PCR Analysis of DNA from Multiple Sclerosis Patients for the Presence of HTLV-I

In 1985, Gessain *et al.* (*1*) showed that human T lymphotropic virus type 1 (HTLV-I) is associated serologically with tropical spastic paraparesis (TSP), a chronic inflammatory disease of the spinal cord that causes progressive paralysis of the legs. The possibility that multiple sclerosis (MS), which in some cases resembles TSP, is associated with infection by HTLV-I or another retrovirus, has also generated considerable interest. The initial evidence from serology and in situ hybridization (*2*) was disputed (*3*). Recently, Reddy *et al.* (*4*) and Greenberg *et al.* (*5*) have reported the amplification by the polymerase chain reaction (PCR) of HTLV-I (*4*) or a related retrovirus (*5*) from the peripheral blood leukocyte (PBL) DNA of a proportion of patients with MS.

We sequenced 172 HTLV-I partial *env* clones obtained by PCR of PBL from ten TSP patients in the United Kingdom of Caribbean origin and two HTLV-I–seropositive patients with adult T cell leukemia (ATL). The results (*6*) showed that no two individuals had an identical sequence of *env*. The frequency of nucleotide changes we observed greatly exceeded that expected from errors made by *Taq* polymerase with the concentrations of Mg^{2+} and dNTPs used (*7*).

We next studied nine white Caucasian adults with clinically definite MS (*8*), six from a routine MS clinic in Canada and three from a rehabilitation clinic in Oxford, United Kingdom. Using HTLV-I *env*-specific primers that consistently amplify HTLV-I from seropositive patients, we amplified a band of the expected size only from the HTLV-I–seropositive leukemic control (Fig. 1). Other primers, from *pol* or other regions of *env*, also failed to amplify MS DNA (*9*).

Reddy *et al.* (*4*) found an identical *env* sequence in all six MS patients between nucleotides 5684 and 6151. However, this is surprising, because this region, as elsewhere in *env*, varies between individual patients' clones at both the nucleotide and amino acid levels, particularly at the 3′ end of the region.

Our results therefore conflict with those of Reddy *et al.* on two counts. First, we cannot reproduce the finding of PCR amplification of HTLV-I from MS patients' DNA. Second, although it is possible that a single sequence variant of HTLV-I is associated with MS, we consider this unlikely because no two patients in the present study had identical *env* sequences.

PCR amplification has two major drawbacks in attempts to isolate low copy number or novel retroviruses. First, its exquisite sensitivity is such that DNA contamination of reaction mixtures is a frequent problem (*10*). Second, PCR readily detects cross-reactive endogenous retroviral sequences (*7*). Our results do not exclude the possibility that a retrovirus is involved in MS, but they suggest that such a virus is unlikely to be HTLV-I.

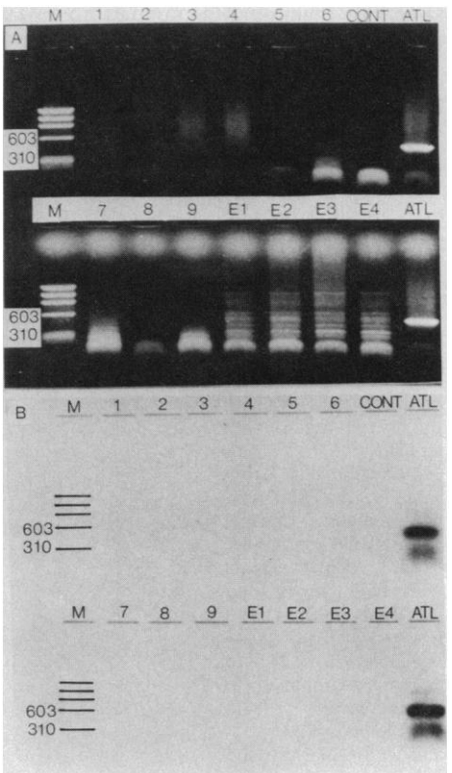

Fig. 1. Amplification products from nine MS and four normal genomic DNA samples are shown after 35 cycles of PCR with *env*-specific primers 6218 to 6237 and 6656 to 6675 (*11*). (**A**) Ethidium bromide–stained 1% agarose gel. (**B**) Autoradiograph of Southern transfer from gel, hybridized to the internal oligoprobes 6246 to 6265. Lanes 1 to 9 are MS samples; CONT is PCR reaction mixture without template DNA; E1 to E4 are normal DNA controls. The ATL is a positive control from which HTLV-I *env* and long terminal repeat sequences have been cloned. The integrity of the DNA samples was demonstrated by amplification with HLA-DR-beta–specific primers (not shown).

CHARLES R. M. BANGHAM
Institute for Molecular Medicine, John Radcliffe Hospital, Oxford OX3 9DU, United Kingdom
SIMON NIGHTINGALE
Queen Elizabeth II Hospital, Edgbaston, Birmingham B15 2TH, United Kingdom
J. K. CRUICKSHANK
Northwick Park Hospital, Middlesex HA1 3UJ, United Kingdom
SUSAN DAENKE
Institute for Molecular Medicine, John Radcliffe Hospital, Oxford OX3 9DU, United Kingdom

REFERENCES AND NOTES

1. A. Gessain *et al.*, *Lancet* **ii**, 407 (1985).
2. H. Koprowski *et al.*, *Nature* **318**, 154 (1985); M. Ohta, K. Ohta, F. Mori, H. Nishitani, T. Saida, *J. Immunol.* **137**, 3440 (1986).
3. S. L. Hauser *et al.*, *Nature* **322**, 176 (1986); A. Karpas, U. Kampf, A. Siden, M. Koch, S. Poser, *ibid.*, p. 177.
4. E. P. Reddy *et al.*, *Science* **243**, 529 (1989).
5. S. J. Greenberg *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **86**, 2878 (1989).
6. S. Daenke, S. Nightingale, J. K. Cruickshank, C. R. M. Bangham, in preparation.
7. C. R. M. Bangham *et al.*, *EMBO J.* **7**, 4179 (1988); A. Shih, R. Misra, M. G. Rush, *J. Virol.* **63**, 64 (1989).
8. C. M. Poser *et al.*, *Ann. Neurol.* **13**, 227 (1983).
9. Data not shown.
10. Y-M. D. Lo, W. Z. Mehal, K. A. Fleming, *Lancet* **ii**, 679 (1988).
11. Amplified DNA was blotted on to a nitrocellulose membrane (Hybond C, Amersham International) in 20× SSC (3*M* NaCl, 0.3*M* trisodium citrate). The membrane was baked at 80°C for 2 hours and hybridized to an oligoprobe end-labeled with ^{32}P-dATP (Amersham International). Two to five nanograms of labeled probe were hybridized to the membrane at 37°C for 1 hour in a buffer containing 0.9*M* NaCl, 90 m*M* tris-HCl (*p*H 8.0), 6 m*M* EDTA, 0.5% (w/v) NP40, 10% (w/v) dextran sulfate, and 100 μg of salmon sperm DNA per milliliter (Sigma). The membrane was washed in 6× SSC at 40°C (2 × 2 min) before exposure to Fuji RX film at −70°C for 2 to 6 hours.
12. We thank J. I. Bell for constant help and advice, M. Yoshida for providing HTLV-I–containing plasmids, and the following for providing blood samples: D. Catovsky, J. K. Cruickshank, G. C. Ebers, S. Nightingale, W. Rosenberg, and D. Wade. C.R.M.B. and S.D. are supported by the Wellcome Trust.

28 July 1989; accepted 15 September 1989

A number of reports have suggested a link between HTLV-I–like retroviruses and multiple sclerosis (MS) (*1–3*). The association of HTLV-I with another chronic neurologic disease, tropical spastic paraparesis (TSP) (*4*), which superficially resembles MS, has added to the speculation regarding retroviruses and MS. The association of a T cell tropic virus with an autoimmune disease such as MS is particularly inviting because of studies indicating some nonspecific defect in T cell immune function in the disease (*5*).

We used the technique of polymerase chain reaction (PCR) to examine mononu-

clear cell (MNC) populations derived from subjects with well-defined MS in geographically separate locals. We performed gene amplification with HTLV-I *gag*, *pol*, and *env* primers that had been previously reported to amplify DNA sequences in MNCs from subjects with MS and also with primers deduced from HTLV-I *gag* and X regions. We used care to prevent cross contamination of viral sequences between samples (*6*) and to select subjects with well-defined cases of both the chronic progressive (CP) and the relapsing remitting forms of MS.

A total of 47 patients with MS (14 from London and 33 from Boston), of whom 15 had CP disease, participated in this study. The average age of the patients was 41 years (ranging from 18 to 72 years); 46 were Caucasian and one was of Afro-Caribbean extraction with parents born in the West Indies. All patients were judged to have clinically definite MS, as evidenced by more than one lesion separated in time of appearance and location in the central nervous system (CNS) (*7*) and by a positive magnetic resonance scan of the brain. These included patients with progressive spastic paraparesis who also had unambiguous lesions in either the brain stem or the optic nerves. In addition, 11 normal individuals and 16 TSP patients with a 5- to 30-year history of chronic spastic paraparesis were tested. None of these patients had clinical signs of brain stem or optic nerve involvement of the CNS. All 16 TSP patients had HTLV-I–reactive antibodies in their sera, as shown by immunofluorescence, ELISA, or immunoblot assays (*8*), while the test results of all the MS patients tested were negative.

We prepared DNA from peripheral blood MNCs and amplified each sample for 30 to 35 cycles using at least three of the six different sets of primers (*9*). In addition to primer sequences described by Reddy *et al.* (*2*) (III and VI) (*9*) and by Greenberg *et al.* (*3*) (IV and V) (*9*), two sets of primers were used which correspond to the *gag* and X region of the HTLV-I proviral genome (I and II) (*9*). Patients from the United Kingdom were tested with *gag* primers I and III and with X-region primers (II) (*9, 10*), while patients from the United States were tested with *gag*, *env*, and *pol* region primers (III–VI). As shown in a representative Southern blot (Fig. 1), MNCs derived from the blood of subjects with TSP gave strong positive signals after PCR, while all of the 47 samples derived from the blood of MS patients were negative when we used the primers outlined above.

The negative reactivity of blood samples from patients with MS may have been due to a lack of sensitivity in the PCR technique. This appears unlikely, however, for two reasons. First, the amplification efficiency of the primers was carefully examined. Using the X-region primers, we detected five copies of HTLV-I diluted in 0.5 μg of DNA (Table 1), and high dilutions of samples from TSP patients gave positive signals with *pol*(IV) and *env*(V) primers (1:10,000 with the *pol* primers and 1:1,000 with the *env* primers, which is equivalent to 10 and 100 peripheral blood MNCs, respectively). We tested the sensitivity of the technique, at the same time examining the samples from MS patients.

Table 1. Primer sensitivity. Primers were tested against genomic DNA from the human T cell line C81-66 (C63/CR_{II}-4) (*11*), which contains approximately five integrated copies of HTLV-I per diploid genome. C81-66 was serially diluted in DNA from uninfected cells, and 0.5-μg samples containing from 10^5 to 0.1 C81-66 genomes were tested.

Primers	C81-66 genomes per sample						
	10^5	10^4	10^3	10^2	10	1	0.1
X as in (*9*)	+	+	+	+	+	+/−	−
Gag as in (*9*)	+	+	+	+/−*	+/−	−	−
Gag as in (*2*)	+	+	+/−	−	−	−	−

*+/− not consistently positive.

Second, two DNA samples were kindly provided by S. Greenberg (National Institutes of Health, Bethesda, Maryland) from patients reported to have definite MS and whom Greenberg and his colleagues had determined to be HTLV-I–positive (*3*). One sample gave strongly positive signals with both the *pol*- and *env*-region primers similar in intensity to those observed in samples from TSP patients, while the second sample was positive for the *pol* region only. Together, these results indicate that lack of sensitivity was not the reason for the negative results in our subjects with MS.

Reddy *et al.* (*2*) indicated, using PCR, that adherent peripheral MNCs were positive for HTLV-I sequences, while nonadherent MNCs were not. In 11 of the patients we examined, MNCs were separated into T cell and non–T cell populations by E rosetting and were stimulated with phytohemagglutinin and lipopolysaccharide, respectively, for 3 days before DNA extraction and PCR analysis. In all 11 patients, resting and activated cell populations were found to be negative for HTLV-I sequences.

In summary, our results fail to demonstrate the presence of HTLV-I DNA in peripheral blood MNCs from a series of patients with definite MS. The amplification of *pol* and *env* sequences from MS patients'

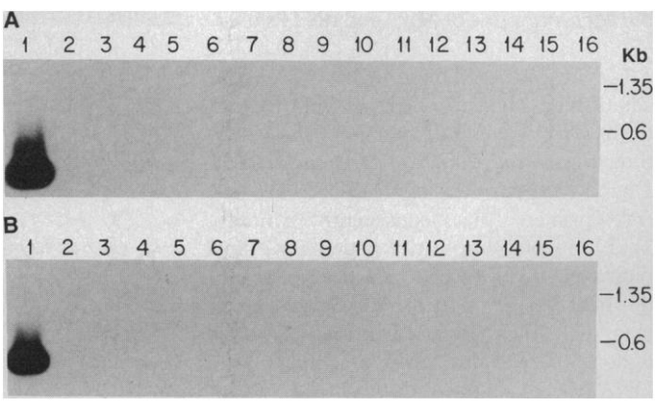

Fig. 1. Representative Southern blots showing *pol* and *env* region gene amplification from MNCs derived from subjects with TSP and MS and from normal controls. DNA samples from T cells of patients with TSP (lane 1) and MS (lanes 2 to 11) and from normal control individuals (lanes 12 to 16) were amplified in the presence of *pol* and *env* region primers (IV and V) for 30 cycles and hybridized with *pol* (**A**) and *env* (**B**) probes. MNCs were obtained by Ficoll density gradient centrifugation and DNA was extracted by cell lysis in a hypotonic buffer (0.2× phosphate-buffered saline) at a cell concentration of 10×10^6 per milliliter and heated to 95°C for 10 min. Proteinase K was added to a final concentration of 0.4 mg/ml, and samples were incubated at 55°C for 30 min, followed by an additional incubation at 95°C for 10 min. Ten microliters of this DNA preparation (equivalent to 100,000 cells) were amplified in a 50-μl reaction for 30 cycles (94°C for 1 min, 55°C for 2 min, 72°C for 3 min) with the use of 1 μg of each primer and 2.5 units of *Taq* polymerase. Serial dilutions of samples from patients with TSP resulted in positive signals with 1:10,000 dilutions of DNA samples and amplification with *pol*-region primers. Twenty microliters of the reaction were separated on 1% agarose gels, transferred to nitrocellulose, and hybridized to ^{32}P-labeled oligonucleotide probes (specific activity of 5×10^8 dpm/μg) in 6× SSC, 0.05% pyrophosphate, 5× Denhardt's solution, 0.5% SDS, denatured salmon sperm DNA (0.1 mg/ml), and labeled probe (2 ng/ml) at 37°C for 18 hours. For probes IV and V, filters were washed at a final stringency of 6× SSC/70°C; for probes III and VI, filters were washed at a final stringency of 6× SSC/55°C and autoradiographed for 2 to 48 hours. Samples from patients with TSP consistently gave strong signals after 2-hour exposures.

MNCs reported by other investigators (*2–3*) suggests there might be a subgroup of MS patients with HTLV-I–like sequences in MNCs. Such sequences may also occur in subjects without MS, and the significance of the observation will require the investigation of larger numbers of MS patients and control subjects. Our studies do not exclude the presence of a distantly related retrovirus or some other infectious agent that cannot be detected with the six primer pairs we used. However, it appears that HTLV-I proviral genome is not commonly found in patients with definite MS.

JENNIFER H. RICHARDSON
Retrovirus Research Group, Clinical Research Center, Harrow, United Kingdom
KAI W. WUCHERPFENNIG
N. ENDO
Center for Neurologic Diseases, Brigham and Women's Hospital, and Division of Neurology, Department of Medicine, Harvard Medical School, Boston, MA 02115
PETER RUDGE
National Hospital for Nervous Diseases, London, United Kingdom
ANGUS G. DALGLEISH
Retrovirus Research Group, Clinical Research Center, Harrow, United Kingdom
DAVID A. HAFLER
Center for Neurologic Diseases, Brigham and Women's Hospital and Division of Neurology, Department of Medicine, Harvard Medical School, Boston, MA 02115

REFERENCES AND NOTES

1. H. Koprowski *et al.*, *Nature* **318**, 154 (1985).
2. E. P. Reddy *et al.*, *Science* **243**, 529 (1989).
3. S. J. Greenberg *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **86**, 2878 (1989).
4. A. Gessain *et al.*, *Lancet* **ii**, 407 (1985); S. Jacobsen, C. S. Raine, E. S. Mingioli, D. E. McFarlin, *Nature* **331**, 540 (1988); M. Osame *et al.*, *Ann. Neurol.* **21**, 117 (1987); M. Newton *et al.*, *Lancet* **ii**, 415 (1987).
5. D. A. Hafler and H. L. Weiner, *Immunol. Rev.* **100**, 307 (1987).
6. S. Kwok and R. Higushi, *Nature* **339**, 237 (1989).
7. C. M. Poser, *Ann. Neurol.* **13**, 227 (1983).
8. A. Dalgleish *et al.*, *AIDS Res. Hum. Retrovir.* **4**, 475 (1988).
9. Primers used for PCR amplification were as follows. (i) *Gag* primers [nucleotides (nt) 1795 to 2030]: 5′-GTCAGACCTGGACCCCCAAAGA-3′, 3′-GTCTCGGTCTCCTTCTACGGGAG-5′; probe: 5′-CCCCAAATCAGCCGTGCTTC-3′; (ii) X-region primers (nt 6824 to nt 7066): 5′-CCTTCTCAGCCCCTTGTCTCCAC-3′, 3′-GCGACGGCTAGTGCTACGCAAAG-5′; probe: 5′-TACTCAGCGGTCTGCTTTTCC-3′; (iii) *gag* region primers (nt 842 to nt 1376) (*2, 12*); (iv) *pol* region primers (nt 2801 to nt 3018) (*3, 12*); (v) *env* region primers (nt 5799 to nt 6106) (*3, 12*); (vi) *env* region primers (nt 5684 to nt 6151 (*2, 12*).
10. DNA was prepared from Ficoll-Paque separated peripheral blood MNCs. PCR reactions were done in a 50-μl volume with 1 μg of DNA, 1 unit of *Taq* polymerase (Cetus Corporation, Norwalk, Connecticut), and 0.5 μg of each primer. The reaction buffer was 50 m*M* KCl, 1.5 m*M* $MgCl_2$; 200 μ*M* of each dNTP, 10 m*M* tris-HCl, *p*H 8.3; and 0.01% gelatin. Samples were denatured for 10 min at 95°C and then given 35 cycles of amplification (93°C for 0.1 min, 60°C for 0.1 min, and 72°C for 0.5 min). Ten microliters of the reaction products were separated by electrophoresis in 2.5% agarose and transferred to Zetaprobe membrane (Bio-Rad) in 0.4*M* NaOH. Filters were incubated for 1 hour at 50°C in 6× SSC, 1% SDS, and 0.5% dried skimmed milk, and then for 2 hours in the same solution containing 5 ng/ml oligonucleotide probe end-labeled with ^{32}P and T4 kinase to a specific activity of 5×10^8 dpm/μg/min. Filters were washed in 6× SSC/1% SDS for 10 min at room temperature and then for 10 min at 50°C and autoradiographed for 0.5 to 48 hours at −70°C.
11. S. Z. Salahuddin *et al.*, *Virology* **129**, 51 (1983).
12. M. Seiki, S. Hattori, Y. Hirayama, M. Yoshida, *Proc. Natl. Acad. Sci. U.S.A.* **80**, 3618 (1983).
13. Supported by a grant from the Multiple Sclerosis Society of Great Britain (J.H.R. and A.G.D.), and National Institutes of Health grants R01 NS 24247 and K08 NS 00981 (D.A.H.). K.W.W. is the Susan Furbacher Conroy Fellow in Multiple Sclerosis Research at the Center for Neurologic Diseases. D. A. H. is a Harry Weaver Scholar of the National Multiple Sclerosis Society.

28 July 1989; accepted 15 September 1989

Response: One argument that Bangham *et al.* make in their comment is that we (*1*) have found an identical *env* sequence between nucleotides 5684 and 6151 in blood samples from all the six MS patients and that this is virtually impossible because, in their opinion, squences of this region *must* vary between individual isolates. In support of their contention, they quote their unpublished data of sequence analysis of polymerase chain reaction (PCR)–amplified material, which I have not had an opportunity to examine. This argument reflects the opinion of Bangham *et al.* and is not supported by the published data on sequence analysis of several cloned proviral HTLV-I genes. In support of our argument that a majority of HTLV-I proviruses have identical sequences in the region we amplified, Fig. 1 (*2*) shows a comparison of the published sequences of three different isolates of HTLV-I. One of the sequences is that of ATK-1 isolated from a patient with adult T cell leukemia (ATL) in Japan in 1982 (*3*). The MT-2 isolate is also from a patient with ATL (*4*). We determined the sequence of the *env* region of this clone. The third isolate, recently isolated and sequenced by Tsujimoto *et al.* (*5*), is from a patient with HAM (HTLV-I–associated myelopathy). The sequence of the *env* region that we have used for amplification is identical in these three isolates, which were obtained at different times and from different disease phenotypes. In addition, we have recently cloned and sequenced the HTLV-I proviral genome from the peripheral lymphocytes of a TSP patient of Caribbean origin (*6*). The *env* region is unaltered in this clone also (*7*). (In fact the sequence of the *env* region amplified from the six MS patients differs from that of these four different isolates from ATL, HAM, and TSP patients). If the argument of Bangham *et al.* were correct, all these published sequences of HTLV-I are incorrect, which is unlikely.

Bangham *et al.* state they cannot find amplification of HTLV-I sequences in the nine MS patients they studied. Richardson *et al.* report that they examined the DNA of several MS patients for the presence of HTLV-I sequences, including those of two DNAs originally found to be positive by Greenberg *et al.* (*8*) with the use of PCR assay; they report that they cannot find amplification of HTLV-I sequences in the MS patients they studied, but they do find

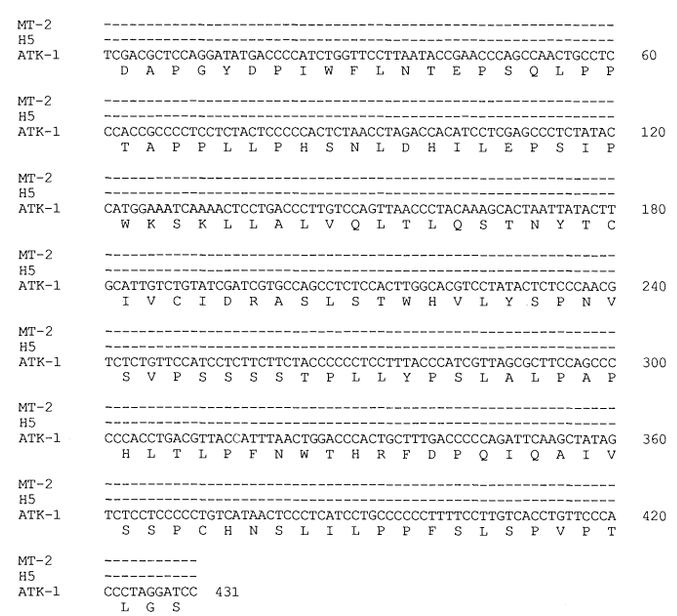

Fig. 1. Comparison of nucelotide sequence of three different HTLV-I isolates. The sequence is from the *env* region that has been used for amplification of DNA from MS patients (*2*).