Failure to demonstrate human T cell lymphotropic virus type I in multiple sclerosis patients

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The polymerase chain reaction (PCR) technique was employed in searching for human T cell lymphotropic virus type I (HTLV-I) gag, env and pol sequences in samples of DNA prepared from two HTLV-I seropositive patients with tropical spastic paraparesis (TSP), the Swedish multiple sclerosis (MS) patients who recently have been reported to be PCR-positive for HTLV-I gag and env sequences, and eight healthy individuals. Precautions were taken in order to reduce the risk of cross-contamination in the PCR. In the two TSP patients strong signals were obtained with gag, env and pol amplification primers and detection probes. In MS patients and healthy individuals, no signals were obtained with gag and env. In occasional experiments, weak signals were seen for the pol segment for a single MS patient and/or healthy individuals, but these signals were not reproducible in subsequent experiments. Thus, the present data do not confirm the presence of HTLV-I sequences in MS patients.

Introduction

Multiple sclerosis (MS) is a demyelinating disease of the central nervous system. The aetiology remains unknown. A possible relationship between a retrovirus related to human T cell lymphotropic virus type I (HTLV-I) and MS has been suggested on the basis of serology and in situ hybridization studies (Koprowski et al., 1985; Ohta et al., 1986), but other studies have failed to confirm this relationship (Hauser et al., 1986; Karpas et al., 1986).

In three recent studies, the DNA in vitro amplification technique, polymerase chain reaction (PCR), has been employed in searching for retrovirus-like agents in MS. Reddy et al. (1989) amplified HTLV-I gag and env sequences from peripheral blood mononuclear cells (PBMNCs) from six Swedish MS patients and could not amplify similar sequences in all but one of 20 healthy individuals. The viral sequences in MS patients were associated with adherent cell populations consisting predominantly of monocytes and macrophages. Only two of the six MS patients had antibodies against disrupted HTLV-I virions in their serum samples, thus indicating lack of correlation between the presence of antibodies reacting with disrupted HTLV-I virions and the presence of HTLV-I sequences. Greenberg et al. (1989) analysed PBMNCs from 21 MS patients and 35 blood donors for sequences homologous to HTLV-I long terminal repeat, gag, env and pol domains using PCR. From six of the 21 MS patients, pol sequences were identified. Three of these six MS patients also tested positive for the env region. Neither gag nor LTR sequences were identified in any MS patient. None of the blood donor samples displayed any HTLV-I-like sequence. All MS patients were uniformly seronegative for antibodies to HTLV-I by ELISA. Most recently, Bangham et al. (1989) have reported failure to amplify HTLV-I env sequences from PBMNCs from any of nine MS patients or controls.

Thus, the attempts to demonstrate a role for HTLV-I or an HTLV-I-like agent in MS using methods from molecular biology have been as conflicting as previous serological studies.

When initiating studies of the possible involvement of HTLV-I in MS, we decided first to use oligonucleotide primers and probes identical to those employed by Reddy et al. (1989) to investigate new samples of DNA prepared from the same Swedish MS patients previously reported by Reddy et al. (1989) to be positive for HTLV-I gag and env sequences. Here, we report the failure to reproduce the detection of HTLV-I gag and env sequences in the six MS patients and the controls using the PCR technique.

Methods

Subjects. Six Swedish MS patients (three males and three females), from the Department of Neurology, University of Lund, Sweden were selected for the study. The MS diagnosis was defined according to
Table 1. Oligonucleotide primers and probes

<table>
<thead>
<tr>
<th>Oligomer</th>
<th>Function</th>
<th>Sequence</th>
</tr>
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<tbody>
<tr>
<td>gag-1</td>
<td>Primer</td>
<td>5'- CGA CGG CCC CGG GGG CTG GCC GCT 3'</td>
</tr>
<tr>
<td>gag-2</td>
<td>Primer</td>
<td>5'- GGT ACT GCA GGA GGT CTG GGA GG 3'</td>
</tr>
<tr>
<td>gag-3</td>
<td>Probe</td>
<td>5'- GAT CCC GTC CGG TCC CGC GCC A 3'</td>
</tr>
<tr>
<td>env-1</td>
<td>Primer</td>
<td>5'- CTC CCT TCT AGT CGA CCG TCC AGG 3'</td>
</tr>
<tr>
<td>env-2</td>
<td>Primer</td>
<td>5'- GCC ACC GGT ACC GCT CGG CGG GAG 3'</td>
</tr>
<tr>
<td>env-3</td>
<td>Probe</td>
<td>5'- GCC TCT CCA GGT ACG TCC 3'</td>
</tr>
<tr>
<td>pol-1</td>
<td>Primer</td>
<td>5'- AAC CCA GTA TCC CCA GTT AA 3'</td>
</tr>
<tr>
<td>pol-2</td>
<td>Primer</td>
<td>5'- AGA ATG TCA TCG ATG TAC TG 3'</td>
</tr>
<tr>
<td>pol-3</td>
<td>Probe</td>
<td>5'- ACC CTT GGG GTA GTA CTT T 3'</td>
</tr>
<tr>
<td>HLA-DPB</td>
<td>Primer</td>
<td>5'- CAG GGA TCC GCA GAG AAT TAC 3'</td>
</tr>
<tr>
<td>HLA-DPB</td>
<td>Primer</td>
<td>5'- GTC CTG CAG TCA TCT ACC TCG GCG 3'</td>
</tr>
<tr>
<td>HLA-DPB</td>
<td>Probe</td>
<td>5'- TTC GAC AGC GAC GTG 3'</td>
</tr>
</tbody>
</table>

DNA extraction. PBMCs were isolated by density gradient centrifugation (Boyum, 1968), cultured overnight at 37°C in tissue culture flasks containing 10% human AB serum and G-CSF at 25 units/ml (AMGen Biologicals) (final concentration), separated into adherent and non-adherent cells and processed for DNA extraction. All DNA handling was done while wearing disposable gloves in order to reduce the risk of cross-contamination. Genomic DNA was extracted by either freezing and boiling (Lench et al., 1988), salting out (Miller et al., 1988), or phenol–chloroform (Böhme et al., 1983). Peripheral blood was drawn twice from six MS patients in 1989 with a 4 month interval, and once from healthy individuals. From the first blood samples from the MS patients and the only blood samples from the Swedish control individuals, DNA was extracted from both adherent and non-adherent cells using the freeze–boil method and the salting out method. From the second blood samples from the MS patients, DNA was extracted from both adherent and non-adherent cells using the phenol–chloroform procedure. From the four Danish healthy individuals and the two TSP patients, DNA was extracted from unseparated PBMC using the phenol–chloroform procedure.

PCR technique. In order to test the sensitivity of our PCR technique, TSP DNA was serially diluted (1:10, 1:100, 1:1000, 1:10000, and 1:100000) with either water or DNA from a healthy individual negative for the presence of HTLV-I sequences. The extracted DNA was amplified by PCR with four different pairs of oligonucleotide primers as shown in Table 1. Each sample was tested at least twice for the presence of gag, env and pol sequences. In order to reduce the risk of cross-contamination, handling and amplification of DNA took place in separate laboratories, while handling and amplification of DNA from TSP patients and of DNA from MS patients and healthy controls took place in two separate buildings using two different thermocyclers. However, in the final experiments, MS and TSP DNA were amplified simultaneously on the same thermocycler to exclude differences between the instruments. Amplifications took place in 100 µl reaction mixtures containing 1 µg genomic DNA in 50 mM KCl, 10 mM Tris–HCl pH 8.4, 1.5 mM MgCl₂, each primer at 1 µM, each dNTP (dATP, dCTP, dTTP, dGTP) at 200 µM, gelatine at 200 µg/ml and 4 units of Taq DNA polymerase (Perkin Elmer Cetus). In separate experiments, the MgCl₂ concentration was varied (1.5, 3 and 5 mM) and in separate experiments new Taq DNA polymerase was added at 2.5 units every tenth cycle. The samples were overlaid with 100 µl of mineral oil to prevent condensation. Samples were subjected to a total of 35, 40 or 45 cycles of amplification in a programmable heat block (Perkin Elmer Cetus). The individual cycles consisted of 2 min at 94°C (DNA denaturing), 2 min at 55°C (primer annealing), and 2 min at 72°C (primer extension). After the last cycle, all samples were incubated for an additional 7 min at 72°C to ensure that the final extension step was complete. Samples were subsequently cooled to 4°C and stored at this temperature for later manipulations (Saiki et al., 1988).

Southern blotting. One µl of the amplified DNA was suspended in one volume of 0.4 M NaOH and spotted manually on Zeta-Probe nylon membrane (Bio-Rad) pre-wetted in distilled water. The DNA was fixed by alkali to the membrane according to the instructions of the manufacturer. Membranes were prehybridized for at least 2 h at 45°C in 5x SSC (1x SSC is 0.15 M sodium chloride, 0.015 M trisodium citrate pH 7.0), 20 µM-sodium phosphate pH 7.0, 10 x Denhardt’s solution, 7% SDS and 100 µg/ml heat-denatured salmon sperm DNA. Hybridization was done overnight in the prehybridization solution supplemented with 50% dextran sulphate to a final concentration of 10% and approximately 5 ng/ml 32p-labelled oligonucleotide probe. The membranes were washed for 1 h at 45°C in 3x SSC, 10 mM-sodium phosphate pH 7.0, 10 x Denhardt’s solution, and 5% SDS, and for 1 h at 50°C in 1x SSC and 1% SDS. Autoradiography was done on X-Omat AR film (Kodak) with an intensifying screen for 2 to 24 h. Southern blotting was done as recently described (Fugger et al., 1989). Briefly, after gel electrophoresis, the amplified DNA was transferred onto Hybond-N nitrocellulose filters (Amersham). The filters were hybridized for 48 h at 42°C in plastic bags with a total volume of 20 ml of 50% (v/v) formamide, 5x sodium chloride–sodium phosphate–EDTA (SSPE) (1x SSPE is 180 mM-sodium chloride, 10 mM-sodium phosphate, 1 mM-EDTA), 0.2% Ficoll, 0.2% polyvinylpyrrolidone, 0.2% bovine serum albumin, 5% (w/v) dextran sulphate, and the radiolabelled probe (10 ng/ml). After hybridization, the filters were washed twice for 5 min each in 2x SSPE and 0.5% SDS at room temperature and twice for 30 min each in 0.2 x SSPE and 0.5% SDS at 65°C.

Oligonucleotide primers and probes. Oligonucleotides were synthesized on a Biosearch model 8600 DNA synthesizer. The design of amplification primers and detection oligonucleotides have previously been reported (see Table 1).
Results

The aim of this study was to try to detect HTLV-I sequences by PCR in MS patients. Our strategy included investigation of two HTLV-I seropositive TSP patients as positive controls, eight healthy individuals as negative controls, and the same six Swedish MS patients as studied by Reddy et al. (1989). Further, we studied the influence of the cellular source of DNA, of the DNA preparation method and, in separate experiments, of the MgCl₂ concentration in the PCR buffer and of replenishment of Taq DNA polymerase every tenth cycle. To ensure that the DNA samples were amplifiable and that components inhibiting the PCR were absent, HLA-DPB-locus-specific primers served as an internal control.

HLA-DPB sequences were readily amplified from all MS patients and healthy individuals irrespective of the DNA preparation method and PBMNC separation (not shown). Strong signals were obtained from both TSP patients with \textit{gag}, \textit{env} and \textit{pol} amplification primers and detection probes (Fig. 1). The sensitivity of our PCR technique was studied in serial dilution experiments which revealed that HTLV-I sequences could be detected at a dilution of 1:10000, i.e. about 100 pg TSP DNA, both when diluted in water and in non-HTLV-I DNA solution. Neither \textit{gag} nor \textit{env} sequences were identified in any MS patient or healthy individual, independent of the DNA preparation method, PBMNC separation and numbers of amplification cycles (35 to 45). All samples were tested at least twice on different days to exclude day to day variation in the PCR technique. In occasional experiments, weak signals were seen for the \textit{pol} segment for a single patient and/or control, but these signals were not reproducible in subsequent experiments.

Discussion

The possible involvement of HTLV-I in MS has recently attracted great attention because of the detection of HTLV-I or HTLV-I-like sequences in MS patients using PCR (Reddy \textit{et al.}, 1989; Greenberg \textit{et al.}, 1989), although this evidence has been disputed (Bangham \textit{et al.}, 1989). Using PCR, we failed to detect HTLV-I \textit{gag} and \textit{env} sequences in six Swedish MS patients previously reported to be positive for the same sequences (Reddy \textit{et al.}, 1989). However, in occasional experiments, weak signals were seen for the \textit{pol} segment for a single patient and/or control, but these signals were not reproducible in subsequent experiments. It seems most likely that the amplified sequences represent endogenous retroviral sequences (Mager \textit{et al.}, 1987), and not artefactual amplification of irrelevant sequences, because they were near the expected size, i.e. about 390 bp (Bangham \textit{et al.}, 1988). This is supported by the fact that the \textit{pol} primers were designed on the basis of \textit{pol} sequences conserved between diverse retroviruses. Furthermore, primers of identical sequence have recently been shown to amplify endogenous retroviruses (Bangham \textit{et al.}, 1988).

In spite of several attempts to optimize the technical details of the PCR, including conditions identical to those reported by Reddy \textit{et al.} (1989), we never detected HTLV-I \textit{gag} or \textit{env} sequences. Thus, PBMNCs from MS patients and Swedish healthy controls were separated into adherent and non-adherent cells, because Reddy \textit{et al.} (1989) reported that the HTLV-I sequences in the MS patients were associated with adherent cells. In order to
exclude the possibility that the procedure for DNA preparation could influence the PCR results, three different methods were employed for DNA preparation. Further, the MgCl₂ concentration in the PCR reaction mixture, which is a critical parameter for successful amplification (Fugger et al., 1989), was varied. Replenishment of the Taq DNA polymerase during the PCR was employed by Reddy et al. (1989), but did not influence our results.

The discrepancy between the observations of Reddy et al. (1989) and our findings might be due to different levels of sensitivity; however, this seems unlikely because we could readily amplify HTLV-I sequences from 100 pg TSP DNA. Although Reddy et al. (1989) do not state the level of sensitivity of their results, they claim that, in general, analysis of samples from all patients showed readily visible bands after ethidium bromide staining and hybridization with the probe.

Another possibility for the divergent findings might be that the MS patients have cleared their detectable retroviral sequences from the blood compartment. This, however, seems unlikely to have happened for all patients.

The extraordinary sensitivity which allows minute quantities of rare sequences to be selectively amplified shows not only the analytical power of PCR, but also entails a potential risk because it may result in the detection of a few contaminating molecules. The magnitude of this risk has only recently become clear (Lo et al., 1989; Kwok et al., 1989). In this context, it is worth noting the close sequence homology between a previously published sequence of a Japanese HTLV-I isolate (Seiki et al., 1983), used as a positive control by Reddy et al. (1989), and their reported HTLV-I sequences of MS origin. Moreover, the isolates from all six patients were almost identical. This observation of sequence similarity is at variance with a recent study by Bangham et al. (1989) who found sufficient variation in the HTLV-I env gene sequence in 10 TSP patients and two with adult T cell leukaemia to distinguish each patient's isolate uniquely. Owing to the potential risk of contamination, most of our studies of MS patients were done in a laboratory where HTLV-I has never been handled, while TSP patients were studied in a separate laboratory in another building.

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References


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