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HTLV-1 in mouthwash cells from a TSP/HAM patient and asymptomatic carriers

Brief Report

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Summary. Using in situ hybridization, the presence of T-cell lymphotrophic virus type I (HTLV-I) was shown in blood lymphocytes of one tropical spastic paraparesis (TSP/HAM) patient and in two asymptomatic carriers. HTLV-I was also detected in epithelial cells derived from mouthwash of the TSP/HAM patient. Mouthwash of one of the carriers showed an infected lymphocyte while mouthwash of the other carrier was negative. The infected epithelial cells stained both in the nucleus and in the cytoplasm, which indicated the presence of the virus in both subcellular compartments. Our observations suggest that saliva cells, lymphocytes and epithelial cells, may potentially participate in oral transmission of HTLV-I.

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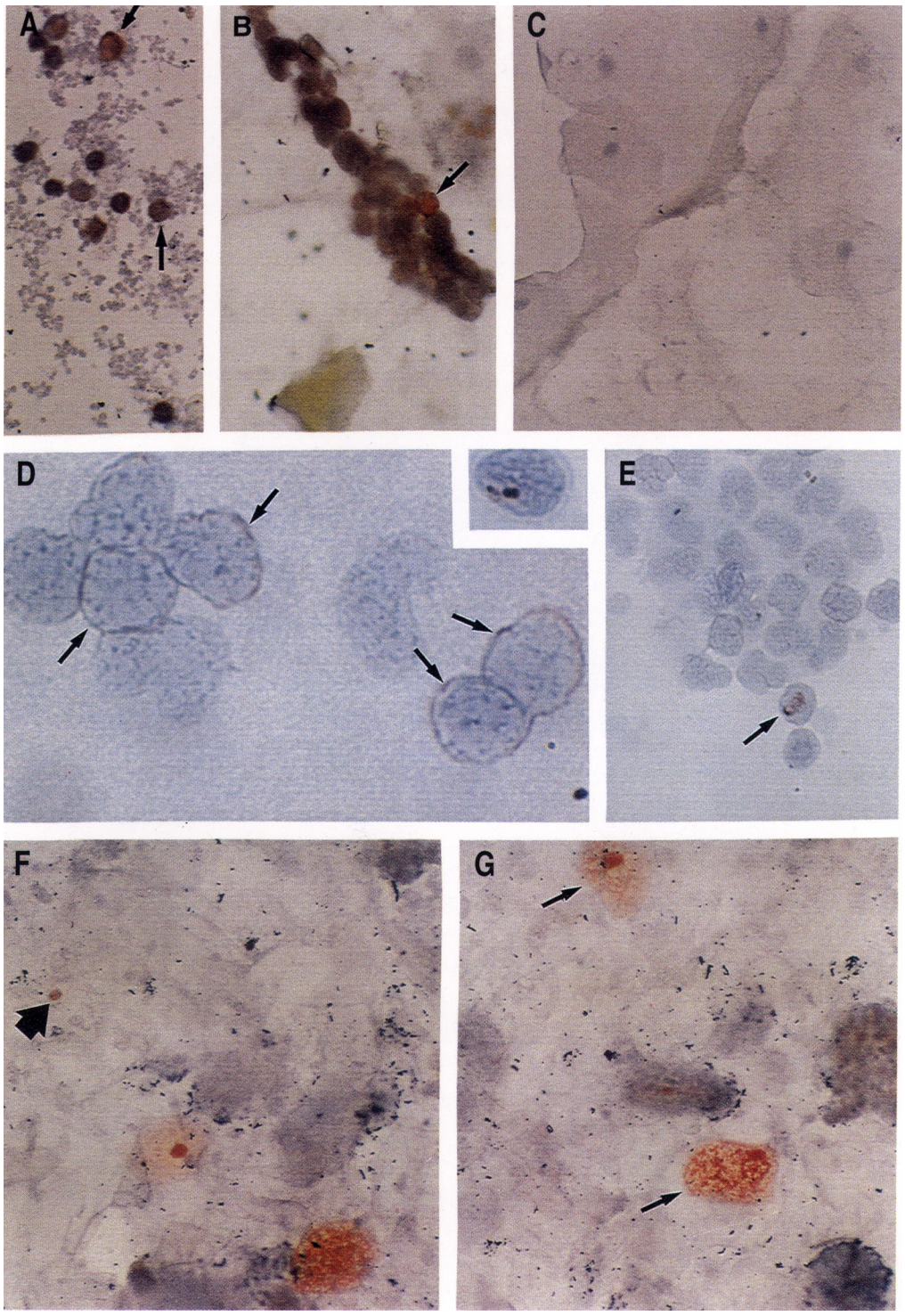
Human T-cell lymphotrophic virus type I (HTLV-I), endemic in several regions, has recently been identified in Jews who immigrated from Mashhad, Iran [1, 2, 9, 15]. The virus is know to cause adult T-cell leukemia (ATL) and/or chronic myelopathy recognized as tropical spastic paraparesis (TSP) or HTLV-I associated myelopathy (HAM). [3]. The known modes of HTLV-I transmission are sexual intercourse, via breast milk, via blood transfusion and by contaminated needles used by drug abusers [11, 12, 14]. The possibility of oral transmission was raised in our previous study in which HTLV-I DNA was detected in mouthwashes of Mashhadi born Jews with TSP and asymptomatic carriers from the same ethnic origin [1, 2]. The present work aimed to identify the cells in the mouthwash in

which the virus could be detected. These cells are suspected to participate in oral viral transmission. The following subjects, were studied.

The TSP/HAM patient (RR) was a 69 year old female born in Mashhad, Iran. One of the asymptomatic carriers (RY) was a 75 year old man, husband of the former, also born in Mashhad. The other carrier (GY) was a 70 year old female born in Mashhad with T-cell lymphoma. A healthy control (KI) was included. Blood and mouthwash samples were taken from all subjects. Mouthwash cell samples were obtained by gargling with sterile saline and centrifugation. HTLV-I antibody titer was assayed in saliva and serum by the particle agglutination test in which the viral antigen was adsorbed on gelatin beads (Fujirebio, Inc., Tokyo, Japan) [9]. In situ hybridzation of blood lymphocytes and of cells isolated from mouthwash samples was performed with biotinylated HTLV-I probe. Cells were fixed on microslides in 4% paraformaldehyde in PBS and stored in 70% ethanol [16]. Microslides were treated for 30 min with 1% triton in PBS, washed with $2 \times$ SSC, acetylated in 0.1M triethanolamine $-$ 0.25% acetic anhydride for 10 min and washed 3 times in PBS and once in $2 \times$ SSC. Endogenous peroxidase was inactivated by 3% H_2O_2 for 30 min at room temperature. Slides were dehydrated with graded alcohols, air dried and covered with 50 μl hybridization buffer (20 μl $20 \times$ SSC; 20 mg BSA, 50 μl 500mM sodium phosphate buffer pH 6.5; 50 μl of 5mg/ml sheared salmon sperm DNA and 0.7ml water) and incubated at 65 °C for 10 min. Biotinylated HTLV-I probe 8.25 Kbp (2.5 μl of stock solution 10 ng/μl, Oncor) was added to another batch of 50 μl hybridization buffer. Coverslips were sealed with rubber cement and the probe and the cells were denatured together at 100 °C for 10 min [18], followed by hybridization at 65 °C overnight. The slides were then washed with $0.1 \times$ SSC containing 0.1% SDS at room temperature for 10 min, and once at 65 °C, followed by 3% BSA in Tris saline for 10 min. The hybridized probe was detected with extravidin peroxidase (Bio Makor, Israel) 1: 50 in PBS added at 37 °C for 30 min. After washing with PBS, AEC peroxidase substrate (Sigma) was added for 30 min at 37 °C. The slides were counterstained with hematoxylin and mounted in glycerol-gelatin (Sigma).

Our previous studies showed that mouthwash samples of the same subjects as in the present study were infected by HTLV-I [1, 2]. The titer of antibodies to HTLV-I both in the serum and in saliva of the TSP/HAM patient was found to be 1:262 and 1:64, respectively. The serum antibody titers of the two carriers were similar (1:256) or less (1:128), but antibody titers in the saliva were undetectable.

Fig. 1. In situ hybridization of blood lymphocytes and mouthwash cells (lymphocytes and epithelial cells) with a biotinylated HTLV-I probe. Positive red signal was detected by peroxidase while blue nuclei (hematoxylin counterstained) were negative. **A** Positive blood lymphocytes of a TSP/HAM patient (RR) $\times$ 200. **B** One positive mouthwash lymphocyte and negative epithelial cells of an asymptomatic carrier (RY) $\times$ 200. **C** Negative mouthwash epithelial cells of a healthy control (KI) $\times$ 200. **D, E** Peripheral and intranuclear staining of blood lymphocytes of another asymptomatic carrier (GY); (**D** $\times$ 1000, **E** $\times$ 400). **F, G** Mouthwash epithelial cells of the TSP/HAM patient (RR) showing either nuclear only (thick arrow) or both nuclear and cytoplasmic staining of variable intensity, $\times$ 200


A
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No antibodies to HTLV-1 could be detected either in blood or saliva of the healthy control.

In situ Hybridization of blood and mouthwash cells with biotinylated HTLV-I probe is demonstrated in Fig. 1 (red staining indicates positive signals). In the TSP/HAM (RR) patient, both blood lymphocytes (Fig. 1A) and mouthwash epithelial cells (Fig. 1F, G) showed positive staining. In some of these epithelial cells only the nuclear DNA was stained while in other cells additional positive cytoplasmic signal of variable intensity could be seen. The carrier (RY) did not show epithelial cell staining but one of the lymphocytes found in the mouthwash was positive (Fig. 1B). In the other carrier (GY) only peripheral blood lymphocytes were stained (Fig. 1D, E). The positive red staining was seen at the periphery of the nuclei organized in discontinuous patches or as intranuclear staining (Fig. 1D insert, E). Mouthwash cells of the healthy control were negative (Fig. 1C).

Our previous studies [1, 2] showed the presence of HTLV-I infection in mouthwash samples from the TSP/HAM patient and from asymptomatic carriers, but could not indicate whether the cells themselves were infected or that cell-free virus was present in the saliva. In the present work, in situ hybridization was used to further determine the cell population of the mouthwash that contained HTLV-I. We showed that HTLV-I could be detected not only in blood lymphocytes but also in mouthwash lymphocytes and in epithelial cells. Furthermore, positive cytoplasmic staining in addition to the positive nuclear signal could be detected in mouthwash epithelial cells. Several studies have shown infected lymphocytes also in gastrointestinal tract [17] and in lower respiratory tract [13]. About 7.4% of blood lymphocytes of asymptomatic carriers were reported to be HTLV-I positive [4]. HTLV-I was found also in non-lymphoid tissue. Using HTLV-I tax RNA probe, Lehky et al. [7] and Osame et al. [12] showed the presence of HTLV-I in the central nervous system of three TSP/HAM patients; the infected cells were characterized as astrocytes. Other studies [5] confirmed the presence of HTLV-I DNA in the brain, including cerebellum, cerebral cortex and thoracic spinal cord. The involvement of HTLV-I in neurodegenerative diseases was deduced from the findings of genomic HTLV-I DNA in cytoplasm and nuclei of thoracic cord cells of TSP/HAM patients [6]. Miyoshi et al. [10] found HTLV-I proviral DNA in six saliva samples of ATL patients and asymptomatic carriers. HTVL-I tax gene sequences were also detected in biopsies from salivary gland of Sjogren's syndrome patients [8]. An interaction between a lymphocyte and a gut epithelial cell was demonstrated by Zacharopoulos [19] suggesting a lymphocyte facilitated infection of the epithelium with HTLV-I. The transmission of HTLV-I via breast milk was also attributed to this process [20]. Our finding of an infected lymphocyte in mouthwash sample from an asymptomatic carrier and heavily infected epithelial cells in mouthwash from a TSP/HAM patient may indicate steps in HTLV-I transmission process. The positive nuclear and cytoplasmic staining of epithelial cells might suggest a possibility of viral proliferation in these cells. Our previous results [1, 2] and the present observations might suggest that both lymphocytes and epithelial cells of the saliva may possibly participate in oral transmission of HTLV-I.

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