HUMAN T-LYMPHOTROPIC VIRUS (HTLV) TYPE I IN VIVO INTEGRATION IN ORAL KERATINOCYTES

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ABSTRACT

Although the infection of HTLV-1 to cell components of the mouth have been previously reported, there was not until this report, a detailed study to show the characteristics of such infection. From 14 Tropical Spastic Paraparesis/ HTLV-1-Associated Myelopathy (HAM/TSP) patients and 11 asymptomatic carrier individuals (AC) coming from HTLV-1 endemic areas of southwest Pacific of Colombia, infected oral mucosa cells were primary cultured during five days. These cell cultures were immunophenotyped by dual color fluorescence cell assortment using different lymphocyte CD markers and also were immunohistochemically processed using a polyclonal anti-keratin antibody. Five days old primary cultures were characterized as oral keratinocytes, whose phenotype was CD3-/CD4-/CD8-/CD19-/CD14-/CD45-/A575-keratin+. From DNA extracted of primary cultures LTR, pol, env and tax HTLV-1 proviral DNA regions were differentially amplified by PCR showing proviral integration. Using poly A+ RNA obtained of these primary cultures, we amplify by RT-PCR cDNA of tax and pol in 57.14% (8/14) HAM/TSP patients and 27.28% (3/11) AC. Tax and pol poly A+ RNA were expressed only in those sIgA positive subjects. Our results showed that proviral integration and viral gene expression in oral keratinocytes are associated with a HTLV-1 specific local mucosal immune response only in those HTLV-1 infected individuals with detectable levels of sIgA in their oral fluids. Altogether the results gave strong evidence that oral mucosa infection would be parte of the systemic spreading of HTLV-1 infection.

Key words: Oral keratinocytes. Oral mucosa. Human T-Lymphotropic virus type 1. Proviral integration. sIgA.

INTRODUCTION

Human T-Lymphotropic virus - HTLV-1 is a human retrovirus initially associated with T-cell malignancies (27). HTLV-1 is a persistent virus, infecting 10 to 20 million people worldwide (28). Although, most of infected individuals remain as asymptomatic carriers, 1 to 2% develops the Tropical Spastic Paraparesis/HTLV-1-associated myelopathy (HAM/TSP) (24), and further 2 to 3% develop an aggressive T-cell leukemia/lymphoma (ATLL) (17). In addition to ATLL and HAM/TSP, HTLV-1 has been associated with a range of chronic or subacute inflammatory conditions in different

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tissues. These associations are weaker than those with ATL and HAM/TSP, and it is not yet agreed that HTLV-1 is the likely cause in each condition (19).

Over the last 25 years the geographical distribution of the HTLV-1 has been identified. The seroprevalence rates differ, according to geographic area, the socio-demographic composition of the population studied and individual risk behaviors. The areas of the world with the highest prevalence rates for HTLV-1 include southwestern Japan, several sub-Saharan African countries, Central and South America, and localized areas of Iran and Melanesia. In the Americas, higher prevalence rates are found in some countries in the Caribbean, such as Jamaica and Trinidad and Tobago. Somewhat lower seroprevalence rates are found in several countries in South America, including Brazil and Colombia (28, 37).

HTLV-1 is transmitted in three ways: (i) from mother to child during prolonged breastfeeding (12, 36); (ii) between sexual partners, mainly from man to woman (10); and (iii) through blood transfusion with HTLV-1-infected cells (22). In areas where the virus is highly endemic, mother-to-child transmission is sometimes the predominant route. Previous reports have suggested as a possible mode of virus transmission the oral fluids of infected individuals (38); recent evidence have documented transmission of HTLV-1 to rats by inoculation with human fluids or cells from infected subjects (32).

Previously we reported that secretory IgA antibody class specific to HTLV-1 proteins in oral fluids of seropositive individuals was correlated with the presence of free genomic RNA in oral fluids of HAM/TSP patients (33). Other studies have shown the detection of viral genomes in salivary secretions and provirus in DNA of salivary lymphocytes (1) and epithelial mouthwash cells (18).

In the present study we showed how several HTLV-1 proviral genomic regions were amplified by PCR from DNA extracted of oral cultured keratinocytes from HTLV-1 infected individuals. Moreover the detection of tax and pol viral poly A+ RNA was correlated with the detection of specific anti-tax-sIgA in oral fluids of HAM/TSP patients. Our results gave strong evidences to propose that HTLV-1 in oral epithelium would be a part of the systemic spreading of virus in the host.

**MATERIALS AND METHODS**

**Patients and samples**

Twenty-five HTLV-1 seropositive individuals from different villages of southwest pacific of Colombia were included in this study. Fourteen were well characterized as HAM/TSP patients according with standard criteria (23) (average age 54.8±11.5); 11 asymptomatic carriers (AC) (average age 45.1 ± 12.8) were also included into the study. As negative controls we included 20 negative HTLV-1/2 negative controls once the ELISA and WB used gave results for both viruses. The study was approved by the Research Ethics Committee of the Health Sciences Faculty of the Universidad del Valle, Cali, Colombia according to the Declaration of Helsinki. All persons enrolled in it were informed about their objectives and consent to donate blood and oral samples only for research purposes. Oral fluid (OF) samples were taken using the FDA approved commercial device Orasure™ (Epitope Inc., Beaverton, OR), following the instructions of the manufacturers. Patient’s blood samples were taken and plasma and lymphocytes were recovered with Hystopaque-1077 (SIGMA) gradients according with the general protocols. Lymphocytes were cryopreserved in DMSO-RPMI1640 (1:1v/v) storage medium at -156°C until their use.

**HTLV-1 ELISA and Western blot**

ELISA Murex HTLV I + II (Murex Biotech Limited, Dartford, UK) diagnostic kit was used for virus screening. Dilutions of 1:40 for plasma and 1:2 for OF were used to test every paired sample. Microplates were washed twice and incubated with goat IgG-antilgG-, IgM- (Sigma Chemical Co, St. Louis, MO) and sIgA (Chapel, Organon Teknika, West Chester, PA) peroxidase labeled conjugate diluted 1:15,000, 1:1000 and 1:2000 respectively. The results were expressed as an OD ratio (OD sample/OD cutoff) and normalized for protein content in OF. Determination of protein concentration in oral
samples was performed with the Bradford colorimetric method (3). An OD ratio \( \geq 1.1 \) was a criterion of seropositivity.

The HTLV-1 confirmation in plasma and OF was performed by western blot using HTLV blot 2.4 assay kit (Genelabs® Diagnostics Pte Ltd. Singapore Science Park. Singapore). OF samples were also analyzed by western blot with a 50-kDa HTLV-1-tax recombinant protein (21).

**Primary cultures of oral epithelial cells**

Using a cotton swabs oral epithelial cells were obtained, they were harvest in transporting minimal essential medium (MEM), L-Glutamine 200 mM supplemented with antibiotics; in all cases the viability of cells was monitored by staining with blue trypan. After that, they were incubated in microplates at 37°C in 5% CO\(_2\) atmosphere in MEM, 15% bovine fetal serum, penicillin (49 μg/ml), amphotericin (4μg/ml), and L-Glutamine 200mM; the culture medium were changed every two days. Cell growing was daily monitored by staining with blue trypan and cell counting was performed with an inverted light microscope. After 5 days (monolayers with more than 60% of confluence), the cultures were submitted to immune staining with a polyclonal anti-keratin antiserum A-575 (Daco, Carpinteria, CA) using the Vectastain Elite ABC kit (Vector, Burlingame, CA). The cultures did begin with 5x10\(^3\) cells/ml and after five days reached they an average cell density of 1.7x10\(^6\) cells/ml.

**Two color flow citometry**

Oral epithelium primary cultures were analyzed by two color fluorescence in a FACSTAR PLUS cell sorter (Beckton-Dickinson, Mountain View, CA) for T, B, macrophage, monocyte and Langerhan cell surface marker expression with monoclonal antibodies to CD3, CD4, CD8, CD19 and CD45 (Becton-Dickinson, Mountain View, CA). The FACS analyses were carried out 24 hours after seeding and also in cultures of five days.

**DNA extraction and PCR protocols**

DNA from primary cultures of oral epithelial cell was obtained according with Ramirez-Solis et al, 1992 protocol (31). Purity of DNA was calculated using the values of O.D\(_{260nm}\)/O.D\(_{280nm}\) ratio; a DNA preparation with O.D ratio \( \geq 1.8 \) was considered pure and suitable for PCR. Each PCR reaction was carried out in a mix containing 1.0 μg of DNA, 0.2 mM of deoxyribonucleoside-5’-triphosphate (dNTPs) mix, 10 μl of 10 X PCR reaction buffer, 1.25 mM MgCl\(_2\), 1μM of HTLV-1-specific primers and 2.5 U of Taq DNA Polymerase (Perkin-Elmer, Cetus Co.) in a total volume of 50 μL. HTLV-1 primers to amplify LTR (737 bp), Pol (189 bp), Tax (159 bp) and a fragment of 1033 bp covering Pol and env proviral regions were used (Figure 1). PCR reactions were performed under the following standardized cycling conditions: once 5 minutes at 94°C followed by 35 cycles of denaturation at 94°C for 2 minutes, 1 minute of annealing to 10°C under the calculated Tm of each pair of primers calculated (26), extension at 72°C for 2 minutes; and a final extension step at 72 ºC for 10 minutes to complete the PCR. DNA of cell line MT2/HTLV-1+ was used as internal control for all PCR reactions. Detection and identification of DNA amplified fragments was performed by southern hybridization using appropriated \([32P]-labeled oligonucleotides as probes\)

**RT-PCR procedures**

Poly A⁺ RNA from approximately 2x10\(^5\) primary cultured of oral epithelial cells/ml of HTLV-1 positive individuals as well as negative controls was extracted using the Dynabeads mRNA DIRECT™ kit (Dynal Biotech ASA, Oslo. Norway) following up the instructions of manufacturers. The Poly A⁺ RNA was used to detect by RT-PCR the mRNA of tax and pol. The cDNA synthesis was carried out in 10 mM Tris-HCl (pH 8.9), 90 mM KCl (1 X RT buffer), 0.9 mM MnCl, 375 μM of each dNTP, and 750 nM of primer for HTLV-1 pol SK111(+) and tax SK44(+), 100 ng of poly A’RNA and 4 U of Tth DNA polymerase (Thermus thermophilus DNA polymerase, Boehringer Mannheim. Germany). The reactions were performed at 70°C for 30 minutes. After that, 40 cycles of a direct PCR was carried out in 10 mM Tris-HCl (pH 8.9), 100 mM KCl, (1 X PCR buffer), 1.25 mM MgCl\(_2\), 0.75 mM EDTA,
Figure 1. Schematic localization along the HTLV-1 proviral genome of several oligonucleotide primers pairs that were used to amplify HTLV-1 proviral sequences from DNA extracted of infected oral keratinocytes. Direction of arrows show the sense of each one of HTLV-1 oligonucleotide primers. Supplementary information about the primers is described in material and methods.

750 nM HTLV-1 primers SK110(+) and SK43(+). The annealing and extension conditions were the same of that previously described for direct PCR. The amplified products were visualized by fluorescence of DNA amplicons with ethidium bromide in agarose gel electrophoresis; the respective HTLV-1 amplified cDNA was identified by southern blot hybridization using appropriated \(^{32}\)P-labeled oligonucleotides as probes (34).

**Statistical calculations**

A Fisher exact test was applied to calculate statistical differences between HAM/TSP and HC for sex, age, proviral region, RNA transcription and immunoglobulin class in plasma and OF.

**RESULTS**

***Reactivity of OF and plasma to viral antigens***

The OD ratios in OF for HTLV-1 antibodies were significantly higher in HAM/TSP patients than in AC (p<0.01). No HTLV-1-specific antibodies were detected in OF and plasma from HTLV-1 seronegative controls. The 71.43% (10/14) of HAM/TSP patients had detectable levels of HTLV-1 specific sIgA in OF in comparison with 18.2% of the AC (P<0.01) (Table 1). Moreover in OF HTLV-1 specific IgG was detected in 100% (14/14) of HAM/TSP patients versus 72.7% (8/11) of AC (P<0.05). No significant differences between HAM/TSP and AC immunoglobulin class and sex and/or age were calculated.

**Table 1. Reactivity of plasma and OF to HTLV-1 antigens as recorded by the ELISA Murex HTLV I + II (Murex Biotech Limited. Dartford. UK) diagnostic kit.**

<table>
<thead>
<tr>
<th>Health Status</th>
<th>Plasma</th>
<th>Oral Fluids</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IgG</td>
<td>IgM</td>
</tr>
<tr>
<td>HAM/TSP</td>
<td>14/14</td>
<td>11/14</td>
</tr>
<tr>
<td>AC (^{(a)})</td>
<td>8/11</td>
<td>2/11</td>
</tr>
<tr>
<td>P (^{(b)})</td>
<td>0.07(^{NS})</td>
<td>0.004</td>
</tr>
</tbody>
</table>

\(^{(a)}\) For AC, n = 11 individuals.
\(^{(b)}\) P value versus HAM/TSP.
\(^{NS}\) Non-significant difference.
Reertoire of HTLV-1 immunoglobulin class in OF

As shown in figures 2a and b, IgG antibodies to p53-55, p19 and r-tax were more frequent in OF from HAM/TSP, in contrast with anti-p28 and p24 which were more commonly registered in AC. Overall specific HTLV-1 sIgA for viral proteins was more frequently detected in OF of HAM/TSP (Figure 2a and b). Furthermore there were significant differences in sIgA between HAM/TSP and AC for p40-Tax (p<0.05), p24 (p<0.05), p19 (p<0.001) and r-tax (p<0.05) proteins (Figure 2c).

Figure 2. Reactivity detected by western blot in plasma and OF against HTLV-1 proteins of one HAM/TSP patient from southwest Colombia. (A). Western blot using the commercial HTLV blot 2.4 assay kit (Genelabs® Diagnostics Pte Ltd. Singapore Science Park. Singapore). (1) plasma control for HTLV-1 included with the kit; (2) plasma control for HTLV-2 included with the kit (3) Anti-IgG HTLV-1 in plasma; (4) Anti-IgG HTLV-1 in OF; (5) Anti-sIgA in OF; (6) Negative plasma control. (B). Western blot with a Tax recombinant protein of a HAM/TSP patient. (1) anti-r-tax -IgG in plasma. (2) anti-r-tax -IgG in OF. (3) anti-r-tax- sIgA in OF (4) Negative OF control. (C). Frequencies of sIgA HTLV-1 specific class reactivity of HAM/TSP patients and AC OF from southwest Colombia against the different HTLV-1 proteins detected by western blot. (HAM/TSP). Tropical Spastic Paraparesis/HTLV-1-Associated Myelopathy. (AC). Asymptomatic Carrier.

Immunophenotyping of oral epithelium cells cultured

As shown in Figure 3 the double labeling CD3/CD4, CD8/CD19 did not show T4, T8 and B lymphocyte positive populations in oral epithelium primary cultures. Additionally the double staining CD14/CD45 also revealed no macrophages and monocytic cells contamination. The immune staining using a polyclonal anti-keratin antibody revealed that oral cells cultures have surface keratins revealing their epithelial origin. Not only by its morphology but also for the absence of CD4, the Langerhan cell contamination was excluded. In summary
the primary cells from the oral epithelium had the phenotype (CD4/CD8/CD14/CD52/A-575-β-keratin*) and were classified as keratinocytes. Furthermore the FACS results did not change in five days primary cultures in comparison with the same at 24 hours after seeding.

**Ex vivo detection of HTLV-1 proviral sequences.**

Figure 4 displays the southern blot results obtained from PCR assays performed in oral keratinocytes DNA by using different sets of HTLV-1 primers; in general the results indicated that HTLV-1 positive samples amplified overall at least one HTLV-1 proviral region integrated in the genome of oral keratinocytes (Table 2). Only 21.4% of proviral DNA of HAM/TSP patients and 27.3% of AC exhibited amplification with all pairs of HTLV-1 primers tested in the study.

**Transcription of viral poly A+ RNA.**

HTLV-1 seropositive individuals who had detectable levels of sIgA in their OF showed an active transcription of tax and pol mRNA. 57.1% (8/14) of HAM/TSP patients amplified by RT-PCR both mRNA and only 27.3% (3/11) of AC (Figure 5a and b). Three AC which expressed viral mRNA corresponded to those that were IgG negatives in plasma but with detectable levels of sIgA in OF. In addition of these results, all HAM/TSP patients included in this study had detectable IgG and sIgA levels of an anti-50kDa recombinant tax protein.

**Figure 3.** Two color flow citometry analyses of pairs of CD antigens as cell membranes markers in primary cultures from oral mucosa cells and keratin detection with the polyclonal antiserum A-575 (Daco, Carpinteria, CA) using the Vectastain Elite ABC kit (Vector, Burlingame, CA) kit. (A to C). FACS analyses in oral mucosa cells after 24 hours of cultured. (D to F). FACS results in oral mucosa cells after five days of cultured. (G) Immune staining of oral mucosa cells cultures after five days with the polyclonal antiserum A-575 (40X). (H). Negative Control IgG (40X).
**Figure 4.** Southern blot hybridization of PCR products obtained from DNA of five days old primary oral keratinocytes from HAM/TSP and AC using different pairs of HTLV-1 oligonucleotide primers. LTR (737bp); Pol (189 bp); Pol-Env (1033 bp); Tax (159 bp). (MT) DNA from MT2 cell line with HTLV-1 provirus. Appropriated $^{[32]}$P-labeled oligonucleotides were used as probes.

<table>
<thead>
<tr>
<th>Proviral region</th>
<th>Length (bp) $^{(a)}$</th>
<th>HAM/TSP</th>
<th>AC</th>
<th>$P$ $^{(b)}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>LTR</td>
<td>737</td>
<td>10/14</td>
<td>6/11</td>
<td>0.43NS</td>
</tr>
<tr>
<td>Pol</td>
<td>189</td>
<td>11/14</td>
<td>7/11</td>
<td>0.65NS</td>
</tr>
<tr>
<td>Tax</td>
<td>159</td>
<td>9/14</td>
<td>5/11</td>
<td>0.43NS</td>
</tr>
<tr>
<td>Pol-Env</td>
<td>1033</td>
<td>9/14</td>
<td>9/11</td>
<td>0.41NS</td>
</tr>
</tbody>
</table>

(a). (bp) base pair. (b). $P$ values calculated with a two tails Fisher exact test. (NS), no statistically significant differences.
DISCUSSION

In the present study we obtained strong evidence to support the existence of a closed association between detectable levels of HTLV-1 specific sIgA in OF and the integration of viral cDNA sequences which promote an active transcription of poly A+ RNA of tax and pol genes in oral keratinocytes. We also demonstrate that HTLV-1 infection in oral cavity is not restricted to the epithelium of HAM/TSP patients, as well as to the asymptomatic carriers; however difference between sIgA levels in OF and expression of tax and pol genes between HAM/TSP patients and AC were observed.

The HTLV-1 integration into DNA of oral keratinocytes which is reported in this work spans the spectra of cell targets for HTLV-1 infection. We previously reported the existence of an association of the presence, in HAM/TSP patients, of free genomic HTLV-1 RNAs with an active production of sIgA in OF (33). The differences recorded between HAM/TSP and AC in the mucosal associated response suggested that not only immunological but cellular changes occur during progression to disease. In general, our results not only supported others previously published but showed that oral keratinocytes are effective cell targets during the systemic spreading of HTLV-1 infection in mucosa (5, 6).

The immune status of those HAM/TSP subjects included in this study, having anti-HTLV-1 IgG and sIgA in OF as well as free viral RNA templates in oral fluids (33), suggest that the infection of HTLV-1 to epithelial targets could occur possibly by contact cell-to-cell with HTLV-1 infected salivary lymphocytes. However still remain to be explain whether those lymphocytes circulate for longer in saliva or they are newly deposit in mouth saliva by extravassing from microcirculation as was previously reported (2, 38). An indirect evidence to explain the infection of oral keratinocytes by cell-to-cell contact between T cells and oral keratinocytes, is that HTLV-1 is primarily transmitted either by breast-feeding (4, 7).
Although the complex receptor for HTLV-1 entry to lymphocytes is partially documented, in other non-lymphocytic cells targets, as keratinocytes, the mechanism of infection is still matter of investigation (25, 13). Several data suggest the ubiquitous glucose transporter GLUT1 act as receptor for Deltaretroviruses HTLV-1 and HTLV-2 envelopes (Env), mediating viral binding and entry (35). GLUT-1 specifically binds a soluble form of both the HTLV-1 and HTLV-1I SU proteins in T cell and non-T cell lines, and was shown to be critical for efficient entry of HTLV-1I-pseudotyped virions (13). The role of GLUT-1 as viral co-receptor for HTLV-1 interaction with target cell and efficient virus spreading correlated largely with heparan sulfate proteoglycan (HSPG) expression on target cells in activated CD4+ T cells and cord blood lymphocytes susceptible to HTLV-1 infection. Altogether these results indicate that GLUT1 and HSPGs are important for an efficient HTLV-1 cell-to-cell transmission but raise concerns on the role of GLUT1 as the HTLV-1 primary binding receptor in lymphocytes (13).

In order to explain the effective integration of HTLV-1 genome in oral keratinocytes, we could argue that a similar complex cell receptor would be important in cell to cell infection. Previous results showed a weak expression of GLUT1 in epithelial cells of the normal mucosa (35, 8); however increasing of GLUT-1 as receptor in this kind of cells could be enhanced by an altered physiological status such as a virus infection. To support this statement, previous data about GLUT-1 localization of expression in the epithelial layers of oral mucosa equivalents, that were grown in a defined plasma-free culture medium without a feeder layer, showed that GLUT-1 was expressed in both basal cell layer and suprabasal cell layer of oral mucosa epithelium (8). Although the evidences could support the hypothesis that GLUT-1 and HSPG would be important molecules playing a role in interaction between HTLV-1 SU proteins and cell target membrane in keratinocytes, it is still matter of discussion.

From the PCR data obtained in this study it was possible to observe that some HAM/TSP and AC keratinocytes DNA did not amplify more than one pair of HTLV-1 specific primers. Although some failure during the PCR process could be responsible for a remote possible mistaken interpretation of results; however another real explanation is that defective proviruses would be generated during the infection process to oral keratinocytes. In order to support the last explanation, previous studies report the existence of defective HTLV-1 provirus in lymphocytic infections not only in ATL (9, 20, 11, 15) but also in HAM/TSP patients (29, 30, 16, 14). In this sense our results would be the first to document the existence of defective HTLV-1 provirus in infected oral keratinocytes. Although not direct evidence have obtained, the presence of defective provirus not only in lymphocytes but in oral keratinocytes, strongly suggest the possibility that low levels of viremia and/or low efficiency in the contact cell-to-cell could possibly favor post integration process that increase the frequency of defective provirus (15); however this statement in oral mucosa keratinocytes needs to be tested.

Although our results indirectly evidence an active infection in oral mucosa which was correlated with an active immune process of mucosa via specific anti-Tax HTLV-1 sIgA, still remains the question weather OF and saliva are really infective fluids. In these sense, inoculation of saliva cells and cell-free saliva from healthy carriers and patients with HAM/TSP into WKA and F344 female rats produced a successful infection (32). By nested PCR from inoculated rat DNA HTLV-1 proviruses in PBMC, spleen, thymus, salivary glands, spinal cord, kidney and brain were detected (32).

In general the results obtained in this study in conjunction with others previously reported, would support the hypothesis that HTLV-1 could primarily exists in oral secretions or it can reach the saliva through the blood after minimal bruises in the oral cavity and potentially could infect cells of the oral epithelium. However, the characteristics of such infection and their effects on the mucosal immune response still remain not fully understood.

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