

# Quantitative Analyses of Messenger RNA of Human Endogenous Retrovirus in Patients with Systemic Lupus Erythematosus

HITOSHI OGASAWARA, TOSHIO NAITO, HIROSHI KANEKO, TAKASHI HISHIKAWA, IWAO SEKIGAWA, HIROSHI HASHIMOTO, YUTARO KANEKO, NORIO YAMAMOTO, NAOKI MARUYAMA, and NAOKI YAMAMOTO

**ABSTRACT. Objective.** Human endogenous retrovirus (HERV) has emerged as a possible causative agent of systemic lupus erythematosus (SLE). To investigate the role of HERV in the etiology of SLE, we performed quantitative analyses of messenger RNA (mRNA) of the HERV clone 4-1 in patients with SLE.

**Methods.** Reverse transcriptase (RT)-polymerase chain reaction (PCR) and real-time quantitative PCR (RQ-PCR; TaqMan methodology) were used in this experiment.

**Results.** The quantities of mRNA of the HERV clone 4-1 *gag* region in patients with SLE were significantly higher than in healthy controls, and the amounts of such mRNA in the patients were decreased by steroid treatment.

**Conclusion.** These phenomena may be related to the production of viral components derived from HERV clone 4-1 and contribute to the pathogenesis of SLE; studies using a larger number of patients are required to confirm these points. (J Rheumatol 2001;28:533-8)

## Key Indexing Terms:

HUMAN ENDOGENOUS RETROVIRUS

SYSTEMIC LUPUS ERYTHEMATOSUS

TAQMAN METHOD

Endogenous retroviruses are known to exist not only in animals, but also in human genomic DNA<sup>1,2</sup>. Human endogenous retroviruses (HERV) generally lack the extracellular phase characteristic of retroviruses and they are inherited as stable Mendelian genes. Since HERV make up 0.1 to 0.6% of human DNA, they contribute substantially to the architecture of the human genome<sup>1</sup>.

Several reports have suggested that expression of HERV proteins induced by the activation of HERV sequences plays an important role in the induction of autoimmune diseases,

including SLE<sup>3-8</sup>. We reported the detection of serum antibodies to HERV clone 4-1 products (p30<sup>gag</sup>) in patients with SLE<sup>9</sup>. In certain patients with SLE, viral component parts of HERV clone 4-1 such as p30<sup>gag</sup> may be produced and contribute to the induction of SLE related immune dysfunction. We performed detection and quantitative analysis of mRNA of the HERV clone 4-1 *gag* region in patients with SLE and examined the effects of steroid treatment on the expression of clone 4-1 *gag* mRNA. We discuss the possible role of HERV clone 4-1 in the induction of SLE.

## MATERIALS AND METHODS

**Patients.** We studied 15 Japanese patients with SLE (women aged 20-48 yrs) diagnosed using the 1982 American Rheumatism Association revised criteria<sup>10</sup>. Control samples were obtained from age and sex matched normal healthy volunteers. The number of patients with cardinal clinical symptoms was as follows: renal disorder 7, hematologic disorder 2, neurologic disorder one, malar rash one, pulmonary disorder one, other symptoms 3. Three patients had not received any steroid and the remaining patients were treated with prednisolone for between one and 24 months. No patient received any immunosuppressive agents before the collection of samples. There were no pregnant subjects.

**Polymerase chain reaction (PCR) and sequencing.** Peripheral blood mononuclear cells (PBMC) were separated from SLE and control samples by centrifugation on a Ficoll-Paque cushion. Total RNA from patients' and controls' PBMC ( $1 \times 10^7$ ) was isolated by phenol and guanidine thiocyanate as described<sup>11</sup>. The cDNA from purified RNA was obtained with a First Strand cDNA Synthesis kit (Amersham International, Buckinghamshire, England) using avian myeloblastosis virus (AMV) RT. Briefly, mRNA (2  $\mu$ g) was incubated with 2.5  $\mu$ g of oligo-dT, 10  $\mu$ l of first strand synthesis reaction buffer, 2.5  $\mu$ l of sodium pyrophosphate solution,

From the Department of Internal Medicine and Rheumatology, and the Department of General Medicine, Juntendo University School of Medicine, Tokyo; the Department of Medicine, Juntendo University Izu-Nagaoka Hospital, Juntendo University School of Medicine, Shizuoka; Ajinomoto Co., Inc.; the Department of Microbiology, Tokyo Medical and Dental University School of Medicine; and the Department of Molecular Pathology, Tokyo Metropolitan Institute of Gerontology, Tokyo, Japan.

Supported by the Japan Human Science Foundation.

H. Ogasawara, MD; H. Kaneko, MD; T. Hishikawa, MD; H. Hashimoto, MD, Professor, Department of Internal Medicine and Rheumatology; T. Naito, MD, Department of General Medicine, Juntendo University School of Medicine; I. Sekigawa, MD, Associate Professor, Department of Medicine, Juntendo Izu-Nagaoka Hospital; Y. Kaneko, PhD, Ajinomoto Co., Inc.; N. Yamamoto, MD, Professor, Department of Microbiology, Tokyo Medical and Dental University School of Medicine; N. Maruyama, MD, Director, Department of Molecular Pathology, Tokyo Metropolitan Institute of Gerontology.

Address reprint requests to Dr. I. Sekigawa, Department of Medicine, Juntendo Izu-Nagaoka Hospital, Juntendo University School of Medicine, 1129 Nagaoka, Izu-Nagaoka-cho, Tagata-gun, Shizuoka 410-2295, Japan.

Submitted May 2, 2000 revision accepted September 27, 2000.

5 µl of dNTP mixture, and 5 µl of human placental ribonuclease inhibitor in the presence of AMV RT (20 units) at 42°C for 50 min.

HERV clone 4-1 was kindly provided by Dr. R. Repaske<sup>2</sup> (Figure 1). Reverse transcription (RT)-PCR of sample cDNA with adequate primers of the clone 4-1 *gag* region was performed using Clontech Amplimer sets (Clontech Laboratories, Palo Alto, CA, USA). The following primers were used: 5'-TCCCTGAGAGAGAGAGCA-3' (*gag* primer 1 in Figure 1) and 5'-TGTGCTTTGACTAACTAAAGC C-3' (*gag* primer 2 in Figure 1). *Gag* primer 1 is derived from the sequence encoding p30<sup>gag</sup> of clone 4-1<sup>2</sup>. *Gag* primer 2 is reported to be a universal primer derived from a highly conserved region of p30<sup>gag</sup><sup>12</sup>. For amplification, PCR involved 35 cycles of 94°C for 45 s to denature, 56°C for 45 s to anneal, and 72°C for 2 min to elongate in the standard PCR mixture (Perkin-Elmer Cetus, Emeryville, CA, USA). To examine the contamination of proviral DNA, samples containing mRNA without RT were also amplified under the same conditions. The PCR products were run on a 2% agarose gel containing 0.5 µg/ml of ethidium bromide.

To investigate the sequences of PCR products in SLE patients, the direct sequencing method was used, as described<sup>13</sup>.

**Quantitative analysis of mRNA of clone 4-1.** Quantitative analyses of mRNA were performed using RQ-PCR, as described<sup>14,15</sup>. TaqMan primers and probes were designed with the computer programs — Primer Express (Perkin-Elmer Applied Biosystems), and EuGen (Daniben System, Cincinnati, OH, USA). The following primers of clone 4-1 were used: 5'-CACATGGTGGAGAGTCGTGTTT-3' (1693–1714 nt) and 5'-GCTTGCGGCTTTTCAGTATAGG-3' (1772–1793 nt). TaqMan probe (Perkin-Elmer Applied Biosystems) of clone 4-1 *gag* region (5'-TTCACCTCTGCGGACCTTCTCAACTGGA-3'; 1729–1756 nt) was labeled with FAM reporter dye (6-carboxyterafluorescein) at the 5' end and with TAMRA (6-carboxyteramethylrhodamine) at the 3' end as the quencher dye. Reaction mixtures of 25 µl contained 10× TaqMan buffer (2.5 µl), 3.5 µM MgCl<sub>2</sub>, 0.5 µl dATP, dCTP, dGTP, and dUTP, 200 nM each primer, 100 nM probe, 0.25 units of AmpliTaq Gold DNA polymerase, 0.125 units of AmpErase uracil N-glucosidase (UNG) (TaqMan PCR core reagent kit, Perkin-Elmer Applied Biosystems), and 100 ng of sample DNA. PCR was completed in an ABI RPISM 7700 Thermal Cycler (Perkin-Elmer Applied Biosystems, Foster City, CA, USA) and included a 2 min 50°C UNG activation, a 10 s 95°C TaqGold activation and predenaturation, and 40 cycles, each consisting of a 15 s 95°C denaturation and 1 min 60°C annealing step. The standard curve composed of 5 points (10<sup>2</sup>, 10<sup>3</sup>, 10<sup>4</sup>, 10<sup>5</sup>, and 10<sup>6</sup> copies) was established using HERV clone 4-1 *gag* and *pol* cDNA (1630–2893 nt). Experiments were performed with duplicates for each data point.

**Statistical analysis.** Statistical analysis was performed using Student's *t* test.

## RESULTS

As shown in Figure 2, mRNA of clone 4-1 *gag* region was detected in SLE patients, but not controls, by RT-PCR using *gag* primers 1 and 2. Our sequence analysis indicated that there were much higher homologous sequences (roughly 95% homology) between this 660 bp mRNA in the SLE patients and the consensus sequence of clone 4-1 (data not shown).

Recently, a novel technology to measure the quantities of mRNA has become available<sup>14,15</sup>. This assay (the TaqMan method) exploits the 5'–3' nuclease activity of the Taq polymerase to detect and quantify specific PCR products as the reaction proceeds. Using this methodology, we performed quantitative analysis of clone 4-1 *gag* mRNA in these SLE patients and controls (Figure 2). As shown in Figure 3, this method also indicated higher quantitative levels of clone 4-1 *gag* mRNA in SLE patients compared with control levels. In the experiment using a greater number of patients, significant quantitative differences of this mRNA between SLE patients and controls were observed (Figure 4).

Furthermore, we examined the influence of steroid treatments on the expression of clone 4-1 *gag* mRNA in patients

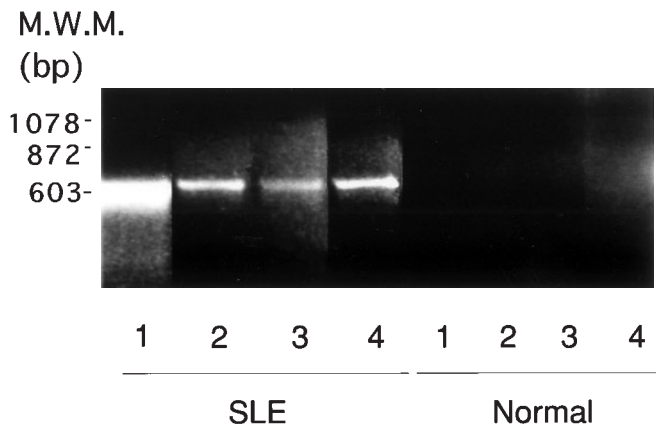


Figure 2. Detection of clone 4-1 mRNA in SLE and healthy individuals. MWM: molecular weight marker.

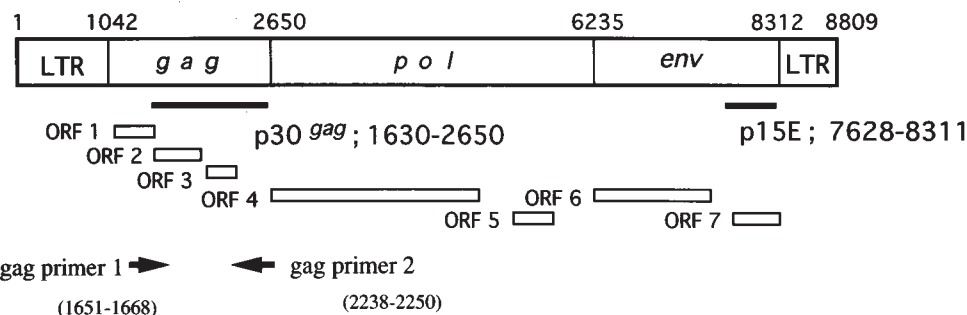


Figure 1. Structure of clone 4-1. Numbers indicate the nucleotide number. ORF: open reading frame.

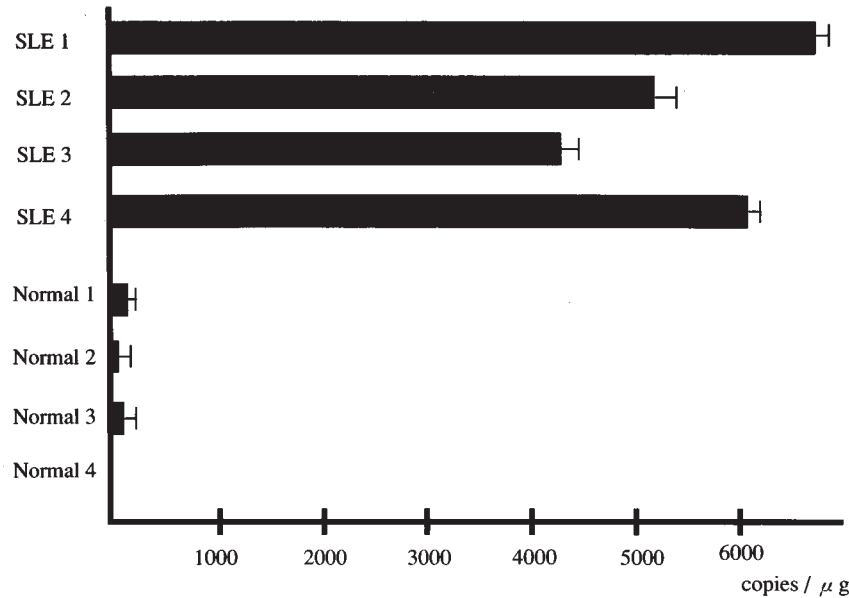


Figure 3. Quantitative analyses of clone 4-1 mRNA in SLE and normal controls. Bars indicate means and standard errors.

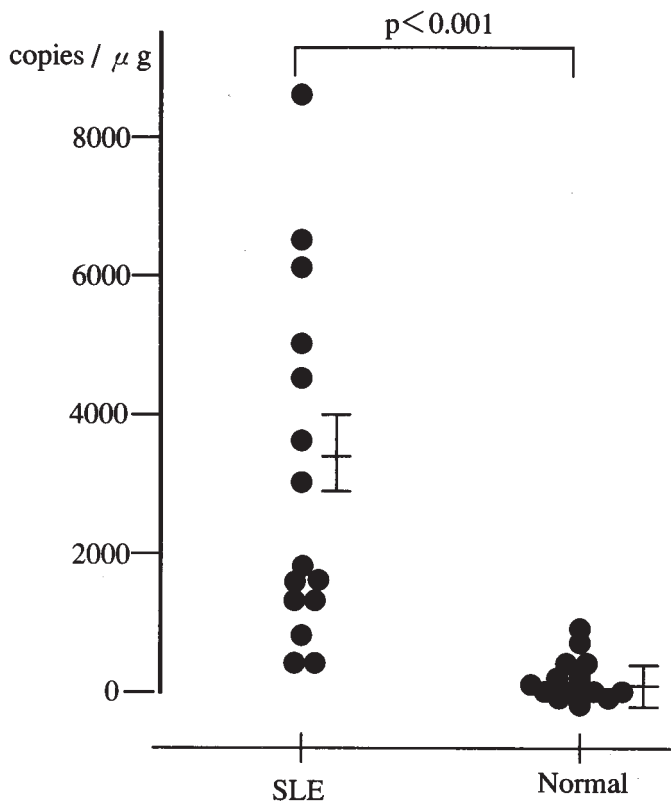


Figure 4. Comparison of the quantity of clone 4-1 mRNA between patients with SLE and normal controls.

with SLE. As shown in Figure 5, levels of such mRNA were decreased by the treatment. In addition, quantitative analyses by the TaqMan method also indicated a decreasing amount of mRNA in the same patients (Figure 6).

## DISCUSSION

A role for retroviruses, including endogenous retroviruses, as an etiologic factor in autoimmune diseases such as SLE is supported by the following evidence: (1) the importance of endogenous retroviruses in mouse models of SLE<sup>16-18</sup>; (2) the detection of antibodies, antigens, and sequences for animal and human retroviral components in the organs and serum of patients with autoimmune diseases such as SLE<sup>3-8,19-24</sup>; (3) electron microscopic detection of unknown retroviral particles in the organs of SLE patients<sup>25</sup>; (4) the finding that viral components derived from endogenous or exogenous retroviruses can induce the immune abnormalities observed in SLE<sup>26,27</sup>; and (5) the similarity of autoimmune manifestations and immune dysregulation between patients with SLE and those infected with known human retroviruses such as human immunodeficiency virus (HIV-1)<sup>28</sup>.

HERV clone 4-1, which widely exists in the genomic DNA of Japanese, is a member of the HERV-E family and has roughly 8.8 kb sequences including open reading frames in the *gag*, *pol*, and *env* regions<sup>2</sup>. There are between 32 and 50 copies of clone 4-1 per haploid genome and 11 integration sites of the HERV in chromosomes 2, 3, 4, 7, 9, 11, 17, 19, and 20<sup>29,30</sup>. Although studies indicate that retroviral p30<sup>gag</sup> proteins derived from nonhuman endogenous retrovirus are deposited in the glomeruli of patients with SLE, and serum antibodies to this nonhuman p30<sup>gag</sup> are detected in SLE patients<sup>19-22</sup>, we detected antibodies to clone 4-1 p30<sup>gag</sup> in 48.3% of Japanese patients with SLE, while the antibody was not detected in healthy control serum<sup>9</sup>. This indicates that the transcription of HERV clone 4-1 genes may be facilitated and virus particles or components may be

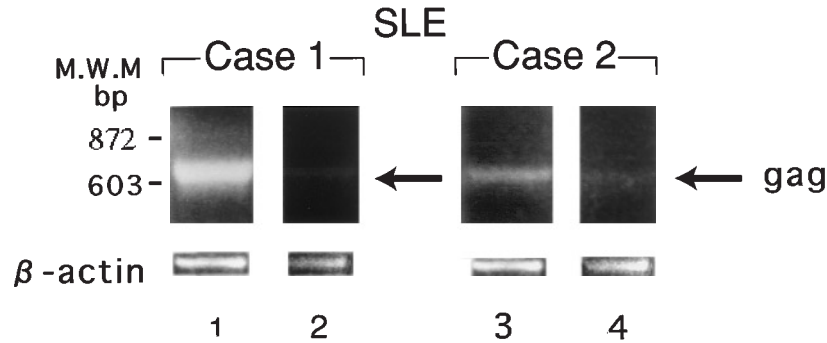


Figure 5. Effects of steroid treatment on detection of clone 4-1 mRNA: before (1, 3) and after (2, 4) steroid treatment in cases 1 and 2.

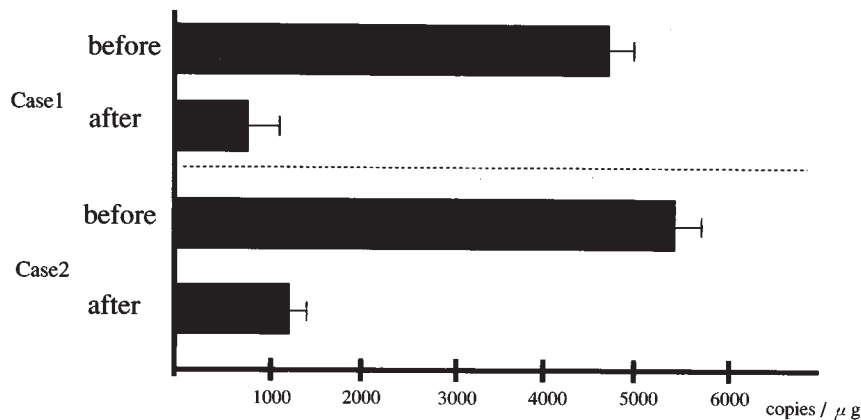


Figure 6. Quantitative changes of clone 4-1 mRNA before and after steroid treatment.

produced in some patients with SLE. Supporting this concept, HERV antigen in the lymphocytes of SLE patients was detected using antibodies to clone 4-1 *gag* products (data not shown), as well as the detection of clone 4-1 mRNA (Figure 2) in our laboratory. Similar data to those for the clone 4-1 *gag* region have also been obtained for the *env* region, although these results are not described here. Further, certain viral component parts derived from animal and human endogenous or exogenous retroviruses (such as p15E) can induce immune dysfunction as observed in patients with SLE, including CD4+ T cell activation and anergy, and polyclonal B cell activation<sup>26,27</sup>. Our recent results also indicated that clone 4-1 derived peptides could induce these cell abnormalities (data not shown). Studies indicate that the molecular mimicry between certain retrovirus components and autoantibodies is important for the development of autoantibody production<sup>22-24</sup>. In addition, such CD4+ T cell abnormalities may contribute to the breakdown of self-tolerance and the resultant induction of SLE related autoimmune phenomena, including autoantibody production, in cooperation with polyclonal B cell activation<sup>31</sup>.

We performed quantitative analyses of mRNA of HERV clone 4-1 and examined the effect of steroid on the expres-

sion of the mRNA using the TaqMan methodology. Several groups have shown that RQ-PCR with the TaqMan technology can be used for quantitative detection of human and animal viruses such as human hepatitis viruses and feline immunodeficiency viruses as an animal model for acquired immunodeficiency syndrome<sup>32,33</sup>. Our results indicated that the amount of mRNA of clone 4-1 *gag* region in SLE patients is greater than that of healthy controls (Figures 3 and 4), and that steroid treatments could reduce the expression of the mRNA (Figure 6). These results suggest that the transcription of clone 4-1 occurred in SLE patients and that steroid could inhibit this process. In addition to the immunosuppressive effects of steroid for lymphocyte functions in SLE, the inhibitory effect of HERV transcription through suppression of the promoter area of HERV may be important for the steroid efficacy in SLE. Indeed, other immunosuppressive agents such as cyclosporin A and FK506 are known to inhibit promoter activity of HIV-1 integrated in human genomes as proviruses and to suppress the transcription from them<sup>34</sup>.

Our computer search of current entries in sequence libraries (GenBank database) indicated that there were much higher homologies (over 95% homology) between the clone 4-1 *gag* region sequence in SLE detected by our methods

and the consensus sequence of clone 4-1 located in chromosome 11 of healthy individuals as compared to the other chromosomes<sup>35</sup>. This finding raises the possibility that the clone 4-1 transcribed in SLE may be derived from chromosome 11, although further sequence studies are required to clarify this<sup>35</sup>.

In controls, clone 4-1 mRNA was not detected using RT-PCR (Figure 2), but quantitative analysis using RQ-PCR revealed the detection of its mRNA, although the amount was extremely small (Figures 3 and 4). This discrepancy may depend on the difference of sensitivity between the RT-PCR and RQ-PCR methods. Further, our preliminary results indicated that 5-AZA-2'-deoxycytidine, a demethylation agent, made it possible to increase the amount of clone 4-1 mRNA in control lymphocytes detected by the TaqMan method, and that the stimuli of mitogens, such as concanavalin A, phytohemagglutinin, and lipopolysaccharide, could not induce the quantitative increase of clone 4-1 mRNA from normal lymphocytes (data not shown). These results indicate that methylation may play an important role in inhibiting the transcription from clone 4-1 genomic DNA to its mRNA in healthy individuals, as reported for other HERV<sup>36</sup>, and the expression of clone 4-1 mRNA is not the consequence of cell activations, although SLE lymphocytes are known to be activated *in vivo*<sup>31</sup>.

To clarify the pathogenic role of HERV clone 4-1, we are now investigating transcription and translation mechanisms of HERV clone 4-1, and relationships between the quantitative expression of clone 4-1 mRNA and clinical symptoms or disease activity in SLE using a larger number of patients.

## REFERENCES

- Leib-Mosch C, Brack-Werner R, Werner T, et al. Endogenous retroviral elements in human DNA. *Cancer Res* 1993;50:5636s-42s.
- Repaske R, Steele PE, O'Neill RR, Rabson AB, Martin MA. Nucleotide sequence of a full-length human endogenous retroviral segment. *J Virol* 1985;54:764-72.
- Herrmann M, Hagenhofer M, Kalden JR. Retroviruses and systemic lupus erythematosus. *Immunol Rev* 1996;152:145-56.
- Nakagawa K, Harrison LC. The potential roles of endogenous retroviruses in autoimmunity. *Immunol Rev* 1996;152:193-236.
- Banki K, Maceda J, Hurley E, et al. Human T-cell lymphotropic virus (HTLV)-related endogenous sequence, HRES-1, encodes a 28-kDa protein: a possible autoantigen for HTLV-1 gag-reactive autoantibodies. *Proc Natl Acad Sci USA* 1992;89:1939-43.
- Brookes SM, Pandolfino YA, Mitchell TJ, et al. The immune response to and expression of cross-reactive retroviral gag sequences in autoimmune disease. *Br J Rheumatol* 1992;31:735-42.
- Blomberg J, Nived O, Pipkorn R, Bengtsson A, Erlinge D, Sturfelt G. Increased antiretroviral antibody reactivity in sera from a defined population of patients with systemic lupus erythematosus: correlation with autoantibodies and clinical manifestations. *Arthritis Rheum* 1994;37:57-66.
- Herrmann M, Kalden JR. PCR and reverse dot hybridization for the detection of endogenous retroviral transcripts. *J Virol Methods* 1994;46:333-48.
- Hishikawa T, Ogasawara H, Kaneko H, et al. Detection of antibodies to recombinant gag protein derived from human endogenous retrovirus like sequence, clone 4-1, in autoimmune diseases. *Viral Immunol* 1997;10:137-47.
- Tan EM, Cohen AS, Fries JF, et al. The 1982 revised criteria for the classification of systemic lupus erythematosus. *Arthritis Rheum* 1982;25:1271-7.
- Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 1987;162:156-9.
- Medstrand P, Lindeskog M, Blomberg J. Expression of human endogenous retrovirus sequences in peripheral blood mononuclear cells of healthy individuals. *J Gen Virol* 1992;73:2463-6.
- Roivainen A, Jalava J, Pirila L, Yli-Jama T, Tiusanen H, Toivanen P. H-ras oncogene point mutations in arthritis synovium. *Arthritis Rheum* 1997;9:1636-43.
- Pongers-Willems MJ, Verhangen OJHM, Tibble GJM, et al. Real-time quantitative PCR for the detection of minimal residual disease in acute lymphoblastic leukemia using junctional region specific TaqMan probes. *Leukemia* 1998;12:2006-14.
- Bièche I, Laurendeau I, Tozlu S, et al. Quantitation of MYC gene expression in sporadic breast tumor with a real-time reverse transcriptase-PCR assay. *Cancer Res* 1999;59:2759-65.
- Yoshiki T, Mellors RC, Strand M, August JT. The viral envelope glycoprotein of murine leukemia virus and the pathogenesis of immune complex glomerulonephritis of New Zealand mice. *J Exp Med* 1974;140:1011-27.
- Izui S, McConahey PJ, Theofilopoulos AN, Dixon FJ. Association of circulating retroviral gp70-anti-gp70 immune complexes with murine systemic lupus erythematosus. *J Exp Med* 1979;149:1099-116.
- Shirai T, Ohta K, Kohno A, et al. Naturally occurring antibody response to DNA is associated with the response to retroviral gp70 in autoimmune New Zealand mice. *Arthritis Rheum* 1986;29:242-50.
- Mellors RC, Mellors JW. Antigen related to mammalian type-C RNA viral p30 proteins is located in renal glomeruli in human systemic lupus erythematosus. *Proc Natl Acad Sci USA* 1976;73:233-7.
- Reynolds JT, Panem S. Characterization of antibody to C-type virus antigens isolated from immune complexes in kidneys of patients with systemic lupus erythematosus. *Lab Invest* 1981;44:410-9.
- Rucheton M, Graafland H, Fanton H, Ursule L, Ferrier P, Larsen CJ. Presence of circulating antibodies against gag-gene MuLV proteins in patients with autoimmune connective tissue disorders. *Virology* 1985;144:468-80.
- Query CC, Keene JD. A human autoimmune protein associated with U1 RNA contains a region of homology that is cross-reactive with retroviral p30 gag antigen. *Cell* 1987;51:211-20.
- Talal N, Garry RF, Schur PH, et al. A conserved idiotype and antibodies to retroviral proteins in systemic lupus erythematosus. *J Clin Invest* 1990;85:1866-71.
- Koshino K, Tokano Y, Hishikawa T, Sekigawa I, Takasaki Y, Hashimoto H. Detection of antibodies to HIV-1 gp41 and HLA class II antigen-derived peptides in SLE patients. *Scand J Rheumatol* 1995;24:288-92.
- Tamura N, Sekigawa I, Hashimoto H, Kira S, Yamamoto N. Syncytial cell formation in vivo by type C retroviral particles in the lung of systemic lupus erythematosus. *Clin Exp Immunol* 1997;107:474-9.
- Haraguchi S, Good RA, Cianciolo GJ, Engelman RW, Day NK. Immunosuppressive retroviral peptides: immunopathological implications for immunosuppressive influences of retroviral infections. *J Leukoc Biol* 1997;61:654-66.
- Kaneko H, Hishikawa T, Sekigawa I, Hashimoto H, Okumura K, Kaneko Y. Role of tumor necrosis factor- $\alpha$  in the induction of HIV-1 gp120-mediated CD4+ T cell anergy. *Clin Exp Immunol* 1997;109:41-6.

28. Sekigawa I, Kaneko H, Hishikawa T, et al. HIV infection and SLE: their pathogenic relationship. *Clin Exp Rheumatol* 1998;16:176-80.
29. Taruscio D, Manuelidis L. Integration site preferences of endogenous retroviruses. *Chromosoma* 1991;101:141-56.
30. Taruscio D, Mantovani A. Eleven chromosomal integration sites of a human endogenous retrovirus (HERV 4-1) map close to known loci of thirteen hereditary malformation syndromes. *Teratology* 1996;54:108-10.
31. Horwitz DA, Stohl W, Gray JD. T lymphocyte, natural killer cells, cytokines, and immune regulation. In: Wallace DJ, Hahn BH, editors. *Dubois' lupus erythematosus*. Baltimore: Williams & Wilkins; 1997:155-94.
32. Mercier B, Burlot L, Ferec C. Simultaneous screening for HBV DNA and HCV RNA genomes in blood donations using a novel TaqMan PCR assay. *J Virol Methods* 1999;77:1-9.
33. Leutenegger CM, Klein D, Hofmann-Lehmann R, et al. Rapid feline immunodeficiency virus provirus quantitation by polymerase chain reaction using the TaqMan fluorogenic real-time detection system. *J Virol Methods* 1999;78:105-16.
34. Karpas A, Lowdell M, Jacobson SK, Hill F. Inhibition of human immunodeficiency virus and growth of infected T cells by the immunosuppressive drugs cyclosporin A and FK506. *Proc Natl Acad Sci USA* 1992;89:8351-5.
35. Ogasawara H, Hishikawa T, Sekigawa I, Hashimoto H, Yamamoto N, Maruyama M. Sequence analysis of human endogenous retrovirus clone 4-1 in systemic lupus erythematosus. *Autoimmunity* 2001 (in press).
36. Groudine M, Eisenman R, Weintraub H. Chromatin structure of endogenous retroviral genes and activation by an inhibitor of DNA methylation. *Nature* 1981;292:311-7.