Prevalence of HTLV-I Tax in a subset of patients with rheumatoid arthritis

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Abstract

Objective

In regions of the world where the human T cell lymphotropic virus type I (HTLV-I) is endemic, it is recognized that infection with this virus is associated with autoimmune diseases such as rheumatoid arthritis (RA). Moreover, mice transgenic for the HTLV-I Tax gene develop a disease akin to RA. The observation that about 8% of healthy American blood donors carry HTLV-I Tax in their lymphocytes (1) prompted studies to determine whether Tax positivity is more prevalent among patients with RA and if so, whether its sequence is homologous with prototypic HTLV-I Tax. This proved to be the case. Of 102 patients with RA tested, one was a carrier of HTLV-I and 25 had the Tax sequences in their mononuclear cells and antibodies to p40 Tax in their sera, while being negative for antibodies to the structural proteins of the virus.

Methods

Blood was collected from 102 RA patients. Lysates of their mononuclear cells were assayed for HTLV-I Tax by PCR/Southern analysis, and in some positive cases Tax sequence analysis was performed. Antibodies to p40 Tax, the gene product of the Tax sequence, were detected by western blot assay using recombinant p40 Tax as antigen.

Results

Of the 102 patients tested, one proved to be a carrier of the virus, having antibodies and sequences for the viral structural proteins, gag and env in addition to p40 Tax. Twenty-five of the 101 HTLV-I/II seronegative patients carried both HTLV-I Tax sequences in their mononuclear cells and had antibodies to p40 Tax. Sequence analysis confirmed homology with HTLV-I Tax.

Conclusion

The data show that the prevalence of HTLV-I Tax positivity among patients with RA is ~3 times higher than among healthy blood donors. Since Tax is known to be involved in the development of numerous autoimmune diseases, the possibility that it is responsible for the development of RA in a subpopulation of patients with this disease is not remote.

Key words

Rheumatoid arthritis, HTLV-I Tax.

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Introduction

Rheumatoid arthritis (RA) is a relatively common disease for which a primary cause has remained elusive. It seems likely that a multiplicity of inciting factors is able to trigger the autoimmune/inflammatory response which is characteristic for the clinical and pathologic manifestations of this condition. In regions of the world where the Human T Cell Lymphotropic Virus Type I (HTLV-I) is endemic, this infectious agent is held to be responsible for a substantial number of cases (2-4). In southern Japan the prevalence of rheumatoid arthritis and Sjögren's syndrome is 5 times higher among patients who are seropositive for antibodies to HTLV-I than among individuals who have no antibodies to this virus (2-5). In the United States infection with HTLV-I is believed to be very low, ranging from 0.016 - 0.1% when this is based on studies measuring antibodies to the structural proteins of the virus (6, 7). On the other hand, the HTLV-I Tax sequence and antibodies to its gene product, p40Tax, have been found to be as high as 8% among a cohort of 250 randomly selected New York City blood donors (1).

It should be realized that Tax and p40-Tax are responsible for the transcription/transactivation of numerous cytokines and growth factors (8-10). Tax has even been shown to stimulate the proliferation of synovial cells *in vitro* (11). Mice transgenenic for the Tax sequence develop a rheumatoid arthritis-like disease which is associated with pathologic alterations such as joint erosions and pannus formation identical to what is seen in man (12-14).

Therefore, it seemed of interest to investigate whether HTLV-I Tax positivity is more prevalent among patients with rheumatoid arthritis than among healthy blood donors.

Materials and methods

Study subjects and specimens

Adult patients diagnosed to have rheumatoid arthritis as defined by the 1987 revised criteria of the American Rheumatism Association (15) were recruited for the study through the arthritis clinics at Bellevue Hospital and the Hospital for Joint Diseases, as well as rheumatologists in private practice. Specimens obtained from 250 randomly selected healthy blood donors who presented at the New York University Medical Center and Bellevue Hospital blood banks, served as controls (1). The specimens consisted of 20 ml of heparinized whole blood which were fractionated into plasma and mononuclear cells by Ficoll/Hypaque gradient centrifugation by methods used routinely in this laboratory (16).

Detection of HTLV-I Tax proviral DNA sequences by PCR/Southern analysis

Preparation of cell lysates and PCR amplification. Whole cell lysates were prepared from ~10^5 peripheral blood mononuclear cells (PBMC) as described (17). Briefly, cells were lysed in autoclave-sterilized distilled water by sonication and boiling, followed by incubation for 1 hr at 55°C in the presence of proteinase K. Samples were then boiled to inactivate the protease and were subjected to two consecutive rounds of 30 cycles of PCR amplification (1 minute at 94°C, 1 minute at 55° C and 1.5 minutes at 72°C per cycle) followed by a final incubation for 10 minutes at 72°C under the buffer and dNTP conditions described. Tax primers consisting of 40 pmoles of SK43 and SK44 (18) (or primers to HTLV-I or -II gag, pol or env) (19, 20) and 4 units of Taq polymerase (Perkin Elmer, Foster City, CA) were included in final reaction volumes of 80 l per sample. Positive and negative control cells for PCR included lysates of the prototypic HTLV-I and -II-infected cell lines, C91PL (HTLV-I) (21) and MoT (HTLV-II) (22), as well as PBMC from HTLV Tax sequence-negative healthy volunteers.

Southern analysis. PCR-amplified products were resolved through 4% agarose gels in the presence of ethidium bromide, followed by denaturation, neutralization and overnight transfer of DNA to nylon membranes, as described (17). DNA was crosslinked to the membranes by incubation for 1 hr at 80°C followed by pre-hybridization and hybridization at 43° C for 2 hours and overnight, respectively, using Tax

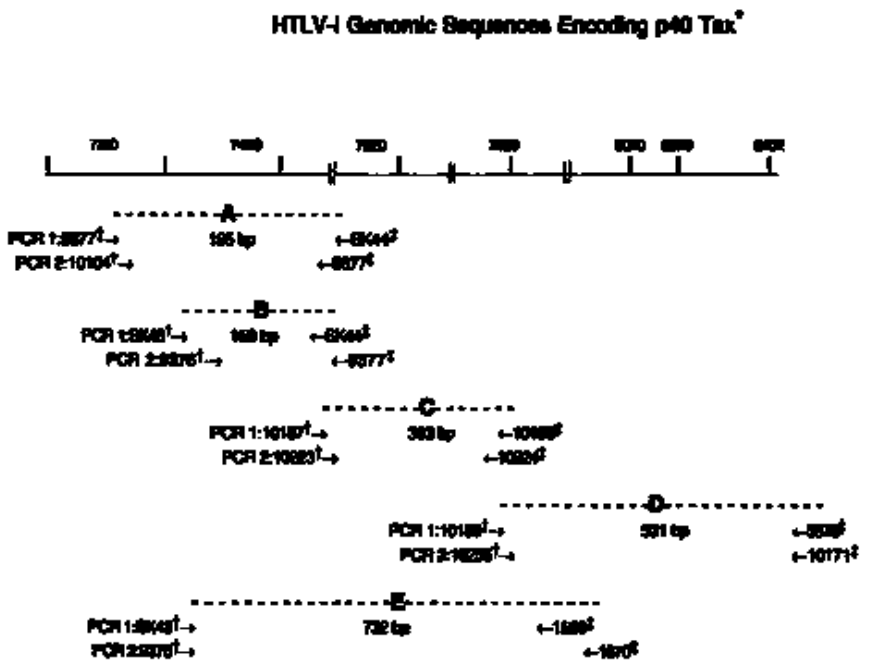

Fig. 1. Sequencing strategy used to identify full-length HTLV-I Tax proviral DNA sequences detected in PBMC of HTLV-I/II seronegative patients with rheumatoid arthritis and healthy blood donors, *Seiki, *et al*., (23). Each segment (A-E) sequenced, was subjected to 2 consecutive rounds of 30 cycles of PCR amplification, using the †sense and ‡antisense primers indicated, followed by direct sequence analysis of the amplified PCR products. The primer sequences are shown in Table 1.

probe SK45 (or probes to HTLV-I/II gag, pol or env [18-20]) that had been tailed at the 3' ends with digoxigenin. Detection of bound probe entailed the use of Fab' fragments of antibodies to digoxigenin conjugated with alkaline phosphatase and the alkaline phosphatase substrates 4-nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP). The reagents for 3'-tailing probes with digoxigenin and detection of bound probe were obtained from Boehringer-Mannheim (Indianapolis, IN).

Sequence analysis

Analysis of the 159 bp Tax proviral DNA sequence detected by PCR/ Southern analysis using primers SK43/ SK44. The Tax sequences, amplified by PCR using primers SK43 and SK44 and detected by Southern analysis using the digoxigenin-tailed probe SK45 (as described above and in ref. 17) in PBMC lysates, from all 25 Tax sequence and p40 Tax antibody-positive patients with rheumatoid arthritis were subjected to sequence analysis as described in detail elsewhere (1) (See Fig. 1, Region B). Briefly, whole cell lysates of ~1 x 10^5 PBMC prepared by Ficoll/Hypaque gradient centrifugation, as described above, were subjected to two consecutive 30 cycle rounds of PCR amplification, each consisting of: 1 minute at 94°C, 1 minute at 55°C, 1.5 minutes at 72°C per cycle and a final 10 minutes incubation at 72°C,under the PCR conditions described (17).

In PCR 1, primers SK43 and SK44 were used. The second PCR was initiated by the transfer of 2 l samples of the PCR products generated in PCR 1 to final PCR reaction volumes of 80 l per sample. The primers used in PCR 2, consisted of the sense and antisense primers, 9376 (5'-CGTGTTTGGAGACTGTGTAC -3') and 9377 (5'-CATCGATGGGGTCCCAGGTG-3') (1). The underlined sequences correspond to the 5 base 3' ends of SK43 and SK44, respectively. The products from PCRs 1 and 2 were resolved through 4% ethidium bromide-containing agarose gels. The DNA in the remainder of any samples that gave visible bands in the gels, was isolated using the reagents and columns contained in the QlAquick PCR Purification Kit (Qiagen, Valencia, CA) and were sequenced directly by the NYU School of Medicine Oligonucleotide Synthesis and Sequencing Facility, using primers 9376 and 9377. Sequences detected in the samples were compared with those published for HTLV-I and -II (23-27). HTLV-I and -II differ in this region by 16 bp. Based on comparative sequence analyses through Genbank and other available databases, HTLV-I Tax has no known homology with endogenous retroviral or human genomic sequences, either at the DNA or amino acid sequence levels.

Analysis of sequences encoded by the p40 Tax open reading frame. Although the Tax-related sequences detected in the PBMC of most of the Tax-positive RA patients tested were identical to or nearly identical to the HTLV-I Tax sequence, this analysis consisted of only 159 bp of the HTLV-I pX region encoding p40 Tax. To ascertain that indeed the entire p40 Tax open reading frame (ORF) which encodes the p40 Tax protein was present and homologous to that of HTLV-I, specimens obtained from 4 different RA patients and 3 healthy Tax-positive blood donors were subjected to further analysis. To this end, a 2-step PCR and sequence analysis were carried out utilizing primer sets spanning the entire p40 Tax ORF (Fig. 1 and Table I).

Detection of Tax mRNA. Reverse transcription (RT)-PCR/Southern analyses were performed as described (28). RT assays were carried out after treatment of lysates of 105 mononuclear cells with DNase I in the presence of M-MLV reverse transcriptase (GIBCO-BRL, Gaithersburg, MD) and HTLV Tax primer SK43, under the conditions described in detail in ref. 28. PCR was performed in the same reaction tubes, after the addition of Taq polymerase and Tax primer SK44, followed by Southern analysis.

Oligonucleotide primer and probe synthesis. All primers and probes used in these studies were synthesized in the Oligonucleotide Synthesis and Sequencing Facility at the NYU School of Medicine.

Table I. Tax primers used in PCR amplifications and sequencing

Region sequenced	PCR	primer	sense	primer	antisense
A*	1	5877	5'-GGC CCA CTT CCC AGG G-3'	SK44†	
	2	10104	5'-CAG GGT5 TTG GAC AGA GTC TT-3'	9377†	
B	1	SK43†		SK44†	
	2	9376†		9377†	
C	1	10187	5'-CGA TGG ACG CGT TAT CGG CT-3'	10188	5'-TTG TAG GGA ACA TTG GTG AG-3'
	2	10223	5'-CGG CTC AGC TCT ACA GTT CCT-3'	10224	5'-GTG AGG AAG GCC CCG AGC TG-3'
D	1	10189	5'-CTC ACC AAT GTT CCC TAC AA-3'	5878	5'-CAG ACT TCT GTT TCG CG-3'
	2	10255	5'-TAC AAG CGA ATA GAA GAA CTC-3'	10171	5'-TCG CGG AAA TGT TTT TCA CT-3'
E	1	SK43†		1569	5'-TAA GGC CTG GAG TGG TGA GG-3'
	2	9376‡		1570	5'-TGA GGG TTG AGT GGA ACG GA-3'

*A-E refer to the Tax sequence regions indicated in Fig. 1. † Primer sequence previously published in ref 18. ‡ Primer sequence previously published in ref 1. All other primer sequences were based on the published HTLV-1 Tax proviral sequence published by Seiki *et al.* (23).

Detection of HTLV-I/II antibodies. In addition to studies carried out in our own laboratory, blood samples from all subjects were sent to the New York Blood Center for blinded, routine screening for antibodies to HTLV-I/II.

Detection of antibodies to HTLV-I p40 Tax. Antibodies to p40 Tax were detected by Western blot analysis utilizing recombinant full-length p40 Tax antigens. The preparation of p40 Tax antigens has been described in detail elsewhere (28). Briefly, recombinant full-length p40 Tax protein was obtained by cloning PCR-amplified proviral DNA sequences spanning the entire p40 Tax open reading frame from the prototypic HTLV-I-infected cell line, C91PL (21), into the glutathione S-transferase (GST) fusion protein expression vector, pGEX-2T (29). The GST-Tax fusion protein was expressed in *E. coli* BL21 cells and purified by chromatography using glutathione linked to Sepharose 4B (Amersham Pharmacia Biotech) and subsequent cleavage with thrombin. Control antigens were prepared similarly, from BL21 cells expressing GST proteins utilizing the GST fusion protein expression vector pGEX-2T lacking p40 Tax sequences. Western blot assays were carried out using 1:10 dilutions of test and control plasmas.

Bound p40 Tax antibody was detected using goat-anti human IgA + IgG + IgM sera conjugated with alkaline phosphatase (Pierce Chemical, Rockford, IL) and the alkaline phosphatase substrates NBT and BCIP (Boehringer-Mannheim). HTLV-positive and -negative human sera were included as controls in each assay. Recombinant p40 Tax and GST-Tax antigens were identified on the blots using molecular weight markers and polyclonal antisera against: (1) recombinant full-length p40 Tax protein expressed in a baculovirus expression system (30), obtained from the NIH AIDS Research and Reference Reagent Program; and (2) schistosomal GST (Amersham Pharmacia Biotech).

Statistical analysis. Statistical analysis was performed using the StatView 4.5 statistical analysis program. A comparison was made between the 25 HTLV-I Tax sequence + p40 Tax antibody-positive RA patients out of the 102 tested versus 23 HTLV-I Tax sequence + p40 Tax antibody-positive healthy blood donors out of 250 tested (1).

Table II. Detection of HTLV-I proviral DNA sequences and antibodies in patients with rheumatoid arthritis.

Detection of HTLV-I proviral DNA sequences in PBMC lysates					
gag-I	gag-II	pol-I	pol-II	env-I	Tax-I/II
1*/10	20/102	1*/102	0/102	1*/102	39/102
Detection of antibodies to HTLV-I/II					
gag and env		p40 Tax			
1*/102		30/102			
Positive for both Tax sequences + Tax antibodies				25/102	
Positive for Tax sequences - Tax antibodies				5/102	

*A single patient accounted for the positive results obtained in each test.

Results

Of the 102 RA patients tested, 1 was positive for proviral DNA sequences encoding HTLV-I pol, p40 Tax and the structural proteins, gag and env, but not HTLV-II gag or pol (Table II). Therefore, this patient may be considered to be infected with HTLV-I. Although the other 101 patients lacked sequences for gag-I and -II, pol I and -II, and env-I, the PBMC lysates from 38 were shown to harbor Tax sequences. Examples of PCR/Southern analyses for HTLV-I and -II gag, pol, env and Tax proviral sequences detected in selected "Tax only"-positive healthy blood donors and RA patients are shown in Figure 2. The only patient shown to be positive for proviral DNA sequences spanning the entire HTLV-I genome was the one found to be seropositive for antibodies to the HTLV-I viral structural proteins, gag and env, when this was tested blindly at the New York Blood Center. This patient originated from an HTLV-I-endemic region. Antibodies to p40 Tax were found in 30 RA patients, including the HTLV-I-positive individual. A representative Western blot assay

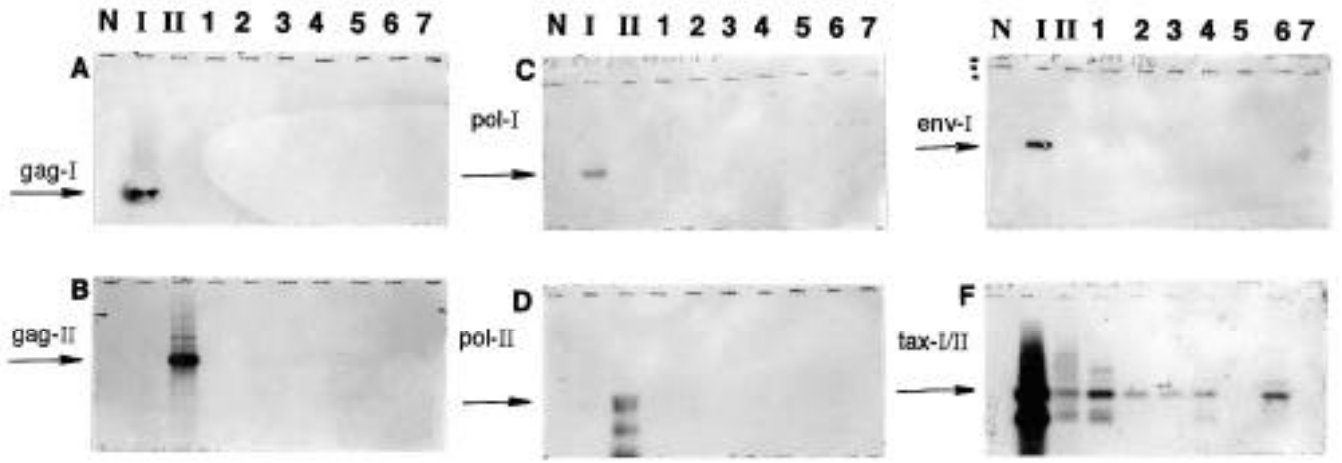

Fig. 2. HTLV-I proviral DNA sequences in lysates of PBMC from healthy blood donors and RA patients detected by PCR/Southern analysis, using the primers and probes described in Methods. The probes used in hybridization were tailed at the 3'ends with digoxigenin and bound probe was detected using Fab' fragments of antibodies to dig oxigenin, conjugated with alkaline phosphatase and the alkaline phosphatase substrates,NBT and BCIP. Sources of cell lysates: lane N, PBMC from a HTLV-I/II -negative volunteer; lane I,HTLV-I-infected cell line, C91PL; lane II,HTLV- II-infected cell line, MoT; lanes 1-3, PBMC from 3 different healthy "Tax only"-positive blood donors; lanes 4-7,PBMC from 4 different RA patients,2 of whom were "Tax only"-positive (Panel F, lanes 4 and 6).

is shown in Figure 3. Of the Tax-positive patients, 25 were positive for both Tax sequences and antibodies to p40 Tax. However, 13 patients who were Tax sequence-positive, and antibodies to the p40 Tax protein. None of the patients'sera reacted with control antigens prepared from lysates of bacteria transformed with the GST protein expression vector lacking p40 Tax coding sequences (data not shown). PBMC lysates from all Tax proviral DNA sequence-positive patients also proved to have Tax mRNA (data not shown). Therefore, the prevalence of Tax sequence + Tax antibody-positivity among this group of RA patients was ~3 times higher than the number observed in a cohort of HTLV-I/II seronegative healthy blood donors (1). These results were statistically significant with a p value ≤ 0.0003.

Five RA patients had antibodies to p40 Tax without demonstrable Tax sequences or Tax mRNA in their peripheral blood leukocytes. In addition,a synovial fluid aspirate from one of the patients who had been found to be Tax sequence and Tax antibody-positive became available for analysis. The synovial fluid mononuclear cells also proved to carry the Tax sequence.

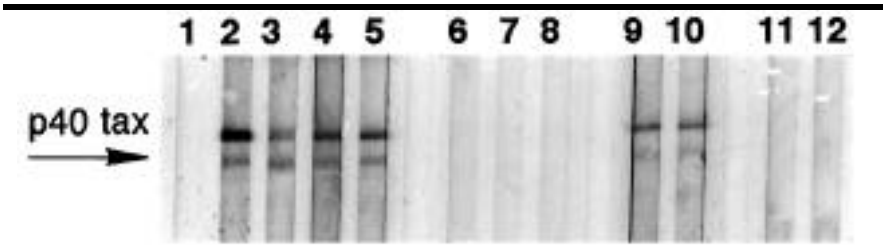

Fig. 3. Antibodies to p40 Tax in plasmas from patients with rheumatoid arthritis and healthy blood donors. Western blot assays were performed using recombinant full-length p40 Tax as antigen. Bound antibody was detected with goat anti-human IgA + IgG + IgM, conjugated with alkaline phosphatase and the alkaline phosphatase substrates NBT and BCIP. In positive samples, the sera reacted with uncleaved GSTp40Tax (upper band) and thrombin-cleaved p40Tax (lower band indicated by the arrow). These bands were identified using a rabbit polyclonal antiserum to recombinant full-length Tax-I, obtained from the NIH AIDS Research and Reference Reagent Program (30) and a goat polyclonal antiserum raised to schistosomal GST (Amersham Pharmacia Biotech,Piscataway, NJ). Sources of plasma samples: lane 1, an HTLV-I/II-negative volunteer; lane 2, an HTLV-I-infected TSP/HAM patient; lanes 3-5, 3 different healthy Tax sequence- positive blood donors; lanes 6-8, 3 different healthy Tax sequence-negative blood donors; lanes 9-12, four different RA patients, (lanes 9 and 10 were from Tax sequence-positive patients; lanes 11 and 12 were from Tax sequence-negative patients).

Sequence analysis was carried out on HTLV-I Tax-related proviral DNA detected by PCR/Southern analysis using primers SK43/SK44 and probe SK45 in cells from all 25 of the RA patients found to be Tax sequence- and Tax antibody-positive. Although 3 of the 25 had single base pair differences in the 159 bp sequence analyzed (see the boldface, underlined region in Figure 4), their sequences were all nearly identical to that published for prototypic HTLV-I (23) and markedly different from prototypic HTLV-II (24-27). Analysis of the entire p40 Tax open reading frame, carried out on 4 RA patients and 3 Tax-positive healthy blood donors demonstrated that: (1) the entire Tax coding sequence is present in cells from both RA patients and blood donors classified as "Tax only"-positive; and (2) the sequences, with only minor, individual variations, are all nearly identical to that of the HTLV-I prototype. The full-length Tax sequence designated RA98 in Figure 4 was derived from analysis of PBMC lysates from the HTLV-I seropositive RA patient.

Discussion

On the basis of the data presented here, it appears that the Tax sequence of HTLV-I is three times more prevalent in the PBMC obtained from HTLV-I/II

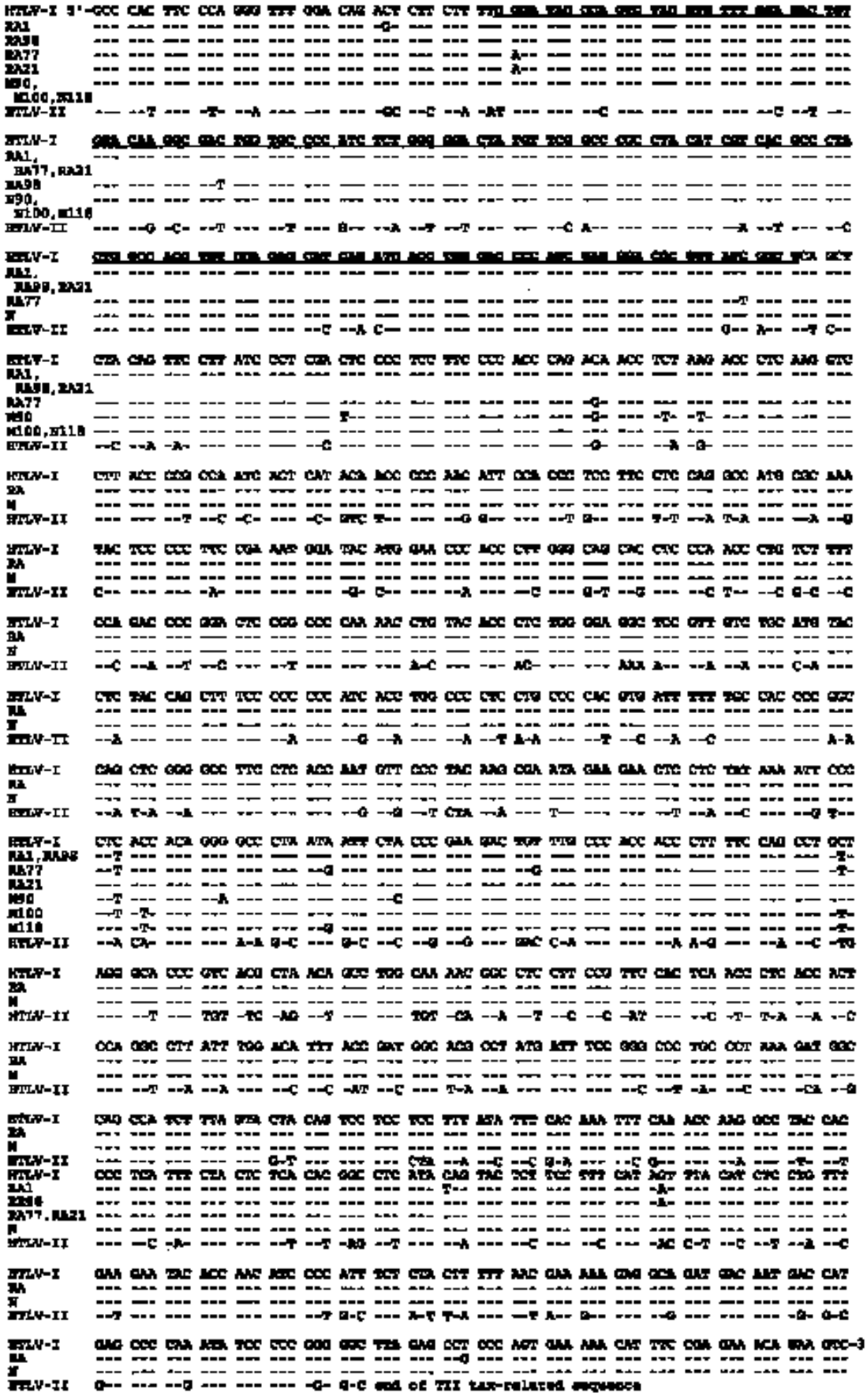

Fig. 4. Analysis of full-length p40 Tax sequences detected in HTLV-I/II seronegative patients with rheumatoid arthritis (RA) and healthy blood donors (N). The prototypic HTLV-I and HTLV-II Tax sequences were published by Seiki *et al.*, (23) and Shimotohno *et al.* (24), respectively. The bolded and underlined sequence is the 159 bp HTLV-I Tax proviral sequence amplified by PCR using primers SK43 and SK44. Dashes represent sequence identity with the prototypic HTLV-I sequence.

seronegative patients with rheumatoid arthritis than in the PBMC of randomly selected healthy blood donors. The sequence homology, now established for the entire open reading frame of the HTLV-I pX region, has strengthened earlier data and conclusions based on only 159 bp (1, 17). Moreover, all Tax sequence-positive patients also had antibodies to p40 Tax, the protein encoded by the Tax sequence. The sera of 5 RA patients had antibodies to p40 Tax, without having demonstrable Tax sequences in their PBMC. Because antibodies to p40 Tax were never found in plasmas of more than 250 healthy blood donors unless their PBMC were Tax sequence-positive as well (1, 31), it is likely that the antibodies generated by the 5 Tax sequence-negative RA patients crossreacted non-specifically with an antigenic epitope not related to HTLV-I. This phenomenon is not unusual in patients with autoimmune diseases such as rheumatoid arthritis and lupus erythematosus (32-34). Moreover, false positive reactions for HTLV-I are known to occur occasionally in recently vaccinated individuals. Most of these were detected by ELISA and proved to be negative by Western blot (35). To preclude such erroneous interpretation, healthy individuals as well as RA patients were considered to be HTLV-I Tax positive only if they had both, *i.e.*, Tax sequences as well as antibodies to p40 Tax.

The data recorded here raise two issues. The first concerns the observation that a large number of RA patients as well as ~8% of healthy blood donors retain the Tax sequence when other genetic components of the virus appear to have been deleted (1). In this regard it should be recalled that in regions of the world where HTLV-I is endemic, "Tax only"positivity is quite common, even among family members of patients who have developed HTLV-I-associated diseases such as adult T cell leukemia and tropical spastic paraparesis/HTLV-I-associated myelopathy (36-39). We have reported a 9-year-old patient with mycosis fungoides, who was "Tax only"-positive, but whose mother was a healthy carrier of the whole virus and had antibodies to its structural proteins when this was determined by routinely used serologic tests (40).

The second issue concerns the possible role of HTLV-I Tax in the development of rheumatoid arthritis. Here an abundance of data can be adduced supporting the concept that Tax stimulates the proliferation and immortalization of CD4+ T lymphocytes, and that it activates IL-2, the IL-2 receptor , IL-6, TNF- , TGF- , INF- , Gm-CSF and many other cytokines (8-10). Synovial cells which contain the Tax sequence are highly proliferative and, when transfected *in vitro* with Tax, synoviocytes lose contact inhibition and acquire the ability to form colonies in semi-solid media (41). However, the most relevant observation is that mice transgenic for Tax develop a chronic inflammatory arthritis which is almost identical to the rheumatoid arthritis seen in man (12-14). A fairly high percentage of the animals also develop Sjögren's syndrome, uveitis, and other autoimmune phenomena (42). It would be of interest to carry out a prospective study, i.e., to follow healthy Tax-positive individuals to determine what percentage of such individuals will develop rheumatoid arthritis.

It has been suggested and we now have good evidence for the likelihood that Tax sequences are episomal in "Tax only"-positive healthy individuals. This has recently been shown by fluorescent *in situ* hybridization (FISH) analysis carried out on metaphase spreads (43). In earlier studies it had been shown that Tax sequences do not necessarily isolate with high molecular weight genomic DNA, and that therefore it is often lost when such preparatory methods are employed (44). The question which is often raised is how such Tax sequences are maintained without being integrated into the cellular genome, *i.e.* in the absence of retroviral machinery. In this regard, it has been well established, that propagation of episomal DNA sequences can take place once per cell cycle - akin to the replication of chromosomal DNA. This process has been best studied for latent Epstein-Barr virus (45; see also the review in ref. 46). For HTLV-I, this phenomenon has been studied extensively by Wattel *et al.* (47).

Over the past 4 decades many infectious agents, including bacteria, mycoplasma and various viruses, have been scrutinized for their possible role in the development of rheumatoid arthritis (48, 49, 34). Among these the Epstein-Barr virus has received the most attention, primarily because this virus is a polyclonal activator of B cells and stimulates the production of rheumatoid factor (50-53). Accumulations of oligoclonal T cells in the joints of patients with RA have also been described repeatedly (54-56). Such cells are mostly CD3+, CD4+, and CD45RO+ (54). As already mentioned, the synovial fluid specimen obtained from one of the Tax-positive RA patients included in this study proved to contain Tax-positive mononuclear cells.

The question as to why not everyone who carries HTLV-I Tax develops RA, and how this inflammatory reaction exacerbates and subsides, has been discussed in considerable detail elsewhere (57).

At the very least, the data presented here have added yet another factor to the multiplicity of agents which may play an etiologic role in the development of rheumatoid arthritis. Recognition that HTLV-I Tax may play a role in a significant number of RA patients is important, because in the future more specific treatment modalities, such as the use of antisense oligonucleotides directed against Tax mRNA, may become available.

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