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Immunohistochemical detection of HIV structural proteins and distribution of T-lymphocytes and Langerhans cells in the oral mucosa of HIV infected patients*

Jürgen Becker[1], Paul Ulrich[2], Rudolf Kunze[2], Hans Gelderblom[2], Angelika Langford[1], and Peter Reichart[1]

[1] Department of Oral Surgery, Faculty of Dentistry, Föhrerstrasse 15, D-1000 Berlin 65
[2] Robert-Koch-Institut des Bundesgesundheitsamtes, Federal Health Administration, Nordufer 20, D-1000 Berlin 65

Summary. Seventeen biopsies taken from oral mucosa of HIV infected patients were analysed for the distribution of $CD4^+/CD8^+$ T-lymphocytes and Langerhans cells. The results were evaluated statistically. An increase in the absolute number of $CD4^+$ and $CD8^+$ cells in HIV infected patients without clinical symptoms, ARC or AIDS was seen in the connective tissue stroma when compared with normal oral mucosa from sero-negative patients. However, the ratio between $CD4^+/CD8^+$ cells was decreased due to the disproportionate increase of $CD8^+$ cells. These findings did not show statistical correlation with the clinical status of the infection. In contrast to the increase in absolute number of $CD4^+/CD8^+$ cells the number of Langerhans cells was unchanged when compared with the control group. Using four different monoclonal antibodies against virus structural proteins ($2 \times$ anti p24, gp41, gp120) two of 26 patients showed labelled cells in consecutive sections in the same connective tissue areas. It was assumed that latently infected mononuclear cells invaded the oral mucosa together with uninfected cells of the cellular immune system.

Key words: AIDS – HIV – Langerhans cells

Introduction

The human immunodeficiency virus (HIV), a member of the lentivirinae subfamily of the retrovirus family, is the aetiologic agent of the acquired immunodeficiency syndrome (AIDS) and related disorders. Soon after the isolation of HIV, the genome was sequenced (Chiu et al. 1985; Sonigo et al. 1985) and virus structural proteins were characterized (for a review see: Wong-Staal and Gallo 1985). It was shown that the virus envelope glycoprotein gp 120 of HIV binds specifically to epitopes of the CD4-receptor molecule (Dalgleish et al. 1984; Klatzmann et al. 1984). The interaction between gp 120 and the cell receptor has been considered to be a necessary and critical step for initiating the reproductive cycle of HIV in its host cell (McDougal et al. 1986).

Clinically, AIDS is characterized by multiple opportunistic infections and/or malignancies, predominantly of the Kaposi sarcoma (KS) type (Friedman-Kien et al. 1982; Reichart et al. 1987). The AIDS-related complex (ARC) encompasses milder forms and sometimes prodromal states of the disease, that is unexplained chronic lymphadenopathy and/or lymphopenia involving $CD4^+$ T-lymphocytes (Masur et al. 1981).

HIV can regularly be isolated from $CD4^+$ lymphocytes of infected persons. However, HIV can obviously replicate in activated B-lymphocytes in addition and in monocyte – macrophage cells, in Langerhans cells (LC) of the skin, in follicular dendritic cells of infected lymph nodes and in brain cells of the monocyte – macrophage or microglia lineage (Montagnier et al. 1984; Levy et al. 1985; Shaw et al. 1985; Gartner et al. 1986; Tenner-Racz et al. 1986; Tschachler et al. 1987). All these cells express low densities of the CD4 surface marker (Wood et al. 1984; Levy et al. 1985; Stewart et al. 1986), which was thought initially to be exclusive to helper lymphocytes (Reinherz et al. 1979).

The oral mucosa contains numerous cells of the immune system in both the epithelium and in the connective tissue. In the epithelium two main groups of immunocompetent cells are present, bone marrow-derived dendritic cells, LC, and T-

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Table 1. Antibodies used

Designation	Reactivity	Reference	Dilution
CD4	Inducer/helper T-lymphocytes	Coulter Immunology, Florida, USA	1:1000[a]
CD8	Suppressor/cytotoxic T-lymphocytes	Coulter Immunology, Florida, USA	1:1000[a]
CD1	Seventy per cent of thymocytes, Langerhans cells	Coulter Immunology, Florida, USA	1:1000[a]
p24	HIV core protein	Dr. R.C. Gallo, NIH, Bethesda, USA	1:10000
p24	HIV core protein	Dr. H. Lutz, Veterinär-Medizinische Klinik Zürich, Switzerland	1:5000
gp41	HIV transmembranous protein	Dr. L. Montagnier, Pasteur Institute, Paris, France	1:200
gp120	HIV envelope protein	Dr. L. Montagnier, Pasteur Institute, Paris, France	1:200

[a] 50 test vial

lymphocytes. In the connective tissue T-lymphocytes, cells of the macrophage system and B-lymphocytes predominate (Becker et al. 1985). In the mucosa and skin LC form a reticular epithelial trap for external antigens and act as antigen-presenting cells for T-lymphocytes. It is well documented that LC carry antigens identical to those of classical macrophages, like HLA-DR (Nagy et al. 1986). Furthermore, they express low densities of the CD4 receptor. Due to the presence of the CD1 antigen they can be differentiated from these macrophages (Murphy et al. 1981). Numerous studies have been performed on the distribution of T-lymphocyte subsets in peripheral blood of ARC and AIDS patients (Weber et al. 1986), but only few studies have focussed on immunopathological changes in skin and oral mucosa during the course of HIV infection. Because intense quantitative phenotype alterations within the skin LC population of ARC/AIDS patients were described by Belsito et al. (1984) the distribution of LC and of T-lymphocyte subsets was studied in the oral mucosa of HIV infected patients to get further insight in the local immune system of the oral mucosa during HIV infection.

Since HIV has recently been demonstrated in skin LC (Tschachler et al. 1987) we attempted to demonstrate the presence of HIV in the oral mucosa using monoclonal antibodies against different virus structural proteins.

Material and methods

For the investigation of $CD4^+$- and $CD8^+$-T-lymphocytes and Langerhans cells 17 patients were divided into three clinical groups, according to the definition of the Centers for Disease Control (1986). The first was those who were HIV infected, clinically without any signs of infection or oral candidiasis ($n=5$), the second ARC ($n=6$) and the third AIDS ($n=6$). HIV infection was diagnosed by ELISA and Western blot. 17 biopsies were taken under local anaesthesia from clinically normal oral mucosa (buccal mucosa: 2, gingiva propria: 9, tongue: 4, hard palate 2), snap frozen in liquid nitrogen and stored at $-75°$ C. All AIDS patients revealed oral candidiasis. In these patients the ratio between $CD4^+/CD8^+$ cells of peripheral blood lymphocytes was determined by flow cytometry using fluorescein-conjugated monoclonal antibodies (Coulter Immunology) and ranged between 0.04–0.4.

For the detection of HIV virus structural proteins (Table 1) biopsies of 26 patients were examined (1. HIV infected, $n=7$, 2. ARC, $n=8$, 3. AIDS, $n=11$). 18 biopsies were taken from oral mucosa, 5 biopsies from oral KS (hard palate) and 3 biopsies from oral hairy leukoplakia (tongue). As a control 10 biopsies of normal oral mucosa of non-HIV infected patients were included (gingiva propria, $n=7$, tongue $n=3$). Two patients were females (control: 4) and 24 were males (control: 6). The average age of the three clinical groups was 34 years (range 24–59 years), that of the control group 20 years (range 10–33 years).

Consecutive cryostat sections of 3–4 µm in thickness were air dried for 2 h and fixed in acetone for 15 min at room temperature. Sections were first incubated with primary monoclonal antibodies (Table 1), then with the unlabelled goat-antimouse bridging antibody (dilution 1:60; Jackson, Avondale, USA) and finally with the alkaline phosphatase – mouse-anti-alkaline phosphatase (APAAP) immune complexes (dilution 1:50; Dianova, Hamburg, FRG). For reference see Cordell et al. (1984). Monoclonal antibodies were diluted with bovine serum albumin (1%) in tris buffered saline (TBS, 0,05 M, pH 7,5). At all steps, sections were incubated for 30 min. The second and third step incubations were each repeated twice for 10 min. After every incubation sections were washed in TBS buffer for 3×5 min. The alkaline phosphatase substrate was prepared as follows: 0.5 ml of a 5% solution of sodium nitrite was added to a solution of 170 ml Tris-HCL (0.05 M, pH 8.7) and 60 ml 0.2 M aminomethylpropandiol containing 90 ml of levamisole, followed by the addition of 125 mg of naphtol AS-BI which had been freshly dissolved in dimethylformamide at 10 mg/ml. This solution was then filtered and used immediately for the staining of slides (15–30 min). The slides were counterstained with haemtoxylin for 1–3 min.

Control reactions were explored for each antibody and biopsy by omitting the primary antibody. Monoclonal antibodies against virus structural proteins were tested for specificity on the H9 and HTLV-IIIB infected H9 T-cell line (Fig. 1) and on tonsillar tissue of HIV sero-negative persons.

The commercially available bridging antibody led to a non-specific background staining in the entire connective tissue. This was probably due to circulating immune complexes, not marked in the control group but pronounced in HIV infected patients. The non-specific reactivity was reduced as follows: 50 µg of a biopsy of a HIV patient, which revealed a pronounced background staining, was homogenized in 1 ml PBS containing 0.02% sodium acide, sedimented by centrifugation and the residue was washed three times in the same buffer. 0.5 ml of the

Table 2. Quantitation of labelled cells (mean values ± SD)

		$CD4^+$	$CD8^+$	$CD1^+$
Normal oral mucosa	E	10,6± 5,2	6,3± 3,0	8,9±5,2
(n=10)	C	17,7±11,1	7,6± 4,6	0,7±1,0
1. HIV infected	E	7,5± 5,6	14,8±14,0	8,3±3,9
(n=5)	C	49,8±23,0	65,1±28,8	0,6±0,9
II. ARC	E	6,7± 6,7	19,0± 9,1	7,6±5,0
(n=6)	C	25,3±19,3	28,4±14,8	2,1±1,1
III. AIDS	E	6,4± 3,8	10,5± 4,7	4,1±2,7
(n=6)	C	40,1±14,7	27,6±26,5	0,8±1,2

E = epithelium; C = connective tissue; n = number of biopsies

original bridging antibody was diluted 1:2 and incubated with the resuspended tissue pellet for 4 h at 4° C by vigorous shaking.

This procedure led to a nearly complete reduction of the unspecific background staining in the connective tissue but not to a reduction of the specific staining. The same dilution of the bridging antibody was used as before.

The epithelium and upper connective tissue stroma were examined separately. In each, 10 randomly distributed fields were evaluated using a Leitz Orthoplan microscope with a primary magnification of ×400. The mean values of the counted cells in ten fields were calculated. Only those cells with clearly recognizable nuclei were counted. For each of the four groups (Table 2) the mean value of each cell type ($CD1^+$, $CD4^+$ and $CD8^+$) with its standard deviation (SD) was calculated. To decide whether there was a significant difference between the groups the values were examined using an analysis of variance (significance level 5%). The number of CD1-, CD4- or CD8-positive cells in the normal oral mucosa of the HIV seronegative control group were compared with the number of positive cells of the oral mucosa of all 3 groups of HIV infected patients, also the 3 individual groups of HIV infected patients were compared against each another.

The mean value and standard deviation (SD) of the ratio between $CD4^+$ and $CD8^+$ cells in the epithelium and in the connective tissue was determined.

Results

The mean values and the SD of the different immunocompetent cells in the four groups investigated are summarized in Table 2. Using an analysis of variance the quantitative increase of CD4- and CD8-positive cells in the connective tissue and the increase of CD8-positive cells in the epithelium of the groups with HIV infection differed significantly compared with the normal oral mucosa of the control group. In contrast, the number of $CD4^+$ cells was decreased in the epithelium of all HIV infected patients (Fig. 2). Statistically, there was no significant difference in the number of CD4- and CD8-positive cells between the individual three groups of HIV infected patients although the pattern of distribution was different. In the first two groups, T-lymphocytes were found predominantly in the upper connective tissue and in the epithelium ($CD8^+$ cells; Fig. 3) and often aggregations of labelled cells were observed. In AIDS patients, however, the labelled cells were often more diffusively distributed in the upper but also in the deeper connective tissue stroma. The ratio between $CD4^+$ and $CD8^+$ cells is shown in Table 3. The mean values and the SD indicated a decreased ratio in the connective tissue of HIV infected patients. This was due to the relatively greater increase of $CD8^+$ cells when compared with $CD4^+$ cells (Table 2).

$CD1^+$ cells were observed only in the upper connective tissue close to basement membrane and within the epithelium itself. Statistically, there was no significant difference in the values of LC between normal oral mucosa and HIV infected patients and between the three individual groups of HIV infected patients itself.

In two out of 26 biopsies of HIV infected patients, labelling for HIV structural proteins was detected. Consecutive sections showed labelled cells in the same connective tissue area positive for all HIV-specific monoclonal antibodies. All antibodies revealed a similar staining pattern. Positive cells could not be detected in the epithelium. One patient revealed brilliantly labelled mononuclear cells in the specific but also in the control reaction in one area of the deeper gingival connective tissue stroma. All other biopsies were negative. The first positive biopsy (Fig. 4) was taken from clinically normal gingiva from a 35-year-old female drug abuser, who developed ARC 4 weeks after biopsy. A second biopsy was taken from an oral hairy leukoplakia (tongue) of a 45-year-old homosexual man. The quantity and staining intensity of labelled cells was more pronounced in the first biopsy, in the second biopsy staining intensity of only few cells was comparable to the first patient (Fig. 5). Neither a higher concentration of primary monoclonal antibodies nor using a mixture of three different antibodies (anti p24, gp41, gp110) resulted in an increase of staining intensity.

Discussion

All covering epithelia and their subepithelial connective tissue stroma contain numerous cells of the immune system. A number of studies have been performed on the distribution of immunocompetent cells in various diseases of the oral mucosa (Becker et al. 1983, 1985). The ratios of T-helper/inducer ($CD4^+$) and T-suppressor/cytotoxic ($CD8^+$) T-lymphocytes in normal oral mucosa in

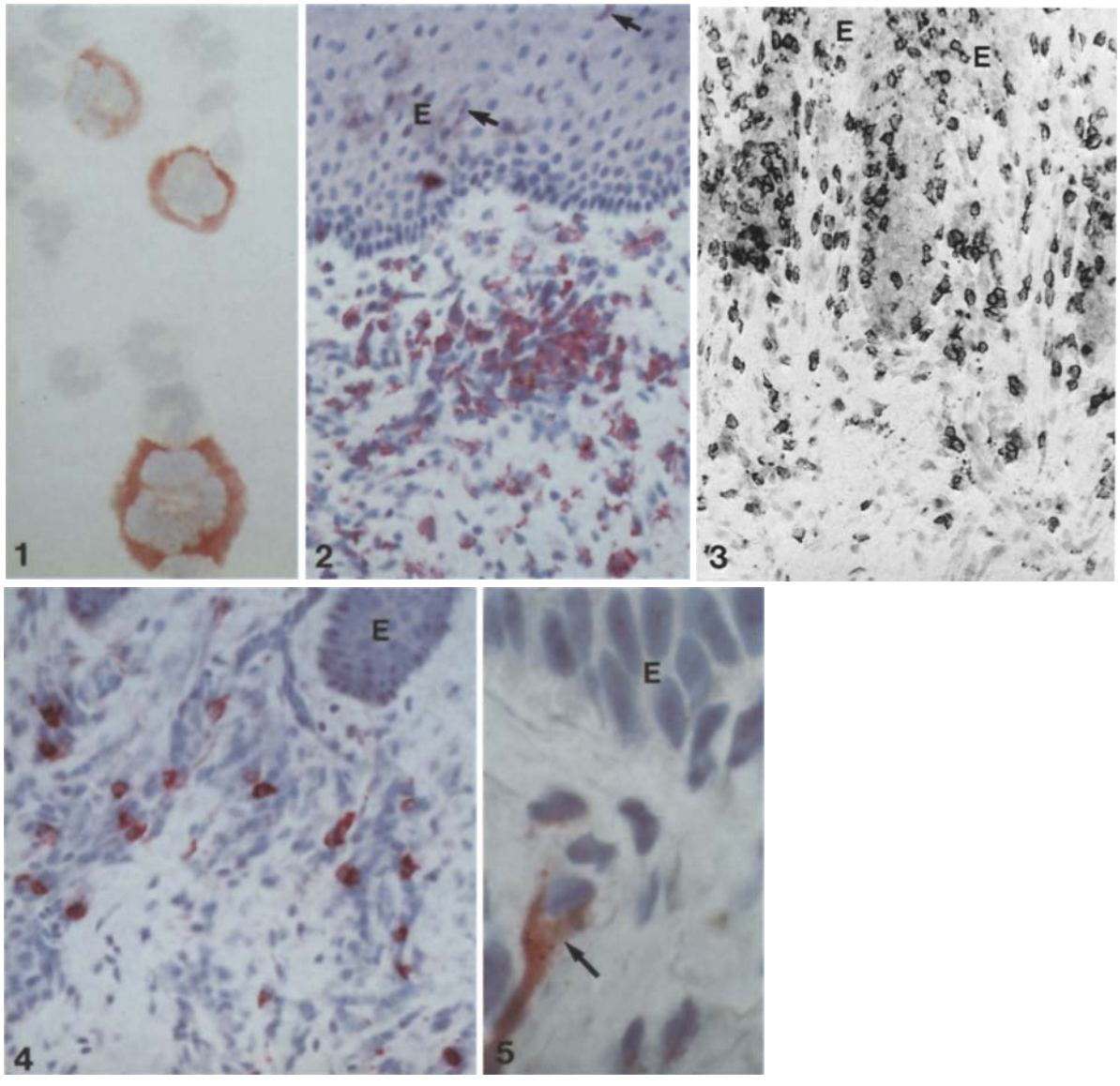

Fig. 1. H9-HTLV IIIB infected H9T-cell line: Positive staining for p24. ×1900

Fig. 2. Numerous $CD4^+$ cells in the connective tissue of an AIDS patient, often forming aggregations of lymphocytes. In the epithelium (*E*) positive cells were rarely observed. ×1200

Fig. 3. Numerous $CD8^+$ positive cells in the epithelium (*E*) and in the connective tissue (same patient as Fig. 3). ×800

Fig. 4. Normal gingiva propria of an HIV infected patient with numerous gp41 positive cells in the connective tissue stroma. ×825

Fig. 5. One cell positive for p24 (*arrow*) in the upper connective tissue of a hairy leukoplakia. (E = epithelium) ×3000

these studies differed when compared to our present data. In this study the ratio of $CD4^+$ and $CD8^+$ cells in the epithelium was 1.76 and 2.53 in the connective tissue. In a previous study (Becker et al. 1985) the ratio was 0.51 in the epithelium and balanced in the connective tissue stroma. These differences might be due to different staining techniques. While in former studies usually the immunoperoxidase technique was used, in the present investigation the APAAP technique was applied. To decide whether different primary monoclonal antibodies with different avidity and/or recognition of epitopes for the CD4-molecule label different quantities of CD4-positive cells in peripheral

Table 3. Ratio between $CD4^+$ and $CD8^+$ cells in the oral mucosa (mean values and SD)

		x SD
Normal oral mucosa	E	1,76±0,69
(n=10)	C	2,53±1,37
I. HIV infected	E	1,06±0,87
(n=5)	C	0,85±0,37
II. ARC	E	0,36±0,29
(n=6)	C	0,82±0,21
III. AIDS	E	0,69±0,44
(n=6)	C	1,82±1,39

tissues would require further double labelling studies. Furthermore, differences in cell distribution, especially in the absolute number of immunocompetent cells, could be related to sampling of different regions of the oral mucosa, as it was shown for Langerhans cells by Daniels et al. (1984). The quantitative increase of labelled CD4- and CD8-positive cells in the connective tissue was found in all locations where biopsies have been taken. This may indicate that the connective tissue stroma of oral mucosa reveals an increased lymphocytic infiltration during the HIV infection. The intense, diffuse infiltration of the deeper connective tissue was only observed in AIDS patients and has not been found in other oral diseases, i.e. oral papillomas, leukoplakias or lichen planus (Becker et al. 1983; 1985). These findings were in contrast to the results found in peripheral blood. While in peripheral blood a continuous decrease of $CD4^+$ cells was observed, in the connective tissue of the oral mucosa the absolute number of CD4- and CD8-positive cells increased. This decreased ratio was obviously due to an disproportional increase of $CD8^+$ cells, but reflects also the decrease of $CD4^+$ cells in peripheral blood.

LC take up foreign antigens and present them together with their own HLA-DR, probably to the CD4 receptor bearing T-lymphocytes (Stewart et al. 1986). In a previous study of oral papillomas and leukoplakias a simultaneous increase of T-lymphocytes and LC was found (Becker et al. 1985). In contrast to these findings the present study revealed an unchanged number of LC in HIV infected/ARC and AIDS patients compared with the control group, although an increase of LC was expected from our previous studies (Becker et al. 1985). Belsito et al. (1984) and Oxholm et al. (1986) observed a reduction of Ia-positive LC in skin biopsies of ARC/AIDS patients and it was suggested that a reduced capacity for Ia antigen and thereby antigen presentation might result in a defective $CD4^+$ helper cell proliferation. This might therefore play a role in the pathogenesis of HIV infection (Belsito et al. 1984). This theory was underlined by the findings of Tschachler et al. (1987), demonstrating that epidermal LC are a target for HIV and that on the electron microscopic level signs of cellular alterations of LC seemed to be due to HIV particles within LC. The unchanged number of LC in the present study, despite the inflammation, may also indicate reduced antigen presentation, but labelling of the CD1 epitope does not give insight in the antigen presenting function of LC.

By in situ hybridisation HIV infected mononuclear cells expressing viral RNA were detected in six of seven lymph nodes and in seven of 14 blood samples of AIDS and ARC patients (Harper et al. 1986). These results demonstrated the presence of only 0,01% to 0,001% positive cells of the cell populations (Harper et al. 1986). It appears, however, that the number of latently infected cells containing the viral genome is at least ten times higher (Kunze et al. 1986).

We showed the presence of labelled cells in the connective tissue of the oral mucosa in two of 26 HIV infected patients. Due to the labelling of cells with monoclonal antibodies against HIV core-(p24)) transmembrane-(gp41) and envelope protein (gp120) it is suggested that these mononuclear cells expressed HIV. Although double labelling studies were not performed, it seems likely that these cells were T-lymphocytes in the first patient (Fig. 4), whereas the stained cells in the second patient (Fig. 5) could be a LC due to the typical dendrite. The presence of HIV expressing cells in the connective tissue of the oral mucosa could be explained by the fact that latently infected cells invaded the oral mucosa together with uninfected cells of the immune system. It has been shown that latently HIV infected lymphocytes can be stimulated like normal lymphocytes and that stimulation is necessary for production of detectable amounts of virus (Zagury et al. 1986). This finding was underlined by the results of Kunze et al. (1986) who detected only one p24-positive cell in unstimulated periperal blood lymphocytes of HIV infected patients only in one out of five patients, while after mitogen stimulation all HIV infected patients revealed 4 to 28 p24-positive cells/1000 peripheral blood lymphocytes. It might be assumed that oral candidiasis or viral infections, (HPV, EBV, CMV) lead to a continuous antigenic stimulation of the local immune system in the oral mucosa. This stimulation may activate latently HIV infected mononuclear cells causing expression of HIV as it was seen in

two out of 26 patients. Furthermore it was demonstrated by Braathen et al. (1987) in tissue culture that HIV infected LC produce immunohistochemically detectable amounts of virus structural proteins after mitogen stimulation. Compared with the large number of HIV positive lymph nodes observed in ARC and AIDS patients by Harper et al. (1986) we could only demonstrate the presence of HIV in a small number of biopsies. This is obviously due to the low number of immunocompetent cells in oral mucosa compared with lymph nodes. The finding of one positive control reaction was unexpected and underlines the necessity to perform control reactions on every biopsy and every antibody. As yet HIV expressing cells were detected in peripheral blood, lymph nodes, brain and LC of the skin. In the present study we were able to demonstrate the expression of HIV in the oral mucosa. Further studies are necessary to explain the probable significance of the presence of HIV in skin and oral mucosa, because this organ is the largest in man and might represent a reservoir for HIV.

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