Increased number of circulating HTLV-1 infected cells in peripheral blood mononuclear cells of HTLV-1 uveitis patients: a quantitative polymerase chain reaction study

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Abstract
Aims—This study aimed to characterise the status of viral infection in patients with HTLV-1 uveitis (HU) by quantifying the circulating HTLV-1 infected cells in the peripheral blood.

Methods—Genomic DNA samples of peripheral blood mononuclear cells (PBMC) were obtained from 25 patients with HU, 14 patients with tropical spastic paraparesis/HTLV-1 associated myelopathy (TSP/HAM), and 21 asymptomatic carriers of HTLV-1. Quantitative polymerase chain reaction (PCR) of the gag region of HTLV-1 provirus DNA was performed on these DNA samples. To confirm the PCR, genomic Southern blot hybridisation was performed to identify integrated HTLV-1 provirus. This procedure detected a few percent of HTLV-1 infected cells in the PBMC.

Results—Most of the HU patients had a significantly increased number of circulating HTLV-1 infected cells (mean (SD) 3·84% (4·45%) of the PBMC), whereas the percentage of infected cells in most asymptomatic carriers was less than 1% (0·54% (1·1%)). Most of the TSP/HAM patients also had a relatively high percentage (11·63% (7·67%)). The differences among these three groups were highly significant by the Mann-Whitney U test.

Conclusion—The results suggested that the increase in the number of HTLV-1 infected cells is one base for the development of inflammatory HU lesions, as it is for TSP/HAM.


The human T cell leukaemia virus type I (HTLV-1) is causatively associated with a T cell malignancy, adult T cell leukaemia (ATL),1-7 and a chronic neurological disorder, tropical spastic paraparesis/HTLV-1 associated myelopathy (TSP/HAM).8-12 Recently, we reported a new disease entity associated with HTLV-1, HTLV-1 uveitis (HU), based on our seroepidemiological, ophthalmic, and virological studies.13-18 HU was tentatively defined as an idiopathic uveitis of otherwise asymptomatic HTLV-1 carriers. Clinically, HU is characterised by a sudden onset of mild iritis, moderate or severe vitreous opacities, and mild retinal vasculitis in one or both eyes.18 Cytological examination of the cells in the anterior chambers from affected eyes disclosed that the infiltrating cells are predominantly lymphocytes with CD3 surface marker (Ono et al, submitted for publication). HTLV-1 infected cells were demonstrated in all the samples examined13 by polymerase chain reaction (PCR).19 The intraocular inflammation responded well to topical and/or systemic corticosteroids and the visual prognosis of HU is good in most patients.16-18

TSP/HAM is a chronic inflammatory neurological disease of the CNS, mainly affecting the thoracic spinal cord resulting in spastic paraparesis.20,21 Because of high levels of serum antibodies against HTLV-1 antigens and the presence of cytotoxic T cells in peripheral blood mononuclear cells (PBMC), the host immune response has been implicated as one of the pathogenetic mechanisms.12-20 One of the characteristic findings is that the number of HTLV-1 infected cells in the PBMC of patients is increased polyclonally or, in some cases, oligoclonally.30-35 Although the mechanism of this increase is not yet understood, it is speculated that the increased number of HTLV-1 infected cells is also relevant to the development of the inflammatory CNS lesions characterised by the infiltration of T lymphocytes.20,21

We tested whether patients with HU, another inflammatory disease caused by HTLV-1, have an increased number of circulating HTLV-1 infected cells. We determined the number of HTLV-1 infected cells in fresh PBMC from these patients using semi quantitative PCR and conventional genomic Southern hybridisation. Our results indicated that the number of HTLV-1 infected cells in PBMCs is significantly increased in most HU patients compared with that of asymptomatic carriers, suggesting a common underlying HTLV-1 infection in HU and TSP/HAM patients.
Materials and methods

PATIENTS WITH HU AND TSP/HAM
All the patients with HU or TSP/HAM and the asymptomatic carriers in this study were residents in the Kyushu area of Japan, where HTLV-1 is endemic. The number of patients with HU was 25 (five males and 20 females, mean age 44-6) and that of TSP/HAM patients 14 (five males and nine females, mean age 54-9). Twenty one asymptomatic carriers (15 males and six females, mean age 48-3) included 14 seropositive blood donors and seven volunteers. In all these groups, no family members were included except for one asymptomatic carrier who is a family member of a TSP/HAM patient. HU was clinically diagnosed according to our criteria.13-18 Briefly, idiopathic uveitis in the otherwise asymptomatic HTLV-1 carriers was diagnosed as HU. Peripheral blood samples of these patients contained no abnormal lymphocytes and the white blood cell count was within the normal range. TSP/HAM was clinically diagnosed using the criteria of Osame et al:23 gradual onset of progressive spastic paraparesis; symmetrical, predominantly upper motor neuron disorders with mild sensory and bladder disturbances; and high antibody titres to HTLV-1 antigens in the serum and cerebrospinal fluid (CSF).

ASSAY FOR ANTIBODIES TO HTLV-1
Serum antibodies to HTLV-1 antigens were tested using a commercially available particle agglutination kit (Fuji Rebio, Tokyo, Japan) and confirmed by an enzyme linked immunosorbent assay kit (Eizai Co Ltd, Tokyo, Japan).

GENOMIC DNA SAMPLES FROM PBMC
With their informed consent, peripheral blood samples were collected from 25 patients with HU, 14 patients with TSP/HAM, and 21 asymptomatic carriers. Fresh PBMC were isolated by density gradient centrifugation with Ficoll-Paque (Pharmacia LKB Biotechnology, Uppsala, Sweden). High molecular weight genomic DNA was extracted by the standard method using SDS/ProteinaseK and phenol chloroform extraction followed by ethanol precipitation. The concentration of the DNA was determined by spectrophotometry.

SEMIQUANTITATIVE PCR ANALYSIS
A primer pair for the HTLV-1 gag region was used to PCR amplify HTLV-1 provirus DNA (Table 1). As a positive control sample having one copy of the HTLV-1 provirus per cell, we used the genomic DNA extracted from the PBMC of an ATL patient which contained more than 90% leukaemic cells. A fivefold dilution series of this ATL DNA was prepared by dilution with genomic DNA of the human T cell line CEM, which is not infected with HTLV-1. The reaction volume of 50 μl contained 0·5 μg of template DNA. The reaction mixture contained 1 μmol/l of each primer, 200 μmol/l of each of the four nucleotide triphosphates, 1 mmol/l of MgCl2 and 1·25 units of Taq polymerase (Biotech International Ltd, Australia). For semiquantitative PCR, template DNA samples for the patients and the control dilution series were amplified simultaneously, and one fifth of these products were resolved by 5% polyacrylamide gel electrophoresis followed by electrotransfer to a charged nylon membrane (Bionde B, Pall BioSupport Company, Glen Cove, NY, USA) using a semi-dry blotter. Thereafter, the membrane was denatured with 0·4 N NaOH and neutralised with 0·5 M TRIS HCl, pH 8·0, and fixed by ultraviolet irradiation. Hybridisation with a 32P labelled internal oligomer probe (G3) was done at 55°C in a hybridisation solution containing 4×SSC, 10×Denhardt solution, and 100 μg/ml of heat denatured E coli DNA. After washing in 2×SSC and 0·1% SDS at 55°C, the hybridised signal was quantified using a Bio-Image Analyser BA100 (Fuji Film, Tokyo, Japan). To estimate the copy number of the HTLV-1 provirus in the template DNA, a standard curve on a semilog scale was constructed from the measured radioactivity in the control dilution series. The samples were assayed by 30 cycles of amplification. When the samples contained more than a few per cent of the infected cells, 25 cycles of amplification were performed for confirmation. The percentage of the infected cells was calculated from the standard curve based on the following assumptions: that the positive control DNA of ATL cells contains one copy of HTLV-1 provirus per cell which was verified by genomic Southern blotting with an HTLV-1 DNA probe; that 0·5 μg of DNA corresponds to 0·75×105 cells; and that HTLV-1 infected cells in vivo have one copy of its provirus per cell.

SOUTHERN HYBRIDISATION OF THE INTEGRATED HTLV-1 PROVIRUS
To test the sensitivity of the blot, a twofold dilution series of the positive control DNA was prepared using ATL DNA and DNA of the HTLV-1 negative human T cell line, Jurkat. Ten μg of genomic DNA of this dilution series and the DNA samples were digested with PstI, which has six recognition sites within the HTLV-1 provirus genome. Following electrophoresis on a 1% agarose gel, the DNA was transferred to a nylon membrane by capillary blotting with 0·4 N NaOH. The membrane was neutralised with 0·5 M TRIS HCl, pH 8·0, fixed by ultraviolet irradiation and hybridised at 65°C in a hybridisation solution containing

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Table 1 Oligomers used for PCR analysis of HTLV-1 provirus

| G1:5'-TTATGCAGGACATCCGCAGT3' (1301-1320) |
| G2:5'-TATCTAGCTCGTGGTATGG3' (1420-1401) |
| G3:5'-GAGACCTCTGCACTACTT3' (1359-1368) |

G1, G2 = primers for gag region of HTLV-1; G3 is the antisense primer. G3 = internal oligomer probe.

Numbers in the parenthesis is the nucleotide position of the original provirus clone, γATK (ref 37).

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The internal oligomer probe, G3, is presented. The membrane of the autoradiogram is exposed. The membrane hybridisation of HTLV-I provirus DNA was amplified by PCR and the products were hybridised with an internal oligomer probe. The radioactivity of each product was measured and plotted on a semi-log scale graph. (A) and (C), autoradiogram of blotted PCR products (A: 25 cycles, C: 30 cycles), (B) and (D) plot of radioactivity of each product (B: 25 cycles, D: 30 cycles).

Results

**SEMIQUANTITATIVE PCR OF THE HTLV-1 PROVIRUS DNA**

The amount of PCR products in the control dilution series was almost linearly related to the copy number of HTLV-1 provirus DNA in the template over the range of a $5^{-2}$ to $5^{-0}$ dilution, by 25 amplification cycles, and of a $5^{-3}$ to $5^{-1}$ dilution by 30 cycles (Fig 1). Therefore, 30 cycles were considered optional for quantifying the products from most of the HU patients and asymptomatic carriers, whereas 25 cycles was used to quantify those in some of the TSP/HAM patients whose PBMC contained more than 10% of infected cells.

Because the efficiency of amplification varied to some extent in each PCR, the control dilution series DNA and the patient samples were amplified simultaneously and analysed on the same gel and membrane. Some of these results are shown in Figure 2, and they demonstrate the increased viral DNA load in HU patients (Fig 2, lanes 6-10) compared with that of asymptomatic carriers (Fig 2, lanes 11-13).

The calculated percentage of HTLV-1 infected cells in the PBMC in the three groups of HTLV-1 carriers was plotted on a semilog scale (Fig 3). Among the 25 HU patients studied, 18 (72.0%) had more than 1% of infected cells. The percentage of HTLV-1 infected cells in the PBMC in HU patients ranged from 0.1% to 17%, with the mean (SD) of 3.84% (4.45%). Among the asymptomatic carriers, 17 of 21 (81.0%) contained less than 1% of HTLV-1 infected cells in the PBMC, with 11 out of 21 (52.4%) having about 0.1% or less (mean (SD) 0.54% (1.11%). It is notable that four of the 21 samples (19%) contained over 1% of infected cells, which contributed to the wide distribution and relatively high mean percentage. The percentage of infected cells in one asymptomatic family member of a TSP/HAM patient was 0.25%. Of the 14 TSP/HAM patients, more than 5% of the infected PBMC were in 12 (85.7%) of them, the highest being 25% (mean (SD) 11.63% (7.67%)). The PBMC of the remaining two patients (14.3%) contained about 1% or less of

![Figure 1](http://bjo.bmj.com/)

**Figure 1**  Semiquantitative amplification of HTLV-1 provirus DNA by gag region PCR. A fivefold dilution series of positive control DNA was amplified by PCR and the products were hybridised with an internal oligomer probe. The radioactivity of each product was measured and plotted on a semi-log scale graph. (A) and (C), autoradiogram of blotted PCR products (A: 25 cycles, C: 30 cycles), (B) and (D) plot of radioactivity of each product (B: 25 cycles, D: 30 cycles).

![Figure 2](http://bjo.bmj.com/)

**Figure 2**  Quantitative PCR of HTLV-1 provirus DNA in the samples. An autoradiogram of the membrane hybridisation of the PCR products (30 cycles) with internal oligomer probe, G3, is presented. The membrane was exposed to X-Omat AR film for 1 hour. (A) Fivefold dilution series of the positive control ($5^{-2}$ to $5^{-4}$ from lanes 1 to 5); (B) samples from HU patients (lanes 6 to 10); (C) samples from asymptomatic carriers (lanes 11 to 13).
Increased population of HTLV-1 infected cells in the PBMC of patients with HU and TSP/HAM. The calculated percentage of HTLV-1 infected cells in each sample was plotted on a semi-log scale. The means and the standard deviations are indicated for each group. (A) TSP/HAM patients (n=14, mean (SD) 11.63 (7.67)), (B) HU patients (n=25, mean (SD) 3.84 (4.45)), (C) asymptomatic carriers (n=21, mean (SD) 0.54 (1.11)). *p=0.0001, **p=0.0008 by Mann-Whitney U test.

Although the range was widely distributed in each group, the increases of the HTLV-1 infected cells in the PBMC of the patients with HU and TSP/HAM were both highly significant when compared with asymptomatic carriers (p=0.0001 for both by Mann-Whitney U test). The difference in the ratio of the infected cells between the patients with HU and TSP/HAM was also significant (p=0.0008 by Mann-Whitney U test).

**Discussion**

In addition to ATL and TSP/HAM, the disorders which have been associated with HTLV-1 include alveolitis, Sjögren syndrome, polymyositis, dermatitis, and arthropathy.38-45 Although these disorders, except for ATL and TSP/HAM, did not have strong epidemiological or clinical data to support their distinction as a clinical entity, a wide spectrum of HTLV-1 pathogenicity has been suggested. Recently, we reported a uveitis associated with HTLV-1 (HU) which has an array of these features and could be considered a third disease caused by HTLV-1 infection.13-18

We showed here, by semiquantitative PCR, that the number of circulating HTLV-1 infected PBMCs was significantly increased to a few percentage points or more in most HU patients, and to about 10% or more in those with TSP/HAM, whereas it was less than 1% in most asymptomatic carriers. We confirmed these results by genomic Southern hybridisa-
tion of the integrated HTLV-1 provirus and dilution studies.

Our results demonstrated that the increase in the HTLV-1 infected cells is a common feature of both HU and TSP/HAM. The percentage of the infected cells, however, showed a rather wide distribution, overlapping that of asymptomatic carriers. Thus the increase itself was not considered to be pathognomonic for these inflammatory disorders. It was already reported that TSP/HAM patients have an increased number of circulating HTLV-1 infected cells in the PBMC.\textsuperscript{30-35} Yoshida et al first reported that detection of the PstI digested internal fragment of the HTLV-1 provirus by conventional Southern blotting indicated the increased replication of the virus in these patients.\textsuperscript{30,31} Using the same method, they later demonstrated an increase in the number of HTLV-1 infected cells in 84, 52, and 20% of the TSP/HAM patients, in seropositive family members of the patients with TSP/HAM and asymptomatic carriers, respectively.\textsuperscript{35} They also found that 19% of the TSP/HAM patients and 16% of their seropositive family members showed oligoclonal or monoclonal expansion of the HTLV-1 infected cells, whereas no clonal proliferation was detected in asymptomatic carriers in the general population. Our Southern blots of HU patients with representative and region specific probes also detected internal fragments of PstI digested HTLV-1 proviral DNA, indicating that more than a few percent of PBMC are infected with HTLV-1. About one third of the HU samples were positive in this assay, which roughly corresponds with the number that were shown by PCR to contain more than 3% infected cells. However, junction fragments containing LTR sequence have not yet been detected (Fig 4B), which suggests that no clonal proliferation at a detectable level has taken place in HU patients. To address this point by a more sensitive method, we are now investigating the clonal population in the PBMC of HU patients using inverse PCR to amplify LTR and flanking cellular DNA.\textsuperscript{46} Our preliminary results suggest that oligoclonal expansion of the HTLV-1 infected cells has taken place in the PBMC of HU patients, but not in that of asymptomatic carriers (Takemoto et al, unpublished observation).

The results of our quantitative PCR of cells from TSP/HAM patients were comparable with the 3–15% found by Gessain et al by Southern blotting with dilution series.\textsuperscript{23} Our results were also similar to those of Kira et al, who performed semiquantitative PCR.\textsuperscript{34} Our
results showed that patients with HU or TSP/HAM have 10–100 times more HTLV-1 infected cells than symptomatic carriers. This increase could be explained either by the proliferation of the HTLV-1 infected cells or by the efficient replication of the virus in these individuals. The presence of a clonal population in the PBMC of TSP/HAM patients\(^3\) and also in that of HU patients (Takemoto et al., unpublished observation) suggests that it resulted from the proliferation of the HTLV-1 infected cells in vivo. This expansion seems to precede the development of TSP/HAM, because 50% of the asymptomatic family members of TSP/HAM also have the increase.\(^3\)

One of the non-structural, regulatory proteins of HTLV-1, Tax1, transactivates the expression of both viral and host cellular genes. Among the genes that are transactivated are those of cytokines such as IL-2, IL-3, IL-4, GM-CSF, and TGF\(_B\).\(^{37–51}\) Thus, it is conceivable that the increased number of HTLV-1 infected T cells may lead to disturbances in the host immune response through the presentation of viral antigens and/or aberrant functions induced by Tax1, which results in inflammatory disorders such as HU and TSP/HAM.

Whether such pathogenetic processes are involved in these two disorders can be tested by characterizing the immunological abnormalities and the status of the viral infection in HU patients and their family members.

In conclusion, we demonstrated that patients with HU and TSP/HAM have much more HTLV-1 infected T cells compared with asymptomatic carriers. These common features of viral infection might be the basis for the development of inflammatory disorders in HTLV-1 carriers.

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Glaucoma Group

DAVID COLE TRAVEL FELLOWSHIP

The David Cole Travel Fellowship, instituted by Merck Sharp & Dohme in memory of Professor David Cole, will assist a visit to a hospital or research centre during the academic year starting 1 October 1995. The award is equivalent to £2000. The purpose of the award is to enable the successful applicant to gain experience and knowledge in pursuit of a specific project related to glaucoma.

Wellcome General Overseas Travelling Research Fellowships 1994–95

The purpose of these fellowships is to allow postdoctoral scientists and medical graduates to gain further research experience by working in leading laboratories in the UK or the Republic of Ireland. Applications are invited from such workers who wish to undertake a research project in any branch of the natural or clinical sciences, which has a bearing on human or veterinary medicine, with the exception of cancer. Applicants may be from any country outside Europe, with the exception of New Zealand and the USA for whom special schemes are available. Awards will be made on the basis of the research proposal. The research proposal should be relevant to the research interests of the candidate in his/her own country. Awards are made in the first instance, although requests for an extension may be considered. Fellowships provide a stipend within the range from £13941 to £27869 per annum, depending on age and experience. They also include the cost of research, attendance at scientific meetings, and return travel. Candidates must be nominated by a sponsor in the UK or the Republic of Ireland, through whom all initial inquiries should be made. A preliminary proposal should include a one or two page outline of the research proposed, the curriculum vitae of the candidate, and a letter indicating that he/she has a position to return to at the end of the fellowship. There are no special deadlines for this scheme and applications may be submitted at any time during the year.

Requests for application forms should be addressed to: Dr J M Wilkinson, The Wellcome Trust, 183 Euston Road, London NWI 2BE; Tel: 0171-6118407.

Candidates from New Zealand and the USA should contact the Health Research Council of New Zealand, Auckland, NZ or the Burroughs Wellcome Fund, Morrisville, NC 27560, USA, respectively, for details of appropriate schemes.

International Society for Clinical Electrophysiology of Vision

The 33rd ISCEV symposium will be held in Athens, Greece, 16–20 June 1995. The congress is organised by the International Society for Clinical Electrophysiology of Vision. Further details: Secretary, Erasmus Conference Centre, International Congress Organisers, 227 Kifissias Ave, 145 61 Kifissia, Greece. (Tel: (01) 6125022/3, 8054004; Fax: (01) 6125021.)

4th European Symposium of Traditional Ophthalmology

The 4th European Symposium of Traditional Ophthalmology will be held in Milan, Italy on 25–26 June 1995. Further details: Dottore Marzio Vanzini, Via Berlotta 22, 40132 Bologna, Italy. (Tel: 39 51 38 24 01; Fax: 39 51 62 32 000).

San Diego Eye Bank

The San Diego Eye Bank is holding its 15th annual current concepts in ophthalmology conference on 11–13 August 1995 at the San Diego Princess Resort. Further details: Britta A Sullaway, Public Relations, San Diego Eye Bank, 3702 Ruffin Road, Suite 100, San Diego, CA 92123, USA. (Tel: (619) 694-0444; Fax: (619) 694-0581.)

The 21st International Pupil Colloquium

The 21st International Pupil Colloquium will be held from 29 August to 2 September 1995 at Schloss Haigerloch, Tübingen, Germany. For further details: Helmut Wilhelm, University Eye Hospital, Department of Pathophysiology of Vision and Neuro-Ophthalmology, D-72076 Tübingen, Germany. (Tel: +497071 294786; Fax: +497071 295038.)

European Strabismological Association

The 22nd meeting of the European Strabismological Association (ESA) will be held in St John’s College, Cambridge, UK on 6–8 September 1995. Application papers, including abstract forms, can be obtained from: Mr J S Elston, MD, FRCS, Oxford Eye Hospital, Radcliffe Infirmary, Woodstock Road, Oxford OX2 6HE, UK.

British and Eire Association of Vitreo-Retinal Surgeons

The next meeting of the British and Eire Association of Vitreo-Retinal Surgeons (BEAVRS) will be held at Cameron House, Loch Lomond, Glasgow on 5–6 October 1995. Members will be contacted with further details in due course; any other doctors wishing to attend should contact Dr H M Hammer or Dr T Barrie, Glasgow Eye Infirmary, 3 Sandyford Place, Glasgow G3 7NB. (Tel: 0141-211 6767; Fax: 0141-211 6770.)

Joint European Research Meeting in Ophthalmology and Vision

JERMOV, the Joint European Research Meeting in Ophthalmology and Vision, will hold its second meeting in Montpellier on 14–18 October 1995. Further details: JERMOV Secretariat – Chairman Agency, Les Portes d’Antigone 43, Place Vauban, 34000 Montpellier, France. (Tel: +33 67 15 99 00; Fax: +33 67 15 99 09.)

The Jules François Prize, 1997

The Jules François Prize of the Belgian ophthalmological societies of SUS 10 000 will be awarded for the sixth time in 1997 to a young scientist who had made an important contribution to ophthalmology. The aim of the prize is to encourage scientific research in ophthalmology. There is no special theme. Fundamental as well as clinical research will be considered. The age limit is 40 years by 31 December 1995. Further details: Dr J D’Haenens, Secretary of the Jules François Foundation, E Beernaerstraat 34, B–8400 Oostende, Belgium.

Correction

Because of a printer’s error the wrong figure appeared as Figure 2 in the paper by Ono et al that was published in the March issue of the journal (BJO 1995; 79: 270–6). The correct figure is given below.

![Figure 2: Quantitative PCR of HTLV-1 provirus DNA in the samples. An autoradiogram of the membrane hybridisation of the PCR products (30 cycles) with internal oligomer probe, G3, is presented. The membrane was exposed to X-Omat AR film for 1 hour. (A) Fivefold dilution series of the positive control (5^(-4) to 5^(-8) from lanes 1 to 5); (B) samples from HIV patients (lanes 6 to 10); (C) samples from asymptomatic carriers (lanes 11 to 13).](image-url)