in epithelial cells of oropharyngeal and genital sites (18-21). The receptor for EBV, which corresponds to the complement fraction 3rd receptor (C3), has been identified on B-cells, certain T cells (22) and on keratinocytes of the middle and upper spinous layer of parakeratinized epithelium (23). In HL epithelial cells with this plasma membrane receptor are known as the site of replicating virus (23).

In the present study 61% (APAAP) resp. 79% (ISH) of oral CS gained from HIV-infected patients contained keratinocytes positive for EBV. Similar results were gained using electron microscopy (negative staining technique), where 53% of HIV-infected patients showed positive results (unpublished), using in situ hybridization, where EBV expression was found in up to 76% of homosexual men (24) or by cytospin in situ hybridization technique in 23/25 cases of HL (25). As described in other studies (12, 27) positive staining was detected in cells with and without phenotypic evidence of viral infection.

Excretion of EBV has been stated as sensitive marker of underlying immunodeficiency. In HIV-infected patients high levels of EBV excretions correlated with a high probability of HIV-progression, prior to significant changes in CD4/CD8 ratios (24), while Alsip and coworkers found higher levels of EBV excretion in AIDS patients than in patients with ARC symptoms or with acute infectious mononucleosis (27). Similar results were found in the present study, where the amount of positive cells/positive case correlated with the stage of HIV associated disease: the highest percentage of positive CS/positive case was found in the group of patients with ARC and AIDS manifestations, suggesting that these patients...
have an altered immunoregulation of EBV.

In contrast, positive results in HIV-seronegative persons examined in the present study have been a very rare finding and positive cases (3/19) revealed a very low number of positive cells. Similar results were described by Alsip et al. (27) while others found low as well as occasionally high excretion of EBV in the general population (13, 14, 16). This may occur due to asymptomatic shedding, which has been demonstrated in up to 60% of healthy adults or due to immunosuppression caused by other factors than HIV-infection (16).

Apart from the clinical stage the positive results for EBV correlated with the clinical diagnosis of HL, and seemed even to preceede the clinical manifestation of HL in three patients: all patients revealing positive results for EA or positive results for VCA or NA with high numbers of positive cells/case showed the presence or later development of HL. This lesion is a frequent and early clinical symptom pointing to an underlying immunodeficiency. It was first described in HIV infected homosexual men, later as well in all other groups at risk for HIV-infection (3, 4). HL has been considered to be a highly predictive lesion for the development of AIDS (28), a fact which has also been stated in the present study. The correlation between the clinical finding or later development of HL with the progression of HIV-associated disease may be reflected in the immunocytochemical results for EBV: while 61% of HIV+ patients and 84% of HIV+ patients with HL showed positive results for EBV, only five of 17 patients without presence or later development of HL were EBV antigen positive. Since the clinical diagnosis of HL may have further consequences for the individual patient, it has been postulated that for confirmation biochemical proof of EBV would be necessary (5). The examination of oral CS, described in this study, offers the possibility of gaining samples from a relatively large area by means of a non-invasive technique. The diagnostic accuracy is comparable with results gained by biopsies (4, 5).

The increased load of EBV found in the oral CS of our patients may have various consequences: since EBV itself is an effective unspecific immunostimulant it may trigger the progression of the disease by activation of CD4+ epithelial cells and thereby coactivating latent HIV which leads to destruction of these cells (17, 29). The decreasing number of CD4 cells may in turn favor further B-cells already latently infected by EBV to complete a full lytic cycle of viral replication without prior immunooelimination. Whether the increased finding of oral lymphomas in HIV-infected patients may be related to the increased EBV expression (30), is yet unknown, but may well relate to similar mechanisms.

CMV can be transmitted via saliva as well as through blood and sexual contact (16). After the initial replication in epithelial cells CMV takes up long-term residence in circulating leukocytes.

### Table 4. Number of cells positive for CMV antigen/positive case (APAAP) and for CMV DNA/positive case (in situ hybridisation).

<table>
<thead>
<tr>
<th>Score</th>
<th>CMV antigen</th>
<th>CMV DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>HIV+</td>
<td>14/39 (36%)</td>
<td>7/39 (18%)</td>
</tr>
<tr>
<td>ASY</td>
<td>3/6 (50%)</td>
<td>0/6</td>
</tr>
<tr>
<td>ARC</td>
<td>4/12 (33%)</td>
<td>5/12 (42%)</td>
</tr>
<tr>
<td>AIDS</td>
<td>3/21 (14%)</td>
<td>6/21 (29%)</td>
</tr>
<tr>
<td>HL</td>
<td>10/19 (55%)</td>
<td>2/19 (10%)</td>
</tr>
<tr>
<td>HIV-</td>
<td>2/19 (11%)</td>
<td>0/19</td>
</tr>
<tr>
<td></td>
<td>16/39 (41%)</td>
<td>9/39 (23%)</td>
</tr>
<tr>
<td>HIV+</td>
<td>4/6 (67%)</td>
<td>1/6 (17%)</td>
</tr>
<tr>
<td>ASY</td>
<td>4/12 (33%)</td>
<td>5/12 (42%)</td>
</tr>
<tr>
<td>AIDS</td>
<td>4/21 (19%)</td>
<td>7/21 (33%)</td>
</tr>
<tr>
<td>HL</td>
<td>9/19 (47%)</td>
<td>1/19 (5%)</td>
</tr>
<tr>
<td>HIV-</td>
<td>4/19 (21%)</td>
<td>0/19</td>
</tr>
</tbody>
</table>

Score +: <5 positive cells/slide; Score ++: 5 to 10 positive cells/slide; Score +++: <10 positive cells/slide.

HIV+: HIV seropositive (HIV+) patients [n=39]; ASY: Asymptomatic HIV+ patients [n=6]; ARC: HIV+ patients with ARC symptoms [n=12]; AIDS: HIV+ patients with AIDS symptoms [n=21]; HL: HIV+ patients with hairy leukoplakia (HL) [n=19]; HIV−: HIV seronegative control persons [n=19].

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**Fig. 7.** MRC-5 cells were negative for CMV DNA, while within nucleus of CMV infected MRC-5 cells positive reactions were observed. ISH ×200.

**Fig. 8.** Mainly intranuclear CMV DNA was detected in 69% of CS of HIV+ patients. CS of HIV+ patient. ISH ×300.
More than 50% of healthy adults and almost 100% of HIV positive homosexuals have serum antibodies to CMV (31). CMV has been isolated from the pharynx in 74% (32) and from parotid (31). CMV has been isolated from the oral CS from HIV-infected patients (33). The examination of oral CS in the present study showed comparable results: 56% (APAAP) resp. 69% (ISH) of CS from HIV-infected patients showed positive results for CMV antigen resp. CMV DNA. In addition to detection of CMV antigen/DNA within cytomegalic cells, positive results were found also in the nucleus and cytoplasm of cells without enlargement or inclusion formation. Similar findings have been described by others in various tissues (34–36).

In contrast to their protective function, elevated serum titers of CMV antibodies seem to be a significant predictor for the development of AIDS (31). While symptomatic infection with CMV occurs very rarely in healthy adults, CMV is a critical opportunistic pathogen in the immunocompromised host (31). Whether the enhancement of local CMV replication correlates with increasing levels of CMV viremia during the progression from ARC to AIDS symptoms and CMV involvement of various organs is under investigation.

For both viruses comparatively low numbers of positive results were found in the group of AIDS patients examined in the present study. This may partly be due to CS of four patients with AIDS symptoms, in which antigens/DNA of EBV and CMV were not detected. In one of these patients treatment with DHPG for a period of 4 months before sampling may be the reason for negative results. The remaining three patients received ACT, were under different antibacterial and antifungal therapies and died shortly after the oral samples were taken. Results in these patients may reflect coincidental changes of viral shedding, may result from prolonged multiple therapies or may be connected with the immunoregulatory breakdown of the late stage of HIV-associated disease.

The oral mucosa allows diagnostic procedures with easy accessibility. The examination of cytologic smears of the oral mucosa provides the benefit of being non-invasive, of gaining material from a relatively large surface compared to biopsy, and of repeated sampling of the same patient during clinical follow-up. However, due to the presence of cell detritus, mucus, bacterial and fungal micro-organisms adhering to the epithelial cells the cytospin technique, used in a prestudy, had resulted in an unequal distribution of epithelial cells. Due to a different technique, which was developed to gain cytologic cell preparations with single cell suspension, the examination of single epithelial cells was possible. In further prospective studies it will be evaluated whether serial samplings of oral CS taken from the same patient can facilitate assessments of the effects of antiviral therapies and give insight into the possible role of these viruses in HIV-associated disease.

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