Int. J. Cancer: **85,** 762–770 (2000)

Publication of the International Union Against Cancer

HUMAN RETROVIRUS 5 SEQUENCES IN PERIPHERAL BLOOD CELLS OF PATIENTS WITH B-CELL NON-HODGKIN'S LYMPHOMA

Modra MUROVSKA[1,2], Sandra LEJNIECE[3], Svetlana KOZIREVA[1,2], Marina KOULIKOVSKA[1,2], Hong YIN[1] and Jonas BLOMBERG[1]*

[1]*Section of Virology, Department of Medical Sciences, Uppsala University, University Hospital, Uppsala, Sweden*
[2]*Department of Oncovirology, August Kirchenstein Institute of Microbiology and Virology, Riga, Latvia*
[3]*National Centre of Hematology, Medical Centre Linezers, Riga, Latvia*

A recently described sequence from a probable 5th human exogenous retrovirus, HRV-5, is related to type A, B and D retroviruses. It was initially detected in a salivary gland biopsy from a patient with Sjögren's syndrome, but it is not consistently associated with this disease. We searched for the HRV-5 sequence in DNA extracted from whole blood of 300 blood donors, 81 patients with hematological malignancy and 21 patients with neurological disease using PCR. While samples from none of the blood donors and the neurological patients became positive, 3 of the 81 patients with hematological malignancy were HRV-5 DNA positive. All 3 had B-cell non-Hodgkin's lymphoma of low grade. The difference in frequency between NHL and controls is statistically significant. HRV-5 DNA was found in DNA from whole blood and in plastic-adherent cells but not in tumor cell DNA. Thus, monocytes/macrophages may be preferred targets for HRV-5. Our result, together with a previous finding of HRV-5 DNA in 2 NHL cases, is compatible with an association between HRV-5 and NHL, whether causal or not. ***Int. J. Cancer*** **85:762–770, 2000.**
© *2000 Wiley-Liss, Inc.*

All retroviruses, including human ones, are potential etiologic agents of hematological malignancies, neurological disorders and disorders of immune regulation. Five exogenous human retroviruses have been described so far. Only 4 of them have been completely characterized at the molecular level. Human T-cell lymphotropic virus types I (HTLV-I; Poiesz *et al.,* 1980) and II (HTLV-II; Kalyanaraman *et al.,* 1982) were first reported in 1980 and 1982, respectively. The lentiviruses human immunodeficiency virus types 1 (HIV-1; Barré-Sinoussi *et al.,* 1983) and 2 (HIV-2; Clavel *et al.,* 1986) were first reported as human lymphotropic virus types III (HTLV-III) and IV (HTLV-IV). A report of a 5th human retrovirus (Manzari *et al.,* 1987) has not been confirmed. The 4 well-known human infectious retroviruses display a tropism for T-lymphocytes and/or macrophages and are comparatively easily detectable in peripheral blood of affected persons. A 932 bp sequence containing part of the *pro* and *pol* genes of a probable new exogenous human retrovirus has been described: the sequence was isolated by polymerase chain reaction (PCR) from sucrose density gradient centrifugation fractions of a salivary gland homogenate from a patient with Sjögren's syndrome (Griffiths *et al.,* 1997). However, this viral sequence is not commonly detected in human DNA, is present in a very low number of copies when detected and is detectable only by sensitive nested PCRs. It therefore has the characteristics of an exogenous human retrovirus and was termed human retrovirus 5 (HRV-5). In the known portion of the conserved *pro* and *pol* genes, it is 60%–70% amino acid identical to endogenous and exogenous types A, B and D retroviruses, most highly to the human endogenous retroviral sequence (HERV) groups HML-3 and HML-4 (around 70%; Andersson *et al.,* 1999). In further studies, only 2 of 92 salivary glands examined were PCR-positive for HRV-5. Neither sample was from primary Sjögren's syndrome (Rigby *et al.,* 1997). These data suggested either that the original isolate was a "passenger" virus unrelated to the pathogenesis of Sjögren's syndrome or that in this disease, the salivary gland is not the main site of HRV-5 replication. Later, 115 lymph node biopsies representing a cross-section of B- and T-cell lymphomas and nonmalignant conditions were examined for the presence of HRV-5 proviral DNA. The rate of detection (4/115, *i.e.,* 3.4%; Rigby *et al.,* 1998) was similar to that in the previous study of HRV-5 DNA in salivary glands (2/92; Griffiths *et al.,* 1997; Rigby *et al.,* 1997). Moreover, for one of the B-cell malignancies positive for HRV-5 by nested PCR, the tumor cells were enriched using magnetic bead separation, and extracted DNA was negative. It is not known which cell is the main target for HRV-5 persistence. However, according to the above indirect data, they may not be lymphatic cells.

HRV-5 DNA has been found in around half of synovial tissues of arthritic joints from patients with rheumatoid arthritis (Griffiths *et al.,* 1999) and to a lesser extent in whole blood from the same patients. Thus, HRV-5 may be involved in several types of disease. However, there are no independent studies using a consistent sample type in patients and more controls, as well as more numerous controls.

Our objective was therefore to investigate the prevalence of HRV-5 infection in a relatively large number of blood donors and to compare this frequency with that of patients with hematological malignancies and neurologic diseases. In this study, we examined DNA samples isolated from whole blood of apparently normal blood donors, patients with different forms of hematological malignancies and patients with neurologic disorders for evidence of HRV-5 proviral DNA. In one hematological malignancy, DNA was also extracted from peripheral blood mononuclear cells (PBMC). Although much more work is needed, our results are compatible with a role of HRV-5 in the genesis of NHL. The absence of amplifiable DNA in tumour cells but presence in plastic-adherent cells do however argue against a direct involvement of HRV-5 in the genesis of NHL.

MATERIAL AND METHODS

Blood donors and patients

Blood samples were collected from 150 randomly selected, apparently normal blood donors during March 1998 in the State Blood Donor Center in Latvia and from 150 randomly selected blood donors during February to March 1998 at the Section of Virology, Uppsala Academic Hospital. Blood samples of patients with various forms of hematological malignancies were collected from 81 randomly selected patients in the National Hematology Center in Latvia from April 1997 to January 1998. Blood samples of patients with different neurological disorders were collected from 21 randomly selected patients in the Department of Neurology, P. Stradin Clinical Hospital, Riga, from April 1997 to January 1998. Blood samples of 10 ml were taken with 0.16% EDTA. Aliquots of whole blood, plasma and Ficoll 400 (Amersham Pharmacia Biotech, Uppsala, Sweden) separated PBMCs were kept at −70°C for further examination.

Grant sponsor: Swedish Institute; Grant number: 380/133, East92; Grant sponsor: Latvian inhabitants and their health. Malignant tumors; Grant number: 2-2.011.

*Correspondence to: Section of Virology, Department of Medical Sciences, Uppsala University, Academic Hospital, SE-751 85 Uppsala, Sweden. Fax: +46-18-665593. E-mail: jonas.blomberg@medsci.uu.se

Received 19 April 1999; Revised 20 October 1999

Preparation of plastic-adherent blood cells

Whole blood (with 0.16% EDTA) was centrifuged at 800 g for 5 min (Lalezari and Khorshidi, 1996). Plasma was replaced with an equal volume of 10% EDTA, and the blood suspension was mixed. Then 0.2 ml of 6% dextran in PBS (pH 7.2) per ml of original blood volume was added, and the blood suspension was mixed well and incubated at room temperature for 15 min. The leukocyte-rich supernatant was transferred to a new glass tube and centrifuged at 800 g for 5 min, then 2 ml of 0.83% NH_4Cl were added to the leukocyte pellet, and the suspension was mixed for 3 min and centrifuged at 800 g for 5 min. The leukocytes were washed twice with RPMI-1640 and cultivated in Petri plates in RPMI-1640 with glutamine (0.3 mg/ml), 20% (v/v) FCS and gentamicin 50 μg/ml) at 37°C, 5% CO_2 for 48 hr. Nonadherent cells were transferred to a new plate. The adherent cells were washed twice with PBS and collected for further DNA isolation. DNA was isolated as described below.

Isolation of tumor cells

Pleural exsudate from patient 117h was diluted with PBS (1:1) and centrifuged at 3000 g for 10 min. The cell pellet was washed twice with PBS and further used for DNA isolation, as described below.

DNA extraction

DNA was extracted from frozen whole blood as well as from PBMC. To extract DNA from whole blood (0.5 ml), the red cells were lysed by adding 1 ml of red cell lysis buffer (0.01M Tris-HCl, 0.005M $MgCl_2$, 0.001M NaCl). Leukocytes were spun down by centrifugation at 20,000 g for 1 min. The leukocyte pellet was washed once with 1 ml of deionized water (dH_2O) and resuspended in 80 μl of 5× proteinase K buffer (0.375M NaCl, 0.12M EDTA pH 8.0), 20 μl of 20% SDS, 30 μl of proteinase K (10 μg/ml) and 240 μl of dH_2O (to the final volume 400 μl) followed by incubation at 55°C for 1 hr. Proteins were then removed by adding 120 μl of 5M NaCl followed by centrifugation at 20,000 g for 7 min. DNA was precipitated with ethanol and dissolved in 50–150 μl dH_2O. PBMC were separated on Ficoll 400, washed twice in PBS and incubated in 500 μl of extraction buffer (0.01M Tris-HCl, pH 8.0, 0.1M EDTA pH 8.0, 0.5% SDS, 20 μg/ml pancreatic RNAase) with proteinase K (100 μg/ml) at 55°C for 24 hr. DNA was extracted by using a standard phenol-chloroform method, precipitated with ethanol and dissolved in 50–150 μl of dH_2O. A globin PCR (Vandamme *et al.,* 1995) was carried out to control the quality of the extracted DNA.

PCR for HRV-5

DNA (0.5–1 μg) was first tested by nested PCR using the primer combination POL1 (Table I; Griffiths *et al.,* 1997). When positive samples were identified, the samples were retested with an additional primer set (POL2, Table I; Griffiths *et al.,* 1997). One more primer set, PRO, targeted to the *pro* region of HRV-5 sequence, was designed and used to test positive samples (Table I) with the following PCR conditions in the first-round reaction: a single cycle of 94°C for 5 min, 40 cycles of 94°C for 1 min, 58°C for 1 min, 72°C for 1 min and final extension at 72°C for 5 min; and with the following PCR conditions in the second round reaction: a single cycle of 94°C for 5 min, 40 cycles of 94°C for 30 sec, 59°C for 30 sec, 72°C for 30 sec and a single cycle of 72°C for 5 min. The PCR reactions were done in a 50 μl volume in the presence of 10 mM Tris-HCl, 50 mM KCl, 200 μM of each deoxynucleoside triphosphate, 10 pM of each oligonucleotide primer, 1U of Taq polymerase and 1.5 and 3 mM $MgCl_2$ for the first and second rounds of PCR, respectively. As a positive control, the plasmid containing HRV-5 919 bp sequence was used (pHRV5.1, a kind gift of Dr. D. Griffiths, London, UK). To prevent and monitor PCR contamination, frequent water controls and negative DNA samples were included (after every 4th sample). The sensitivity of nested PCRs for HRV-5 was detected using a dilution series of the positive control (4 fg, 400 ag, 40 ag, 4 ag and 0.4 ag of plasmid DNA). The plasmid DNA was amplified in the presence of 500 ng of human genomic DNA. The sensitivity of POL1 and POL2 were 4 ag (approximately 1 molecule of pHRV5.1 per reaction) and of PRO 40 ag (approximately 10 molecules of pHRV5.1 per reaction). Water controls were included between each sample. The amplicons were analyzed by agarose gel electrophoresis (AGE) on 1.4% agarose and stained with ethidium bromide. All DNA samples were positive in a globin PCR, confirming that they were of sufficient quality for further PCR examination (Vandamme *et al.,* 1995).

Cloning and sequencing

PCR products were cloned using the TOPO TA Cloning Kit (Invitrogen, Groningen, The Netherlands) according to the manufacturer's protocol. The plasmid clones were sequenced on an ABI 310 capillary electrophoresis system (Perkin Elmer Applied Biosystems, Foster City, CA) using the PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer). Sequences were further analysed using BLAST 2.0.5 (Altschul *et al.,* 1997) and the Clustal W 1.6 alignment program.

Southern blot analysis

Cellular DNA (10 μg) was digested either by Eco RI or Hind III and subjected to AGE on 0.8% agarose (22 V, 24 hr). AGE was performed in Tris-borate-EDTA (TBE) buffer. The gel was stained with ethidium bromide (0.5 μg/ml). DNA fragments were transferred to nylon membrane (Hybond N^+, Amersham Pharmacia Biotech, Aylesbury, UK) in 10× SSC by using standard capillary blotting (Sambrook *et al.,* 1989). After fixation with UV irradiation for 7 min, the membrane was prehybridized for 3 hr at 50°C in 6× SSC, 1% SDS, 5× Denhardt's solution and 100 μg/ml of herring sperm DNA and hybridized with ^{32}P-labelled specific probe. As a specific probe, the HRV-5 sequence containing 931 bp Eco RI-Hind III fragment cut from the plasmid pHRV5.1 was used and labelled with [α-^{32}P]dCTP (Amersham Pharmacia Biotech) by using the Ready-To-Go labelling kit (Amersham Pharmacia Bio-

TABLE I – HVR-5 PCR PRIMERS USED

Nucleotide of combination	Gene	Function	Sequence	Positions
POL1	*pol*	Outer sense	5′-GCCATGACACCATCAAGAAGTGCT-3′	94–117
		Outer antisense	5′-TGCTTTGGGATCATAGTAGGAAC-3′	679–657
		Inner sense	5′-ATTAGGCTCCAGAGAAGGCAGAAG-3′	306–329
		Inner antisense	5′-CCGGGAGTCCAGGTTGTAATG-3′	657–637
POL2	*pol*	Outer sense	5′-CCATCACATTATGGGGAAGAGACA-3′	185–208
		Outer antisense	5′-GAATGTCTTGTTCATGTAGAGGTAT-3′	747–723
		Inner sense	5′-GCCATTGTCATGGCTGGACAACAA-3′	407–430
		Inner antisense	5′-CCTTCAGATCGAGTACTATTAATGG-3′	708–684
PRO	*pro*	Outer sense	5′-GATTGGCCGAAGTCATGGCC-3′	34–53
		Outer antisense	5′-CCTTCTCTGGAGCCTAATCC-3′	323–304
		Inner sense	5′-GCGGCCATGACACCATCAAG-3′	91–110
		Inner antisense	5′-AAAATGTCTCTTCCCCATAA-3′	212–193

tech) according to the manufacturer's procedure. Hybridization was performed overnight at 50°C in fresh hybridization solution containing radiolabelled probe of 5×10^6 cpm/ml (specific activity 0.3×10^9 dpm/μg). The membrane was washed under conditions of high stringency (the final wash was performed in 0.1× SSPE, 0.1% SDS at 68°C) and analyzed in a Cyclone phosphorimager (Packard, Meriden, CT).

Cell cultures and co-cultivation

Cord blood lymphocytes were purified by Ficoll gradient and cultured for 72 hr in growth medium (RPMI 1640 medium [GIBCO-BRL, Bethesda, MD] with 10% [v/v] FCS) supplemented with phytohemagglutinin (5 μg/ml, Sigma, St. Louis, MO). Such pre-activated cord blood lymphocytes were co-cultivated with Ficoll-purified PBMC from the HRV-5-positive patient in ratios 1:1, 1:2, 1:4 and 1:8. Co-cultures were then maintained in growth medium containing 100 U/ml of human recombinant interleukin-2 (Biogene, Riga, Latvia). The cell morphology and growth activity were observed by light microscopy daily during 20 days.

RESULTS

Frequency of HRV-5 PCR positivity in peripheral blood of blood donors

A nested HRV-5-specific PCR was used to detect the presence of HRV-5 proviral sequence in DNA isolated from whole blood of 150 Latvian and 150 Swedish blood donors, of which all were randomly selected. DNA quality and HRV-5 PCR sensitivity were monitored. All were negative in the POL1 PCR for the HRV-5 homologous sequences (Table II).

Frequency of HRV-5 sequence in peripheral blood cells DNA from patients with neurologic disorders

DNA was isolated from whole blood of 21 patients with different pathologies of the central and peripheral nervous system (Table II). Thirteen of them had demyelinating diseases (7 multiple sclerosis and 6 Guillain-Barré syndrome); 8 had other neurologic disorders. All samples were positive in a globin PCR but negative in PCR with primer set POL1. Positive controls were positive in all amplifications.

TABLE II – DNA SAMPLES ISOLATED FROM PERIPHERAL BLOOD CELLS STUDIED FOR PRESENCE OF HUMAN RETROVIRUS-5 (HRV-5) DNA

Sample/origin/diagnosis	Number examined	Number positive for HRV-5 DNA	P^1
Apparently normal blood donors			
Latvian	150	0	
Swedish	150	0	
Total	300	0	
Patients with hematological malignancies			
Acute leukemia	9	0	
Chronic lymphocytic leukemia	30	0	
Non-Hodgkin's lymphoma	22	3	0.0003
Hodgkin's disease	5	0	
Other non-lymphoid malignancies	15	0	
Total	81	3	
Patients with nervous system disease			
Demyelinating diseases	13	0	
Other neurological disorders	8	0	
Total	21	0	

[1] Comparison of the frequency of HRV-5 DNA positivity of NHL samples with those of blood donors using Fisher exact test.

Detection of HRV-5 sequence in peripheral blood cells DNA from patients with various forms of hematological malignancy

DNA was isolated from whole blood of 81 Latvian patients with different forms of hematological malignancy. All samples were positive using a globin PCR. In a nested POL1 PCR, 3 samples (3.7%) were positive for HRV-5 proviral DNA (Table II). All 3 were also positive in the somewhat less sensitive PCR with PRO primers (Table I). Although the sensitivity of POL2 primers was identical to that of POL1 in plasmid titrations, all 3 positive samples were negative in PCR with POL2 primers (Griffiths *et al.*, 1997) (Fig. 1). The amplified sequences were 98% identical to the original HRV-5 sequence (Genbank U46939) and showed 7 (113h) to 12 (222h) unique nucleotide differences in comparison with the sequence of plasmid pHRV5.1, which was used as a positive control (Fig. 2). They were 95% identical to each other. In the RT amplimers, relative to the U46939 nucleotide sequence, 113h had 3 substitutions, all ambiguous. 117h had 7 substitutions, of which 2 resulted in amino acid substitutions. 222h had 8 substitutions, of which none resulted in amino acid exchanges. In the protease amplimers, 113h had 1, 117h had 2 and 222h had 1 substitution, resulting in 1, 2 and 2 amino acid substitutions, respectively. Disregarding the ambiguous substitutions, no mutations into stop codons were observed in both amplimers.

From one of the patients (117h), DNA was isolated from whole blood and purified PBMC simultaneously. DNA isolated from whole blood was repeatedly positive in PCR by using POL1 and PRO primers. In contrast, DNA isolated from purified PBMC was negative with all sets of primers, while plasmid controls were positive (Fig. 3). This patient was initially diagnosed as a case of chronic lymphatic leukemia and had an elevated leukocyte count due to circulating malignant cells. We estimate that approximately 80% of the total leukocyte count (20×10^9/L) was due to his disease. In addition, a more pure population of tumour cells was obtained from pleural exsudate of patient 117h. DNA isolated from them was negative in PCR with all 3 HRV-5-specific primer sets (POL1, POL2 and PRO). As with the other PCRs, 1 μg of DNA was used in the PCR. This absence of PCR signal therefore indicated that the HRV-5 sequences were not integrated in a majority of the malignant lymphocytes. However, DNA from cells adhering to plastic selected out of the peripheral blood of patient 117h was positive in PCR for HRV-5 with primer set POL1. For this amplification, 95 ng of isolated DNA was used. DNA samples isolated from the whole blood and PBMC of patient 117h as well as DNA isolated from whole blood of an HRV-5 PCR-negative blood donor were then digested by restriction enzymes Eco RI and Hind III and analyzed in Southern blot hybridization. HRV-5-containing plasmid in dilution was used as a positive control. After hybridization with the 931 bp-specific probe (Eco RI-Hind III cut from pHRV5.1) labelled to a specific activity of 0.3×10^9 dpm/μg under high-stringency washing conditions, a smear of hybridization was observed in the tracks with DNA isolated from the whole blood of the HRV-5 PCR-positive patient 117h (Fig. 4). Bands or a smear were observed neither with DNA samples isolated from PBMC of the same patient nor with the DNA samples isolated from the whole blood of a blood donor.

Co-cultivation of PBMC of patient 117h with phytohemagglutinin (PHA) pre-activated cord blood lymphocytes in ratios 1:1, 1:2, 1:4 and 1:8 in presence of T-cell growth factor (IL-2) showed neither morphological changes reminiscent of a cytopathic effect nor growth alterations suggestive of immortalization compared with uninoculated controls.

The HRV-5-positive patients with hematological disease

All 3 HRV-5-positive patients were inhabitants of Latvia. They were diagnosed to have a B-cell non-Hodgkin's lymphoma of low-grade malignancy. The Kiel lymphoma (Chabner *et al.*, 1980; Stansfeld *et al.*, 1988) and other (Vose *et al.*, 1996) classification systems were followed. One was a female (113h) and 2 were male (117h, 222h) aged 45, 64 and 59 years, respectively.

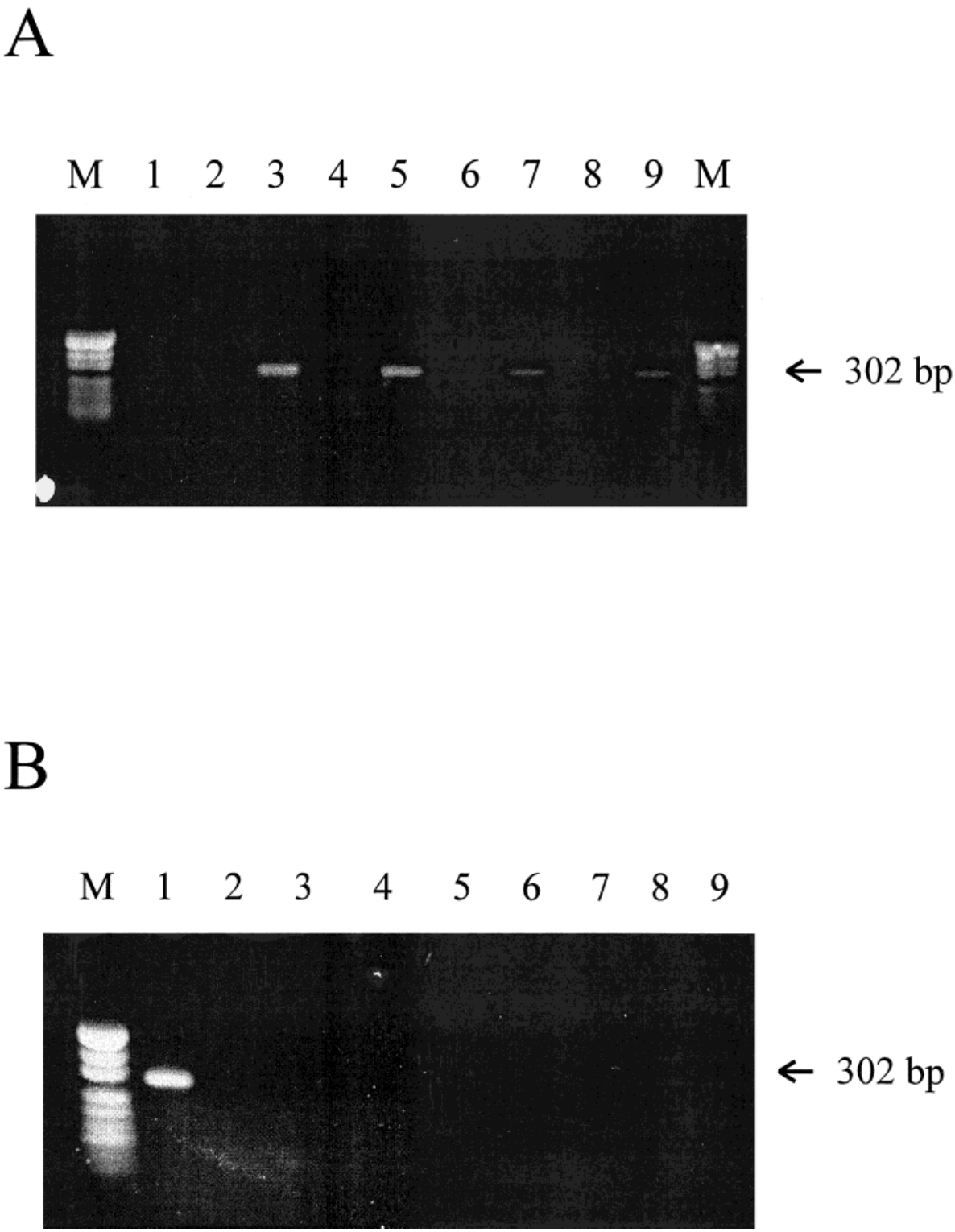

FIGURE 1 – (*a*) Sensitivity of nested PCR for HRV-5 (primer set POL2 according to Table I; Griffiths *et al.*, 1997). Amplification products with serially diluted plasmid pHRV5.1 are shown. Lane 1 contained products of a reaction with 0.4 ag; lane 3, 4 ag; lane 5, 40 ag; lane 7, 400 ag; lane 9, 4 fg of pHRV5.1. Lanes 2, 4, 6, 8 are water controls handled as the plasmid-containing samples. Lane M, pUC19 DNA digested with MspI (Fermentas, Vilnius, Lithuania). Plasmid DNA was amplified in the presence of 500 ng of human genomic DNA. The sensitivity of detection using nested PCR with POL2 thus was 4 ag, which approximately corresponds to 1 molecule of HRV-5 per reaction. (*b*) Nested PCR for HRV-5 (primer set POL2 according to Table I) on DNA from whole peripheral blood of hematological patients. Lane 1, positive control plasmid; lanes 2, 4, 6, 8, DNA samples from blood donors; lanes 3, 5, 7, DNA samples from patients 222h, 113h and 117h, respectively; lane 9, water control; lane M, pUC19 DNA digested with MspI (Fermentas). Although the POL2 primer set was as sensitive as primer set POL1 in sensitivity titrations, it could not amplify from NHL patient DNA, in contrast with primer sets POL1 and PRO, which were positive in all 3 NHL patients.

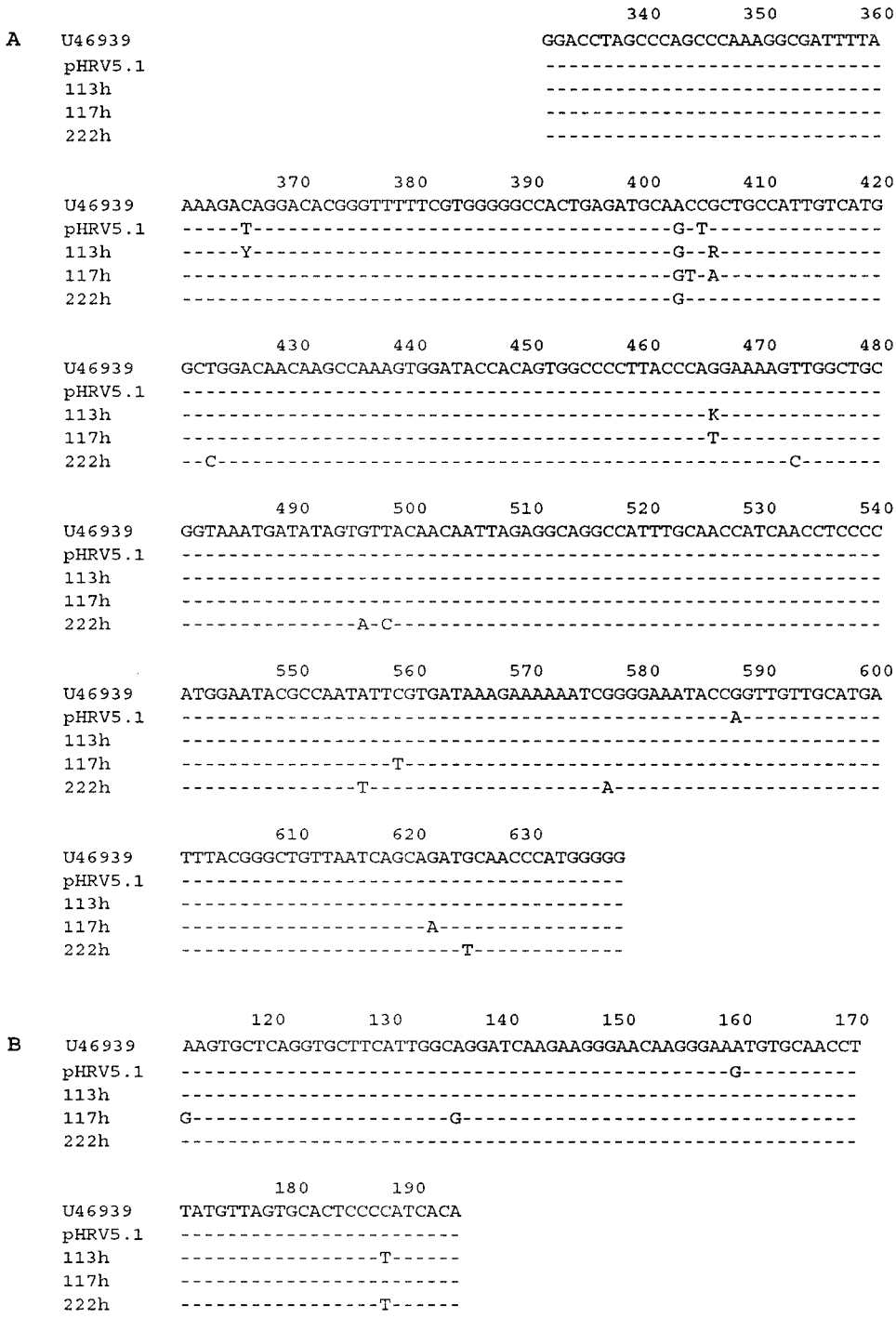

FIGURE 2 – Nucleotide sequence and deduced amino acid sequences of human retrovirus-5 (HRV-5) *pol* region amplified from peripheral blood DNA of patients 113h, 117h and 222h with the POL1 primer set. The DNA sequence is shown aligned with the original HRV-5 clone 6 and positive control plasmid pHRV5.1. A dash indicates identity with the original clone. Primer sequences are omitted. Y indicates C or T, R indicates A or G and K indicates G or T. (*b*) Nucleotide and translated amino acid sequences from HRV-5 *pro* region amplified from peripheral blood cell DNA of the same patients with the PRO primer set. The DNA sequence is shown aligned with the original HRV-5 clone (Griffiths *et al.,* 1997) and positive control plasmid pHRV5.1. Primer sequences are omitted.

Patient 113h had a decreased leukocyte count (4.5×10^9/L) with 67.8% (3.05×10^9/L) lymphocytes, 6.7% (0.3×10^9/L) granulocytes and 25.5% (1.15×10^9/L) monocytes. Blood analyses were performed by using the Roche (Basel, Switzerland) Micros analyzer. Lymph nodes in the abdominal cavity were enlarged from 2–4 cm to 5–7 cm, as recorded by ultrasound examination. Enlargement of intrathoracal lymph nodes was also observed using X-ray examination. The spleen was enlarged to 16×6 cm using ultrasound examination. Using X-ray examination, destructive changes in the first lumbal vertebra were observed. The change in the vertebra was not studied histologically because the patient declined investigation. The diagnosis, low-

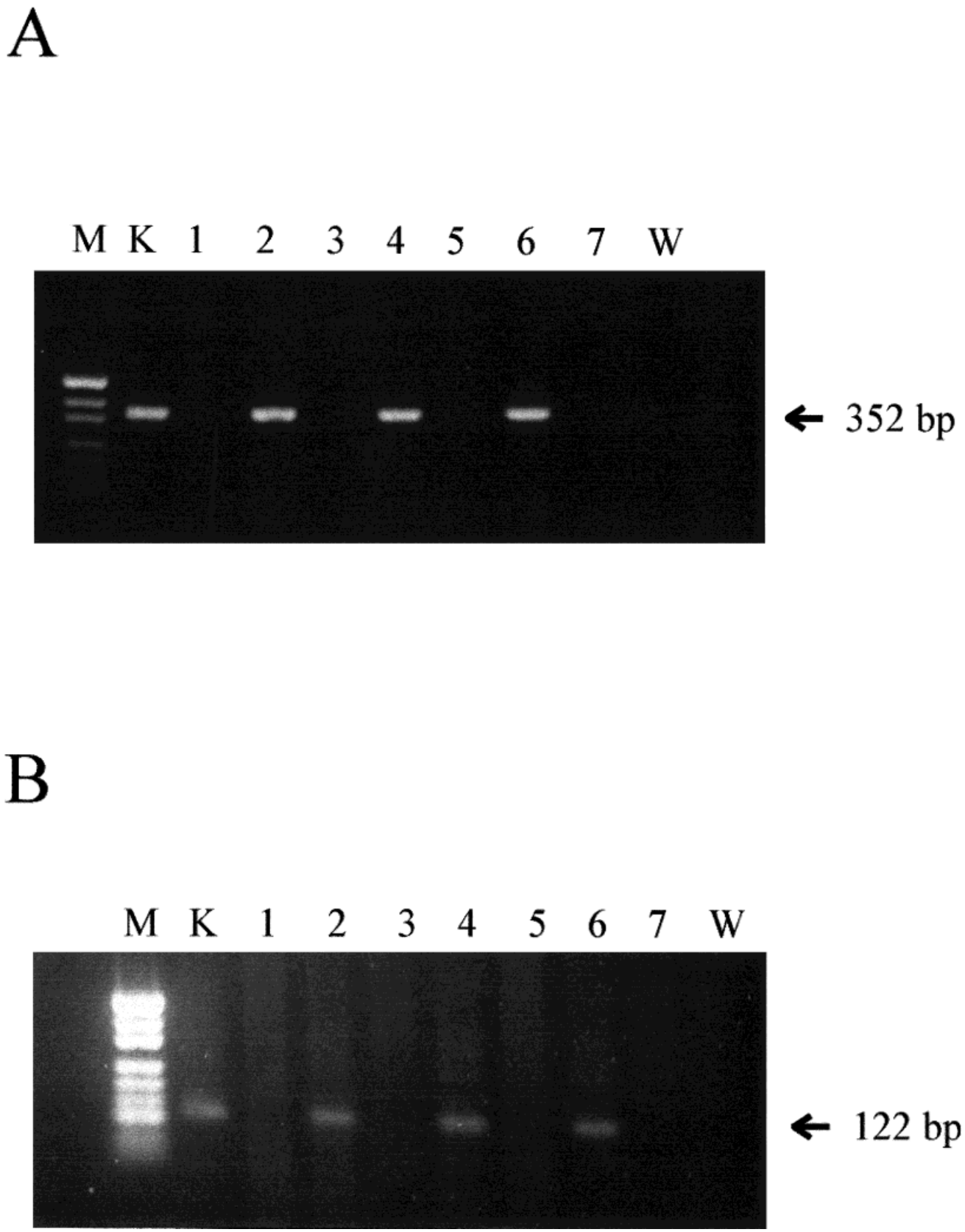

FIGURE 3 – Nested PCR for HRV-5 on DNA samples from hematological patients. (*a*) The results of PCR with primer set POL1 (Griffiths *et al.*, 1997) according to Table I. (*b*) The results of PCR with primers targeted to *pro* region of HRV-5 (primer set PRO according to Table I). Lanes 2, 4, 6 contain amplification products from whole peripheral blood DNA of patients 222h, 113h and 117h, respectively. Lanes 1, 3, 5 contain products from whole peripheral blood DNA samples from 3 Swedish blood donors. Lane 7, amplification product from DNA isolated from purified PBMC of patient 117h; lane K, amplification product from the positive control plasmid; lane W, water control; lane M, pUC19 DNA digested with MspI (Fermentas).

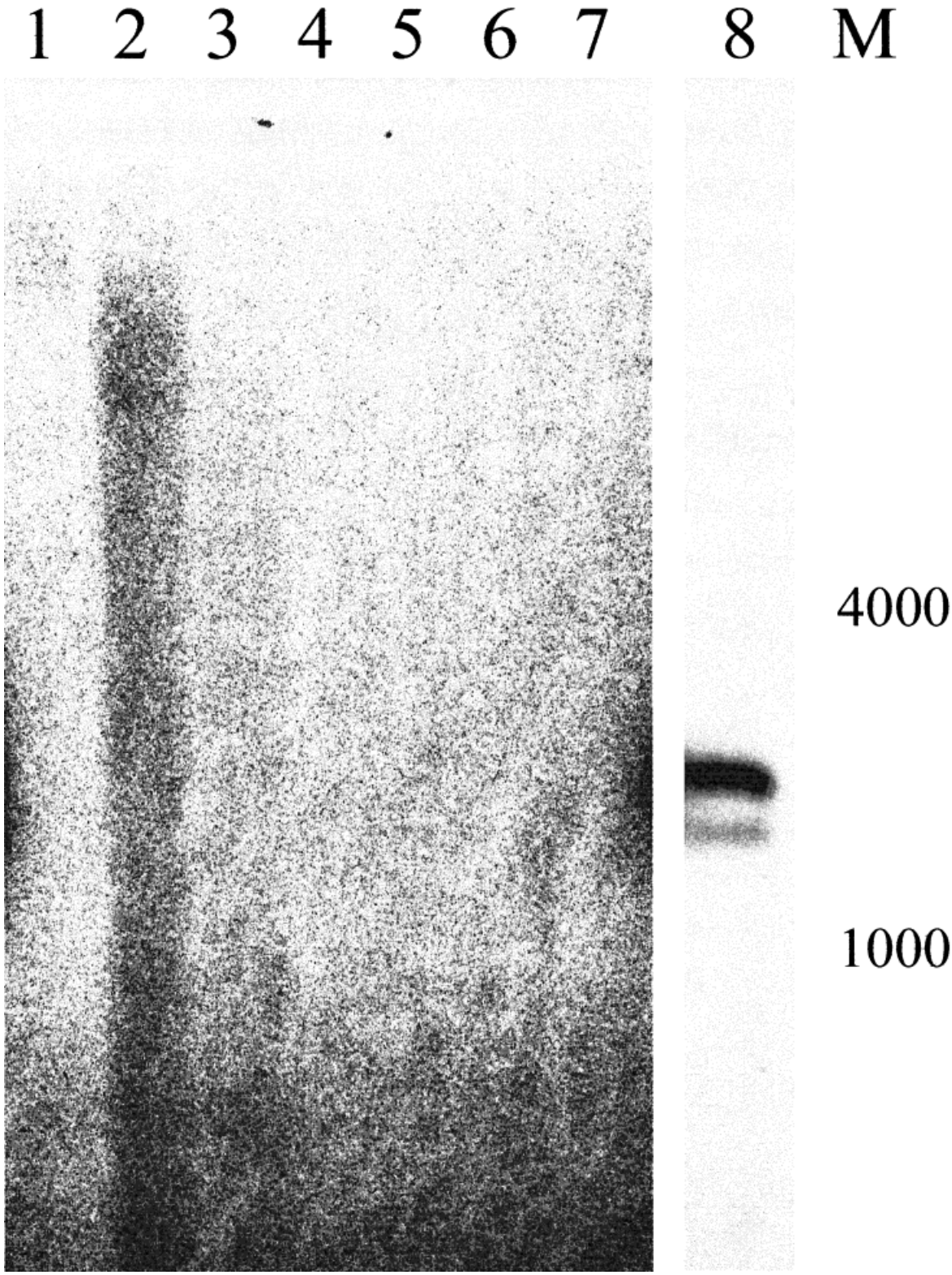

FIGURE 4 – Southern blot of human genomic DNA isolated from whole blood and PBMC of patient 117h. The plasmid pHRV5.1 was labelled by ^{32}P, hybridised and washed under high stringency. Lane 1, undigested DNA from whole blood of patient 117h; lanes 2, 3, DNA from whole blood of patient 117h digested with Eco RI and Hind III, respectively; lanes 4, 5, DNA from PBMC of the same patient digested with Eco RI and Hind III, respectively; lanes 6, 7, DNA from whole blood of a blood donor digested with Eco RI and Hind III, respectively; lane 8, 100 pg of cloned HRV-5 sequence (pHRV5.1). M, approximate positions of molecular sizes derived from the known size of the control plasmid (3,879 bp) and a marker (Amersham Pharmacia Biotech; product 27-4001). Lanes from larger amounts of plasmid were edited out of the picture because of their very strong hybridisation signals. The intensity levels were adjusted in the phosphorimager to bring out weak signals in the patient DNA lanes and to decrease the intensity of plasmid signals.

grade non-Hodgkin's lymphoma, was proven by histology and immunohistochemical examination of bone marrow ($CD20^+$, $CD3^-$). The patient died 4 years after onset of the disease. No autopsy was carried out.

Patient 117h had an evaluated leukocyte count (19.7×10^9/L) with 78.0% (15.35×10^9/L) lymphocytes, 15.0% (2.95×10^9/L) granulocytes and 7.0% (1.4×10^9/L) monocytes. An enlargement of all groups of superficial lymph nodes was observed. The spleen was palpable 4 cm below left costal margin and the liver 2 cm below it. A chest radiograph showed fluid in both pleural spaces. A pleural biopsy showed infiltration with small lymphocytes. The diagnosis, low-grade non-Hodgkin's lymphoma, was proven by bone marrow histology and immunohistochemical examination ($CD20^+$, $CD3^-$).

Patient 222h also had an elevated leukocyte count (17.5×10^9/L) with 69.2% (12.7×10^9/L) of lymphocytes, 15.2% (2.65×10^9/L) granulocytes and 15.6% (2.78×10^9/L) monocytes. Splenomegaly (14×5 cm) and lymph node enlargement in the abdominal cavity (parapancreatical, para-aortal and perisplenic) were observed upon ultrasound examination. The diagnosis, low-grade non-Hodgkin's lymphoma, was proven by histology and immunohistochemical examination of the lymph node and bone marrow ($CD20^+$, $CD3^-$).

None of the 3 patients had a history of arthritis.

DISCUSSION

Of 402 persons investigated for the presence of HRV-5 DNA in whole blood, 3 were positive. All 3 had a non-Hodgkin's lymphoma (NHL). They had the following common features: 1. the lymphoma was of low-grade malignancy; 2. all were of B-cell origin; 3. they all had a proliferation of malignant cells in the spleen and abdominal lymph nodes, leading to splenomegaly and abdominal lymph node enlargement; 4. 2 of them had metastases of lymphoma in atypical sites: the pleural cavity and lumbal vertebrae.

Counting with sequences amplified by both functional nested primer sets (POL1, PRO, Table I), the 3 sequences were 98% identical with the original HRV-5 sequence (Genbank U46939). However, the difference to it, to the sequence of plasmid pHRV-5.1 used as a positive control and the differences between the 3 NHL-derived sequences were sufficient to ascertain that all 3 were independent HRV-5 sequences and not a result of PCR contamination. The percent of identity between themselves was 96% and 95% in *pro* and *pol* regions, correspondingly. In addition, the negative results in PCR with one of the primer sets targeted to the *pol* region of HRV-5 (POL2, Table I) in the presence of a positive plasmid pHRV5.1 control advocated against our new clones being PCR contaminants. Judging from the sensitivity of the primer set designed by ourselves and targeted to the *pro* region of HRV-5 (PRO, Table I), which is 40 ag (equivalent to 10 molecules of HRV-5) per PCR reaction, we concluded that each of the patients had at least 10 HRV-5 molecules per 1×10^5 peripheral blood cells. The nearly negative Southern blot results with whole blood DNA from patient 117h, while the probe hybridized to a positive-control plasmid, also showed that HRV-5 DNA was present in low abundance. After a long exposure in the phosphorimager, a weak high m.w. spot was seen with undigested DNA, while a weak diffuse smear of hybridization was detected in whole blood DNA after Eco RI and in a lower m.w. range after Hind III digestion. The completeness of restriction enzyme digestion demonstrated by ethidium bromide staining and the sharp bands obtained with control plasmid indicate that this interpretation of the weak signals is valid. Similar results were obtained in 2 experiments. Although the rest of the HRV-5 genome is not yet published, there are no Eco RI and Hind III restriction sites within the 932 bp stretch of HRV-5 (U46939; DNASTAR Windows MapDraw version 3.04a). Using the phosphorimager software (Optiquant), the signals of the undigested, EcoRI and HindIII digested DNAs were estimated to be approximately 10,000-fold less than that of the 100 pg positive control plasmid. This would indicate that roughly 10 fg (2,500 molecules) HRV-5 DNA was present in the lanes containing whole blood DNA from patient 117h. Although the interpretation of the SB pattern is admittedly tenuous, it is compatible with a random integration of HRV-5 in a few blood cells. A similar conclusion has been reached by Griffiths *et al.,* (1997) and Rigby *et al.,* (1997, 1998) using PCR with salivary gland DNA from Sjögren's syndrome patients and lymph node biopsy DNA from 3 non-Hodgkin's lymphoma patients, although no Southern blot signals were detected. Judging from the absence of both PCR and Southern blot signals from PBMC DNA but presence in total blood DNA of patient 117h, the target cell for HRV-5 could be one of the minor blood cell populations, *i.e.,* granulocytes or monocytes. Our finding of HRV-5 DNA in DNA extracted from adherent cells of patient 117h's blood suggested that the preferred targets for HRV-5 may be monocytes/macrophages. This is also compatible with the absence of morphological or growth alterations suggestive of cytopathic effect or immortalization after co-cultivation of 117h PBMC with cord blood lymphocytes.

The absence of HRV-5 DNA in DNA extracted from PBMC and cells of pleural exsudate of patient 117h, which were dominated by malignant cells, indicates that HRV-5 is not directly causing the lymphoma through insertional *cis*-activation of oncogenes. The data could, however, be compatible with an indirect, *trans*-activating mechanism.

All 3 HRV-5-positive B-cell non-Hodgkin's lymphoma patients are inhabitants of Latvia. In our investigation, 3 of 22 patients (14%) with non-Hodgkin's lymphoma were positive for HRV-5 DNA. The HRV-5 DNA-positive were 3.7% of all examined patients with hematological malignancies. At the same time, no one of the 300 blood donors, 150 from Latvia and 150 from Sweden, as well as no one of 21 Latvian patients with various diseases of the nervous system were positive for HRV-5 DNA. The difference in frequency of HRV-5 DNA positivity between blood donors and NHL patients was statistically significant (0/300 *vs.* 3/22 gives a p value of 0.0003 judged by the Fisher exact test). In an earlier publication (Rigby *et al.,* 1998), HRV-5 DNA was found in lymph node biopsies from 3 of 51 NHL patients but also in a lymph node biopsy from one healthy individual. The number of patients and controls were too low to allow conclusions regarding an association between NHL and HRV-5. Adding their data to ours, there are totally 6 HRV-5 DNA-positive cases known in 73 NHL patients, *i.e.,* a frequency of 8.2% in NHL patients. It has earlier been suggested that HRV-5 may occur in few individuals in the normal population (Griffiths *et al.,* 1997; Rigby *et al.,* 1997, 1998). This was primarily based on salivary gland and lymph node biopsies. Our lack of detection in whole blood from 300 blood donors may be reconciled with these data if one assumes that salivary glands and lymph nodes tend to contain more virus than peripheral blood. The recent report of a 4- to 5-fold lower frequency of detection of HRV-5 DNA in whole blood compared with arthritic synovial tissue in rheumatoid arthritis patients (Griffiths *et al.,* 1999) lends some support to this interpretation. Thus, although we may have missed some blood donors with low levels of HRV-5 DNA, the finding of HRV-5 DNA in whole blood of 3 NHL patients could mean that the virus is more abundant in these patients than in asymptomatic carriers. Our ability to obtain a weak Southern blot signal from one of them fits with this explanation. Our data support that HRV-5 is an exogenous retrovirus present in low levels in some humans. However, the tools for demonstrating the presence of HRV-5 are incomplete. The complete genome must be cloned and sequenced to create reliable serologies using recombinant proteins or synthetic peptides. Optimal virus culture techniques must be developed.

It is too early to consider whether there is a causal association between HRV-5 DNA positivity and low-grade NHL with the features described above. The clarification of this issue is important because of the increasing incidence of NHL in many countries.

In Sweden, the incidence increased 4-fold from 1960 to 1995. The incidence per 100,000 inhabitants in Latvia in 1997, according to data from the Latvian Cancer Incidence Register, has been 3.1 for non-Hodgkin's lymphoma and 6.3 for chronic lymphocytic leukemia. As a comparison, the incidence per 100,000 inhabitants in Sweden during 1995 was 16.8 for non-Hodgkin's lymphoma, excluding chronic lymphatic leukemia, and 4.4 for chronic lymphocytic leukemia (Swedish National Board of Health and Welfare 1998). The reasons for this increase are largely obscure. The reported association between rheumatoid arthritis and NHL (see *e.g.,* Kinlen, 1992) is of special interest here because of an association between HRV-5 and rheumatoid arthritis (Griffiths *et al.,* 1999). In addition, blood transfusion has been associated with an increased risk of NHL and skin cancer (Blomberg *et al.,* 1993; Brandt *et al.,* 1996), although this has been disputed by another study performed under somewhat different conditions (Adami *et al.,* 1997). There is therefore a great need to ascertain whether HRV-5 is a factor behind NHL.

ACKNOWLEDGEMENTS

We thank Dr. D. Griffiths for the gift of plasmid pHRV5.1. We are indebted to Drs. G. Nemceva and I. Danilane, the State Blood Donor Center, Riga, Latvia, for access to the donors' blood samples and to Dr. I. Logina, Department of Neurology, P. Stradin Clinical Hospital, for access to the blood samples of patients with neurologic diseases.

REFERENCES

ADAMI, J., NYREN, O., BERGSTROM, R., EKBOM, A., MCLAUGHLIN, J.K., HOGMAN, C., FRAUMENI, J.F. JR. and GLIMELIUS, B., Blood transfusion and non-Hodgkin's lymphoma: lack of association. *Ann. intern. Med.,* **127,** 365–371 (1997).

ALTSCHUL, S.F., MADDEN, T.L., SCHAFFER, A.A., ZHANG, J., ZHANG, Z., MILLER, W. and LIPMAN, D.J., Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.,* **25,** 3389–3402 (1997).

ANDERSSON, M-L., LINDESKOG, M., MEDSTRAND, P., WESTLEY, B., MAY, F. and BLOMBERG, J., Diversity of human endogenous class-II-like sequences. *J. gen. Virol.,* **80,** 255–260 (1999).

BARRÉ-SINOUSSI, F., CHERMANN, J.C., REY, F., NUGEYRE, M.T., CHAMARET, S., GRUEST, J., DAUGUET, C., AXLER-BLIN, C., VEZINET-BRUN, F., ROUZIOUX, C., ROZENBAUM, W. and MONTAGNIER, L., Isolation of a T-lymphotropic retrovirus from a patient at risk for acquired immune deficiency syndrome (AIDS). *Science,* **220,** 868–871 (1983).

BLOMBERG, J., MÖLLER, T., OHLSSON, H., ANDERSSON, H. and JONSSON, M., Cancer morbidity in blood recipients. *Europ. J. Cancer,* **29A,** 2101–2105 (1993).

BRANDT, L., BRANDT, J., OLSSON, H., ANDERSSON, H. and MÖLLER, T., Blood transfusion as a risk factor for non-Hodgkin's lymphoma. *Brit. J. Cancer,* **73,** 1148–1151 (1996).

CHABNER, B.A, FISHER, R.I., YOUNG, R.C. and DEVITA, V.T., Staging of non-Hodgkin's lymphoma. *Semin. Oncol.,* **7,** 285–291 (1980).

CLAVEL, F. AND 12 OTHERS, Isolation of a new human retrovirus from West African patients with AIDS. *Science,* **233,** 343–346 (1986).

GRIFFITHS, D.J., VENABLES, P.J.W., WEISS, R.A. and BOYD, M.T., A novel exogenous retrovirus sequence identified in humans. *J. Virol.,* **71,** 2866–2872 (1997).

GRIFFITHS, D.J. and 13 OTHERS, Detection of human retrovirus 5 in patients with arthritis and systemic lupus erythematosus. *Arthritis Rheum.,* **42,** 448–454 (1999).

KALYANARAMAN, V.S., SARNGADHARAN, M.G., ROBERT-GUROFF, M., MIYOSHI, I., GOLDE, D. and GALLO, R.C., A new subtype of human T-cell leukemia virus (HTLV-II) associated with a T cell variant of hairy cell leukemia. *Science,* **218,** 571–573 (1982).

KINLEN, L.J., Malignancy in autoimmune diseases. *J. Autoimmun.,* **5** (Suppl A), 363–371 (1992).

LALEZARI, P. and KHORSHIDI, M., Neutrophil and platelet antibodies in immune neutropenia and thrombocytopenia. *In:* N.R. Rose, E. Conway de Macario, J.L. Fahey, H. Friedman and G.M. Peun (eds.), *Manuals of clinical laboratory immunology,* pp. 344–350, American Society for Microbiology, Washington, D.C. (1996).

MANZARI, V., GISMONDI, A., BARILLARI, G., MORRONE, S., MODESTI, A., ALBONICI, L., DE MARCHIS, L., FAZIO, V., GRADILONE, A., ZANI, M., FRATI, L. and SANTONI, A., HTLV-V: a new human retrovirus isolated in a Tac-negative T cell lymphoma/leukemia. *Science,* **238,** 1581–1583 (1987).

POIESZ, B.J., RUSCETTI, F.W., GAZDAR, A.F., BUNN, P.A., MINNA, J.D. and GALLO, R.C., Detection and isolation of type C retrovirus particles from fresh and cultured lymphocytes of a patient with cutaneous T-cell lymphoma. *Proc. nat. Acad. Sci. (Wash.),* **77,** 7415–7419 (1980).

RIGBY, S.P., GRIFFITHS, D.J., JARRETT, R.F., WEISS, R.A. and VENABLES, P.J.W., A new human retrovirus: a role in lymphoma? *Amer. J. Med.,* **104,** 99–100 (1998).

RIGBY, S.P., GRIFFITHS, D.J., WEISS, R.A. and VENABLES, P.J.W., Human retrovirus-5 proviral DNA is rarely detected in salivary gland biopsy tissues from patients with Sjögren's syndrome. *Arthritis Rheum.,* **40,** 2016–2021 (1997).

SAMBROOK, J., FRITSCH, E.F. and MANIATIS, T., Analysis of genomic DNA by Southern hybridization. *In:* N. Ford, C. Nolan and M. Ferguson (eds.), *Molecular cloning,* sections 9.31–9.62, Cold Spring Harbor Laboratory Press, New York (1989).

STANSFELD, A.G., DIEBOLD, J., NOEL, H., KAPANCI, Y., RILKE, F., KELENYI, G., SUNDSTROM, C., LENNERT, K., VAN UNNIK, J.A., MIODUSZEWSKA, O. and WRIGHT, D.H., Updated Kiel classification for lymphomas. *Lancet,* 1, 292–293 (1988).

THE SWEDISH CANCER REGISTRY. Cancer incidence in Sweden 1995. Swedish National Board of Health and Welfare, Stockholm (1998).

VANDAMME, A.M., FRANSEN, K., DEBAISIEUX, L., MARISSENS, D., SPRECHER, S., VAIRA, D., VANDENBROUCKE, A.T. and VERHOFSTEDE, C., Standardisation of primers and an algorithm for HIV-1 diagnostic PCR evaluated in patients harbouring strains of diverse geographical origin. *J. Virol. Meth.,* **51,** 305–316 (1995).

VOSE, J.M., BIERMAN, P.J. and ARMITAGE, J.O., Diagnosis and treatment of non-Hodgkin's lymphoma of adults. *In:* P.H. Wiernik, G.P. Canellos, J.P. Dutcher and R.A. Kyle (eds.), *Neoplastic diseases of the blood,* pp. 907–911, Churchill Livingstone, New York (1996).