

HTLV Tax gene expression in patients with lymphoproliferative disorders

E A Cardoso, N Miranda, P Gameiro, M J Frade, M Figueiredo, A Parreira

Abstract

Aims—To study the expression of the human T lymphotropic virus (HTLV) *Tax* gene in peripheral blood mononuclear cells.

Methods—Blood was collected from 72 patients with lymphoproliferative disorders. Serum from all patients was assayed for antibodies directed against HTLV-I structural proteins by ELISA and western blotting. RNA was purified from fresh blood cells and amplified by reverse transcription polymerase chain reaction (RT-PCR). After Southern blotting, the PCR products were hybridised with a ³²P end-labelled probe specific for the *Tax* gene.

Results—All samples were seronegative. A specific band for the *Tax* gene was found in five samples. Each of the patients positive for *Tax* gene expression had a different type of lymphoproliferative disorder.

Conclusions—Infection by HTLV-I cannot be assessed solely by immunological assays, particularly when only disrupted virions are used. Sensitive molecular biology assays are essential for detecting viral gene expression in fresh blood cells.

(*J Clin Pathol* 1996;49:938-941)

Keywords: HTLV, lymphoproliferative disorders, *Tax* expression.

Human T-cell leukaemia virus type I (HTLV-I) has been implicated in the aetiology of adult T cell leukaemia (ATL)^{1,2} and tropical spastic paraparesis/HTLV-I associated myelopathy (TSP/HAM).³ Another retrovirus, HTLV-II, has been isolated from a hairy cell leukaemia (HCL) of T-cell phenotype.⁴ Both viruses are closely related and indistinguishable by common serological assays.⁵ Seroepidemiological studies indicate that only 1 to 2% of people infected with HTLV-I will eventually develop the disease. Other disorders have been tentatively associated, direct or indirectly, with these viruses, including Sjögren's syndrome,⁶ chronic infective dermatitis,⁷ polymyositis,⁸ T-cell alveolitis,⁹ retinitis and uveitis,¹⁰ B-cell chronic lymphocytic leukaemia (CLL),¹¹ and lymphoma mimicking Hodgkin's disease.¹² The relation with virus infection was based on serological or molecular tests. However, there are insufficient data to judge whether these findings are fortuitous or whether there is a genuine association with HTLV.

Although T-cells seem to be the preferential target for the virus, other haemopoietic cells

may carry integrated viral DNA.^{13,14} A broad range of cell lines is also susceptible to infection.¹⁵ These cells contain the HTLV genome and synthesise viral RNA, but are restricted in their expression of viral structural proteins. Efficient expression of the HTLV genome requires both virus and host specific proteins and is dependent on the viral long terminal repeat (LTR). Of particular importance is the viral *Tax* gene, localised in the pX region, which has been implicated in tumorigenesis.¹² It transactivates viral and several cellular genes¹⁶⁻¹⁸ and is capable of immortalising cells in vitro.¹⁹ It has been suggested that the deregulation of these cellular genes is a critical step for the *Tax* mediated transformation process in HTLV leukaemogenesis.^{14,20} Several host specific cellular protein factors have been shown to interact with the virus LTR and presumably influence the expression of the viral genome and several cellular growth regulatory genes and cytokines.²¹⁻²³ It was found that a significant fraction of patients with ATL carry deleted viral genomes in their cells¹⁷ with preferential retention of the *Tax* gene. These data apparently suggest that the HTLV oncogenic effect may be observed in the absence of viral replication. However, the significance of different *Tax* mRNA levels, in infected cells, is not fully understood.²⁴

Except for those with TSP/HAM, most carriers of and patients with HTLV-I associated disease have low antibody titres.^{25,26} In fresh blood cells the presence of antigens or expression of viral genes cannot be detected using conventional methods, such as immunofluorescence or northern blotting.²⁷ It is possible that estimates of HTLV associated disease will be increased in the light of molecular, rather than just serological evidence of infection. The reverse transcription polymerase chain reaction (RT-PCR) seems to be the most suitable assay for this purpose.^{28,29}

Previous seroepidemiological studies from our group indicate that Portugal is not an endemic area for HTLV and the prevalence of seropositivity is 0.55% in the general population.³⁰ To our knowledge there were no reported cases of ATL in Portugal and the few known cases of TSP/HAM were described in black people native to former Portuguese African colonies.³¹

The aim of the present study was to investigate whether molecular evidence of HTLV infection could be found in patients with different types of lymphoproliferative disorders, even in the absence of detectable antibody.

Laboratório de
Biologia Molecular
(CIPM), Instituto
Português de
Oncologia, Lisboa,
Portugal
E A Cardoso
P Gameiro

Serviço de
Hematologia
N Miranda
M J Frade
A Parreira

Laboratório de
Virologia
M Figueiredo

Correspondence to:
Dr E A Cardoso,
Laboratório de Biologia
Molecular, Instituto
Português de Oncologia,
Rua Prof. Lima Basto,
1070 Lisbon, Portugal.

Accepted for publication
27 June 1996

Table 1 Clinical and histological classification of patients

Classification	Number of patients
All patients	72
Age (mean (range))	54.26 (16–89)
Male/female	34/38
All patients by diagnosis	
Hodgkin's disease	16
Non-Hodgkin's lymphoma	
B-cell	34
T-cell	7
Hairy B-cell leukaemia	2
Acute lymphoblastic leukaemia	
B-cell	1
T-cell	1
Chronic lymphocytic leukaemia	
B-cell	11

Methods

The study population comprised 72 patients with a cytological or histological, or both, diagnosis of lymphoproliferative disease (Hodgkin's disease or non-Hodgkin's lymphoma). Blood samples were collected over a period of 18 months. The demographic and clinicopathological details of the patients are shown in table 1. Risk factors for HTLV infection (blood transfusions, drug misuse, sexual promiscuity and/or residence in endemic regions) were noted for each patient.

Thirteen patients had been transfused in the past. One patient was a drug addict and HIV positive. Seven patients lived, for variable periods of time, in areas endemic for HTLV. One heterosexual patient had a history of sexual promiscuity.

IMMUNOLOGICAL ASSAYS

Serum from all patients was tested for the presence of anti-HTLV antibodies by ELISA (Sanofi-Pasteur and Diagnostic Biotechnology commercial kits) with disrupted virus and confirmatory tests were carried out by western blotting (DB).

PREPARATION OF RNA

Peripheral blood mononuclear cells were isolated on a Ficoll-Hypaque density gradient and lysed by the addition of guanidine thiocyanate. After ultracentrifugation in caesium chloride, the RNA was quantified, divided into aliquots and stored at -70°C pending analysis. The same procedure was carried out with HUT 102 cells, which served as a positive control. To avoid contamination, RNA extraction and RT-PCR were performed in Biohazard laminar flow hoods, in different rooms. All material used was treated with diethyl pyrocarbonate and sterilised. Sterile micropipette tips had filters to prevent contamination of the micropipette with aerosols. No more than two samples were handled at a time.

Integrity of the RNA was analysed by electrophoresis in formaldehyde containing agarose gels.

RT-PCR

RT-PCR was performed as recommended by the manufacturer (Perkin-Elmer Cetus). Reverse transcription was performed in a 20 μl volume, containing 1 μg total RNA, at 42°C for 45 minutes, followed by five minutes at 99°C and five minutes at 5°C . Reverse transcriptase

from M-MLV (BRL) was used in some cases. Random hexamer primers were used for the RT-PCR assay. Controls without reverse transcriptase, RNA and Taq polymerase were always included in each test.

PCR PRIMERS AND PROBES

Primers SK43 and SK44 recognise Tax regions 7358–7377/7496–7516 in HTLV-I and 7248–7267/7386–7406 in HTLV-II. They were probed with SK45, which is common to both viruses. Primers SK54 and SK55 recognise positions 3365–3384 and 3465–3483 of the HTLV-I pol gene. They were probed with SK56, which is specific for the HTLV-I pol gene. SK58 and SK59 recognise positions 4198–4217 and 4281–4300 of the HTLV-II pol gene. They were probed with SK60, which is specific for the HTLV-II pol gene.

PCR was performed in a final volume of 100 μl , including 20 μl of the reverse transcription product, in a DNA Thermal Cycler (Perkin-Elmer/Cetus) for 30 cycles. PCR conditions were as follows: 15 seconds at 94°C (denaturation), 30 seconds at 53°C (annealing), 30 seconds at 68°C (elongation), followed by a further 10 minutes at 68°C . Twenty microlitres of each sample were tested for DNA integrity by electrophoresis. In the confirmatory tests these volumes were increased to 40 μl for the patients' samples and reduced to 0.5 μl for the positive control, as the signal was too strong when 20 μl was used. Positive controls were run in slots away from the patient samples to avoid contamination.

SOUTHERN BLOT ANALYSIS

Hybridisation was performed using the standard method with slight modifications. After denaturation and neutralisation, DNA was transferred to nylon membranes (Hybond N+, Amersham). Pre-hybridisation was performed for at least two hours, followed by overnight hybridisation (incubation). Solutions were composed of sodium phosphate buffer 20 mM, 5 \times SSC, 10 \times Denhart's solution, 7% SDS, 100 $\mu\text{g}/\text{ml}$ salmon testes DNA. Dextran sulphate, at final concentration of 10%, was added to the hybridisation solution. Probes were ^{32}P end-labelled using T4 kinase. Hybridisation and stringency temperatures were calculated for each probe.³² Washes were carried out in 2 \times SSC for 10 minutes, 2 \times SSC plus 0.1% SDS three times for 10 minutes and finally 1 \times SSC plus 1% SDS for 10 minutes. Cronex 4 film (Dupont) was used for autoradiography, with exposure for two hours at room temperature and for 24 hours at -70°C .

TREATMENT WITH DNASE

All positive samples were treated with 75 units of RNase free DNase (Sigma) following published protocols.³² This amount of DNase completely abolished the amplification of 1 μg RNA from HUT 102 cells, in the absence of reverse transcriptase (data not shown). DNase treatment was performed at different times for each sample. RT-PCR and hybridisation were repeated for these samples.

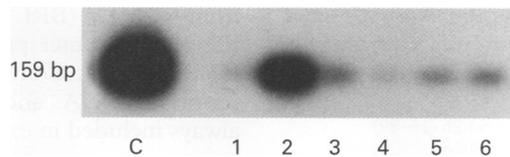


Figure 1 Detection of HTLV *Tax* gene expression by RT-PCR, followed by Southern blot hybridisation. Lane C, positive control; lanes 1–6, patient samples.

Results

HTLV antibodies were detected in only one of 72 patients tested. “Positive” samples from this patient were negative when analysed by western blotting.

In RT-PCR tests, for the patients’ RNA, none of the primers used gave rise to detectable bands when stained with ethidium bromide. The appropriate primers showed the presence of a single, strong band, with the expected molecular weight, when RNA from HUT 102 cells was tested. No bands were seen when primers specific for HTLV-II were used. Hybridisation of RNA from six patients with the *Tax* probe showed a specific band of 159 base pairs, the size expected with the *Tax* primers used (fig 1). In a few instances, the bands were visible after exposure for just two hours. No signal was seen with the *pol* probes for either HTLV-I or HTLV-II, even after exposure for four days. The positive patient samples were found in different screening assays. No background amplification was found, at any time, in the negative controls.

After treatment with DNase, five of the six cases remained positive, although fainter bands were produced. Amplification due to remnant provirus fragments was ruled out as treatment with DNase completely degrades any DNA present in HUT 102 cells, as described in Methods. The only case giving a negative result was the serological false positive. This patient was an 82 year old man with a high grade T-cell non-Hodgkin’s lymphoma with bone involvement. He died during the course of this study (patient 4, table 2). None of the six patients listed in table 2 had been in endemic areas. Patient 5 had a history of sexual promiscuity; patient 2 had been transfused previously and had two associated benign neoplasms (melanocytoma of the left eye and mixed tumour of the parotid) as well as leukaemia (T-ALL).

Discussion

Of the 72 patients with lymphoproliferative disorders analysed in this study, only one had detectable antibodies directed against HTLV; this result was not confirmed on western blotting. These results are in agreement with the

known data about the low seroprevalence for this virus in Portugal, which is similar in most western countries. However, we detected, in this series, molecular evidence of HTLV infection in five (6.9%) patients in whom the expression of, at least, one viral gene could be demonstrated. Our results also indicate that in this series, as *pol* gene expression was not detected, viral replication seems to be absent.

The disagreement between the serological and molecular techniques may be explained by the lack of sensitivity of the former method. Alternatively, infection by a defective virus expressing the *Tax* gene only could explain this discrepancy. The *Tax* gene protein was not included in the serological tests used. The RT-PCR assay system is a sensitive method for the detection of target gene expression, although it does not provide useful additional information about integrated proviral DNA, as does classic Southern blot analysis. Studies on cellular DNA would indicate which virus is involved and whether it is defective.

The positive RT-PCR result in patient 4 became negative after DNase treatment. This observation could result from a provirus not expressing the genes we looked for and from which DNA fragments contaminated the RNA sample; alternatively, the level of expression may be below the detection threshold of the RT-PCR method used.

Two patients with B-cell chronic lymphoproliferative disorders (CLL and HCL) were positive, although we were unable to identify the cellular target of viral infection. HTLV-I infection has been associated indirectly with B-cell CLL,³³ but never, to our knowledge, with B-cell HCL; only the rare form of T-lineage HCL was reported as probably resulting from HTLV-II infection.⁴ It has been suggested that genes from the pX region of the human retroviruses could act upon non-infected cells.³⁴ This effect could lead to continuous immunological stimulation and ultimately contribute to the emergence of a neoplastic clone.³³ Whether this phenomenon is related to the occurrence of B-CLL or is just a fortuitous finding, is a matter of conjecture. Alternatively, these patients may be infected by an as yet unknown retrovirus with great homology for that region. Human retroviruses of this group have a well conserved genome, especially in the pX region.⁵ Endogenous human DNA sequences, similar to these viruses, have already been reported.³⁵ Although the present series is relatively small, no particular association with any lymphoproliferative disorder could be identified, reinforcing the concept that apart from ATL, there is no causal relation between HTLV infection and haematological malignancy.

The real incidence of *Tax* gene expression in healthy and serological negative populations is not yet known and therefore we cannot determine the true significance of our findings. We also cannot infer that *Tax* gene expression has any oncogenic potential in these patients.

Our results indicate that infection with HTLV cannot be assessed solely by immunological assays, particularly when only disrupted

Table 2 Clinical and histological details of patients expressing the HTLV *Tax* gene

Patient number	Age (years)	Sex	Diagnosis	Risk factors for HTLV
1	44	Male	Hairy cell leukaemia (B-lineage)	No
2	17	Female	T-cell acute lymphoblastic leukaemia	Yes
3	50	Female	B-cell chronic lymphocytic leukaemia	No
4	82	Male	High grade T-cell non-Hodgkin’s lymphoma	No
5	64	Female	High grade B-cell non-Hodgkin’s lymphoma	Yes
6	67	Female	B-cell centrocytic non-Hodgkin’s lymphoma	No

virions are used, as has also been shown by others.^{17 26 36} Sensitive molecular biology assays are essential for detecting viral gene expression in fresh blood cells.

This work was supported by grants obtained from the Junta Nacional de Investigação Científica (JNICT) and the Portuguese Ministry of Health.

We thank Dr M Robert-Guroff for reading the manuscript and Ms Leonor Rebelo for technical assistance.

- 1 Poiesz BJ, Ruscetti FW, Gazdar AF, Bunn PA, Minna JD, Gallo RC. Detection and isolation of type C retrovirus particles from fresh and cultured lymphocytes of a patient with cutaneous T-cell lymphoma. *Proc Natl Acad Sci USA* 1980;77:7415-19.
- 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;79:2031-5.
- 3 Gessain A, Barin F, Vernant JC, Gout O, Maurs L, Calender A, et al. Antibodies to human T-lymphotropic virus type-I in patients with tropical spastic paraparesis. *Lancet* 1985;ii:407-10.
- 4 Kalyanaraman VS, Sarngadharan MG, Robert-Guroff M, Miyoshi I, Golde D, Gallo RC. A new subtype of human T-cell leukemia virus (HTLV-II) associated with a T-cell variant of hairy cell leukemia. *Science* 1982;218:571-3.
- 5 Shaw GM, Gonda MA, Flickinger GH, Hahn BH, Gallo RC, Wong-Staal F. Genomes of evolutionarily divergent members of the human T-cell leukemia virus family (HTLV-I and HTLV-II) are highly conserved, especially in pX. *Proc Natl Acad Sci USA* 1984;81:4544-8.
- 6 Vernant JC, Buisson G, Magdelaine J, de Thore J, Jouannelle A, Neisson-Vernant C, et al. T-lymphocyte alveolitis, tropical spastic paraparesis and Sjögren's syndrome. *Lancet* 1988;ii:177.
- 7 Lagrenade L, Hanchard B, Fletcher V, Cranston B, Blattner W. Infective dermatitis of Jamaican children: a marker for HTLV-I infection. *Lancet* 1990;336:1345-7.
- 8 Morgan OSC, Rodgers-Johnson P, Mora C, Char G. HTLV-I and polymyositis in Jamaica. *Lancet* 1989;ii:1184-7.
- 9 Sugimoto M, Nakashima H, Watanabe S, Uyama E, Tanaka F, Ando M, et al. T-lymphocyte alveolitis in HTLV-I associated myelopathy. *Lancet* 1987;ii:1220.
- 10 Yamaguchi K, Mochizuki M, Watanabe T, Miyata N, Tajima K, Mori S, et al. Human T-lymphotropic virus type I uveitis. *Leukemia* 1994;8(Suppl 1):S88-90.
- 11 Blattner WA, Gibbs WN, Saxinger C, Robert-Guroff M, Clark J, Lofters W, et al. Human T-cell leukemic/lymphoma virus-associated lymphoreticular neoplasia in Jamaica. *Lancet* 1983;ii:61-4.
- 12 Duggan DB, Ehrlich GD, Davey FP, Kwok S, Sninsky J, Goldberg J, et al. HTLV-I-induced lymphoma mimicking Hodgkin's disease. Diagnosis by polymerase chain reaction amplification of specific HTLV-I sequences in tumour DNA. *Blood* 1988;71:1027-32.
- 13 Franchini G, Mann DL, Popovic M, Zicht RR, Gallo RC, Wong-Staal F. HTLV-I infection of T and B cells of a patient with adult T-cell leukemia-lymphoma (ATLL) and transmission of HTLV-I from B cells to normal T cells. *Leuk Res* 1985;9:1305-14.
- 14 Longo DL, Gelmann EP, Cossman J, Young RA, Gallo RC, O'Brien SJ, et al. Isolation of HTLV-transformed B-lymphocyte clone from a patient with HTLV-associated adult T-cell leukemia. *Nature* 1984;310:505-6.
- 15 Nagy K, Clapham P, Cheingson R, Popov, Weiss RA. Human T-cell leukaemia virus type I: induction of syncytia and inhibition by patients' sera. *Int J Cancer* 1983;32:321-8.
- 16 Dittmer J, Gitlin SD, Reid RL, Brady JN. Transactivation of the P2 promoter of parathyroid hormone-related protein by human T-cell lymphotropic virus type I Tax 1: evidence for the involvement of transcription factors Ets 1. *J Virol* 1993;67:6087-95.
- 17 Wano Y, Feinberg M, Hosking JB, Bogerd H, Greene WC. Stable expression of the Tax gene of type I human T-cell leukemia virus in human T cells activates specific cellular genes involved in growth. *Proc Natl Acad Sci USA* 1988;85:9733-7.
- 18 Inoue J-I, Seiki M, Taniguchi T, Tsuru S, Yoshida M. Induction of interleukin 2 receptor gene expression by p40^x encoded by human T-cell leukemia virus type 1. *EMBO J* 1986;5:2883-8.
- 19 Grassmann R, Dengler C, Muller-Fleckenstein I, Fleckenstein B, McGuire K, Dokhalar M-C, et al. Transformation to continuous growth of primary human T lymphocytes by human T-cell leukemia virus type I X-region genes transduced by a Herpesvirus saimiri vector. *Proc Natl Acad Sci USA* 1989;86:3351-5.
- 20 Koyanagi Y, Itoyama Y, Nakamura N, Takamatsu K, Kira J, Iwamasa T, et al. In vivo infection of human T-cell leukemia virus type I in non-T-cells. *Virology* 1993;196:25-33.
- 21 Fuji M, Tsuchiya H, Chuhjo T, Ninamino T, Miyamoto K-I, Seiki M. Serum response factor has functional roles both in indirect binding to the CARG box and in the transcriptional activation function of Human T-cell Leukemia Virus Type I Tax. *J Virol* 1994;68:7275-83.
- 22 Gitlin SD, Dittmer J, Shin RC, Brady JN. Transcriptional activation of the human T-lymphotropic virus type I long terminal repeat by functional interaction of Tax 1 and Ets 1. *J Virol* 1993;67:7307-16.
- 23 Suzuki T, Hirai H, Murakami T, Yoshida M. Tax protein of HTLV-I destabilises the complexes of NF-kappa B and I kappa B-alpha and induces nuclear translocation of NF-kappa B for transcriptional activation. *Oncogene* 1995;10:1199-207.
- 24 Furukawa Y, Osame M, Kubota R, Tara M, Yoshida M. Human T-cell leukemia virus type-1 (HTLV-I) Tax is expressed at the same level in infected cells of HTLV-I-associated myelopathy or tropical spastic paraparesis patients as in asymptomatic carriers but at lower level in adult T-cell leukemia cells. *Blood* 1995;85:1867-70.
- 25 Dalglish A, Richardson J, Matutes E, Cruickshank K, Newell A, Sinclair A, et al. HTLV-I infection in tropical spastic paraparesis: lymphocyte culture and serologic response. *AIDS Res Hum Retroviruses* 1988;4:475-85.
- 26 Ehrlich GD, Glaser JB, Abbott MA, Slamon DJ, Keith D, Sliwkowski M, et al. Detection of anti-HTLV-I Tax antibodies in HTLV-I enzyme-linked immunosorbent assay-negative individuals. *Blood* 1989;74:1066-72.
- 27 Franchini G, Wong-Staal F, Gallo RC. Human T cell leukemia virus (HTLV-I) transcripts in fresh and cultured cells of patients with adult T cell leukemia. *Proc Natl Acad Sci USA* 1984;81:6207-11.
- 28 Korber B, Okayama A, Donnelly R, Tachibana N, Essex M. Polymerase chain reaction analysis of defective human T-cell leukemia virus type I proviral genome in leukemic cells of patients with adult T-cell leukemia. *J Virol* 1991;65:5471-6.
- 29 Setoyama M, Fujiyoshi T, Mizoguchi S, Katahira Y, Yashiki S, Tara M, et al. HTLV-I messenger RNA is expressed in vivo adult T-cell leukemic/lymphoma patients: an in situ hybridisation study. *Int J Cancer* 1994;57:760-4.
- 30 Cardoso EA, Robert-Guroff M, Franchini G, Gartner S, Moura-Nunes JF, Gallo RC, et al. Seroprevalence of HTLV-I in Portugal and evidence of double retrovirus infection of a healthy donor. *Int J Cancer* 1989;43:195-200.
- 31 Sá J, Coelho MH, Cardoso EA, Terrinha AM, Moura-Nunes JF. Mielopatia tropical associada a HTLV-I. *Revista Portuguesa Doenças Infecciosas* 1991;14:51-5.
- 32 Sambrook J, Fritsch EF, Maniatis T. In: *Molecular cloning: a laboratory manual*. 2nd edn. Cold Spring Harbour: Cold Spring Harbour Laboratory Press, 1989.
- 33 Mann DL, DeSantis P, Mark G, Pfeifer A, Newman M, Gibbs N, et al. HTLV-I associated B-cell CLL: indirect role for retrovirus in leukemogenesis. *Science* 1987;236:1103-6.
- 34 Ensoli B, Barillari G, Salahuddin SZ, Gallo RC, Wong-Staal F. Tat protein of HIV-1 stimulates growth of cells derived from Kaposi's sarcoma lesions of AIDS patients. *Nature* 1990;345:84-6.
- 35 Banki K, Maceda J, Hurley E, Ablonczy E, Mattson DH, Szegedy L, et al. Human T-cell lymphotropic virus (HTLV)-related endogenous sequence, HRES-1 encodes a 28-Kd protein: a possible autoantigen for HTLV-I gag reactive autoantibodies. *Proc Natl Acad Sci USA* 1992;89:1939-43.
- 36 Garin B, Gosselin S, de The G, Gessain A. HTLV-I/II infection in a high viral endemic area of Zaire, Central Africa: comparative evaluation of serology, PCR, and significance of indeterminate western blot pattern. *J Med Virol* 1994;44:104-9.