eastern and Southwest oncology group protocol (abstr.). *Proc Am Soc Clin Oncol* 1990, **9**, 132.
30. Motzer RJ, Gulati S, Bajorin D, *et al.* VAB6 +/− high dose carboplatin plus etoposide plus autologous bone marrow rescue for poor risk germ cell tumor patients (abstr.). *Proc Am Assoc Cancer Res* 1990, **31**, 186.
31. Ozols RF, Ihde DC, Linehan WM, *et al.* A randomized trial of standard chemotherapy versus a high dose chemotherapy regimen in the treatment of poor prognosis non seminomatous germ cell tumors. *J Clin Oncol* 1988, **6**, 1031–1040.

Acknowledgements——The authors thank Mrs M. Belleteste and M. Moussé for their excellent secretarial help and Mrs L. Saint-Ange for her assistance in the preparation of the manuscript.

This study was supported in part by Institut Gustave-Roussy Grant 84D13.

Eur J Cancer, Vol. 27, No. 7, pp. 835–838, 1991.
Printed in Great Britain

0277–5379/91 $3.00 + 0.00

Serological and Molecular Evidence of Infection by Human T-cell Lymphotropic Virus Type II in Italian Drug Addicts by Use of Synthetic Peptides and Polymerase Chain Reaction

Anita De Rossi, Fabrizio Mammano, Annarosa Del Mistro and Luigi Chieco-Bianchi

Infection with human T lymphotropic virus type I (HTLV-I) is associated with specific forms of tumours and neurological disorders, but the pathogenic activity of HTLV-II is not yet established. Moreover, due to high crossreactivity between the two viruses, differential diagnosis is not readily achieved. To discriminate between HTLV-I and HTLV-II infections, we employed synthetic peptides specific for HTLV-I and HTLV-II env regions, and the polymerase chain reaction (PCR). In a series of 962 intravenous drug addicts (IVDAs) and 50 patients with haematological malignancies, 51 and 2 samples, respectively, were reactive against HTLV-I proteins; among these, HTLV-I infection was confirmed only in 1 patient with adult T-cell lymphoma, while HTLV-II infections were identified in 6 out of 14 PCR-tested IVDAs. These findings provide evidence of HTLV-II infection among Italian IVDAs. The differentiation between HTLV-I and HTLV-II infections may contribute to a better understanding of HTLV-II pathogenicity in man.
Eur J Cancer, Vol. 27, No. 7, pp. 835–838, 1991

INTRODUCTION

HUMAN T-CELL lymphotropic viruses type I (HTLV-I) and II (HTLV-II) have been associated with specific forms of malignancy in man. HTLV-I, the aetiological agent of adult T-cell leukaemia/lymphoma [1, 2], is also related to neurological disorders, known as tropical spastic paraparesis or HTLV-I-associated myelopathy [3, 4]. HTLV-II has been isolated in rare cases, including subjects with a T-cell variant of hairy cell leukaemia [5], but a conclusive association with this or other diseases has not yet been established.

Besides the endemic areas [6, 7], antibodies reactive to HTLV-I antigens have also been found world-wide in subjects at risk for AIDS, such as intravenous drug addicts (IVDAs), homosexuals and haemophiliacs [8–12]. In this regard, we previously found that 4–5% of HIV-1 seropositive IVDAs living in north-eastern Italy were also seroreactive for HTLV-I [13, 14].

However, antibody crossreactivity between HTLV-I and HTLV-II, due to the high level of genomic and aminoacid sequence homology between the two, makes it difficult to differentiate one infection from the other on the basis of standard serological tests [15]. To this end, molecular methods might be more appropriate, and the polymerase chain reaction (PCR), which specifically amplifies short DNA sequences [16], has recently been applied to discriminate between HTLV-I and HTLV-II infection [17, 18]. This differential diagnosis is important for epidemiological and public health studies, as well as prospective clinical analysis of HTLV-infected persons in order to understand better the natural history and pathogenesis of these infections.

In this study we investigated whether HTLV-II infection could be identified and distinguished from HTLV-I in a series of IVDAs living in the Veneto region of Italy, using the synthetic peptides and the PCR techniques.

MATERIALS AND METHODS

Patients

Serum samples were obtained from 962 IVDAs and from 50 patients with haematological malignancies, consisting of 1 case

Correspondence to A. De Rossi.
The authors are at the Institute of Oncology, University of Padova, Via Gattamelata 64, 35128 Padova, Italy.
Revised 21 Mar. 1991; accepted 9 Apr. 1991.

of adult T-cell lymphoma (ATL), 28 adult cases of non-Hodgkin lymphoma (NHL), and 21 children with acute lymphoblastic leukaemia (ALL). All the sera were tested for the presence of antibodies to HIV-1 and HTLVs. When seroreactivity to HTLV was detected, the subject was re-examined, and following his informed consent, 5 ml of blood was drawn for PCR analysis.

Serological analysis

Antibodies to HIV-1 were detected by ELISA (Dupont de Nemours, Wilminghton, Maryland) and western blot (Dupont). HTLV-antibody presence was first evaluated with a commerical ELISA (Dupont); reactive sera were then assayed by western blot (WB, HTLV-I kit, Dupont). All sera showing antibody reactivities to at least one HTLV gene product were further analysed by SynthEIA HTLV-I and SynthEIA HTLV-II (Olympus Immunochemicals, Olympus Corporation, Lake Success, New York); the antigens in these assays are 34 oligomer synthetic peptides that correspond to a highly antigenic and conserved portion of the envelope protein gp 46 of HTLV-I and HTLV-II viruses, respectively [19]. The assay was performed following the manufacturer's instructions; samples were considered positive when optical density (OD) values were higher than cut-off values, calculated as follows: OD negative control + (0.5 × OD positive control).

PCR

Peripheral blood lymphocytes (PBL) were obtained by Ficoll–Hipaque gradient separation of 5 ml heparinised blood, and washed in phosphate-buffered saline (PBS); 2×10^6 cells were lysed with 0.5 ml of TE buffer (10 mmol/l Tris-HCl, pH 8, 0.5 mmol/l EDTA) containing 0.001% Triton-X 100, 0.0001% sodium dodecyl sulphate (SDS) and 600 μg/ml proteinase K (Boehringer Mannheim). Amplification by PCR was accomplished in 100 μl reaction mixture containing 25 μl cell lysate (10^5 cells), 20 nmol of each deoxynucleotide triphosphate (dNTP), 100 pmol of each primer, 2.5 U *Taq* polymerase (Perkin-Elmer, Norwalk, Connecticut), 50 mmol/l KCl, 10 mmol/l Tris-HCl (pH 8.3), and 1.5 mmol/l $MgCl_2$. Target DNA was amplified using a DNA thermal cycler (Perkin-Elmer), and 30 cycles (each of 1 min at 92°C, 1.5 min at 50°C, and 1.5 min at 72°C) were carried out. 30 μl of each amplified sample was electrophoresed on 2% Nusieve and 1% agarose gel and then transferred to filters (Hybond N, Amersham International). Hybridisation was achieved with a 5′ end ^{32}P-labelled oligonucleotide probe specific for the amplified fragment. Filters were exposed to X-ray film for 12 h [20]. The primer pairs used in this set of experiments recognised the 5′–3′, 7455–7474, 7574–7595 and the 5′–3′ 7344–7364, 7464–7484 tax/rex sequences of HTLV-I and HTLV-II, respectively [21, 22]. Differential diagnosis between the two viruses was achieved by using specific oligonucleotides; ATCGGCTCAGCTCTACAGTT for HTLV-I, and GTCAGCTCTCCTCTCCAATA for HTLV-II. PCR specificity was tested in 10 DNA samples obtained from healthy donors; PCR sensitivity was evaluated on 10-fold serial dilutions of MT2 and C344MO cells, which carry the HTLV-I and HTLV-II viral genomes, respectively.

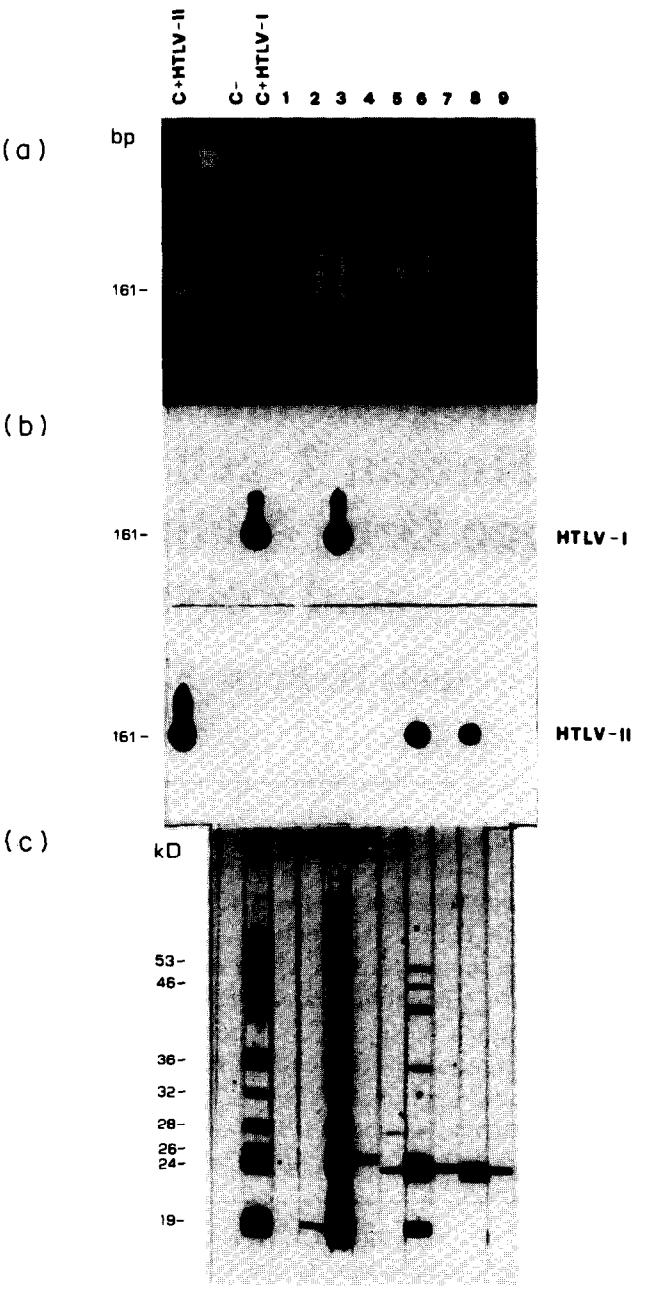

Fig. 1. Serological and molecular analysis of representative samples. Agarose gel electrophoresis of DNA samples amplified with tax/rex primer set (a). Autoradiography (12 h exposure) after Southern blotting and hybridisation with HTLV-I probe, and with HTLV-II probe (b). Lanes: c+ HTLV-II = 10^5 C344MO cells, c− = reaction buffer, c+ HTLV-I = 10^5 MT2 cells, 1 = healthy donor, 2 = patient with ALL, 3 = patient with ATL; lanes 4–9 = IVDA subjects. Panel (c) shows western blot of corresponding patients' sera; first two lanes = negative and positive HTLV-I reference sera, respectively.

RESULTS

Reactivity against HTLV-I proteins on western blot assay was detected in 51 of the 962 IVDA samples, and in 2 of the 50 samples from patients with haematological malignancies. The patterns of serum reactivity, however, were dissimilar. Only 5 sera, 1 from the ATL patient and 4 from IVDAs, reacted against almost all HTLV-1 proteins and could be defined as western blot positive; 48 sera, 1 from an ALL patient and 47 from IVDAs, reacted only against HTLV-I core proteins, and were defined as indeterminate on western blot [23, 24]. All these sera were further evaluated by SynthEIA HTLV-I and HTLV-II assays. Serum from the ATL patient was positive for HTLV-I, while serum from the ALL patient was negative for both HTLV-I and HTLV-II. Among the sera from IVDAs, only 1 was positive for HTLV-I, while 13 were positive for HTLV-II (Table 1).

Table 1. HTLV-I and HTLV-II infection among the studied subjects

Sample code	Status	HTLV-I western blot	SynthEIA HTLV-I	SynthEIA HTLV-II	PCR HTLV-I	PCR HTLV-II
1	Healthy control	–	–	–	–	–
2	ALL	p19	–	–	–	–
3	ATL	p19,24,26,28,32,36,46,53	+	–	+	–
4	IVDA/HIV+	p24	–	–	–	–
5	IVDA/HIV+	p24,28	–	–	–	–
6	IVDA/HIV+	p19,24,36,46,53	–	+	–	+
7	IVDA/HIV+	p24	–	+	–	–
8	IVDA/HIV+	p24	–	+	–	+
9	IVDA/HIV+	p24	–	–	–	–
10	IVDA/HIV+	p19	–	–	–	–
11	IVDA/HIV–	p19	–	+	ND	ND
12	IVDA/HIV+	p19	–	–	–	–
13	IVDA/HIV+	p19	–	+	ND	ND
14	IVDA/HIV–	p24	–	+	ND	ND
15	IVDA/HIV+	p24	+	–	–	–
16	IVDA/HIV+	p24	–	+	–	+
17	IVDA/HIV+	p24	–	+	ND	ND
18	IVDA/HIV+	p24	–	+	ND	ND
19	IVDA/HIV+	p19,24,26,28,36,53	–	+	–	+
20	IVDA/HIV+	p19,24,26,28,53	–	+	–	+
21	IVDA/HIV–	p19,24,26,28,36,53	–	+	–	+
22	IVDA/HIV+	p24	–	+	ND	ND
23	IVDA/HIV–	p24	–	–	–	–

ND = not done.

Samples from both the ALL and ATL patients were available for PCR assay. Due to low subject compliance, PCR could be performed in only 14 IVDAs (Table 1 and Fig. 1). The primer pair sequences were chosen from HTLV-I and HTLV-II tax/rex regions that were sufficiently similar to allow detection of either DNA. The use of these primers generated a PCR product of 161 nucleotides (Fig. 1A), which was then hybridised with two different oligonucleotide probes that recognise specific HTLV-I and HTLV-II viral sequences (Fig. 1B); thus a differential diagnosis between the two viruses could be achieved. As expected, the sample from the ATL patient was strongly positive for the HTLV-I genome, while the sample from the ALL patient was negative for both viruses. The 14 IVDA samples, including case no. 15, which was positive on SynthEIA HTLV-I, were all negative for HTLV-I; however, 6 cases (nos 6, 8, 16, 19, 20 and 21, Table 1), which were positive on SynthEIA HTLV-II, were also positive for HTLV-II genomic sequences.

DISCUSSION

This study reports serological and molecular evidence of HTLV-II infection in Italian IVDAs showing seroreactivity against HTLV-I proteins. In a series of 962 IVDAs, sera from 51 were reactive against HTLV-I proteins on western blots; of these, 4 were positive for both core and env proteins, while 47 reacted only against core proteins. PCR and synthetic peptide assays confirmed HTLV-II infection in all 4 western blot positive cases (nos 6, 10, 20 and 21, Table 1), and in 2 (nos 8 and 16, Table 1) out of 10 cases indeterminate on western blot which had been tested in PCR. PCR analysis and synthetic peptide assay results were concordant in 14 samples out of 16 that were tested by both methods (Table 1). 2 samples were negative on PCR for both viruses, but were positive for HTLV-II and HTLV-I on synthetic peptide assay (nos 7 and 15, respectively; Table 1). In this regard, it is worth mentioning that the sensitivity limit of the PCR technique in our hands is the presence of at least 10 HTLV carrier cells in the sample under examination, so the above discrepancy could reflect a very low viral load. Moreover, it seems important to stress that, to date, a positive result on synthetic peptide assay cannot be taken as a confirmatory criterion for HTLV-I or HTLV-II infection; consequently, we considered only the cases with positive PCR results as unequivocally infected by HTLV-II. However, once the sensitivity and specificity of the env-derived synthetic peptides are ascertained, this assay will probably prove very useful when cell samples are not available, and for retrospective serum study.

We obtained clear evidence of HTLV-II, but not HTLV-I infection in the IVDA population studied. A differential PCR sensitivity could be reasonably excluded as the same sensitivity was observed in the HTLV-I and HTLV-II positive control cell lines; moreover, the positive PCR result for HTLV-I in the ATL patient confirmed the technique's efficiency in detecting HTLV-I. Our findings are also consistent with recent studies in which the application of PCR demonstrated that a high percentage of HTLV-I seroreactive IVDAs are actually infected by HTLV-II [17, 18].

Most of the HTLV-carrier subjects were also HIV-1 infected. Although a correlation between HTLV-II and specific disease in humans has not yet been found, HTLV-II, as well as HTLV-I, are known to facilitate HIV-1 expression and infection *in vitro* [25]. The use of PCR analysis, and possibly env-derived synthetic peptides, to discriminate between HTLV-I and HTLV-II infections will offer an opportunity to understand the pathogenicity of HTLVs and their cofactorial role in AIDS progression.

1. Poiesz BJ, Ruscetti FW, Gadzar AF, Bunn PA, Minua JD, Gallo RC. Detection of type-C retrovirus particles from fresh and cultured lymphocytes of a patient with cutaneous T-cell lymphoma. *Proc Natl Acad Sci USA* 1990, 77, 7415–7419.
2. Yoshida M, Miyoshi I, Hinuma Y. Isolation and characterization of retrovirus from cell lines of human adult T-cell leukemia and its implication in the disease. *Proc Natl Acad Sci USA* 1982, **79**, 2031–2035.
3. Gessain A, Barin F, Vernant JC, *et al.* Antibodies to human T-lymphotropic virus type I in patients with tropical spastic paraparesis. *Lancet* 1985, **ii**, 407–410.
4. Bhagavati S, Ehrlich G, Kula RW, *et al.* Detection of human T-cell lymphoma/leukemia virus type I DNA and antigen in spinal fluid and blood of patients with chronic progressive myelopathy. *N Engl J Med* 1988, **318**, 1141–1147.
5. Kalyanaraman VS, Sarngadharan MG, Robert-Guroff M, *et al.* A new subtype of human T-cell leukemia virus (HTLV-II) associated with a T-cell variant of hairy cell leukemia. *Science* 1982, **218**, 571–573.
6. Gallo RC. The human T-cell leukemia/lymphotropic retroviruses (HTLV) family. Past, present and future. *Cancer Res* 1985, **45**, 4524s–4533s.
7. Blattner WA. Epidemiology of HTLV-I and associated diseases. In: Blattner WA. *Human Retrovirology: HTLV*. New York, Raven Press, 1990, 251–256.
8. Robert-Guroff M, Safai B, Gelmann EP, *et al.* HTLV-I specific antibody in AIDS patients and others at risk. *Lancet* 1984, **ii**, 128–130.
9. Tedder RS, Shanson DC, Jeffries DJ, *et al.* Low prevalence in the U.K. of HTLV-I and HTLV-II infection in subjects with AIDS, with extended lymphoadenopathy, and at risk of AIDS. *Lancet* 1984, **ii**, 125–127.
10. Robert-Guroff M, Weiss SH, Giron JA, *et al.* Prevalence of antibodies to HTLV-I, -II, and -III in intravenous drug abusers from an AIDS endemic region. *JAMA* 1986, **255**, 3133–3137.
11. Born M, von der Helm K, Wernicke D, Deinhardt F. Presence of antibodies to Human lymphoma-Leukemia virus (HTLV-I) in Germans with symptoms of the acquired immunodeficiency syndrome (AIDS). *J Med Virol* 1985, **15**, 57–63.
12. Manzari V, Barillari G, Albonici L, French D, De Marchis L, Frati L, Human T-lymphotropic retrovirus infection in Italy. *Ann NY Acad Sci* 1987, **511**, 401–405.
13. De Rossi A, Dalla Gassa O, Del Mistro A, Faulkner Valle G, Chieco-Bianchi L. HTLV-III and HTLV-I infection in population at risk in the Veneto Region of Italy. *Eur J Cancer Clin Oncol* 1986, **22**, 411–418.
14. De Rossi A, Bortolotti F, Cadrobbi P, Chieco-Bianchi L. Trends of HTLV-I and HIV infections in Drug Addicts. *Eur J Cancer Clin Oncol* 1988, **24**, 279–280.
15. Anderson DW, Epstein JS, Pierik LT, *et al.* Development by the Public Health Service of Criteria for Serological Confirmation of HTLV-I/II infections. In: Blattner WA, ed. *Human Retrovirology: HTLV*. New York, Raven Press, 1990, 391–396.
16. Saiki RK, Scharf S, Faloona F, *et al.* Enzymatic amplification of beta-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. *Science* 1985, **230**, 1350–1354.
17. Lee H, Swanson P, Shorty VS, Zack JA, Rosenblatt JD, Chen ISY. High rate of HTLV-II infection in seropositive IV drug abusers in New Orleans. *Science* 1989, **244**, 471–475.
18. Kowk S, Gallo D, Hanson C, McKinney N, Poiesz B, Sninsky JJ. High Prevalence of HTLV-II among intravenous drug abusers: PCR confirmation and typing. *AIDS Res Hum Retroviruses* 1990, **6**, 561–565.
19. Hosein B, Fang CT, Wang CY. Synthetic peptides to distinguish HTLV-I from HTLV-II infection (abstr.). International Retrovirus Conference on Current Issues in Human Retrovirology: HTLV, 1991.
20. De Rossi A, Ades AE, Mammano F, *et al.* Antigen detection, virus culture, polymerase chain reaction, and in vitro antibody production in the diagnosis of vertically transmitted HIV-1 infection. *AIDS* 1991, **5**, 15–20.
21. Seiki M, Hattori S, Hirayama Y, Yoshida M. Human adult T-cell leukemia virus: Complete nucleotide sequence of the provirus genome integrated in leukemia cell DNA. *Proc Natl Acad Sci USA* 1983, **80**, 3618–3622.
22. Shimotohno K, Takahashi Y, Shimizu N, *et al.* Complete nucleotide sequence of an infectious clone of human T-cell leukemia virus type II: an open reading frame for the protease gene. *Proc Natl Acad Sci USA* 1985, **82**, 3101–3105.
23. Khabbaz RF, Shulman G, Lairmore MD, Hartley TM, De B, Kaplan JE. Significance of positive and indeterminate HTLV-I serologic results in blood donors. In: Blattner WA, ed. *Human Retrovirology: HTLV*. New York, Raven Press, 1990, 397–400.
24. WHO. Proposed WHO criterias for interpreting results from Western blot assays for HIV-1, HIV-2, and HTLV-I/HTLV-II. *WHO Wky Epidem Rec* 1990, **37**, 281–283.
25. Montefiori DC, Mitchell WM. Persistent coinfection of T lymphocytes with HTLV-II and HIV and the role of syncitium formation in HIV-induced cytopathic effect. *Virology* 1987, **2**, 372–378.

Acknowledgements—We thank G. Miazzo Scapin for technical work, and P. Segato for help in preparing the manuscript.

This work was supported by grants from Istituto Superiore della Sanità, Progetto AIDS 1990 and Associazione Italiana per le Ricerche sul Cancro.