Acta Neuropathol (1996) 92 : 358–361

REGULAR PAPER

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The main HTLV-I-harboring cells in the muscles of viral carriers with polymyositis are not macrophages but CD4$^+$ lymphocytes

Received: 12 February 1996 / Revised, accepted: 11 April 1996

Abstract We have analyzed muscle biopsy specimens from polymyositis patients who are also positive for human T cell lymphotropic virus type I (HTLV-I) using both immunohistochemistry for surface antigens of lymphocytes and macrophages and in situ polymerase chain reaction for HTLV-I proviral DNA on the same sections. We found HTLV-I in CD4$^+$ cells but not in macrophages. This finding suggests that most of the HTLV-I-containing CD4$^+$ cells are not macrophages but lymphocytes.

Key words In situ polymerase chain reaction · Immunohistochemistry · Human T cell lymphotropic virus type I · Proviral DNA · Polymyositis

Introduction

Patients with polymyositis (PM) in Jamaica and Kagoshima, Japan exhibit a seropositivity to human T cell lymphotropic virus type I (HTLV-I) that is higher than in the general population [3, 14]. A direct HTLV-I infection of muscle fibers was detected by in situ hybridization (ISH) in a patient dually infected with HIV and HTLV-I [18]. Using ISH alone, we were not able to detect HTLV-I in patients with PM who were infected with HTLV-I [3]. However, employing an in situ polymerase chain reaction (PCR), we found HTLV-I provirus in some of the CD4$^+$ infiltrating cells in HTLV-I-positive PM patients but not within the muscle fibers [4]. We therefore performed both immunohistochemistry for CD4 or macrophage and in situ PCR for HTLV-I provirus on the same sections from muscle biopsy samples from HTLV-I-positive PM patients to determine which cell types contained HTLV-I.

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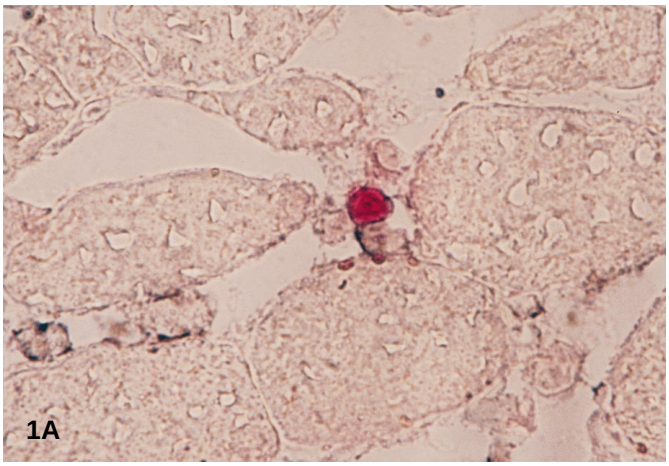
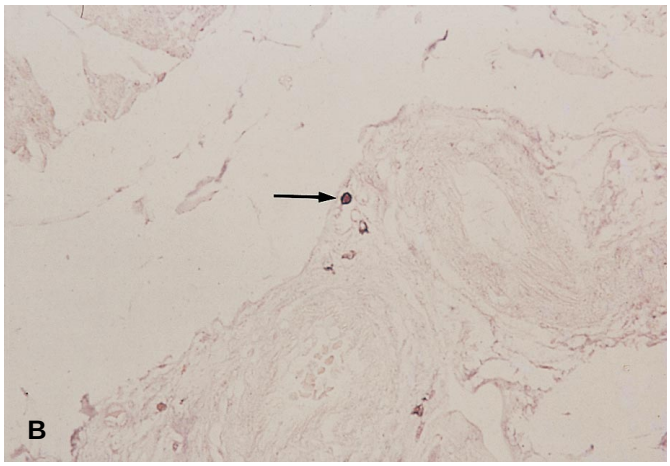
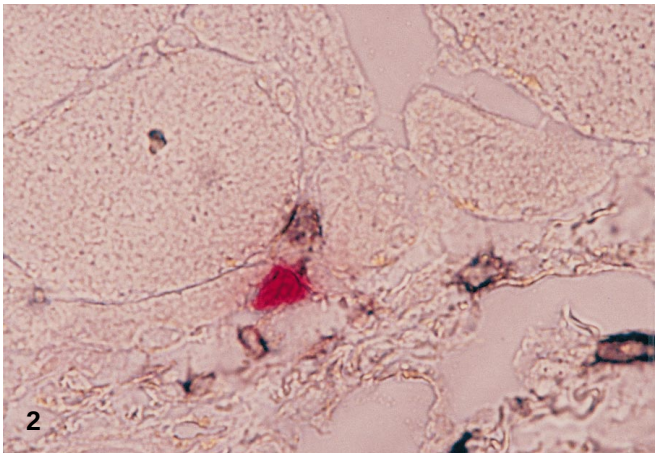
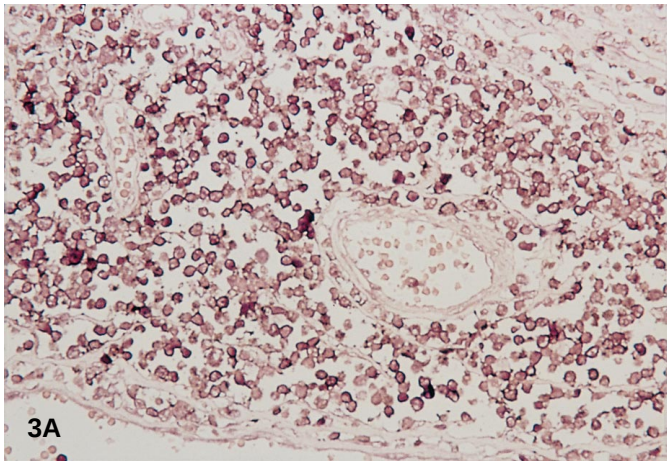
Materials and methods

We obtained muscle biopsy specimens from two HTLV-I-positive PM patients (patients 1 and 2 in [4]) and two HTLV-I-negative PM patients (patients 5 and 6 in [4]), a muscle autopsy specimen from a patient suffering from HTLV-I-associated myelopathy (HAM) (patient 4 in [4]), and a lymph node from a patient (patient 8 in [4]) with adult T cell leukemia (ATL) as a positive control. One part of each muscle specimen and the lymph node were frozen and sectioned. The frozen sections were picked up on aminosilane-coated slides. To distinguish the types of cell that harbored HTLV-I, we analyzed sections from HTLV-I-positive cases using both immunohistochemistry and in situ PCR. Initially, we performed immunogold silver staining (IGSS) using anti-CD4 monoclonal antibodies (Dako) or anti-macrophage monoclonal antibodies (Dako, KP-1; or Becton Dickinson, Ber-Mac) and AuroProbe LM secondary antibodies (Amersham) on the frozen sections. Subsequently, we performed the following in situ PCR on the same frozen sections. . This procedure, which increased the specificity of the reaction, was a modification of a previously reported in situ PCR [1, 12, 16]. Frozen sections (8 μm thick) were cut, picked up on 2% aminosilane-coated slides, and fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 30 min. After washing in PBS and dehydration in an ethanol series, the sections were digested with trypsin (Sigma) at 0.5 mg/ml in 100 mM TRIS-HCl (pH 7.4) for 20 min at 37° C. After digestion, the slides were washed twice in TRIS-HCl (pH 7.4) for 5 min, dehydrated in a graded ethanol series, and then air dried. For amplification in situ, tissue sections were preheated to 82° C for 5 min, and then a solution comprising PCR buffer [50 mM KCl, 10 mM TRIS-HCl (pH 8.3), 1.5 mM $MgCl_2$, 0.01% gelatin, 200 mM dNTPs, 10 mM digoxigenin-dUTP], 1 mM primers and 0.02 U/ml Taq polymerase was applied. Two primer sets spanning nucleotides 7302–7504 of the HTLV-I *pX* region, and nucleotides 5021–5340 of the HTLV-I *pol* and *env* region were used separately. The primers comprised bases 7302–7326 and 7504–7481 of the *pX* region, and bases 5021–5040 and 5340–5321 of the *pol* and *env* region. The sections were then each overlaid with a coverslip, and the slides were put on the heating block of an Atto thermal-cycler with preheated mineral oil to prevent evaporation during thermal cycling. After initial denaturation at 93° C for 3 min, and 20 cycles of denaturation at 93° C for 1.5 min, annealing at 45° C for 2 min, and polymerization at 72° C for 2 min, the slides were removed from the cycler and









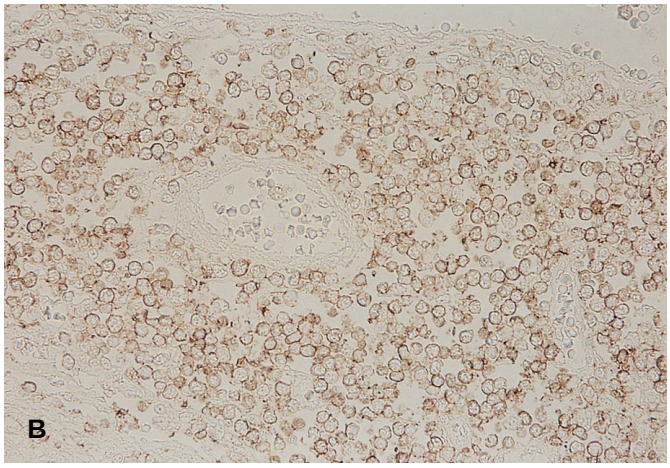


Fig. 1 **A** Combination of immunohistochemistry for CD4 and in situ polymerase chain reaction (PCR) for human T cell lymphotropic virus type I (HTLV-I) *pX* on the same section from a muscle biopsy of a patient with HTLV-I-positive polymyositis (PM). One HTLV-I-positive cell (*red signal*, colored with alkaline phosphate substrate) can be seen in endomysium, which is $CD4^+$ (*black signal*). **B** Double staining on the same section of the muscle from a patient with HTLV-1-associated myelopathy. One HTLV-I-positive cell (*blue signal* colored with nitro blue tetrazolium, *arrow*) can be seen near the vessel, which is $CD4^+$ (*black signal*). **A** × 1000, **B** × 300

Fig. 2 Combination of immunohistochemistry for macrophage and in situ PCR for HTLV-I *pX* on the same section from the muscle from a patient with HTLV-I-positive PM. One HTLV-I-positive cell (*red signal*) can be seen in perimysium, which is not a macrophage (*black signal*). × 1000

Fig. 3 **A** Combination of immunohistochemistry for CD4 and in situ PCR for HTLV-I *pX* on the same section of the lymph node from the patient with adult T cell leukemia (ATL). Many HTLV-I-positive cells (*blue signal*) can be seen, which are $CD4^+$ (*black signal*). **B** No positive signal for HTLV-I can be seen on the $CD4^+$ cells (*black signal*) in the lymph node from the same ATL patient using in situ PCR with irrelevant primers. **A**, **B** × 300

washed twice for 2 min each in xylene to remove residual oil. After removing the coverslips, the sections were washed twice in absolute ethanol and then rehydrated in a graded ethanol series. Post-in situ PCR washing was performed by mild agitation in 0.1 × SSC twice for 15 min at 45° C. Immunological detection of the digoxigenin-labeled amplified DNA was accomplished with a polyclonal anti-digoxigenin antibody conjugated to alkaline phosphatase (AP; Boehringer Mannheim) at room temperature for 30 min. The color reaction was performed with AP substrate tablets of the Multicolor detection set or with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate (Boehringer Mannheim). Controls for in situ amplification included known negative (frozen muscle of an HTLV-I-negative PM patient) and positive (frozen lymph node of ATL patient) test samples, the omission of Taq DNA polymerase or primers, and the use of irrelevant primers (for HIV).

Results

Using both IGSS and in situ PCR we found that some of the $CD4^+$ cells in the skeletal muscles from two HTLV-I-positive PM patients and a HAM patient contained HTLV-I provirus (Fig. 1). We did not find HTLV-I in macrophages (Fig. 2), which were identified using two different antibodies (KP-1 and Ber-Mac). None of the sections from HTLV-I-negative PM patients contained HTLV-I provirus. In addition, no HTLV-I was detected within the muscle fibers or endothelial cells. We found that most $CD4^+$ cells in the lymph node from an ATL patient (positive control) contained the HTLV-I provirus (Fig. 3A); no positive signal for HTLV-I on the $CD4^+$ cells in the lymph node from the same ATL patient was found using in situ PCR with irrelevant primers (negative control, Fig. 3B).

Discussion

Leon-Monzon et al. [11] studied skeletal muscles from six HTLV-I-positive patients by immunohistochemistry, solution-phase PCR, and co-cultures of the patients' lymphocytes with their homologous muscles. They concluded that amplified HTLV-I sequences are related to scattered HTLV-I-positive endomysial macrophages. However, we were unable to find HTLV-I in macrophages using a combination of immunohistochemistry for macrophages and in situ PCR for HTLV-I provirus on the same sections. This finding suggests that most of the HTLV-I-containing $CD4^+$ cells are not macrophages but lymphocytes. Several previous studies have reported that $CD4^+$ lymphocytes are the major reservoir for HTLV-I. In addition, the amount of HTLV-I proviral DNA has been shown to correlate with that in the infiltrating $CD4^+$ lymphocytes in the spinal cord from patients with HAM [10]. Although Leon-Monzon et al. [11] concluded using immunohistochemistry with antibodies against HTLV-I p19 and p24 that endomysial macrophages contain HTLV-I, the antibody against HTLV-I core protein is reported to cross-react to normal human tissue [7, 15, 17]. Hence, it is necessary to confirm the results either by immunohistochemistry using antibody against HTLV-I tax, which is more specific to HTLV-I, or by methods such as ISH. For a definite identification double immunostaining using antibodies to HTLV-I and to $CD4^+$ lymphocytes is necessary.

As HTLV-I has been shown to infect a wide variety of cell types in vitro [2, 5, 6, 8, 14, 19, 20] and in vivo [9], we cannot at present rule out the possibility that rare macrophages or other cells in the muscle may contain HTLV-I. Therefore, we conclude that the main HTLV-I-harboring cells in the muscle from most patients with HTLV-I-positive PM are $CD4^+$ lymphocytes.

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