ARTIGO/ARTICLE

Establishment of HTLV-I-infected cell lines from peripheral blood mononuclear cells of Brazilian patients

Estabelecimento de linhagens celulares infectadas por HTLV-I a partir de células mononucleares periféricas de pacientes brasileiros

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ABSTRACT

To investigate epidemiological and pathogenetic features of HTLV-I infection, a cohort of carriers has been followed at the USP Teaching Hospital since 1991. This study describes the establishment of cell lines from peripheral blood mononuclear cells (PBMC) of infected subjects. Ex vivo *PBMC were cultured with those from a seronegative donor and morphologic evidence of cell transformation was obtained after 90 days with detection of multinucleated cells exhibiting cerebriform nuclei. Integration of HTLV-I proviral DNA and expression of viral antigens was demonstrated in culture by PCR and immunofluorescence. Cell lines were maintained for 240 days, gradually weaned from exogenous IL-2. Immunophenotyping of cell lines on flow cytometry yielded evidence of cell activation. Establishment of HTLV-I-infected cell lines from* ex vivo *PBMC is feasible and may be useful for studies on lymphocyte phenotypic changes and on mechanisms of HTLV-induced cell proliferation. Moreover they may be used with diagnostic purposes in immunofluorescence tests.*

Key-words: *HTLV-I. Culture. Cell line. Brazil.*

RESUMO

Para investigar a epidemiologia e patogênese da infecção por HTLV-I seguimos coorte de portadores dessa retrovirose no HC-FMUSP desde 1991. Este estudo descreve o estabelecimento de linhagens celulares a partir de células mononucleares periféricas (CMP) de indivíduos infectados. As CMP foram cultivadas com as de doador soronegativo, verificando-se após 90 dias evidência morfológica de transformação celular com detecção de células multinucleadas com núcleos cerebriformes. Demonstrou-se integração do DNA proviral e expressão in vitro *de antígenos virais pela PCR e imunofluorescência. As linhagens celulares transformadas foram mantidas por 240 dias, com retirada gradual de IL-2 exógena. A imunofenotipagem por citometria de fluxo revelou ativação celular. O estabelecimento de linhagens celulares infectadas por HTLV-I a partir de CMP* ex-vivo *é exeqüível e pode ser útil na investigação de alterações fenotípicas linfocitárias e dos mecanismos de proliferação celular induzida por esse retrovírus. Podem ainda ser utilizadas com intuito diagnóstico em reações de imunofluorescência.*

Palavras-chaves: *HTLV-I. Cultura. Linhagens celulares. Brasil.*

Human T-lymphotropic virus type I (HTLV-I), an exogenous retrovirus, is etiologically linked with two major diseases, adult T-cell leukemia/lymphoma and a chronically progressive neurologic disease, known as HTLV-I-associated myelopathy/tropical spastic paraparesis (HAM/TSP)[9,19]. This retroviral infection is endemic in several geographic regions of the globe, that include southern Japan, Central Africa, the Caribbean basin and South America, as well as the Melanesian islands[4]. In Brazil, infected individuals have been more frequently diagnosed, since 1993, when national compulsory

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seroscreening of blood donors was implemented. Overall, HTLV-I seroprevalence rates among Brazilian blood donors vary from 0.08 to 1.35%, according to geographic origin[8]. Even though markers of disease progression are presently unavailable for the follow-up of HTLV-I asymptomatic carriers, the lifetime risk of developing an associated disease has been estimated to vary between 0.2% and 4%[12 14 15]. Both ATL/L[17] and HAM/TSP[2] have been reported in nationwide surveys of Brazilian patients from distinct geographic origins, but particular patterns of viral transmission, as well as the host's genetic background and local environmental factors may possibly account for differences in the epidemiological profile of Brazilian cohorts of HTLV-I-associated disease[6].

Since 1991, a cohort of HTLV-I-infected individuals has been followed at the outpatient clinic of the Department of Infectious and Parasitic Diseases, University of São Paulo School of Medicine for clinical, epidemiological and laboratory investigation purposes. HTLV-I infection in these individuals is more often diagnosed by recognition of seroreactivity to specific *gag* and *env* viral antigens in serologic tests that include screening enzyme immunoassays and confirmatory and discriminative Western blot tests. Additionally detection of proviral DNA in peripheral blood mononuclear cells by polymerase chain reaction provides further evidence of persistent retroviral infection[10]. The present study, approved by the Institutional Review Board (CAPPesq), describes the establishment of HTLV-I-infected cell lines from peripheral blood mononuclear cells (PBMC) of seropositive patients. Though time-consuming and labor-intensive, viral cultures, carried out under proper biosafety requirements, provide valuable information on the biological behavior of HTLV-I-infected cells and may eventually lead to the establishment of infected lymphocytic cell lines.

MATERIAL AND METHODS

After informed consent, blood specimens were collected from two seropositive women, assisted at the outpatient clinic of the University hospital in São Paulo, Brazil. The first patient was a 47-year-old white housewife, who complained of progressive weakness of her lower limbs and urinary retention for 3 years. At first medical visit, she already required a walking aid, but no other abnormality was noticed on clinical examination. Spastic paraparesis with pyramidal signs was detected on neurologic examination and CSF analysis revealed mild mononuclear pleocytosis (10 cells/mm^3) with normal protein levels (37mg/dl). Specific anti-HTLV-I/II antibodies and HTLV-I proviral DNA were detected in CSF, confirming the diagnosis of HAM/TSP[18]. The second patient was a 34-year-old saleswoman, who reported diffuse alopecia and infiltrated skin plaques on the anterior abdominal wall. A skin biopsy was performed in the affected area and yielded dermatotropic cutaneous T-cell lymphoma. Her total white blood cell and lymphocyte counts were normal (6,700 and 2,037 cells/mm^3, respectively). Cytometric immunophenotypic analysis of her peripheral blood mononuclear cells (PBMC), using monoclonal antibodies was normal for CD4+ and CD8+ subsets, as well as for CD25 expression. Serum lactic dehydrogenase (LDH) and calcium levels were within normal limits and therefore, she was diagnosed with smoldering ATL/L[13].

PBMC from both patients were separated from EDTA-treated blood samples by Ficoll® gradient centrifugation and $2x10^6$ cells were cultured in RPMI 1640 medium, supplemented with 20% heat-inactivated fetal bovine serum and 10% partially purified recombinant human interleukin 2 (IL-2), in the presence of penicillin (100IU/ml), streptomycin (100μg/ml), amphotericin B, and glutamine (2nmol/l) on 24-well plates. After 72-hour incubation at 37°C in a 5% CO_2 atmosphere, an equal number of lymphocytes previously stimulated with phytohemagglutinin (2μg/ml) from a seronegative donor were added to HTLV-infected PBMC. Cocultures were then maintained under these experimental conditions and fed, every 3 to 4 days, with fresh medium to provide appropriate expansion, according to *in vitro* cell growth. Subsequently cultures were added with PHA-stimulated PBMC from the same seronegative donor every 2 weeks.

RESULTS

After 90 days in culture, morphologic evidence of cell transformation was obtained in cocultures developed from both patients' PBMC, with identification of multinucleated cells (Figure 1) that exhibited cerebriform nuclei on Giemsa staining. Long-term cell lines were maintained for up to 240 days, after being gradually weaned from exogenous IL-2. In order to demonstrate proviral integration in established cell lines, nested PCR amplification of HTLV-I *tax* sequences was carried out, as previously described[10]. Briefly, cultivated cell lysates were obtained by proteinase K digestion and subsequently underwent genomic amplification, using consensus oligonucleotide primers (SK 43 and SK44), that allow detection of both HTLV-I and HTLV-II proviral sequences. A second round of amplification was then performed, with primers nt 7375-7394 and nt 7486-7502, that are complementary to sequences that lie internally to the edges of amplicons generated in the first round, producing a DNA fragment of 128bp (Figure 2). Discrimination between HTLV-I and HTLV-II sequences was achieved by restriction enzyme digestion of nested PCR products, with *Taq* I and restricted length polymorphism (RFLP) analysis was carried out visually, after electrophoresis in 2% agarose gels[20]. In both studied patients, RFLP analysis yielded a 122bp DNA fragment, compatible to HTLV-I infection (data not shown). Cells from the seronegative donor, used in cocultures, yielded in contrast no HTLV proviral sequences.

In vitro production of viral antigens was sought after by direct immunofluorescence (IF), as previously reported[1]. For this purpose, cultures were harvested and submitted to 2000rpm centrifugation for 10 minutes. Cell pellets were subsequently resuspended in sterile PBS, spotted onto IF slides and cells fixed with cold 1:1 acetone/methanol solution for 15 minutes. PBMC from the same HTLV seronegative donor that provided PBMC for cocultures were used as negative cell controls. Dried slides were kept at -20°C until the detection step was carried out with a 1:10 diluted serum sample from a HTLV-I seropositive patient. PBS and a sample of HTLV-I-seronegative serum were used as

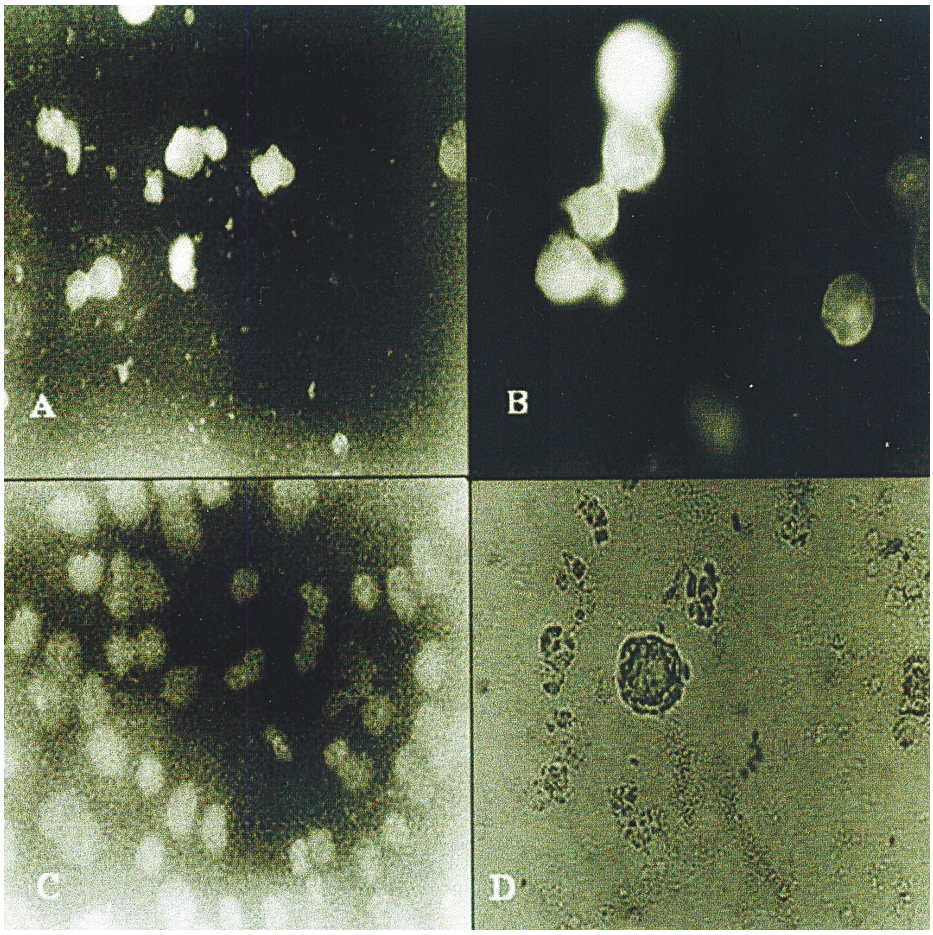

Figure 1 - Direct immunofluorescence, using polyclonal anti-HTLV-I serum, detects HTLV-I antigen expression in cultured ex vivo *peripheral blood mononuclear cells (PBMC) from a patient with smoldering adult T-cell leukemia (A) and in lymphocytes from an HTLV-I-infected established cell line – MT-2 (B); no antigen expression is seen in PBMC from the HTLV-I-seronegative donor that were used to feed cocultures (C); HTLV-I-induced multinucleated cells in* ex vivo *PBMC of a patient with HAM/TSP after 90 days in culture.*

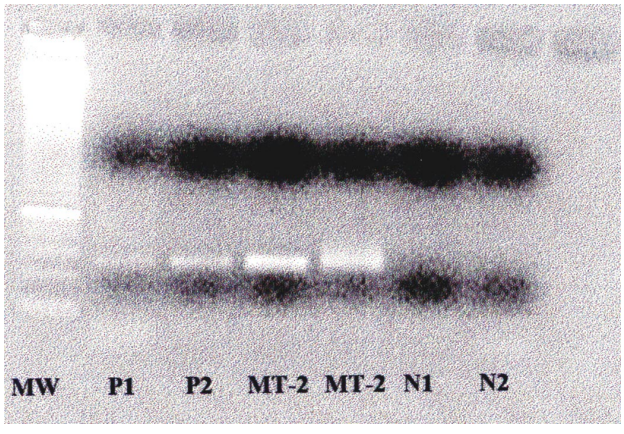

Figure 2 - Nested PCR amplification of proviral tax *sequences after electrophoresis in ethidium bromide-stained 2% agarose gels. Positive samples yield a 128 bp DNA fragment. MW – 100 bp ladder; P1 – DNA obtained from cultured* ex vivo *PBMC from a patient with smoldering ATL; P2 – DNA obtained from cultured* ex vivo *PBMC from a patient with HAM/TSP; MT-2 – DNA obtained from an HTLV-I-infected established cell line – MT-2; N1 – DNA obtained from* ex vivo *PBMC from the HTLV-I-seronegative donor that were used to feed cultures; N2 – negative control (water).*

additional negative controls. Slides were then incubated with a 1:100 dilution of sheep anti-human IgG fluorescein isothiocyanate conjugate at 37ºC for 45 minutes and examined for detection of viral antigens. Immunofluorescence, using polyclonal antiserum, revealed expression of viral antigens in a large proportion of cultivated cells from both established cell lines (Figure 1).

Lymphocyte immunophenotyping of established cell lines was performed after 90 days in culture, using a FACSCalibur flow cytometer. Briefly, for 4-color multiparameter flow cytometric analysis, cells were stained in separate tubes, using monoclonal antibodies (BDIS) to cell surface markers CD3, CD4, CD8, CD19, CD25 (IL-2 receptor), CD56 and HLA-DR, in a 15-minute incubation in the dark. After washing in PBS, cells were run in flow cytometry and data analysis performed with the CellQuest computer software. A predominance of CD3+ CD4+ lymphocytes was verified in HTLV-I-infected cell lines and increased expression of surface markers of cell activation (HLA-DR$^+$) could be demonstrated (mean fluorescence intensity of 927 and 537 for HTLV-I-infected cell lines and of 212 and 118 for respective controls). No difference was noticed in CD25 expression.

DISCUSSION

Infection with HTLV-I is known to induce *in vitro* spontaneous lymphocyte proliferation in the absence of mitogen or antigen stimulation and this proliferative response is believed to be dependent on the transactivation proprieties of the proviral *tax* gene product ($p40^{tax}$). Lymphocyte proliferation eventually evolves to cell immortalization and *in vitro* transformation[16].

However HTLV-I-induced leukemogenesis *in vivo* is so far not fully understood. HTLV-I-mediated T-cell transformation in infected individuals presumably arises from a multistep oncogenic process resulting in accumulation of genetic defects and dysregulated growth of infected cells leading to development of ATL/L in a minority of HTLV-I carriers[7,11]. Nevertheless further research is still warranted for a better understanding of ATL/L pathogenetic mechanisms.

The establishment of HTLV-I-infected cell lines from *ex vivo* PBMC of HTLV-I-infected individuals provides useful investigative tools for studies on phenotypic changes of infected lymphocytes and may thus help in further elucidation of the mechanisms involved in HTLV-induced cell proliferation.

In the present study we have demonstrated that the establishment of HTLV-I-infected $CD4^+$ cell lines from *ex vivo* PBMC is feasible under proper biosafety requirements in Brazilian laboratories, as previously described abroad[5].

Apart from their potential use in pathogenetic studies of HTLV-I-induced cell transformation, these infected cell lines may also be helpful in the confirmatory diagnosis of HTLV-I infection. Current serological algorithms for the diagnosis of HTLV-I infection usually recommend the use of screening enzyme immunoassays, followed by serological confirmation and discrimination based on seroreactivity to viral *gag* and *env*-coded antigens on Western blot assays[3,21]. However given the overall high cost of commercially available Western blot kits, serodiagnostic algorithms in resource-poor settings may consider the possibility of using HTLV-I infected cell lines with diagnostic purposes in confirmatory immunofluorescence tests.

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