J Neurol (1990) 237:352–355

Search for retroviral sequences in peripheral blood mononuclear cells and brain tissue of multiple sclerosis patients

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Received June 16, 1990 / Accepted July 4, 1990

Summary. DNAs from peripheral blood mononuclear cells (PBMCs) of 21 patients with multiple sclerosis (MS), 1 patient with tropical spastic paraparesis (TSP) as well as DNAs from brain and spinal cord of 5 MS cases and 3 controls were examined for human T-cell lymphotropic virus (HTLV)-related sequences by polymerase chain reaction. The primers used were derived from the HTLV-I *gag, env* and *tax* genes. Amplified products were separated on agarose gels, blotted onto nylon membranes and hybridized to specific radiolabelled oligonucleotides. The sensitivity of amplification and hybridization was one copy of target DNA in 10^5 cellular genomes. None of the specimens was positive for HTLV-I sequences except the TSP probe. These negative data are all the more significant because brain material from MS patients was used in these studies. Our studies thus fail to support speculations that HTLV-I is involved in the aetiology of multiple sclerosis.

Key words: Multiple sclerosis – HTLV-I – Polymerase chain reaction

Introduction

Multiple sclerosis (MS) is a remitting and relapsing demyelinating disease whose aetiology is still unknown. Epidemiological data and virus-included MS-like diseases in laboratory animals are suggestive of a viral infection in MS [26]. In particular, the association of human immunodeficiency virus (HIV) in AIDS encephalopathy and human T-cell lymphotropic virus I (HTLV-I) in tropical spastic paraparesis (TSP) has given support to the hypothesis that human retroviruses may also be involved in MS. Moreover, following serological and in situ hybridization studies a possible relationship between HTLV-I related viruses and MS has been suggested [8, 13]. However, other investigators obtained negative results and could not confirm this association [5, 7].

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More recently the polymerase chain reaction (PCR) has been applied to search for retroviral sequences in MS, taking advantage of this technique to detect minute amounts of a certain nucleic acid [20]. Reddy et al. [18] reported the amplification of HTLV-I *gag* and *env* sequences from macrophages in 6 Swedish MS patients, while 19 out of 20 control samples remained negative. Greenberg et al. [3] found HTLV-I *pol* sequences in 6 of 21 MS patients; 3 of these were also positive for HTLV-I *env*, whereas 35 controls remained negative. In contrast, Bangham et al. [1], Richardson et al. [19], and Nishimura et al. [12] failed to detect HTLV-I sequences in a total of 76 MS patients. Furthermore, Fugger et al. [2] could not confirm the results of Reddy et al. [18] re-examining the 6 Swedish MS patients.

Since human retroviruses infect the central nervous system (CNS) and cause lesions as shown in AIDS encephalopathy or probably also in TSP, we have studied DNA extracted from 5 MS brains as well as from peripheral blood mononuclear cells (PBMCs) and peripheral macrophages from 21 MS patients, for the presence of HTLV-I sequences to find out whether MS brains may harbour HTLV-I or related sequences. We applied PCR using primers of HTLV-I *gag* and *env* genes as well as from the *tax* gene, since the latter gene is highly conserved among human retroviruses [11]. Except for positive controls, we failed to obtain a positive hybridization signal in any of our test samples. This indicated that the genome of HTLV-I or a related virus is not present in the DNA of mononuclear cells or brain from patients with MS.

Subjects and methods

Patients and DNA extraction

The data of the patients are summarized in Table 1. All patients had clinically definite MS according to the criteria of Poser et al. [16]. Ten normal control persons were matched for sex and age. Blood samples were coded so that the laboratory personnel performing DNA extraction and PCR had no knowledge of the iden-

tity of the samples. PBMCs were prepared from heparinized blood by Ficoll-Hypaque gradient centrifuging [4]. Half of the sample was used to extract DNA directly, while from the second half adherent cells consisting of monocytes/macrophages were prepared as described [18], and were subsequently used to extract DNA. The extraction of DNA from cells and deep-frozen (−80°C) brain tissue followed established procedures [10]. Brain specimens were derived from 5 MS cases, 2 of these with a short clinical course, 6 months and 1 year. From each MS brain samples were taken from the centre and periphery of several plaques as well as from areas histologically unaffected. In addition, 3 control brains were included, representing brain material from patients dying as a result of heart failure, brain tumour and amyotrophic lateral sclerosis (ALS). From the latter patient spinal cord material was also tested. TSP DNA was kindly provided by Dr. S. Nightingale (Birmingham, UK) and Dr. C. Bangham (Oxford, UK). MT-2 cell line [27] containing several HTLV-I genomes was obtained from Dr. B. Zorr (Berlin) and the HTLV-I *tax* gene carrying plasmid pKCR42 [6] from Dr. M. Hatanaka (Kyoto, Japan).

Table 1. Clinical data of the multiple sclerosis patients studied

No.	Sex	Age (years)	Duration of disease (years)	EDSS[a]	Course of disease[b]
1	F	40	1	2.0	2
2	M	28	3	3.0	2
3	F	29	7	1.5	1
4	F	34	9	6.5	3
5	M	51	21	2.5	2
6	M	31	9	3.5	3
7	F	35	17	6.5	4
8	F	39	4	3.0	2
9	F	50	17	1.5	1
10	F	48	7	4.0	3
11	M	34	5	1.5	1
12	F	41	9	1.5	1
13	M	49	3	2.5	2
14	F	47	11	3.5	4
15	F	45	2	2.0	2
16	F	52	10	6.5	3
17	F	53	33	6.5	4
18	M	25	2	2.0	2
19	M	45	2	1.0	1
20	M	51	11	6.0	4
21	M	53	5	3.5	4

[a] Expanded disability status scale [9]
[b] 1, Relapsing with complete remissions; 2, relapsing with incomplete remission; 3, relapsing-progressive; 4, secondary chronic progressive

Primers and probes

The primers and probes used for PCR analysis are summarized in Table 2. *gag* and *env* primers are the same as those published by Reddy et al [18]. *tax* primers and probe are more than 80% homologous to the HTLV-II sequence [23]. All oligonucleotides were synthesized using phosphoramidite chemistry on a Cyclone DNA synthesizer (Milligen, Eschborn, FRG) and purified by polyacrylamide gel electrophoresis.

PCR analysis

Amplification was carried out with 1 μg or 2 μg DNA in a reaction volume containing 16.6 m*M* $(NH_4)_2SO_4$, 67 m*M* TRIS (pH 8.8), 6.7 m*M* $MgCl_2$, 100 μg/ml bovine serum albumin (BSA), 10 m*M* β-mercaptoethanol, 0.2 m*M* of each dNTP (Pharmacia, Uppsala, Sweden), 1 μ*M* of each oligonucleotide primer, and 1.25 units Taq DNA polymerase (New England Biolabs, Schwalbach, FRG). After an initial denaturation step 40 cycles were run in a thermal cycler (Biomed, Theres, FRG) as follows: 1 min 60°C, 2 min 71°C, 1 min 94°C. One-fifth of each reaction was subjected to agarose gel electrophoresis and transferred on to nylon filters (Gene screen plus, Du Pont, Dreieich, FRG) by alkaline transfer. Filters were prehybridized in 6 × SSPE [1 × SSPE is 0.18 *M* NaCl, 10 m*M* NaH_2PO_4/Na_2HPO_4 (pH 7.7), 1 m*M* ethylenediaminetetra-acetate (EDTA), 10 × BFP (1 × BFP is 0.2 g/l BSA, 0.2 g/l Ficoll 400, 0.2 g/l polyvinylpyrrolidine), 0.07 g/l tRNA and 1% sodium dodecyl sulphate (SDS) at 42°C for 3 h. Hybridization was carried out overnight at 63°C–73°C (depending on the melting temperature of the oligo probe) in a fresh buffer containing 6 × SSPE, 1% SDS and 1–4 × 10^6 cpm/ml ^{32}P-end labelled oligo probe. Filters were washed twice in 6 × SSPE, 1% SDS at room temperature (10 min), once in 1 × SSPE, 1% SDS at the hybridization temperature (1 min) and exposed to Du Pont Cronex films at −70°C with an intensifying screen. Labelling of oligonucleotides with γ^{32}P-ATP (Amersham, Braunschweig, FRG) and T4 polynucleotide kinase (Boehringer, Mannheim, FRG) was carried out as described previously [10].

Results

Crucial for the detection of minute amounts of a certain DNA sequence is the sensitivity of the PCR. In the case of *gag* and *env* primers the amplification efficiency was

Table 2. Oligonucleotide primers used for HTLV-I polymerase chain reaction

Oligo	Function	Location [21]	Sequence (5′–3′)
gag 1	Primer (+)	841– 864	CGACCGCCCCGGGGGCTGGCCGCT
2	Primer (−)	1375–1353	GGTACTGCAGGAGGTCTTGGAGG
3	Probe	1080–1101	GATCCCGTCCCGTCCCGCGCCA
env 4	Primer (+)	5662–5685	CTCCCTTCTAGTCGACGCTCCAGG
5	Primer (−)	6129–6106	GCCACCGGTACCGCTCGGCGGGAG
6	Probe	5877–5897	GCCTCTCCACTTGGCACGTCC
tax 7	Primer (+)	7358–7377	GGAGACTGTGTACAAGGCGA
8	Primer (−)	7516–7496	AGGGATAAGGAACTGTAGAGC
9	Probe	7447–7468	AGAGCATCAGATCACCTGGGAC

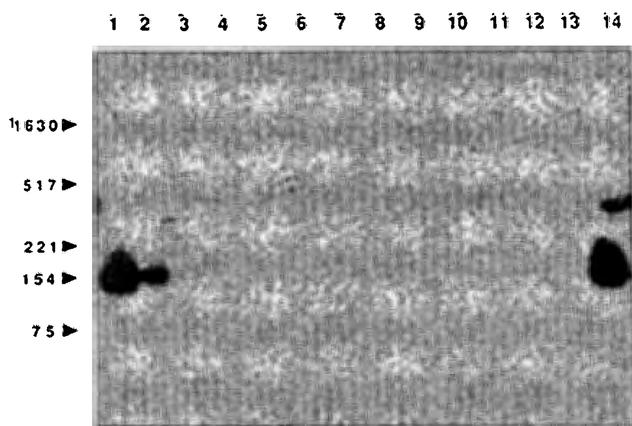

Fig. 1. Polymerase chain reaction with HTLV-I – *tax* primers and brain tissue from MS patients and controls. *tax* primers yield a 159 bp fragment. *Lanes 1–3:* 300, 30, 3 copies of *tax* target DNA each diluted in 1 µg HTLV-negative control DNA; *lane 4:* HTLV-negative control DNA; *lane 5:* reaction without any DNA; *lanes 7–9, 11, 12:* DNAs from inflammatory plaques fo 5 multiple sclerosis brains; *lanes 6, 10, 13:* DNAs from control brains; *lane 14:* tropical spastic paraporesis DNA

determined by amplifying serial tenfold dilution of MT-2 cell DNA, which harbours approximately 10 HTLV-I genomes/infected cell. In our assay, one copy of MT-2 DNA was constantly detected in 10^5 copies of uninfected cellular genomes with *env*-specific primers, while with *gag*-specific primers only one copy of MT-2 DNA was demonstrable in 10^3 copies of cellular genomes. The sensitivity of *tax* primers was measured using the plasmid pKCR42, revealing one target copy in 10^5–10^6 copies of uninfected genomes (Fig. 1). Thus, amplification efficiency with *env* and *tax* primers was comparable to published studies applying PCR technology, while the efficiency of *gag* primers was poor. By applying the PCR to our MS and control specimens, none of the DNA derived from blood samples reacted positively with *gag* and *env* or *tax* primers, except the TSP DNA. Moreover, all brain-tissue-derived DNAs were negative with either primer pair tested (Fig. 1).

Discussion

The recent reports from two laboratories [3, 17], that sequences of HTLV-I are present in DNA of patients with MS have led to a resurgence of interest in the possible viral aetiology of MS. In this context, studies were carried out to confirm the association between HTLV-I and MS and also to find out whether other retroviruses may be associated with this disease. Using the PCR technique, we failed to detect HTLV-I sequences in DNA of mononuclear cells from 21 MS patients. These negative data agree with findings from four other laboratories [1, 2, 12, 19]. Negative PCR findings in the past have been explained by the proponents of the HTLV-I theory as lacking in sensitivity [17]. However, the sensitivity of the *env* and *tax* primers in our study was in the range of other studies employing PCR technology [20]. This holds true especially for the *tax* primers that were able to detect one copy of target DNA from a highly conserved region in up to 10^6 cellular genomes. Thus, a lack in sensitivity is unlikely to be the reason for the failure to detect HTLV-I sequences in our MS and control probes studied.

To search for HTLV-I-related viruses in MS material we included primers from the *tax* gene of HTLV-I in our analysis. The *tax* gene was chosen because it is crucial for HTLV-I replication, since it encodes for the transacting activity [24]. In general, transactivating genes are highly conserved among retroviruses [11]. Therefore, using primers from the *tax* gene should allow the detection not only of HTLV-I sequences, but also of other related exogenous retroviruses.

In contrast to other groups searching for HTLV sequences in MS, we also analysed DNA extracted from MS brain tissue of five MS patients, since in other retrovirus infections with a neurological symptomatology viral footprints are detectable in CNS tissue. In murine leukaemia [14] or visna [25] virus infection of the CNS virus can be found in brain tissue. Similar observations have been made in HIV infection where viral DNA can be detected in brain material by Southern blotting [22] or PCR [15]. If in MS HTLV-I infection occurs in PMBCs one may expect viral sequences also to be detectable in inflammatory lesions in the CNS of MS patients, but the PCR results were also negative, indicating that HTLV-I or related virus was not associated with these cases.

Our data suggest that HTLV-I is not commonly found in patients with definite MS either in peripheral blood mononucleocytes or in brain tissue. However, we cannot exclude the possibility that a distantly related retrovirus or some other infectious agent that cannot be detected with our primer pairs may still be involved in the pathogenesis of MS.

Acknowledgements. We thank Dr. S. Siddell and T. Holtschke for oligonucleotide synthesis, B. Geyer for help with photography, and H. Kriesinger for typing the manuscript. This work was supported by grants from the Bundesministerium für Forschung und Technologie and Hertie-Stiftung.

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