3 Results

Assuming that retroviruses do have a role in the etiology of certain autoimmune diseases, different retroviruses might be expected: new or not-yet detected exogenous viruses – for example zoonotically transmitted – or aberrantly expressed endogenous viruses. Different approaches are therefore necessary to assess the involvement of exogenous and endogenous retroviruses: Generic methods for the detection of exogenous retroviruses and specific detection methods, including quantification for the expression of endogenous retroviruses, have to be applied.

Electron microscopy is the classical virological catch-all method, and has been used in the past to screen AID samples for virus particles. Plasma samples from multiple sclerosis patients and long-term cell cultures derived from MS patients have been examined, as well as plasma and tissue samples from patients with other autoimmune diseases. Unusual structures were detected in some cells, but no retroviral particles were found (Gelderblom, personal communication). Electron microscopy has the advantage for detecting all viruses, even categorizing them according to particular morphological features, but has at the same time the major disadvantage of a relatively high detection limit (10^5 to 10^6 viral particles per mol; Biel and Gelderblom, 1999, Gentile and Gelderblom, 2005). Negative results are therefore not a proof of absence; these results may be due to low virus titers. In consequence, two more sensitive catch-all retrovirus methods, the differential display reverse transcriptase PCR (DDRT-PCR) and a reverse transcriptase activity assay (RTA), were adapted and applied to samples from adult patients affected by the following rheumatic disorders: spondylitis ankylosans, systemic lupus erythematosus, and scleroderma, as well as to healthy controls.

Expression of human endogenous retrovirus sequences (HERVs) might be observed in healthy, asymptomatic, as well as diseased individuals; however, different expression levels may be the key to disease development. In order to determine aberrant HERV expression, several real-time PCRs were established. Blood samples from patients with autoimmune diseases (SLE, scleroderma, or spondylitis ankylosans) as well as blood samples from different groups of MS patients were analyzed. Three groups of MS patients were included in the study: (1) children and adolescents with early onset MS (EOMS), defined as disease onset before age 16, who had all been in remission for the previous 6 months; (2) children and adolescents with EOMS who were seen between 24 to 48 hours after the onset of an acute episode of MS (‘active MS’); and (3) adults with MS, all receiving immunomodulatory therapies. A control group of children and adolescents with neurological symptoms of inflammatory and non-inflammatory origin, as well as healthy individuals (children, adolescents and adults) were examined for specific expression of endogenous retrovirus sequences by quantitative real-time PCR. All samples were also tested for proviral load of HTLV or expression of HTLV*tax*.

3.1 Generic approaches

3.1.1 Differential display

The original differential display (DD) using oligo(dT) primer in combination with degenerate random primers (Liang and Pardee, 1998) was not suitable as general screening method for the expression of retroviral sequences due to the enormous amount of PCR products to be analyzed. The mRNA population can be divided into subsets in order to render subtractive analysis possible. Either oligo(dT), one base- or two base-anchored primers can be employed, leading to one, three or nine cDNA syntheses, which have to be performed in parallel. The cDNAs have then to be combined independently with different random primers. Simplification was a prerequisite for the use of DD as a general screening tool with which to compare patient samples to healthy controls. Modifications of the DD using reverse primers specific to the retroviral primer binding site (PBS) led to a strong decrease in the amount of PCR products that had to be analyzed in order to identify retroviral sequences. In this investigation, the DD was adapted as a pan-retrovirus screening method for cell culture supernatants using HIV and SMRV as model viruses (Uhlenhaut et al., 2001). The efficiency and reproducibility was shown for three different retroviruses (HIV, HTLV and SMRV) using infected and non-infected cell culture supernatants in comparison. If all tRNA sequences which are used by animal retroviruses should be included in the screening, eight different primers have to be used in combination with eight random primers (Lys1,2, Lys3, Pro, Trp, His, Leu, Arg, and Glu, see addendum 5.12.4 and 5.13 for a compilation of tRNAs used as primers**).** This modified assay was applied to blood samples from spondylitis ankylosans patients (n=2) in comparison to blood samples from healthy donors (n=2). In contrast to cell culture supernatant, this more complex approach led to several hundred PCR products to be analyzed for the retrovirus-specific primers. Sequence analysis of these PCR products led to no known retroviral or unknown sequences, but to human genes. Different PCR products of exactly the same length were frequently detected, these bands can not be separated by PAGE and thus the DD approach is impossible, since optical differentiation due to the banding pattern itself can not be accomplished. As a consequence, this outcome rendered the assay using PBS-specific primers too complex as a general screening method to be used with whole blood of patients and healthy controls. Comparison of infected and non-infected cell cultures leads to far fewer PCR products to be analyzed than comparison of human samples. Besides the individual variations of different human beings, this effect may be caused by the broad variety of cell types that are analyzed. While cell cultures consist in general of one type of cell only, many different cell types are present in patients' samples. Another factor to be considered is the probable multifactorial nature of AIDs; a person without genetic predisposition for a certain disease may well be infected with the triggering virus without developing any symptoms. Thus, infected and yet asymptomatic individuals may be chosen as negative controls, leading to the exclusion of relevant sequences by the use of

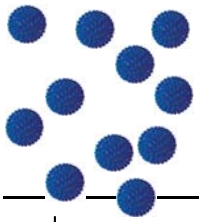
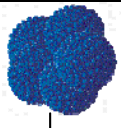
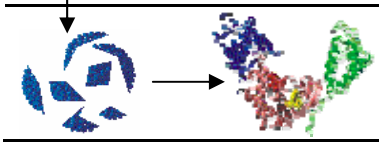
subtractive techniques. Still, given well characterized samples pre-screened for retrovirus infection, the DD should be a suitable method to gain first sequence information of the virus. In order to pre-select samples, an additional pan-retrovirus screening method was established: the reverse transcriptase activity assay.

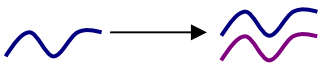
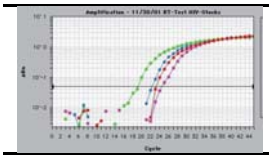
3.1.2 Reverse transcriptase activity assay (RTA)

3.1.2.1 A catch all-method

The entire retrovirus family is characterized by the activity of a unique retrovirus-specific enzyme, reverse transcriptase (RT), which, during the retroviral infection cycle, transcribes viral genomic RNA into a double-stranded DNA provirus. Reverse transcriptases from several retroviruses have been well characterized and shown to require an RNA template, primer, nucleotide triphosphates, and bivalent cations. The appropriate conditions provided, a retrovirus-containing sample might be used as exogenous source for reverse transcriptase activity. Reverse transcriptase molecules from different viruses favor specific reaction conditions that should be accounted for to achieve an optimized assay design. In the absence of other exogenous RT activity, the detection of cDNA would serve then as proof of retroviral reverse transcriptase activity. The presence of cDNA can be detected and quantified, for example, with a real-time PCR assay. The assay established in this investigation is a modification of published assays. Briefly, an exogenous RNA template (genomic MS2 phage RNA) is used as template in combination with a sample (e.g. retrovirus producing cell culture supernatant). The cDNA synthesis is performed, and cDNA is detected and quantified in a MS2-specific real-time PCR assay (tab. 5).

Tab. 5: RTA flowchart

	Step	**Analyzed conditions**
 Virus particles in cell culture supernatant or plasma	Sample preparation Removal of cells and cellular debris by centrifugation	Storage of samples, conditions for plasma sampling Other preparations (PEG)
	Purification	Filtration Sucrose cushion
	Ultracentrifugation	
	Lysis of virus particles/release of RT	Cell culture lysis buffer Different detergents

	Step	Analyzed conditions
Addition of cDNA buffer including MS2 RNA template  RNA cDNA	cDNA synthesis	Buffer conditions BSA Protease inhibitors Tris concentration DDT concentration RNasin Mg^{2+}/Mn^{2+} concentrations
 Detection	Quantitative real-time PCR	Specific for MS2
Trouble shooting:	Elimination of background signals Assay range	RNase digestion DNA trap

In the following, reverse transcriptase activity is either given in nU using commercially available RT for calibration, or in relation to the threshold cycle (ct) value obtained with real-time PCR. 45 PCR cycles were performed for each PCR, thus negative samples have 45 as given ct value. Since lower RT values indicate higher concentration of template, the RT activity is given as 45-ct value. Giving the RT activity as 45-ct value allows direct comparison with other detection methods where usually higher values represent higher yields.

3.1.2.2 Establishing optimized assay performance

3.1.2.2.1 Sample preparation

Retroviruses are enveloped viruses and can easily be inactivated by detergents or heat. Adequate sample preparation is therefore crucial for successful detection of reverse transcriptase activity. Viral particles were obtained from different infected cell culture supernatants and plasma from HIV infected individuals. Cell debris was removed by centrifugation. Retroviral particles were pelleted by ultracentrifugation. Filtration of cell culture supernatants and plasmas to purify the samples has been previously described (Pyra et al., 1994, Khan et al., 2001, Sears et al., 1999, Khan and Sears, 2001, Voisset et al., 2001, Andre et al., 2000). Since viruses are defined as filterable agents, this purification should have no reductive impact on the assay (2nd Koch's postulate). However, it could be shown that filtration reduced the RT activity considerably, especially filtration of plasma (fig. 10).

3.1.2.2.1.1 Filtration reduces RT activity in HIV-positive plasma

A substantial reduction of reverse transcriptase activity was observed for HIV-positive plasma in filtrated compared to non-filtrated samples (fig. 10).

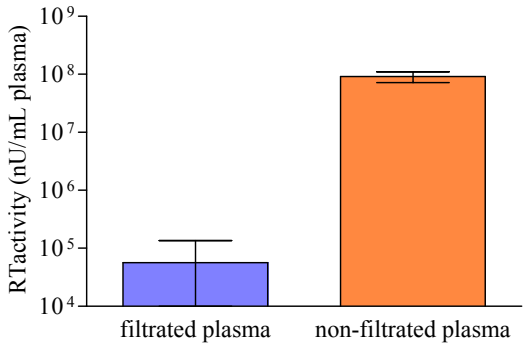


Fig. 10: Effect of filtration on the reverse transcriptase activity in HIV-positive plasma. RT activity for filtrated (0.45 µm) and non-filtrated plasma of an HIV-positive individual was analyzed. The RT activity assay was performed in triplicate, using Mg^{2+} buffer. The RT activity is given as nU/mL plasma**.**

Non-filtrated and filtrated (0.45 µm) aliquots of HIV-positive plasma were analyzed. The determined reverse transcriptase activity was 9.1×10^7 nU/mL plasma for the non-filtrated and 5.6×10^4 nU/mL plasma for the filtrated sample; one of three filtrated samples tested negative. Filtration accounted for a loss of reverse transcriptase activity of approximately 3 $\log_{10}$ in this plasma sample.

The diminishing effect on the reverse transcriptase activity resulting from filtration had to be linked to some kind of aggregation that prevented the viral particles from passing through the filter pores. To examine whether the inhibiting factor inherent in the HIV-positive plasma was HIV-specific, HIV-positive plasma and HIV-negative plasma were spiked with the same amount of HIV particles as well as with animal retrovirus particles (Squirrel monkey retrovirus, SMRV) in parallel tests and RT activity was determined (fig. 11).

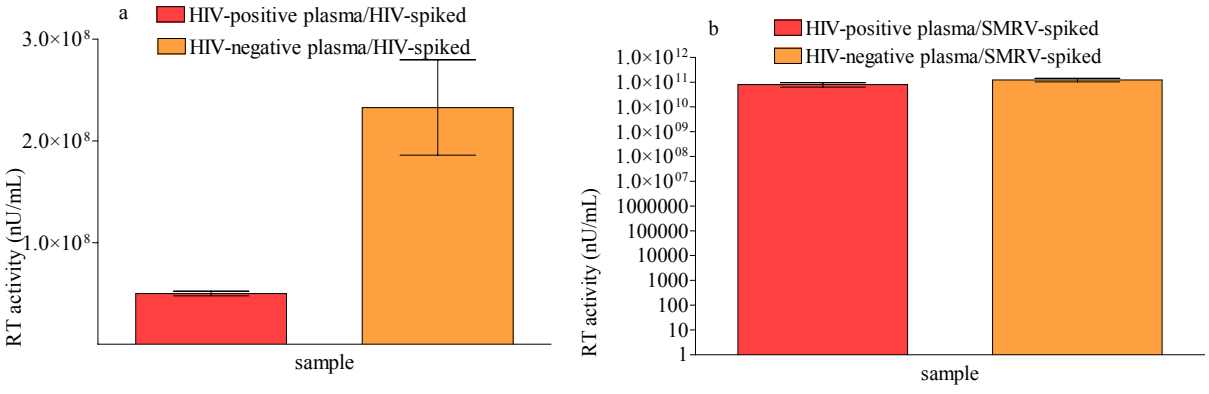


Fig. 11: Loss of reverse transcriptase activity in HIV-positive plasma. (a) HIV-negative and HIV-positive plasma were spiked with HIV. The samples were subjected in parallel to the reverse transcriptase activity assay. (b) Loss of SMRV-RT activity in HIV-positive plasma compared to HIV-negative plasma. The same amount of SMRV particles were spiked onto HIV-negative and HIV-positive plasma. All samples were tested in triplicate; the data shown was obtained with Mg^{2+} buffer.

Comparison of the RT activity in HIV-positive and HIV-negative plasma spiked with the same amount of cell culture derived HIV stock showed 4.7 times lower RT activity for the HIV-positive sample. The control experiment using the animal retrovirus SMRV showed no significant differences regarding RT activity for the HIV-positive sample compared to the SMRV spiked HIV-negative plasma. The observed reduction of RT titers was thus specific for HIV and HIV-positive plasma. This finding led to the assumption that antibodies directed against HIV particles but not SMRV were responsible for this effect. In the following, the effect of antibodies adhering to virus particles was analyzed. HIV-positive plasma was tested directly as well as following an antibody-stripping procedure.

3.1.2.2.1.2 RT activity assay without antibody-stripping

HIV-positive plasma with 50 pg p24 per mL (as determined by antigen capture assay) and with a relatively low antibody titer (as determined by Western blot and ELISA) was analyzed directly or after spiking with HIV particles from cell culture supernatant. HIV-negative plasma was spiked with the same amount of HIV particles and analyzed in parallel. Approximately the same level of RT activity was detected for HIV-negative and HIV-positive plasma spiked with the same amount of HIV particles (fig. 12)

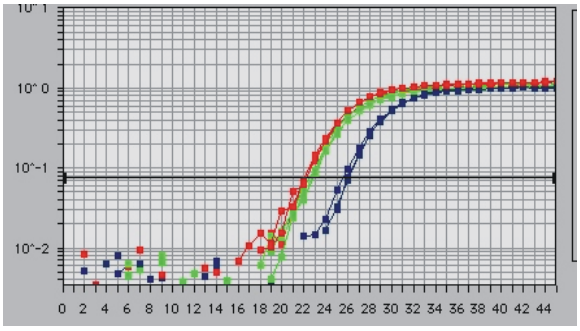


Fig. 12: Reverse transcriptase activity assay without antibody-stripping
Red: HIV-negative plasma spiked with HIV
Blue: HIV-positive plasma
Green: HIV-positive plasma spiked with HIV
All samples were tested in triplicate; data given was obtained with Mg^{2+} buffer.

HIV-positive plasma without additional HIV spiking showed RT activity (fig. 12: blue curve). HIV-negative and HIV-positive plasma spiked with the same amount of HIV particles (fig. 12: red and green curve, respectively) showed approximately the same RT activity. No additive effect of the added HIV particles on the detected level of RT activity was found for HIV-positive plasma.

3.1.2.2.1.3 RT activity assay including antibody-stripping

An antibody-stripping solution based on a glycine buffer used for antibody-stripping performed with Western blot analysis was used (final concentration: 0.1 M glycine, 0.02 M magnesium acetate, 0.05 M potassium chloride, pH 2.2) to remove antibodies adhering to virus particles prior to the performance of the reverse transcriptase activity assay (fig. 13). A 2-fold concentrate of the glycine buffer was mixed with the same volume of plasma. The mixture was immediately put onto a sucrose step gradient (20%, 25% and 30% sucrose) and subjected to ultracentrifugation.

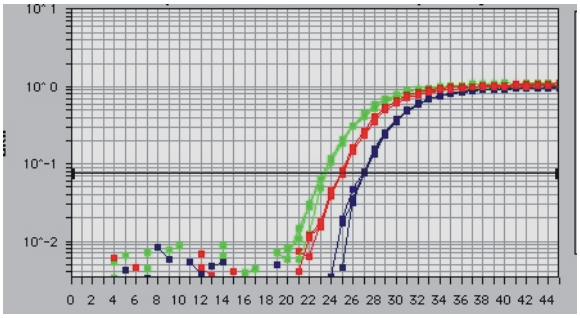


Fig. 13: Reverse transcriptase activity assay with antibody-stripping
Red: HIV-negative plasma spiked with HIV
Blue: HIV-positive plasma
Green: HIV-positive plasma spiked with HIV
All samples were tested in triplicate; data given was obtained with Mg^{2+} buffer.

The RT activity of HIV-positive plasma increased relative to the RT activity in HIV-negative plasma with antibody-stripping buffer treatment prior to centrifugation. The HIV-positive plasma spiked with additional HIV particles now displayed a higher level of RT activity than the HIV-negative plasma spiked with HIV particles, as should be expected due to the additive effect of the initial viral load of the HIV-positive plasma and the spiked virus (fig. 13: green curves). Antibody-stripping resulted in restoration of RT activity. It was assumed that HIV particles were linked by HIV antibodies, forming aggregations larger than 0.45 µm (pore size). However, the diminishing effect was also observed in non-filtrated samples, thus the observed effect could be due, at least in part, to antibodies directed against HIV reverse transcriptase molecules themselves.
Although the antibody-stripping strengthened the hypothesis that HIV-specific antibodies were responsible for diminishing effects on reverse transcriptase detection in HIV-positive plasma, the harsh conditions required for the separation of antibodies and viral particles reduced the sensitivity of RT activity assay considerably. Therefore antibody-stripping was not performed for analytical assays in order to maintain high assay sensitivity.

3.1.2.2.1.4 Comparison: filtration and sucrose cushion

An alternative purification step using sucrose cushions instead of filtration was developed. A comparison of the two methods was carried out with supernatant from SMRV infected 293 cell culture. Since no antibodies against SMRV were present in the supernatant, filtration should not have a diminishing effect on the RT activity. Filtrated supernatant was examined in the reverse transcriptase activity assay, in parallel filtrated and non filtrated supernatant further purified through a 20% sucrose cushion, and one sample was tested without any further purification except the removal of cells and cell debris before ultracentrifugation (fig. 14).

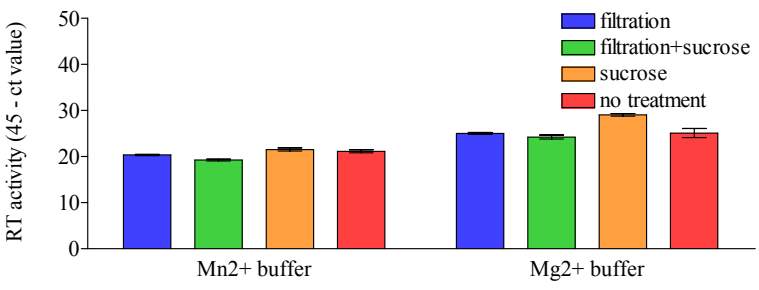


Fig. 14: Purification: filtration versus sucrose cushion. All samples (SMRV infected 293 cells) were collected on the same day and stored at –70°C. RT assays were carried out in triplicate for Mg^{2+} and

Mn^{2+} buffer in parallel at different time points. The data shown represents the RT activity contained in 0.025 µL supernatant. Differences for Mg^{2+} and Mn^{2+} buffer are due to the Mg^{2+} preference of the SMRV reverse transcriptase. RT activity is given as 45-ct value; 3.3 ct value is equivalent to 1 $\log_{10}$ RT activity.

Filtration-only as well as filtration followed by centrifugation through a 20% sucrose cushion resulted in slightly lower RT activity for Mg^{2+} and Mn^{2+} buffer for the SMRV reverse transcriptase compared to untreated controls. The relatively lower RT activity of filtrated samples could be explained by a loss of sample volume on the filter membrane. The control samples without treatment showed the same RT activity as the samples purified by sucrose. Thus, purification was accomplished by centrifugation through a 20% sucrose cushion instead of filtration and was performed routinely for all RT assays.

3.1.2.2.2 Lysis of viral particles

The members of the retrovirus family share not only the particularities of reverse transcription but also a similar virus structure. The capsid, or core, is covered by an envelope that is derived from the cell plasma membrane through budding. Retroviral enzymes, including the reverse transcriptase, are located within the core. Detection of reverse transcriptase activity requires liberation of the enzyme from the viral core without affecting the enzymatic activity. Therefore mild lysis conditions are crucial. The virus pellet obtained by ultracentrifugation can be disintegrated by non-ionic detergents. Different detergents were tested in combination with M-MLV reverse transcriptase for their effect on RT activity. It was shown that the respective detergent or buffer did not influence the performance of reverse transcriptase. Cell Culture Lysis buffer (Promega) did not reduce the reverse transcriptase activity of M-MLV, but did affect the subsequently performed real-time PCR. Thus, non-diluted samples resulted in too low RT activity compared to the diluted samples, probably due to an inhibiting detergent concentration inherent in this buffer (commercial buffer, composition unknown). As a result, the cDNA obtained had to be diluted at least 100-fold prior to the real-time PCR. To avoid this dilution and thus to enhance the sensitivity of the assay, an alternative buffer system was developed: The virus pellet was lysed directly using the cDNA buffer containing the appropriate concentration of detergent of either Triton X-100 or Igepal. A buffer using Triton X-100 (56 mM Tris/HCl, 56 mM KCl, 5.6 mM DTT, 0.5% Triton X-100, final concentration) worked in combination with Mg^{2+} buffer (5 mM), but resulted in precipitations with Mn^{2+} (0.8 mM and 0.4 mM), which rendered a real-time PCR using the cDNA as template impossible. An alternative 2-fold buffer was designed replacing Triton X-100 with Igepal (0.6% final concentration); the detergent concentration was used according to Heneine et al., 1995. The buffer using Igepal as lysis agent could be used with either cation (Mn^{2+} or Mg^{2+}) and did not reduce the RT activity of M-MLV reverse transcriptase, as was shown by comparison of the manufacturers' buffer (first strand buffer and DTT, Gibco) and the developed lysis/cDNA buffer (fig. 15).

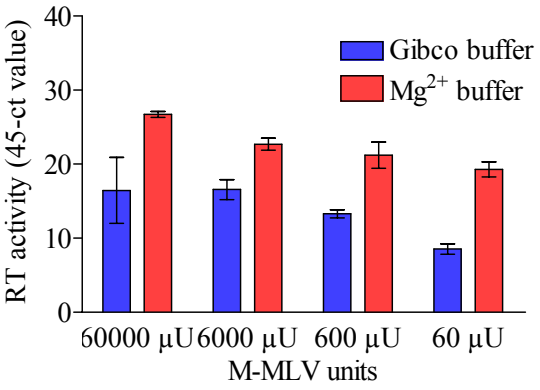


Fig. 15: Comparison of cDNA buffers. Four dilutions of M-MLV reverse transcriptase were compared using either the Gibco buffer or lysis/cDNA buffer (Mg^{2+}). The comparison was carried out using Mg^{2+} because M-MLV is dependent on this cation. All samples were tested in triplicate. 60,000 µU correspond to a 1:10,000 dilution of the enzyme. RT activity is given as 45-ct value; 3.3 ct value corresponds to approximately 1 $\log_{10}$ RT activity.

The Gibco buffer (blue bars) showed 2 to 3 $\log_{10}$ lower activity compared to the lysis/cDNA buffer. 20000 µU correspond to a 1:10000 dilution of the enzyme as delivered by the manufacturer. In the following, the cDNA lysis buffer (containing Igepal) was used for lysis of pelleted virus particles and cDNA syntheses, using either Mg^{2+} or Mn^{2+} as cation.

3.1.2.2.3 Buffers

3.1.2.2.3.1 Buffer 1: cDNA buffers and bivalent cations

Reverse transcriptases of retroviruses can be divided into categories based on cation preference. Some enzymes prefer either Mg^{2+} or Mn^{2+} as cation, while other enzymes use both cations with the same efficiency. Testing for just one cation could lead to negative results for reverse transcriptases. The ion-dependency was used to develop an RT assay system that can not only detect and quantify minute amounts of reverse transcriptase activity, but also can determine the ion specificity of the respective enzyme at the same time.

Therefore different buffers and buffer conditions were tested using commercially available M-MLV reverse transcriptase. Published Mg^{2+} concentrations for RT activity assays varied from 5.5 mM to 9.0 mM (Andre et al., 2000, Voisset et al., 2001, Sears et al., 1999, Pyra et al., 1994, Lovatt et al., 1999). In some of the samples the buffer containing 9 mM Mg^{2+} led to precipitations, thus rendering PCR analysis impossible. Comparisons showed no difference in sensitivity for 5 mM to 9 mM Mg^{2+}. The standard buffer was set up with a final concentration of 5 mM Mg^{2+}. No current data regarding appropriate manganese concentration was available. Concentrations of 5 mM to 9 mM turned out to be inapplicable due to immediate and strong precipitations interfering with cDNA synthesis and PCR performance. In 1974 K. Mölling published a Mn^{2+}optimum of 0.4 mM for Friend Murine Leukemia virus reverse transcriptase (Moelling, 1974). In the following, Mn^{2+} concentrations ranging from 0.2 mM to 1.2 mM were tested. The optimal concentration was determined to be 0.8 mM Mn^{2+} (fig. 16).

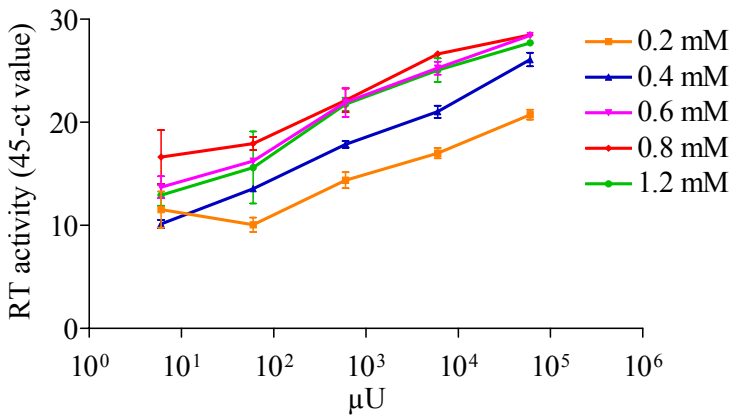


Fig. 16: Determination of optimized Mn^{2+} concentration. The cDNA syntheses were carried out in triplicate using a serial dilution of M-MLV RT. RT activity is given as 45-ct value; 3.3 ct value corresponds to approximately 1 $\log_{10}$ RT activity.

Thus, samples of unknown reverse transcriptase activity and samples of known retroviruses were tested in parallel using Mn^{2+} (0.8 mM) or Mg^{2+} (5.0 mM) in order to detect RT activity as well as to determine the ion specificity. After determination of the cation preference for HIV (Mg^{2+}), only Mg^{2+} buffer was used for further analyses.

3.1.2.2.3.2 Buffer 2: cDNA buffers and BSA

Some of the published reverse transcriptase activity assays contain bovine serum albumin (BSA; 0.13 µg/µL; Andre et al., 2000, Voisset et al., 2001, Pyra et al., 1994). Buffers for cDNA synthesis with either cation were tested with and without BSA in parallel. The addition of BSA had no effect on the Mg^{2+} buffered cDNA synthesis. However, combinations of different Mn^{2+} concentrations (0.1, 0.4, 0.8, and 1.2 mM Mn^{2+}) and 0.13 µg/µL BSA led to precipitations. In order to optimize the assay conditions, BSA (10 ng/µL) was tested in combination with different Mn^{2+} concentrations. This lower protein content did not result in precipitations, but controls with Tris buffer or water instead of template tested positive in real-time PCR, whereas no difference was observed for M-MLV dilutions or SMRV reverse transcriptase. Since the comparison of buffer with and without BSA showed no difference for Mg^{2+}, but rather precipitations for higher concentrations of BSA or false positive results for control samples in combination with Mn^{2+}, BSA was not added to the cDNA buffer.

3.1.2.2.3.3 Buffer 3: cDNA buffers and Tris concentration

Tris was used as buffer in most published cDNA buffers with concentrations ranging from 10 to 56 mM (Lovatt et al., 1999, Pyra et al., 1994, Voisset et al., 2001, Andre et al., 2000). Final Tris concentrations of 10 mM and 56 mM were tested. Mg^{2+} buffer showed a higher sensitivity with 56 mM Tris/HCl (45-ct value = 17.30 for 6000 µU) compared to 10 mM Tris (45-ct value = 23.60) corresponding to approximately 2 $\log_{10}$ reduced activity. Four different Mn^{2+} concentrations (0.1, 0.4, 0.8, and 1.2 mM) were tested in combination with both Tris concentrations. The 10 mM Tris concentration resulted in no detectable reverse transcriptase activity in combination with Mn^{2+}, while the expected RT activity was

detected for 56 mM Tris in combination with different Mn^{2+}concentrations. The standard Tris/HCl (pH 8.5) concentration used for the cDNA buffer was 56 mM.

3.1.2.2.3.4 Buffer 4: cDNA buffers and DTT-concentration

Dithiothreitol (DTT) is used in protein assays to protect free sulfhydryl groups from oxidation and to reduce disulfide bonds quantitatively. Due to its protein-stabilizing nature, DTT is also used in protein storage buffers. The reducing nature of DTT was used to imitate the reducing milieu of mammalian cells (Kosower and Kosower, 1978). DTT concentrations used for different reverse transcriptase activity assays varied from 1.6 mM to 11.2 mM (Voisset et al., 2001, Andre et al., 2000, Pyra et al., 1994, et al., Lovatt et al., 1999). In addition, an adverse effect on cDNA efficiency has also been described for DTT (Lekanne et al., 2002).

Therefore concentrations of 0.56 mM and 5.6 mM DTT were compared, using commercially available M-MLV reverse transcriptase and two cell culture supernatants derived from the porcine endogenous retrovirus (PERV) producing PS cell line, and SMRV infected 293 cells as sources for RT activity (fig. 17). For M-MLV, the 5.6 mM DTT in combination with Mn^{2+} resulted in a higher sensitivity compared to the 0.56 mM DTT (shifts in ct value of 2.5 to 3.4; corresponding to approx. 1 $\log_{10}$ of RT activity). The sensitivity for the Mg^{2+} buffer was independent of the DTT concentration, and the standard deviations were also comparable.

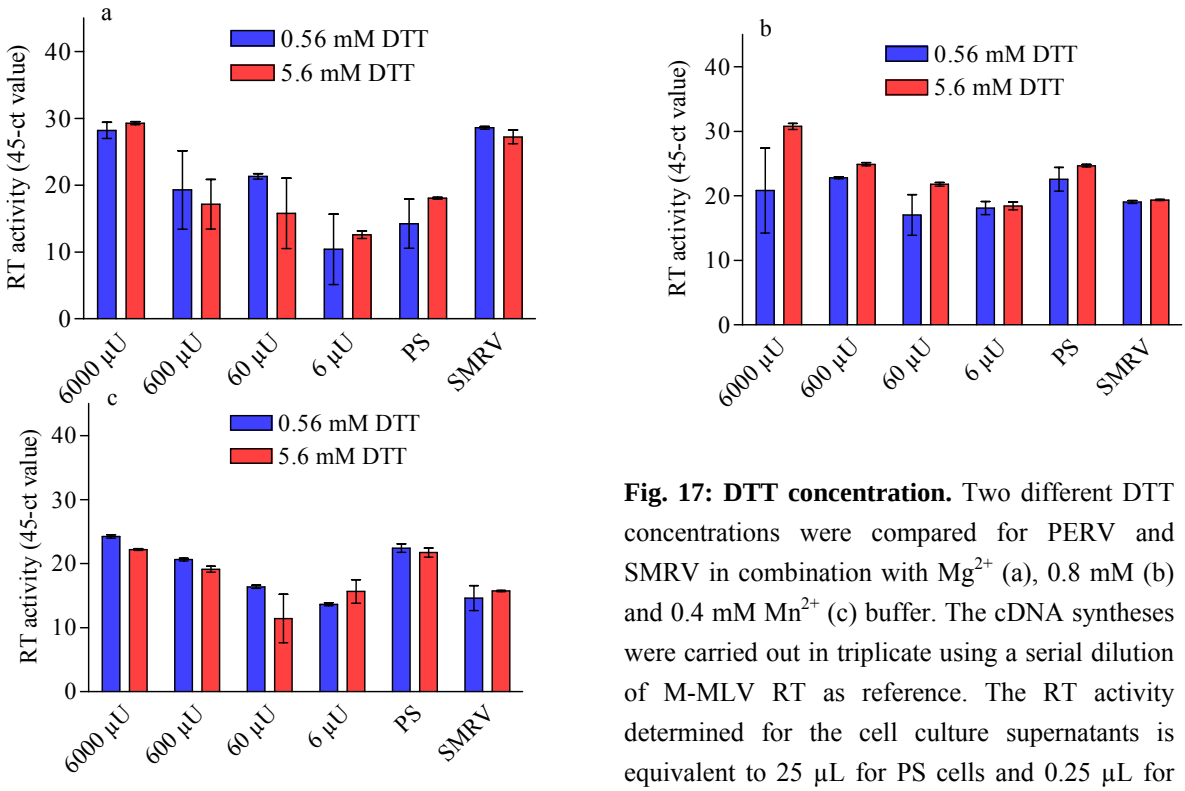


Fig. 17: DTT concentration. Two different DTT concentrations were compared for PERV and SMRV in combination with Mg^{2+} (a), 0.8 mM (b) and 0.4 mM Mn^{2+} (c) buffer. The cDNA syntheses were carried out in triplicate using a serial dilution of M-MLV RT as reference. The RT activity determined for the cell culture supernatants is equivalent to 25 µL for PS cells and 0.25 µL for SMRV infected 293 cell culture supernatant. RT activity is given as 45-ct value; 3.3 ct value corresponds to approximately 1 $\log_{10}$ RT activity.

There was no significant difference for the two DTT concentrations with both virus lysates regardless of the cation used, although the porcine endogenous retroviruses showed a slightly higher RT activity with the higher DTT concentration in combination with 0.8 mM Mn^{2+} and Mg^{2+} buffer. The M-MLV RT showed higher RT activity for 5.6 mM DTT in combination with 0.8 mM Mn^{2+}. Furthermore, the standard deviations were lower for the buffer with a higher DTT concentration. As a result, 5.6 mM DTT was included in the standard cDNA buffer.

3.1.2.2.3.5 Buffer 5: storage buffer for enzyme standard dilution

A serial dilution of M-MLV reverse transcriptase was used as an internal standard. Since the dilution had to be stored for several weeks or months, the stability of the enzyme during storage had to be ensured in order to obtain comparable results. The storage buffer for the serial dilution of the M-MLV standard reverse transcriptase was set up in a manner similar to the manufacturer buffer (20 mM Tris/HCl pH 7.5, 100 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 0.01% (v/v) Igepal, 100 µg/mL BSA, 50% (v/v) glycerol). The reverse transcriptase activity was determined for the freshly diluted enzyme and after 14 weeks of storage (fig. 18).

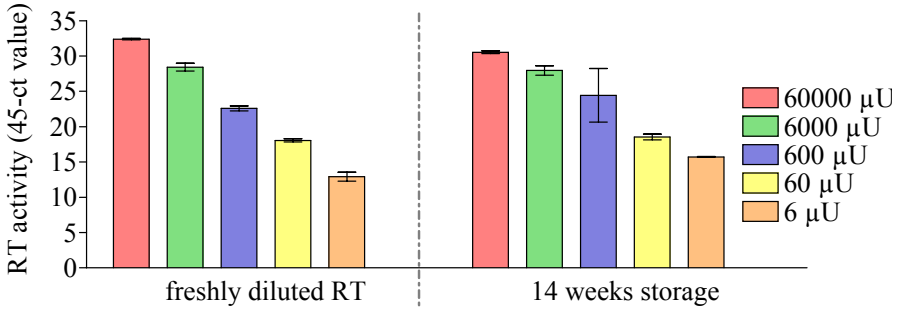


Fig. 18: Storage of enzyme standard dilution. Serial dilutions of RT were made as described above and cDNA syntheses were performed with Mg^{2+} buffer in triplicate using a serial dilution of M-MLV reverse transcriptase. The first cDNA synthesis was carried out immediately after dilution; the second cDNA synthesis was carried out after 14 weeks of storage at -20°C. RT activity is given as 45-ct value; 3.3 ct value corresponds to approximately 1 $\log_{10}$ RT activity.

The RT activity of M-MLV remained stable over a period of 14 weeks, even though the dilution was used 17 times as a standard and thus had been kept at 4°C for about 30 minutes while cDNA syntheses were prepared. No dilution was stored and used for more than four months.

3.1.2.2.4 Storage of samples

Proteins may be affected in their structural integrity and, consequently, in their biological function by freezing without protective additives. Freezing and thawing as well as storage at -70°C may lead to a reduction of RT activity and thus produce too low or negative results due to physico-chemical damage of the enzyme. Glycerol storage could provide more gentle storage conditions and therefore result in stabilization of RT activity.

The effect of glycerol was studied using HTLV-1-positive MT2 cell culture supernatant (Morozov and Weiss, 1999). One aliquot of freshly harvested supernatant was stored directly, while the other was mixed with the same volume of glycerol; both samples were stored at –70° for 10 days before they were tested in Mg^{2+}- and Mn^{2+}-buffered RT assays (fig. 19).

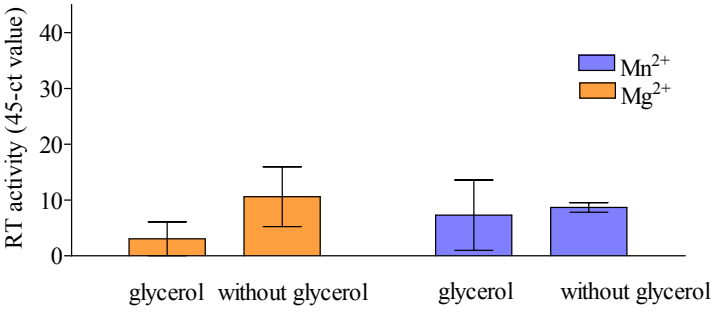


Fig. 19: Assay protection: storage with and without glycerol. The cDNA synthesis was performed in triplicate with Mg^{2+} and Mn^{2+} cDNA buffer in parallel. The equivalent of 0.5 µL HTLV-producing cell culture supernatant was used as an exogenous source for reverse transcriptase activity. The dilution with glycerol has been accounted for. RT activity is given as 45-ct value; 3.3 ct value corresponds to approximately 1 $\log_{10}$ RT activity.

In the presence of Mn^{2+} cations the samples stored without glycerol showed a slightly higher RT activity than samples stored with glycerol (fig. 19). The Mg^{2+} assay without glycerol showed a 100-fold higher sensitivity than samples stored with glycerol. Moreover, with glycerol two of three samples were negative for RT activity. This comparison showed that storage with glycerol could render the results of retrovirus-positive samples negative. Furthermore, when freshly harvested SMRV-positive 293 cell culture supernatant was compared to supernatant stored without glycerol at -70°C, no difference in RT activity was observed (fig. 20). These findings indicate that protective additives were not only superfluous, but furthermore that the addition of glycerol can have a negative effect on RT activity.

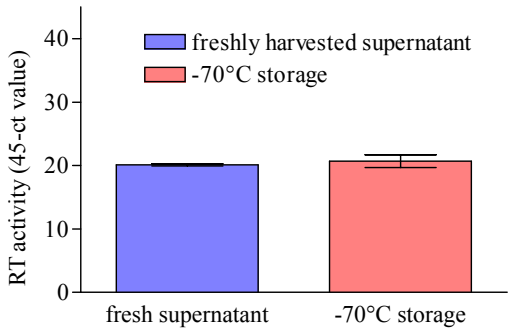


Fig. 20: Comparison of RT activity for freshly harvested and stored supernatant. SMRV positive 293 cell culture supernatant was used. The data shown is related to RT activity per 0.05 µL supernatant detected with Mg^{2+} buffer. RT activity is given as 45-ct value; 3.3 ct value corresponds approx. to 1 $\log_{10}$ of RT activity.

3.1.2.2.5 Assay range and internal control

The range of the established real-time PCR extends over 6 $\log_{10}$. In addition to the non-diluted lysate, 10- and 100-fold dilutions of virus lysate were applied. The parallel application of three different dilutions of one sample broadened the effective detection range of the assay to 8 $\log_{10}$. Moreover, high virus titers observed in some infected cell

culture supernatants rendered dilutions of 1:1000 and 1:10000 necessary in order to assess the actual reverse transcriptase activity. In addition to increasing the range of the assay, analyzing different 10-fold dilutions in parallel served as control. Potential false positive signals due to contamination of the real-time assay with PCR product or RT activity of the DNA-dependent polymerase would give constant results and not follow the logarithmic dilution of the lysate. Inhibiting factors inherent within a sample would also be detected, since they would result in reduced RT activity for the non-diluted sample in comparison to dilutions.

3.1.2.2.6 Assay protection and background elimination

Absence of amplifiable DNA is crucial to avoid false positive signals. Genomic MS2 phage RNA was chosen as template, since the replication cycle of this phage does not involve a DNA intermediate (Pyra et al., 1994). A systematic contamination of the template by amplifiable DNA or inadvertent introduction of such DNA by a sample was thus impossible. To eliminate possible carry-over contamination, the uracil N-glycosylase (UDG, Invitrogen) protocol was used for the TaqMan assay (Hartley and Rashtchian, 1993, Sambrook and Russell, 2001c). As a heteropolymer, the MS2 RNA has the further advantage of being much less efficiently used by eukaryotic DNA-polymerases than the synthetic homopolymeric templates usually employed for RT tests (Sarngadharan et al., 1978). Thus the risk of false positive signals due to contaminating cellular DNA polymerases in cell cultures supernatants was reduced.

3.1.2.2.6.1 Influence of protease inhibitors

Residual cellular or other contaminating proteases could result in negative or too low results in the RT assay due to degeneration of reverse transcriptase before or during cDNA synthesis. In order to avoid such a loss of enzyme activity, the effect of protease inhibitors (Complete mini, Boehringer Mannheim) was tested. A comparison was carried out for SMRV infected cell culture supernatant (293 cells) with and without protease inhibitors added to the cDNA buffer. As a control, protease inhibitors were also added to cDNA mix without addition of any exogenous source for RT activity (RT-negative control; fig. 21).

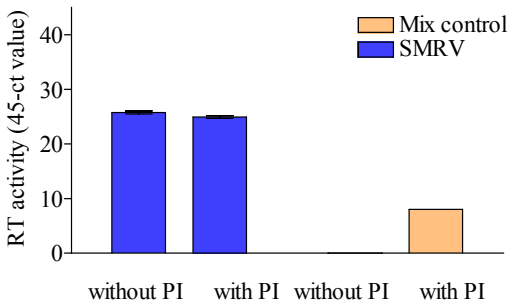


Fig. 21: Assay protection: use of protease inhibitor. The cDNA syntheses were carried out in triplicate with Mg^{2+} cDNA buffer using SMRV infected cell culture supernatant as source for RT activity. No exogenous source for RT activity was added to the mix control. The protease inhibitor was used according to the manufacturer's instructions and added to the lysis buffer prior to particle lysis. RT activity is given as 45-ct value; 3.3 ct value corresponds to approximately 1 $\log_{10}$ RT activity.

The results for the SMRV infected 293 cell culture supernatant showed no significant difference with and without added protease inhibitor. Protease inhibitor added to the control reaction created a detectable signal in the absence of any genuine reverse transcriptase activity. Thus, addition of protease inhibitor could result in false positive signals. Therefore protease inhibitor not was used for analytical RTA.

3.1.2.2.6.2 Lysate stability

In order to assess the stability of reverse transcriptase activity, HIV particles derived from cell culture supernatant were spiked into HIV-positive and HIV-negative human plasma as a model for retrovirus-infected patient samples. The samples were subjected to RTA. The remaining lysate of the virus preparation was stored at 4°C and used again after six days as an exogenous source for RT activity in a cDNA synthesis to assess the stability of the enzymatic activity (fig. 22).

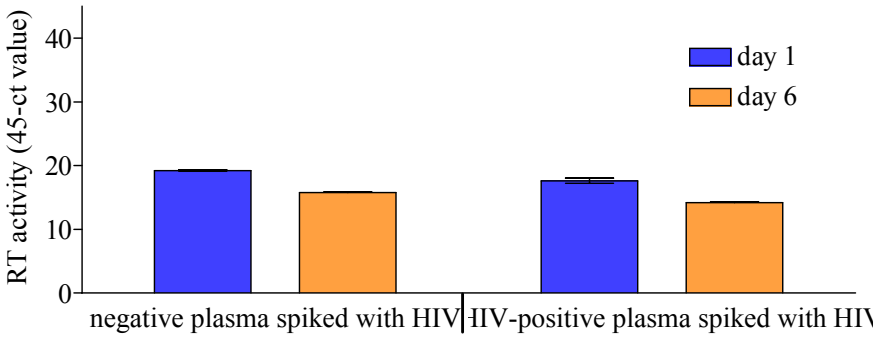


Fig. 22: Lysate stability. HIV-negative human plasma as well as HIV-positive human plasma was spiked with HIV particles and RT activity was determined. Aliquots of the preparations were stored at 4°C for 6 days and used as an exogenous source for cDNA synthesis. The cDNA synthesis was carried out in triplicate, using Mg^{2+} buffer. The data shown represents the RT activity of 0.25 µL spiked plasma. RT activity is given as 45-ct value; 3.3 ct value corresponds to approximately 1 $\log_{10}$ RT activity.

After six days of storage at 4°C both samples showed a 10-fold decrease in RT activity, which correlates to the described loss of infectivity for HIV in patient samples (0.5 $\log_{10}$ to > 2 $\log_{10}$; Moudgil and Daar, 1993). No difference in reverse transcriptase activity was observed for HIV-positive and HIV-negative plasma. Since adverse effects for protective additives such as glycerol or protease inhibitors were shown, the reverse transcriptase activity assay was always performed using freshly lysed particle preparations.

3.1.2.2.6.3 RNase digestion of RNA template

The RT assay displayed background signals besides the RNasin-related activity. Non-infected samples and buffer controls were tested weakly positive, especially for Mn^{2+} buffer derived cDNAs. The observed difference in the background signals obtained with the two different buffers could be linked to a target change of DNA-dependent polymerases to RNA as template in the presence of Mn^{2+}, resulting in low level DNA synthesis during PCR (Myers and Gelfand, 1991). Therefore the RNA template had to be removed or destroyed prior to PCR, and RNase digestion was performed after cDNA

synthesis using Ribonuclease A, directly followed by real-time PCR. Two different RNase concentrations were compared, 0.25 and 0.5 µg, and optimal results were obtained using 0.5 µg RNase A for 10 µL cDNA for 30 minutes at 37°C (fig. 23).

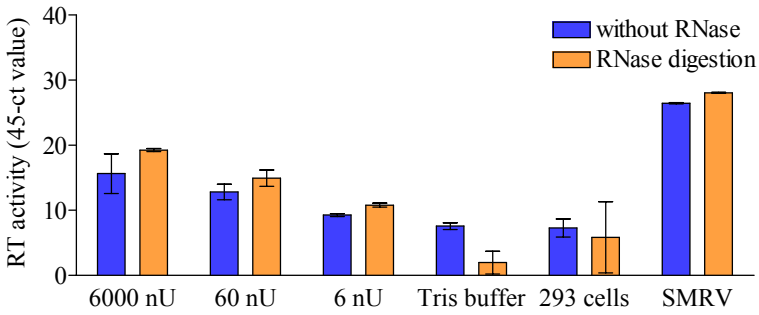


Fig. 23: Background elimination: RNase digestion. The cDNA syntheses were carried out in triplicate for Mg^{2+} buffer derived cDNAs. Three different dilutions of M-MLV reverse transcriptase, Tris buffer and non-infected 293 cell culture supernatant as negative control as well as SMRV infected 293 cell culture supernatant were tested in parallel with and without RNase digestion of cDNA prior to the PCR. RT activity is given as 45-ct value; 3.3 ct value corresponds to approximately 1 $\log_{10}$ RT activity.

All three dilutions of M-MLV tested displayed a 3- to 7-fold increased RT activity (corresponding to 1 to 2 ct value) following RNase digestion. The SMRV infected 293 cell culture supernatant showed a 2-fold increase in RT activity for the assays treated with RNase A. Although two of three samples of non-infected 293 cell culture supernatant showed a residual signal after RNase digestion, the mean background signal was also reduced. While the Tris-negative control was slightly positive without RNase treatment, this background activity was strongly reduced upon RNase treatment. Since the digestion of RNA prior to real-time PCR led to an increased sensitivity for samples with inherent RT activity and to a reduction of false positive signals, all samples of the RTA were treated with RNase prior to real-time PCR.

3.1.2.2.6.4 Effects of RNase inhibitor RNasin

One of the backbones of the RTA is the ample availability of RNA template. In order to avoid loss of RNA template before or during cDNA synthesis reaction, adequate conditions for RNA stability and protection were applied: all buffers, chemicals, and equipment used were RNase free. Nevertheless, addition of RNase inhibitor could be a useful measure of precaution to protect the RNA template during cDNA synthesis in case of an inadvertent RNase contamination, and has been described in many assays (Pyra et al., 1994, Lovatt et al., 1999, Voisset et al., 2001, Andre et al., 2000). However, there were indications that false positive signals may be produced by RNasin in combination with brome mosaic virus (BMV) RNA template (Lugert et al., 1996). The effect of RNasin (Promega) added to pre-mix containing MS2 phage RNA was tested in a comparison to a non-infected Molt 4/8 and a virus-producing cell culture supernatant (SMRV infected 293 cells). Without RNasin, the non-infected supernatant showed an RT activity of less than 10 nUs, with one out of three samples being completely negative. Addition of RNasin

(20 U) to the cDNA mix resulted in an increase of the measured activity to several hundred nU. The same effect was observed for the SMRV infected cell culture supernatant; the activity increased about 1.5 $\log_{10}$. Without RNasin, the detected activity was 1.0×10^5 nUs; RNasin increased the measured activity to about 6.0×10^7 nUs. As a result of these observations, RTA was carried out without RNasin or any other RNase-inhibitor.

3.1.2.2.6.5 A DNA trap for background activity

The exclusion of protease and RNase inhibitors as well as the RNase digestion of the RNA template prior to PCR improved the quality of the assay – regarding sensitivity and background signals – considerably. Nevertheless, minute background activity was still observed in negative controls (data not shown). It was presumed that this activity was generated by the DNA-polymerase used for the PCR assay. Although the MS2-template is much less efficiently used by eukaryotic DNA polymerases than the synthetic homopolymeric templates usually employed for RT tests (Sarngadharan et al., 1978), small amounts of non digested RNA could lead to these persistent background signals. Appropriate conditions provided, different DNA dependent polymerases, such as DNA polymerase I from *E.coli* and eukaryotic enzymes like DNA polymerase α and γ, have RT-like activities, also DNA polymerase β and Klenow enzyme show minor activities (Ricchetti and Buc, 1993). Since RT background activity might depend on the source of *Taq* polymerase, two DNA-polymerases were compared, *Taq* polymerase (Invitek) and *AmpliTaq Gold* (Universal Master Mix, Roche). Both assays displayed the same level of background activity. Since PCR conditions were optimized for the *Taq* polymerase and different extents of RT activity were described for other commercially available, heat-stable DNA-polymerases as well (e.g. for natural DNA polymerase such as *Thermus flavis* [Tfl pol] as well as for genetically produced DNA polymerase, such as *Thermus thermophilus* [Tth pol], and since DNA polymerases without RT activity such as *Pwo pol* were quite expensive, the suppression of RT-like activity was aspired to, instead of exchanging the DNA-polymerase (Newton and Graham, 2001, Myers and Gelfand, 1991). The RT-like activity appears only in the case of very little or no cDNA or DNA as target. To suppress the RT-like activities of the DNA polymerase, a surrogate template – activated calf thymus DNA (Sigma) – was offered during PCR (Lugert et al., 1996). Since no matching primers or probes for the PCR assay were available within the mix, this target could not result in any signal in the real-time PCR.

A cDNA-mix containing MS2 RNA as template was compared to a mix containing MS2 RNA and calf thymus DNA as nucleic acid template in the TaqMan-PCR assay without prior cDNA synthesis. The mix containing MS2 RNA only resulted in a signal corresponding to 700 nU of RT activity, while the mix including MS2 RNA and calf thymus DNA (DNA trap) resulted in no signal, thus indicating suppression of the RT activity displayed by the Taq polymerase. The Tris buffer controls, cell culture media, and additional negative controls subsequently tested negative in combination with the DNA

trap and Mg^{2+} cDNA buffer (data not shown). This modification of the RTA led to the complete inhibition of aberrant but not of specific RT activities with Mg^{2+} buffer. Therefore calf thymus DNA was added to every cDNA mix (500 ng per reaction).

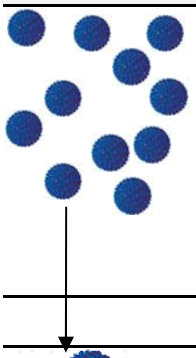
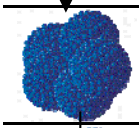
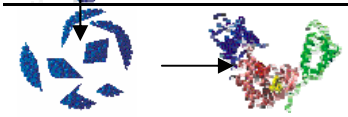
3.1.2.2.6.6 Background of manganese buffer derived cDNA

The fact that the *Taq* polymerase used for PCR develops an affinity for RNA as template with low concentrations of Mn^{2+} results in a steady background signal for cDNA synthesized with Mn^{2+} buffers, in spite of the calf thymus DNA trap and the RNase digestion of the template prior to PCR. The Mn^{2+} inherent in the cDNA mix was passed on to the PCR mix, leading to a final concentration of 0.4 mM Mn^{2+}. This mix resulted in a background signal (ct > 30). This background corresponds to an equivalent of 1000 nU of M-MLV RT activity obtained with Mg^{2+} buffer. Nevertheless, the Mn^{2+} buffer was employed in parallel to the Mg^{2+} buffer in order to determine the ion dependency of the respective retrovirus. In the presence of Mn^{2+}dependent RT this background signal was negligible, since the ct values derived from reverse transcription ranged well below 30. All reverse transcriptases tested were able to use both cations, Mn^{2+} and Mg^{2+}, but with different efficiencies.

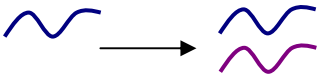
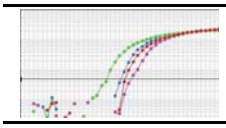
3.1.2.2.7 Assay conditions

The optimized conditions are listed in the following table (tab. 6):

Tab. 6: RTA flowchart: optimized conditions

	Step	Optimized conditions
 Virus particles in cell culture supernatant or plasma	Sample preparation Removal of cells and cellular debris by centrifugation	Storage at -70°C after removal of cellular debris, no protective additives. If required: PEG precipitation for larger volumes
	Purification	20% sucrose cushion
	Ultracentrifugation	70,000 × g 1.5 hours
	Lysis of virus particles/release of RT	Lysis and cDNA buffer: Tris 56 mM, KCl 56 mM, DTT 5.6 mM, Igepal 0.9% final concentration
Addition of cDNA buffer including MS2 RNA template	cDNA synthesis	Buffer conditions No protective additives (i.e. BSA, protease inhibitors or RNasin).

cont'd

	Step	Optimized conditions
 RNA cDNA		Mg^{2+} buffer (final concentration): MS2 RNA 300 ng/reaction, CT DNA 500 ng/reaction, MS2 reverse primer 500 nM, $MgCl_2$ 5 mM, dNTP 500 µM each Mn^{2+} buffer (final concentration): MS2 RNA 300 ng/reaction, CT DNA 500 ng/reaction, MS2 reverse primer 500 nM, $MnCl_2$ 0.8 mM, dNTP 1 mM each
	Quantitative real-time PCR	Specific for MS2
Trouble shooting:	Elimination of background signals	RNase digestion prior to PCR DNA trap included in cDNA mix Assay range >6 log_{10}

3.1.2.2.8 Assessment of the RTA assay performance

3.1.2.2.8.1 Standardization and quantification

Quantification of the RT activity can be accomplished with different standards. (1) A plasmid standard was cloned and used to quantify the amount of synthesized cDNA as copies per milliliter. (2) A serial dilution of a SMRV virus stock with quantified (RT real-time PCR) amount of particles per mL was used as lysis control and allowed to estimate the amount of viral particles in comparison to unknown stocks and supernatants using SMRV as model retrovirus. (3) A serial dilution of commercially available reverse transcriptase (M-MLV) with a defined activity of 200 units per µL was used in parallel to the samples and negative controls in triplicate for each assay. The stability of the activity over time was shown (fig. 18). The SMRV virus stock (2) was prepared and the lysate was tested in parallel with M-MLV reverse transcriptase in various assays, showing the reproducibility of the assay.

Since the M-MLV RT prefers Mg^{2+} to Mn^{2+}, direct comparison and quantification in RT units were carried out for RTs which also preferred Mg^{2+} only. The RT activity is thus either given in nU of reverse transcriptase activity (Mg^{2+}-preferring enzymes), or in relation to the ct value (45-ct), when RT activities were obtained with Mn^{2+} or with both cations in parallel.

3.1.2.2.8.2 Correlation of reverse transcriptase activity and viral load

Two different retrovirus producing cell culture supernatants were examined to assess the correlation of viral load determined by virus-specific quantitative RT real-time PCR assays and by using the retrovirus-generic RT assay. Viral load of supernatants of HIV infected

293T cells and Squirrel monkey retrovirus (SMRV) infected 293 cells were quantified with both assays (fig. 24).

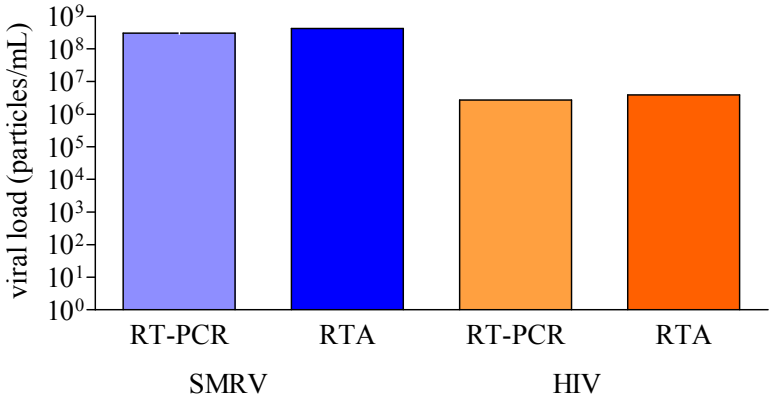


Fig. 24: Quantification of viral load by determination of RNA genomes and RT activity. Both assays were performed using aliquots of supernatant harvested at the same time and stored under the same conditions. The RTA was performed in triplicate with Mg^{2+} cDNA buffer. The quantitative real-time PCR was performed in duplicate. Viral load is given as particles per mL of cell culture supernatant.

The amount of RT molecules and viral particles was calculated based on known units of commercially available M-MLV detected with the RTA. According to Sears and Khan, 10 pU of RT activity are equivalent to one RT molecule (Sears and Khan, 2003). For HIV, approximately 100 RT molecules have been estimated per virion (Telesnitsky and Goff, 1997). This estimation was also used to calculate the viral loads of other retroviruses tested, thus 1000 pU correspond to 100 RT molecules and are equivalent to one retrovirus virus particle. The results for RNA genome equivalents compared to the calculation of virus particles based on determination of RT activity gave equal viral loads for the respective virus, indicating that quantification of virus particles can be reliably determined by RTA.

3.1.2.2.8.3 Reproducibility

The inter- and intra-assay variance was determined with repeated RTAs using infected cell culture supernatants of two retroviruses, SMRV (293 cells) and HTLV-1 (MT2 cells). The supernatants varied in viral load; while the SMRV viral load was relatively high (3×10^8 particles per mL, determined by RT-PCR), the viral load of the HTLV-1 infected cell culture supernatant was considerably lower (9×10^5 particles per mL, determined by RT-PCR; fig. 25).

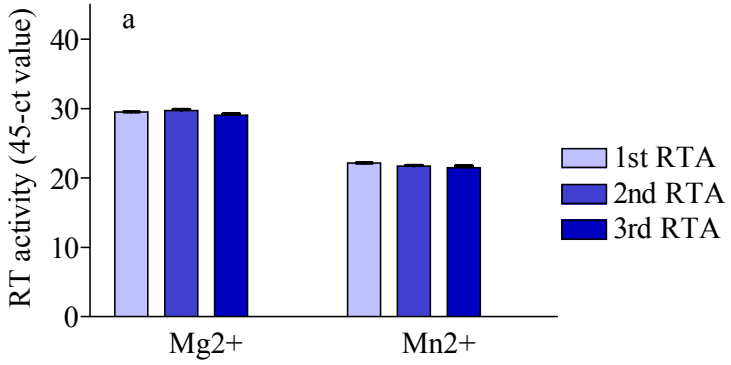


Fig. 25: Reproducibility of the RTA. (a) SMRV: The data shown represents the mean ct values and the respective standard deviations of three different RTAs carried out with aliquots of the same SMRV infected cell culture supernatant (293 cells) at different time points.

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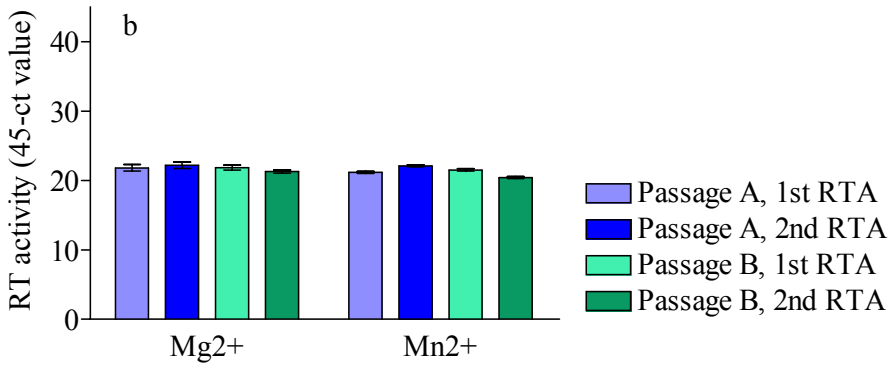

(b) HTLV: Supernatant of MT2 cells was harvested at two different points of time (passages A and B; B harvested 2 weeks or 4 passages later); samples were stored at -70°C. Both supernatants were tested twice in two independent RTAs with different cDNA buffers (Mg^{2+} and Mn^{2+}); each sample was tested in triplicate. The data shown represents ct values obtained with the equivalent of 0.025 µL SMRV infected and 25 µL HTLV-1 infected cell culture supernatant. RT activity is given as 45-ct value; 3.3 ct value corresponds to approx. 1 $\log_{10}$ RT activity.

Nine cDNAs independently derived from three SMRV preparations showed minute standard deviations. The inter assay variance for the RTA was assessed by analyzing aliquots of the same stock in parallel (mean 45-ct value and standard deviation for Mg^{2+}: 29.36 +/- 0.28; for Mn^{2+}: 21.75 +/- 0.36). The RTA was performed for both cations Mn^{2+} and Mg^{2+}. While the inter assay variance was low for both cations, the total RT activity was almost 250 times higher with Mg^{2+} than with Mn^{2+}, demonstrating the Mg^{2+} preference of SMRV reverse transcriptase.

Supernatant of two different passages of MT2 cell culture was tested in two independent RTAs in parallel in order to compare the RT activity of different passages of the same cell culture, as well as to assess the inter and intra assay variability. The comparison of reverse transcriptase activity for different RTAs carried out with the same supernatant, as well as for supernatants harvested at different time points, gave comparable results for all four tests. The stable RT activity over time also indicates constant expression of HTLV by MT2 cells.

Considering the workflow from harvesting the supernatant, to particle preparation and lysis, cDNA synthesis and PCR, the assessed inter and intra assay variances for the RTA are fairly small.

3.1.2.3 Analysis of RT activity in cell culture supernatants

The general performance of the RTA was tested for various cell culture supernatants: non-infected, HIV, SMRV, or HTLV infected supernatants. Furthermore, cell lines with unknown infection status for retroviruses were investigated: cancerous cell lines, such as human leukemia, human melanoma or teratocarcinoma derived cell lines as well as animal cell lines and cell lines established from PBL of MS patients.

3.1.2.3.1 RT activity in non-infected cell culture supernatant

Since several compounds showed RT activity, non-infected cell cultures supernatants were analyzed for background RT activity. Supernatant of non-infected CEM, 293 Graham, HEpG2 and Vero cells was collected (when adherent cells were confluent or suspension cell lines reached a density of 1×10^6 cells per mL) and stored at -70°C (fig. 26).

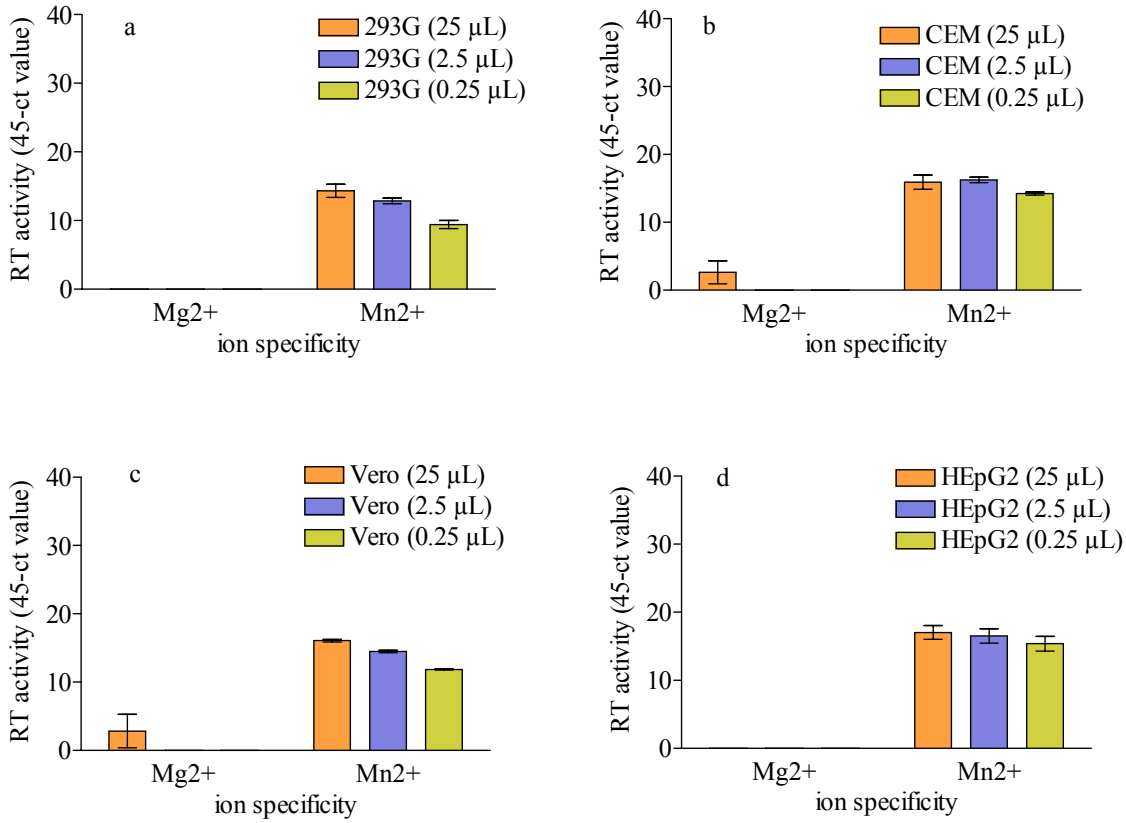

Fig. 26: RT activity in non-infected cell culture supernatants. The cDNA syntheses were carried out using Mg^{2+} and Mn^{2+} buffer in parallel, three dilutions of the respective lysate (equivalent to 25 mL, 2.5 mL, and 0.25 µL supernatant) were tested in triplicate. RT activity is given as 45-ct value; 3.3 ct value corresponds to approx. 1 $\log_{10}$ RT activity; a: 293 Graham, b: CEM, c: Vero, d: HepG2 cells.

The cell culture supernatants showed no evidence of Mg^{2+}-dependent RT activity. With Mg^{2+}, minute signals for Vero and CEM cells were detected in one of three non-diluted supernatants, and are probably caused by residual cellular polymerases (the measured ct values correspond to approximately 0.05 nU units of RT activity, or 0.05 calculative retrovirus particles). Mn^{2+}-related RT activities were observed for all cell culture supernatants and reflect the background inherent in all Mn^{2+}-dependent RTAs. The 10-fold dilution of lysates was not reflected by the signals obtained in the TaqMan assay; this was indicative of background signals rather than retroviral RT activity. Genuine RT activity derived from retrovirus particles present in a cell culture supernatant would be increased by the concentration of virus particles prior to lysis and cDNA synthesis, e.g. by

ultracentrifugation of larger volumes of supernatant or by particle preparations such as polyethylene glycol (PEG) precipitation. To determine whether the observed RT activities were retrovirus-related or representing Mn^{2+}-related background signals, two non-infected cell culture supernatants of 293 Graham and Molt cells were subjected to a polyethylene glycol (PEG) precipitation in order to pellet potentially existing retrovirus particles (Yamamoto et al., 1970). Suspensions of these preparations corresponding to 100 times the volume of the original supernatant were analyzed in parallel with both cell culture supernatants. No increased RT activity was observed for PEG precipitation in comparison to cell culture, indicating that the observed weak signals for Mn^{2+} buffer were not representing particle-associated RT activity, but rather background signals.

3.1.2.3.2 RT activity in four different human melanoma cell lines

Malignant melanomas arise from uncontrolled proliferation of melanocytes; they are the second most common kind of cancer in Caucasians. Exposure to sunlight is considered to be a major risk factor for the induction of transformation, and the expression of human endogenous retroviruses, including HERV-K, can be induced by UV light (Hohenadl et al., 1999). This co-occurrence led to the hypothesis that HERV-K might be involved in tumor genesis. Four cell lines derived from human melanomas were tested for reverse transcriptase activity that should be present in the case of the expression of HERV-K or any other retrovirus.

Supernatant of G361, MelJuso, Mewo and G-RM cells (all melanoma derived cell lines) were collected and stored at -70°C before being applied to the RTA (fig. 27).

The cell culture supernatants showed no evidence for Mg^{2+}-dependent RT activity. Observed signals for MelJuso (d) and Mewo (b) represented 10 nU to 30 nU, corresponding to 10 to 30 retroviral particles, and are probably linked to contamination of the sample with residual cellular enzymes, incomplete RNase digestion, or assay interference. This assumption is based on the fact that the 100-fold dilution of virus lysate resulted in the highest RT activity detected for Mewo (b), and that RT activity was only detected in the 10-fold dilution but not in undiluted supernatant for MelJuso (d). Mn^{2+} buffer derived cDNAs showed a higher RT activity for the non-diluted supernatant than for the 10-fold and 100-fold dilutions, but the differences in ct values do not reflect the expected 1 $\log_{10}$ difference for G361 (c). The dilution pattern was reflected by the RT activity detected in G-RM (a). However, the RT activity in these samples was even lower than the background signals of non-infected cells, and was categorized as background activity that is always present with Mn^{2+} buffer. Larger volumes of melanoma-derived cell culture supernatant could not be obtained and tested for particle associated RT activity. The four melanoma- derived cell lines did not show higher RT activity than other human cell lines tested.

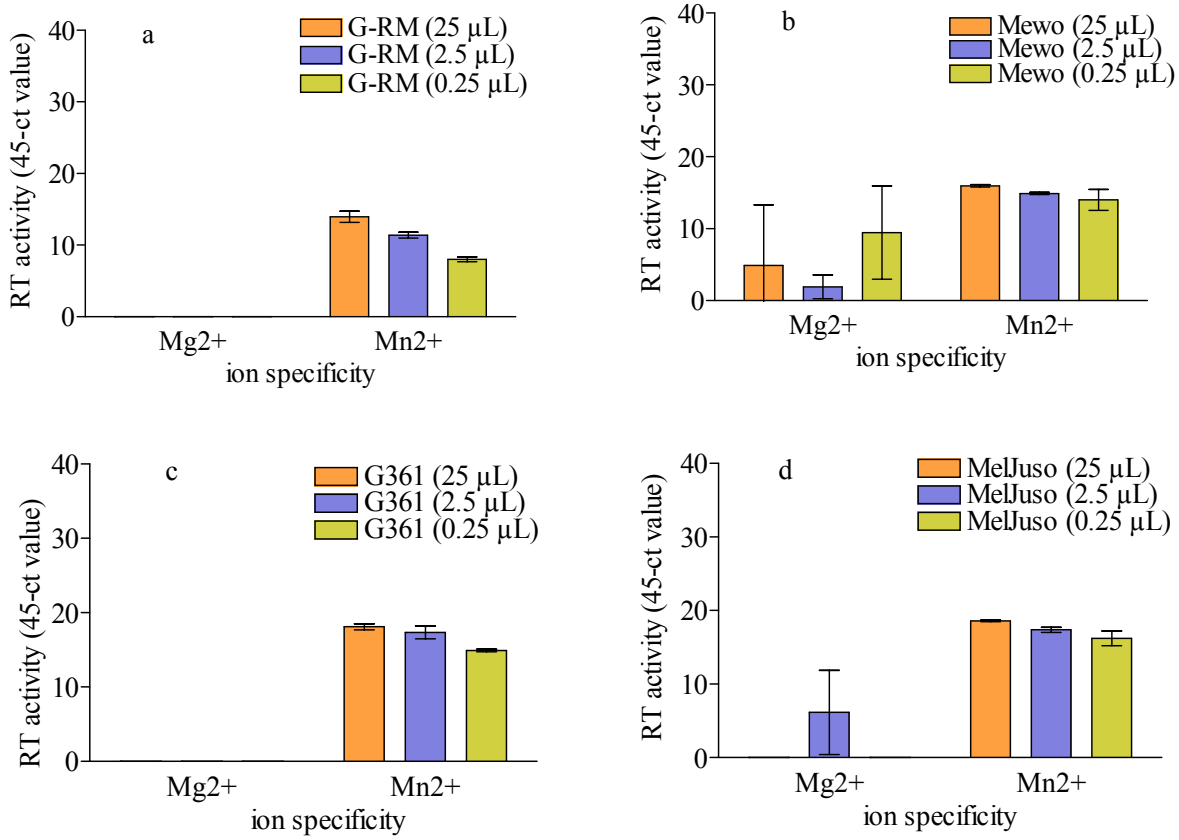

Fig. 27: RT activity in four melanoma-derived cell culture supernatants. The cDNA syntheses were carried out using two different cation buffers (Mg^{2+} and Mn^{2+}), in three dilutions of the respective lysate and in triplicate for each sample. The equivalent of 25 µL, 2.5 µL, and 0.25 µL supernatant was used for each assay as an exogenous source for reverse transcriptase activity. RT activity is given as 45-ct value; 3.3 ct value corresponds to approx. 1 $\log_{10}$ RT activity. (a) G-RM; (b): Mewo, (c): G361, (d): MelJuso

3.1.2.3.3 RT activity in a HERV-K mRNA expressing cell line

The human teratocarcinoma cell line GH was established in Roswitha Löwer and Johannes Löwer's group at the Paul-Ehrlich-Institut. Expression of different mRNA species of the human endogenous retrovirus K (HERV-K) was detected within GH cells: Small *env* mRNAs, a double-spliced *env* transcript, coding for cOrf, a product that is functionally related to *rev* (HIV), and Gag proteins were detected with immunofluorescence. Furthermore, a high expression of HERV-K mRNA was observed (Tonjes et al., 1996).

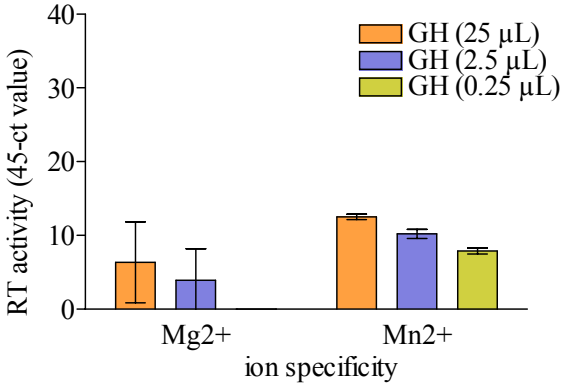

Fig. 28: RT activity in a HERV-K expressing teratocarcinoma cell line. Cell culture supernatant of GH cells was stored at -70°C. The RTA was carried out in triplicate for three dilutions of virus lysate with two different cDNA buffers (Mg^{2+} and Mn^{2+}). The equivalent of 25 µL, 2.5 µL, and 0.25 µL of supernatant was used as an exogenous source for the reverse transcriptase activity. The RT activity is given as 45-ct value; 3.3 ct value corresponds to approximately 1 $\log_{10}$ RT activity.

The comparison of other cell culture supernatants and the teratocarcinoma cell culture supernatant showed a low but explicit reverse transcriptase activity for the GH supernatant (fig. 28). The detected RT activity with Mg^{2+} buffer was equivalent to 6800 particles per mL, while the 10-fold diluted sample resulted in an RT activity equivalent to 200 particles per mL, based on calculation of HIV, HTLV, and SMRV viral load. A weak Mn^{2+}-dependent RT activity was observed which was within the range of the non-infected control cell lines; the 10-fold dilutions are not reflected by the ct values. The sample used for the RTA had been stored at -70°C for 26 months before analysis; storage may have attenuated the RT activity.

3.1.2.3.4 RT activity in Kasumi-1 cell culture supernatant

Retroviruses can be causative agents for leukemias, e.g. HTVL-I can induce the adult T-cell leukemia (ATL) in humans, the feline leukemia virus (FeLV) can cause not only lymphosarcomas, but also bone marrow tumors and immunosuppression in cats. In case of a retroviral factor for the onset of leukemia, a cell line established from a patient with leukemia may still express the respective retrovirus. Kasumi-1 is a cell line established from the peripheral blood of a 7-year old Japanese child with acute myeloid leukemia (AML). This cell line was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ), being declared as RT activity positive in an ELISA assay while testing negative for HIV and HTLV. The RTA was carried out in triplicate for three dilutions of virus lysate with two different cDNA buffers (Mg^{2+} and Mn^{2+}; fig. 29).

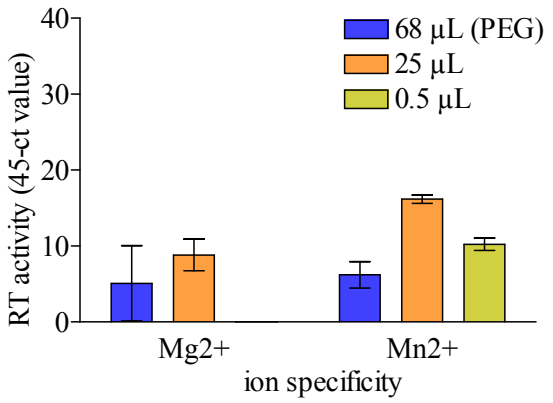

Fig. 29: RT activity in Kasumi-1 cell culture supernatant. The RTA was carried out in triplicate using different dilutions of virus lysate prepared from cell culture supernatant and 1000-fold concentrated PEG precipitation of particles with two different cDNA buffers (Mg^{2+} and Mn^{2+}). The data shown represent the RT activity in 0.5 µL, 25 µL, and 68 µL supernatant. RT activity is given as 45-ct value; 3.3 ct value corresponds to approximately 1 $\log_{10}$ RT activity.

RT activity was measured with both assays, the Mg^{2+} related activity corresponding to approximately 240 virus particles per mL supernatant. In order to determine whether the detected RT activity was particle associated or caused by the interference of other compounds, different dilutions of virus lysate, including a PEG precipitation, were analyzed. No Mg^{2+} related RT activity was detectable for the equivalent of 0.5 µL supernatant. The RT activity derived from particles after PEG precipitation was lower compared to the equivalent of 25 µL supernatant. The same effect was observed for the Mn^{2+} buffered RTA; the ct values obtained for 0.5 µL and 25 µL supernatant reflected the

dilutions, while lower activity was determined for the equivalent of 68 µL obtained following PEG precipitation. This relative loss of activity was probably caused by the PEG precipitation itself, since the PEG incubation was carried out o/n, the following preparation and ultracentrifugation lasted for several hours at 4°C, and the concentrated particles were frozen at -70°C again until the RTA was performed. Nevertheless, these results showed that, despite an observed loss due to sample treatment, RT activity in Kasumi-1 cells was particle-associated and exhibited a slight Mn^{2+} preference.

3.1.2.3.5 RT activity in porcine cell culture supernatant

Endogenous retroviruses and related elements not only constitute a considerable part of the human genome, but also the genomes of other vertebrates. While most human endogenous retroviruses are truncated and non-functional, endogenous retroviruses are expressed as infectious particles in some animals. Porcine endogenous retroviruses (PERVs) are integrated into the genome of all pigs. They belong to the gammaretroviruses and are closely related to feline, murine, and gibbon ape leukemia viruses that can induce leukemia and immunodeficiencies in the infected host (Patience et al., 2001). At least two subtypes of PERV, PERV-A and PERV-B, infect human cells in vitro (Specke et al., 2001, Patience et al., 1997a). Expression of PERVs has been shown in several pig tissues at the level of mRNA production (Akiyoshi et al., 1998, Clemenceau et al., 1999), and viral particles have been shown to be released by mitogen stimulated pig peripheral blood mononuclear cells (PBMC; Wilson et al., 1998, Tacke et al., 2000), by cultured aorta endothelial cells (Martin et al. 1998), by pig pancreatic islet cells (van der Laan et al., 2000), and by several pig cell lines, including the kidney cell line PK-15 (Lieber et al., 1975).

The release of PERVs from PK-15 and from PS cell lines, both porcine epithelial kidney derived cell lines, was shown by subjecting supernatants of these cell lines to the RTA (fig. 30a and b, respectively).

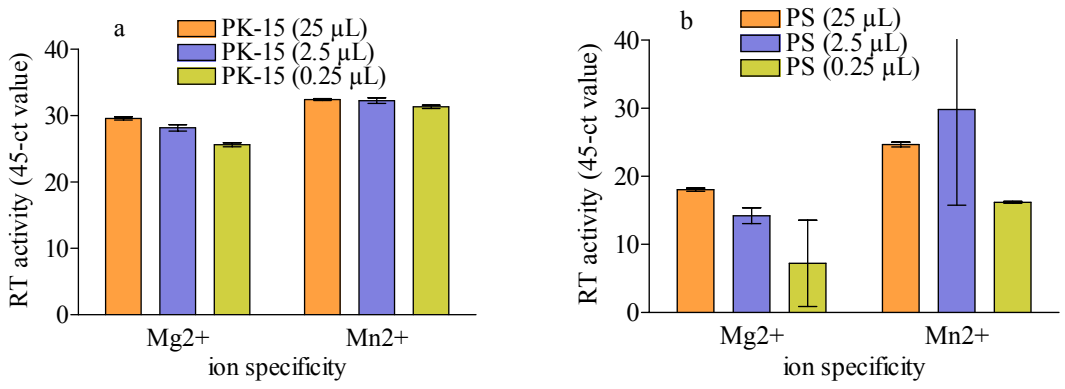

Fig. 30: RT activity in two porcine cell culture supernatants. Cell culture supernatants were stored at -70°C. The RTA was carried out in triplicate for three dilutions of virus lysate (representing 25 µL, 2.5 µL, and 0.25 µL of cell culture supernatant) with different cDNA buffers (Mg^{2+} and Mn^{2+}). RT activity is given as 45-ct value; 3.3 ct value corresponds to corresponding to 1 $\log_{10}$ RT activity. (a): PK-15; (b): PS cell culture supernatant.

Both porcine cell culture supernatants exhibited relatively high levels of reverse transcriptase activity. The ct values for the different PK-15 dilutions were too close and did not reflect the 10-fold dilutions, especially for Mn^{2+} (fig. 30a). Due to technical requirements of the instrument, reliable ct values can be obtained starting at a ct value of 15; the values obtained for earlier PCR cycles (usually 3 to 15) are used to define the threshold for the respective assay. The high viral load in PK-15 supernatant leads to ct values of 12; this was not within the linear range of the TaqMan® assay. Thus, a high level of RT activity was detected but exact quantification not performed. RT activity for PS cell culture supernatant was about 3 $\log_{10}$ lower than for the PK-15 supernatant (fig. 30b). The ct values for PS supernatant obtained with Mg^{2+} reflected the 10-fold dilutions of the lysate, while the ct values for the 10-fold dilution obtained with Mn^{2+} showed large deviations. The lower reverse transcriptase activity, probably caused by lower numbers of retroviral particles observed for the PS cell line, could later be linked to an accidental infection with swine fever that was not yet detected at the time RTA was performed. After deduction of the background signal observed with Mn^{2+}, the reverse transcriptases of PERVs in porcine cell culture supernatants showed a clear Mn^{2+} preference.

3.1.2.3.6 Reverse transcriptase activity in two MS cell lines

MS has been associated with many different viruses (addendum 5.4 and 5.5). In recent years retroviruses have been in the focus of interest. Different groups in Denmark, France and Italy described RT activity, expression of retrovirus sequences (endogenous sequences) or retrovirus particles. Spontaneously formed B lymphoblastoid cell lines from long-term cultured peripheral blood mononuclear cells (PBMNCs) have been established from multiple sclerosis patients in Denmark. These cell lines have been previously described to exhibit RT activity (Christensen et al., 1999). Two of these cell lines (MS1533 and MS1845) were cultivated, supernatant was collected, and stored at -70°C. The RTA was performed in parallel with a PEG precipitation corresponding to a 1000-fold concentration in order to test whether any detected RT activity was particle- associated. In this case, the RT activity should increase by about 1000-fold through by PEG precipitation prior to the RT assay (fig. 31).

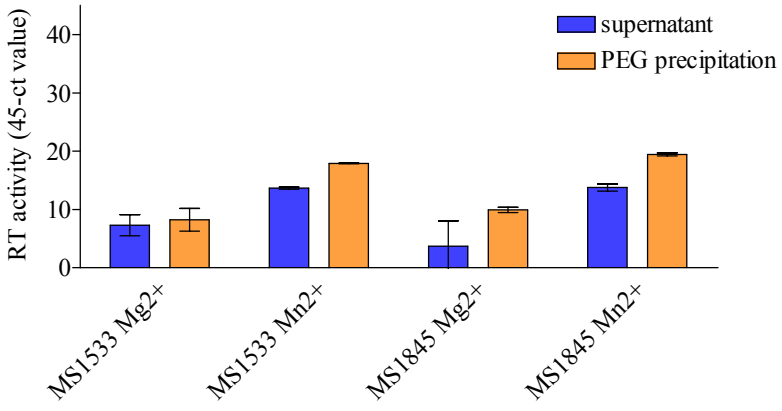

Fig. 31: RT activity in MS cell culture supernatant and PEG precipitation. Supernatant of two MS patient-derived cell cultures was collected and a 1000-fold concentration of potentially existing retroviral particles was performed by PEG precipitation. The equivalent of 25 µL cell culture supernatant was directly compared to the equivalent of

2.5 mL supernatant obtained by a 10-fold dilution of the PEG precipitation. The assay was carried out in triplicate with Mn^{2+} and Mg^{2+} buffer in parallel. RT activity is given as 45-ct value; 3.3 ct value corresponds to approximately 1 $\log_{10}$ RT activity.

The MS1533 cell line showed RT activity with Mg^{2+}, however, the PEG precipitation (100-fold concentration of the supernatant) showed only a 2-fold higher RT activity (240 and 460 nU/mL, respectively). The Mn^{2+}-dependent RT activity was also only slightly increased for the PEG precipitation in comparison to the supernatant. A low Mg^{2+} related RT activity was detected in MS1845 supernatant. The PEG precipitation (100-fold concentration of the supernatant) showed a 6-fold higher RT activity (140 and 890 nU/mL, respectively). The observed differences for supernatant compared to PEG precipitation could be linked to assay variances, especially if the relatively low overall activity is considered. However, they were not linked to particle associated RT activity: if the activity was particle associated, the 100-fold concentration of supernatant would have resulted in equally higher activity instead of 2- and 6-fold elevated activity. The RT activities determined for MS cell lines with the RTA were far below that described by Christensen et al. (tab. 7).

3.1.2.3.6.1 Induction of virus growth

In order to explain the observed differences between measured RT activity and the published data, the MS cell cultures were stimulated with butyrate to test whether virus production could be induced. Supernatant of the butyrate-treated cell culture was tested in parallel to non-treated supernatant (fig. 32).

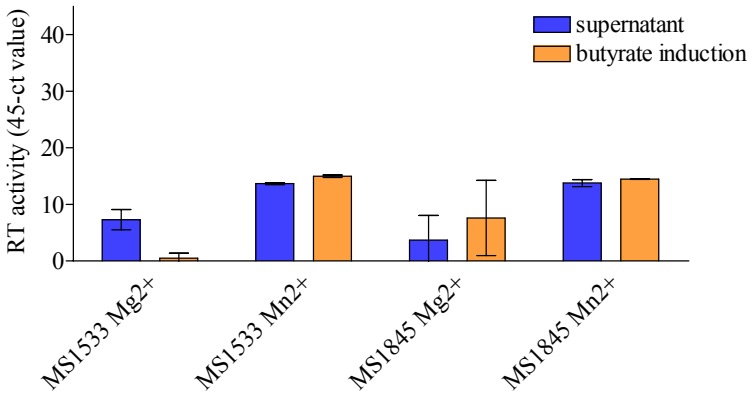

Fig. 32: RT activity in MS cell culture supernatant with and without butyrate treatment. Supernatant of non-treated and butyrate-treated MS derived cell cultures was collected and stored at -70°C. The equivalent of 25 µL cell culture supernatant was used as an exogenous source for RT activity. The assay was carried out in triplicate with Mn^{2+} and Mg^{2+} buffer in parallel. RT activity is given as 45-ct value; 3.3 ct value corresponds to approximately 1 $\log_{10}$ RT activity.

The MS1533 cell culture exhibited lower RT activity with the butyrate treatment; there was no detectable RT activity with Mg^{2+} for butyrate-treated samples. The Mn^{2+}-dependent RT activity showed a decline of about 1 $\log_{10}$ for the treated cell culture supernatant in comparison to the control. Butyrate induction resulted in low Mg^{2+}-dependent RT activity for MS1845 supernatant; the effect of butyrate induction on the Mn^{2+}-dependent RT

activity was not significant. Using butyrate as a means of virus induction did not enhance detectable reverse transcriptase activity in the examined cell cultures MS1533 and MS1845 (tab. 7).

Tab. 7: Reverse transcriptase activity* in MS cell lines

Cell line	RT activity in supernatant (nU/mL)	RTA following PEG precipitation (nU/mL) **	RTA following butyrate treatment (nU/mL)	Christensen et al., 1999 (nU/mL)
MS1533	248	460	Not detectable	1.4×10^2–1.2×10^{15}
MS1845	142	891	740	1.5×10^8–2.5×10^{10}

* Mg^{2+}-dependent reverse transcriptase activity

** 100-fold concentration of supernatant

3.1.2.3.7 RT activity in cell culture supernatants infected with human retroviruses

3.1.2.3.7.1 RT activity in HIV-1 infected cell culture supernatant

The reverse transcriptase activity of the human immunodeficiency virus (HIV) was analyzed in different assays. HIV is the best characterized human retrovirus at present. Various sensitive and specific detection methods for HIV have been developed (Western blot, ELISA, RT-PCR, p24 antigen capture assay). The HIV reverse transcriptase has also been studied extensively. This enzyme is one of the key targets for antiretroviral therapy (highly active antiretroviral therapy, HAART); mutations within this enzyme are one of the reasons for therapy failure. The accessible knowledge of HIV RT and the fact that it is a human retrovirus makes it a suitable virus to validate the RTA and to assess the potential of this optimized assay.

3.1.2.3.7.1.1 Determination of viral load with the RTA compared to titration

Supernatant of HIV infected CEM cell culture supernatant was used for reverse transcriptase activity analysis, while the determination of $TCID_{50}$ by titration according to Spearman and Kärber (Spearman and Kärber, 1974) was performed in parallel. The viral load was calculated on the basis of M-MLV RT units as 3.2×10^9/mL and the infectious titer was determined as 1.7×10^7/mL. Not all generated HIV particles are infectious; this is the case for only one in 100 to 1000 particles (Dimitrov et al., 1993). Thus, different from determination of the infectious titer ($TCID_{50}$), non-infectious particles can be detected by the reverse transcriptase activity assay, provided they can be precipitated and contain active reverse transcriptase molecules. The correlation of virus load and reverse transcriptase activity was shown (fig. 24).

3.1.2.3.7.1.2 Comparison of reverse transcriptase activity of drug-resistant and sensitive HIV

Mutations in the reverse transcriptase can lead to resistance of HIV-1 to RT inhibitors but are often associated with reduced viral fitness. Thus, resistant viruses are expected to be less replication competent, and are often overgrown by wild type virus once the therapeutic pressure is released (Collins et al., 2004). These mutations may also affect the efficiency of the reverse transcriptase. The influence of resistance associated mutations on the enzymatic activity of the HIV-1 reverse transcriptase was analyzed using AZT/ddC/d4T, AZT/ddC/d4T/3TC and AZT/DLV multiresistant recombinant viruses in comparison to NL4.3 as wild type control. The results for $TCID_{50}$ and p24 antigen assay for the recombinant clones and for the wild type clone, as well as the respective cell culture supernatants, were obtained from S. Duwe, 2003; fig. 33).

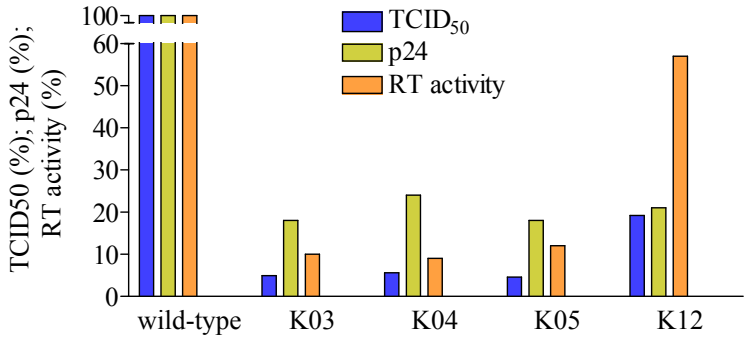

Fig. 33: Replication efficiency of drug-resistant and sensitive HIV-1 clones. The data obtained for the wild type clone was defined as 100% for $TCID_{50}$, p24 antigen capture assay, and RT activity, respectively. Supernatant of the infected cell cultures was stored at -70°C. The equivalent of 0.5 µL supernatant was used as an exogenous source for RT activity. AZT: azidothymidine, ddC: didesoxycytidine; d4T: didesoxythymidine; 3TC: cytidine analogue; DLV: Delvavirdin. AZT, ddC, d4T and 3TC are nucleoside reverse transcriptase inhibitors (NRTI; dideoxynucleosides); DLV is a non-nucleoside reverse transcriptase inhibitor (NNRTI).

Titration and p24 antigen assay showed diminished fitness for the clones with resistance-associated mutations. Three samples (K03, K04 and K05) exhibited 10-fold lower RT activity (K03=10%, K04=9% and K05=12%) compared to the wild type clone. These findings corresponded to the lower $TCID_{50}$ results (5%), while the p24 value indicating the amount of viral antigen was slightly higher (18%-24%). Sample K12 had a similar p24 value (21%), but showed a 4-fold higher $TCID_{50}$ as well as an increased RT activity representing 57% of the RT activity measured for wild type virus. Comparison of p24 values to $TCID_{50}$ and RT activity suggested that different resistance-associated mutations of the RT could result in different levels of diminished viral fitness, depending on the mutation.

3.1.2.3.8 Results of the analysis of reverse transcriptase activity detected in cell culture supernatants

3.1.2.3.8.1 Comparison of reverse transcriptase activity in different cell lines

The results obtained for non-infected as well as retrovirus infected cell lines, for human and animal cell lines, as well as for cell lines of diseased individuals were tested. The data are compiled in tab. 8. The RT activity is given in µU/mL cell culture supernatant. These units do not reflect equal activities for Mg^{2+} and Mn^{2+} as discussed above. However, RT activities for different cell lines can be compared independently for Mg^{2+} and Mn^{2+}.

Tab. 8: Compilation of RT activity detected in different cell lines

Cell line	Origin	Mg^{2+}-dependent RT activity		Mn^{2+}-dependent RT activity	
		µU/mL	Standard deviation	µU/mL	Standard deviation
MT2	Human; HTLV-transformed T cells	1169.16	104.12	1053.92	73.88
293T/HIV infected	Human kidney; HIV infected	50.90	39.20	208.80	69.28
293/SMRV infected	Human kidney; SMRV infected	$5.26x10^8$	$1.07x10^7$	$8.10x10^4$	443.80
293 Graham	Human kidney	0	0	1.48	0.14
CEM	Human lymphoblast	0.02	0.01	32.24	17.20
HEpG2	Human carcinoma	0	0	4.24	3.64
Vero	Monkey kidney	0.07	0.05	40.20	3.28
G-RM	Human melanoma	0	0	13.12	5.08
Mewo	Human melanoma	1.97	2.79	3.64	0.28
G361	Human melanoma	0	0	11.32	2.12
MelJuso	Human melanoma	0	0	15.60	0.56
GH	Human teratocarcinoma	0.20	0.16	3.48	0.01
Kasumi-1	Human leukemia	0.425	0.04	6.36	1.80
PK15	Porcine kidney	$3.48x10^5$	$4.42x10^4$	$5.73x10^6$	$7.24x10^5$
PS	Porcine kidney	496.00	13.64	$1.39x10^4$	$1.93x10^3$
MS1533	Long- term PBL from an MS patient	0.25	0.13	1.34	0.13
MS1845	Long- term PBL from an MS patient	0.14	0.19	1.53	0.45

3.1.2.3.8.2 Determination of cation preference

All samples were tested with two different cDNA buffers in order to determine the ion preference of the respective reverse transcriptase (fig. 34).

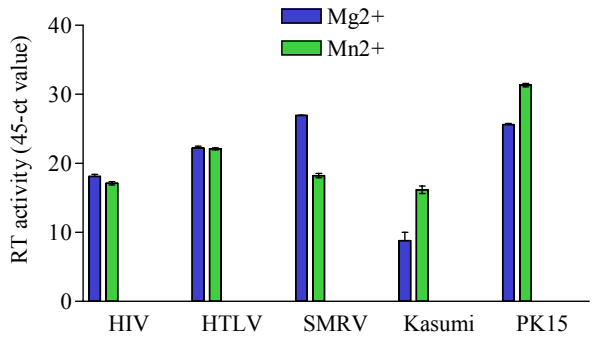

Fig. 34: Effects of cations on the activity of different reverse transcriptases. The cation preference of all analyzed RTs was determined by using Mg^{2+} and Mn^{2+} buffer for cDNA synthesis in parallel. The data shown were compiled from RTAs performed at different time points. The comparison of the RT activity (given as 45-ct value) indicates the ion preference of the respective reverse transcriptase. The data shown were obtained with different volumes of supernatant: HIV: 0.5 µL; HTLV: 25 µL; SMRV: 0.0025 µL; Kasumi: 25 µL; and PK15: 0.025 µL.

Tab. 9: Quantitative comparison of ion preferences

Virus	Mg^{2+} (mean ct value)	Mn^{2+} (mean ct value)	Δct	Difference in RT activity	Ion preference
HIV	26.93 (+/-0.38)	27.88 (+/-0.20)	0.95	0.288 $\log_{10}$	Mg^{2+} (Weak)
HTLV	22.80 (+/-0.37)	22.90 (+/-0.12)	0.10	0.030 $\log_{10}$	None
SMRV	18.05 (+/-0.04)	26.78 (+/-0.26)	7.73	2.645 $\log_{10}$	Mg^{2+}
Kasumi-1	36.17 (+/-1.69)	28.83 (+/-0.45)	7.34	2.224 $\log_{10}$	Mn^{2+}
PK15	19.37 (+/-0.24)	13.67 (+/-0.21)	5.70	1.727 $\log_{10}$	Mn^{2+}

All RTAs were carried out as 3-fold values; mean ct values and standard deviations are given. The calculation of different RT activities is based on the relation of 1 ct corresponding to 3.3 $\log_{10}$ copies of PCR product.

The HIV reverse transcriptase had a slight preference for Mg^{2+} but was almost as efficient using Mn^{2+}; this result corresponded to earlier findings (Telesnitsky and Goff, 1997). A second human retrovirus, HTLV-1, had no clear preference for either of the two cations; reverse transcription was performed with equal efficiency with either cation. This is in contrast to the previously described Mg^{2+} preference of the HTLV-1 reverse transcriptase (Owen et al., 1998), however, a shift in preference to Mn^{2+} from Mg^{2+} has been described with HTLV-1 RT when the template/primer were changed (Rho et al., 1981) Squirrel monkey retrovirus RT displayed a clear Mg^{2+} preference, while the RT activity of the Kasumi-1 cell culture supernatant showed a slight Mn^{2+} preference if the Mn^{2+}-related background and the low overall reverse transcriptase activity are taken into account. PERVs expressed by porcine cell cultures also showed an explicit Mn^{2+} dependency. The quantification of RT activity in units was defined by using a commercially available Mg^{2+}-dependent enzyme (M-MLV); therefore a direct comparison of RT activity obtained for

Mg^{2+}- and Mn^{2+}-dependent enzymes given as units may be questionable. In order to allow a quantitative comparison of the ion preferences of the analyzed reverse transcriptases, differences for RT activity are given as Δct values (tab. 9).

3.1.2.4 Reverse transcriptase activity assay employing plasma samples

Since the reverse transcriptase activity assay allows the detection of all retroviruses provided that these are present as extra-cellular particles, the assay should be able to detect traces of retroviral infections in plasma. The RTA was established using cell-free virus obtained from infected cell cultures, but preliminary tests with plasma showed that the detection of reverse transcriptase in plasma requires conditions other than those used for cell culture supernatant. Besides the diminishing effect of antibodies (fig. 10-13), other plasma components disturbed the assay performance and resulted in real-time PCR data that could not be analyzed. A 10-fold dilution of the plasma in 10 mM Tris prior to the first centrifugation was introduced (Heneine et al., 1995). As a result, the negative effects of plasma components on the assay performance were eliminated and reproducible results were obtained.

3.1.2.4.1 Detection of RT activity during HIV seroconversion

In general, HIV diagnostic is performed on plasma by detection of antibodies against viral proteins. This leads to a diagnostic window during which an individual may be infected, but has not yet developed antibodies against the virus. Application of PCR offers earlier detection of HIV infection, but still requires a certain amount of viral particles. The RTA was used to examine six plasma samples from an HIV-1 infected individual during seroconversion. The focus of analysis was whether the non-specific assay would be able to detect the infection as well as to determine the approximate diagnostic window of the assay (fig. 35). All samples had been previously tested with different HIV-specific assays. The time of infection was not known. No virus could be grown from the first sample and p24 antigen capture assay (Dupont) was also negative (day 1). Virus cultivation was successfully performed on the second sample; the p24 value was 29 pg/mL (day 17). The third sample had 50 pg/mL p24 (day 23). The following samples tested negative for p24 antigen.

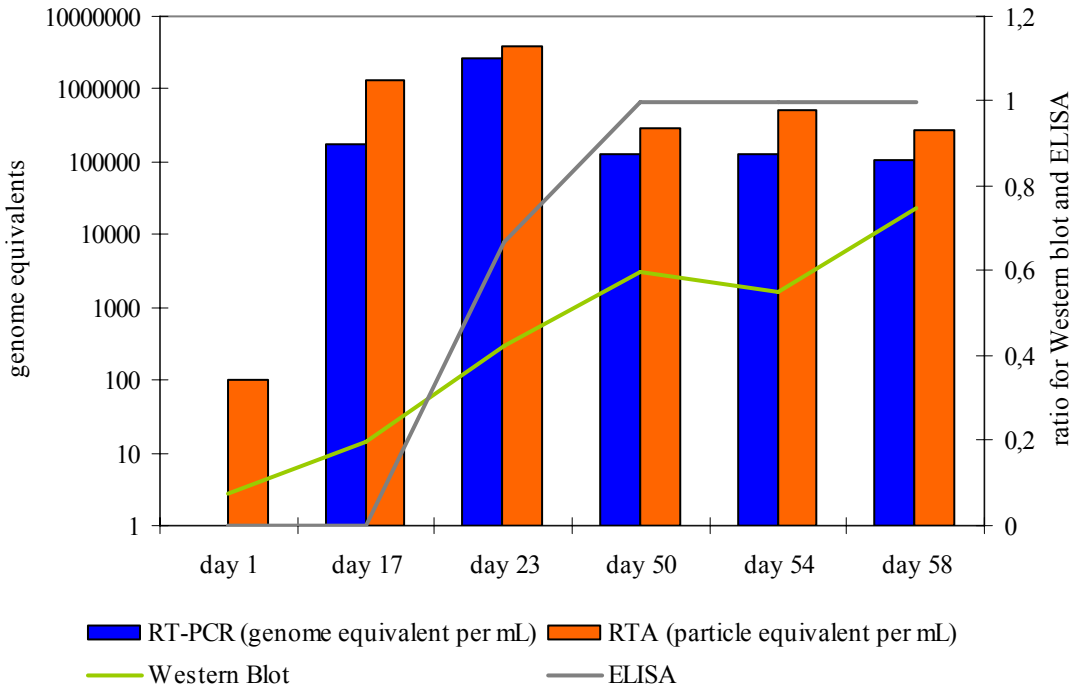

Fig. 35: HIV detection during seroconversion. Four different assays were used to analyze the samples. RT-PCR was performed for five different regions of the HIV-genome; the genome equivalents given are based on quantitative env-RT-PCR. Other HIV-specific assays applied were Western blot and ELISA. The curve given (grey) represent detected bands in relation to ten bands obtained for the last sample (day 58) for the Western blot. Three different ELISAs were used; the curve given shows the positive ELISA results divided by three (for all possibly positive ELISAs). The RTA was performed as a generic retrovirus assay. Day 1 represents the first sample. The time of infection is not known. The RT-PCR, p24 antigen capture assay, virus cultivation, ELISAs and Western blot analyses were performed by C. Kücherer and S. Neumann.

The first sample was negative for RT-PCR in five regions of the HIV-genome. The sample obtained at day 17 tested positive, as were all following samples with different viral loads. Western blot analysis (Pasteur) was indeterminate for the first two samples; the first plasma showed a weak p24 band, which can also be seen with some HIV-negative samples. The second sample revealed two weak bands and was also considered to be indeterminate. The number of bands obtained with Western blot analysis rose in the course of time; all samples from day 23 on were classified as HIV positive. Three different ELISAs (Wellcome, Biochrom, Pasteur) were performed on the plasmas. The first two samples were negative in all three ELISAs. The sample obtained on day 23 tested positive with two ELISAs (Wellcome, Pasteur) and from day 50 on all three assays tested positive. The RTA was performed on these samples using an equivalent of 10 µL plasma as an exogenous source for reverse transcriptase activity. The earliest sample showed an RT activity that was low but clearly detectable, corresponding to approx. 100 nU/mL plasma; all following samples were positive for reverse transcriptase activity (fig. 35). The viral load determined by RTA was higher than quantification by RT-PCR. This discrepancy was most likely linked to differences in sample preparations. Virus pellet used for RNA extraction was obtained by centrifugation of 14,000 × g for 90 minutes. Loss of template

during RNA preparation and the efficiency of cDNA synthesis had also to be taken into account. The virus pellet used for the RT assay was obtained by ultracentrifugation at 70,000 × g for two hours. Since no additional preparation steps were necessary for RTA-performance, there was no loss of activity due to processing.

3.1.2.4.2 RT activity in plasma from autoimmune patients compared to healthy blood donors

3.1.2.4.2.1 Analysis of RT activity in healthy blood donors

The RTA was set up as a generic screening method for retroviruses particularly in samples obtained from patients affected by autoimmune diseases (AIDs). A role of retroviruses in the etiology of AID has been suggested repeatedly, although proof is still lacking. The etiological link between virus infection and disease is especially difficult to prove in slowly progressive diseases in which the infection may have taken place a long time before the onset of symptoms. The methods applied so far involve specific methods which need as a matter of course either sequence information of the respective virus, knowledge about detectable viral protein structures, or antibodies against the virus. Generic methods such as electron microscopy have a relatively high detection limit, thus virus infections may not be detected. The established RTA combines generic and sensitive detection of retroviruses.
Samples from patients with scleroderma, SLE, or spondylitis ankylosans were obtained as EDTA-KE blood. Samples were stored at 4°C or room temperature for up to 2 hours. Plasma and cells were separated by centrifugation (2000 × g for 10 minutes). Control citrate plasma that was obtained from blood bank samples was treated differently. Cells were removed from these samples at the time of blood collection and completely cell-free citrate plasma was obtained. This difference in processing may influence the performance of the RTA. Storage of samples as whole blood at 4°C or room temperature or during transportation could lead to the disruption of cells and the release of cellular enzymes. Contamination with cellular polymerases could result in false positive background signals. To assess the influence of the different modes of sample collection, blood from healthy (asymptomatic) individuals was collected and left at room temperature for 2 hours before centrifugation and freezing of plasma. The reverse transcriptase activity was determined for plasma of blood bank samples and other negative controls in parallel (fig. 36).

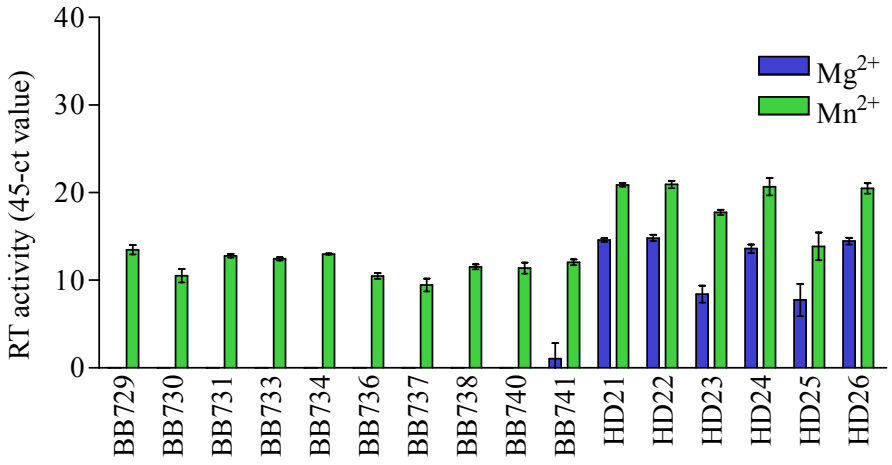

Fig. 36: Reverse transcriptase activity in plasma from healthy donors. BB indicates cell free citrate plasma samples obtained from the blood bank, while samples from healthy individuals (EDTA plasma, HD) were not completely free from enzymatic background RT activities. RTA was performed in triplicate for each sample using Mg^{2+} and Mn^{2+} buffer in parallel. RT activity is given as 45-ct value; 3.3 ct value is equivalent to 1 $\log_{10}$ RT activity.

Profound differences were detected for the samples depending on the mode of sampling. Cell-free plasma showed absolutely no RT activity with Mg^{2+} and only weak background signals most likely related to the Mn^{2+} induced template switch of the DNA-polymerase used for real-time PCR. These signals were below the cut-off defined with non-infected cell culture supernatant. This switch from DNA to RNA as template can be induced by Mn^{2+} added with the cDNA. The observed Mn^{2+}-related background for blood bank samples corresponds to the background observed for non-infected cell culture supernatants (fig. 26). Samples that had remained at room temperature or 4°C for some time before separation of plasma and blood cells showed a low but clearly detectable RT activity with the Mg^{2+} buffer and an increase in RT activity for the Mn^{2+} buffer when compared to the samples that were processed directly after blood collection. Since cellular polymerases can use RNA as template, it is probable that cellular enzymes released during storage and transportation accounted for this background signal, which was unavoidable due to the manner of sampling.

It was shown that virus spiked into the plasma of healthy controls (sampled like the analyzed AID patient samples) can be detected reliably down to 300 virus particles (based on Mg^{2+}-preferring RT, data not shown). Values below this threshold obtained from ETDA whole blood samples can not be securely linked to retroviral enzymatic activity. Therefore the application of the RTA as a screening method for patient plasma is limited.

3.1.2.4.2.2 RT activity in plasma from autoimmune disease patients

Plasma samples from autoimmune disease patients, spondylitis ankylosans, SLE, and scleroderma, were analyzed in comparison to healthy (asymptomatic) controls.

3.1.2.4.2.2.1 RT activity in plasma of spondylitis ankylosans patients

Although a strong association between an HLA allele (HLA-B27; Zhang et al., 2004) and the development of spondylitis ankylosans is seen, obviously more factors play a role in the development of disease. However, identification of a certain infectious agent or other factors could not as yet be accomplished. Samples from six patients with spondylitis ankylosans were analyzed for particle-associated reverse transcriptase activity in plasma (fig. 37).

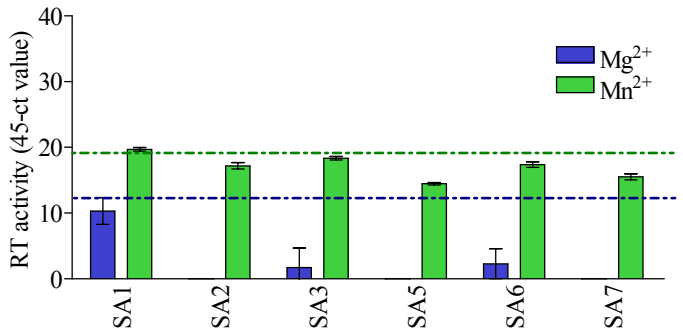

Fig. 37: Reverse transcriptase activity in plasma from spondylitis ankylosans patients. All samples were tested in triplicate, using two different buffers (Mg^{2+} or Mn^{2+}) for cDNA synthesis and three dilutions of the lysate (non-diluted, 1:10, and 1:100 diluted). The data shown represent the mean RT activity per 7.5 µL plasma. Horizontal lines indicate the mean RT activity obtained with plasma from healthy donors either with Mg^{2+} (blue) or Mn^{2+} (green) buffer. Only ct values of controls which were treated in the same way as the patient samples were averaged; blood bank samples were excluded. RT activity is given as 45-ct value; 3.3 ct value is equivalent to 1 $\log_{10}$ RT activity.

None of these plasmas showed a higher RT activity than the average of reverse transcriptase activity detected in samples from the healthy (asymptomatic) controls.

3.1.2.4.2.2.2 RT activity in plasma of SLE patients

Systemic lupus erythematosus (SLE) has been associated with different retroviruses, especially with HERVs. The detection of antibodies which were reactive with segments of several HERVs as well as with HIV and HTLV antigens suggested the possibility of cross-reactions to other retroviruses (Hermann et al., 1996, Ranki et al., 1992, Bengtsson et al., 1996, Nelson et al., 1994, Li et al., 1996). Only one of 19 samples tested was obtained from a male patient. All samples were analyzed for particle associated RT activity in plasma (fig. 38).

One sample (9) showed a higher Mn^{2+}-related RT activity compared to asymptomatic controls. However, a major standard deviation was observed and the detected result was not reproducible. Sample 14 showed a higher Mg^{2+} related RT activity compared to the mean RT activity observed in asymptomatic controls. The RT activity in sample14 was not significantly higher compared to controls. In sum, plasma of SLE patients showed no elevated RT activity for any of the samples analyzed in comparison to asymptomatic controls.

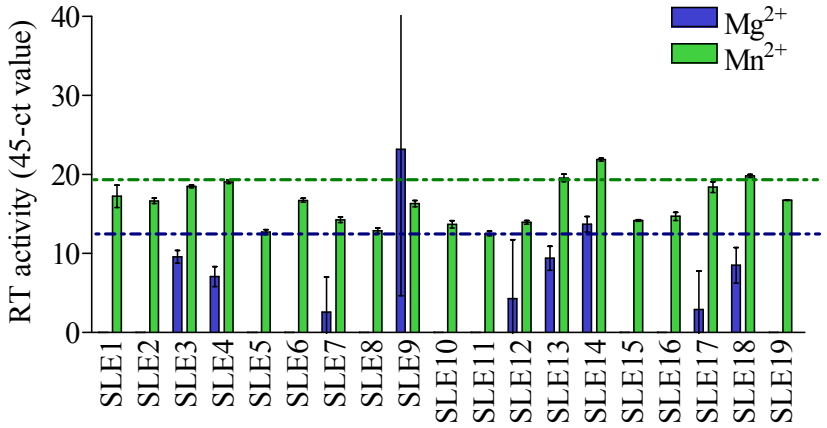

Fig. 38: Reverse transcriptase activity in plasma from SLE patients. All SLE plasmas were tested in triplicate, using two different buffers for cDNA synthesis (Mg^{2+} or Mn^{2+}) and in three dilutions each (non-diluted, 1:10, and 1:100 diluted). The data shown represent the mean RT activity per 7.5 µL of plasma for the non-diluted samples. Horizontal lines indicate the mean RT activity obtained with plasma from healthy donors. Only ct values of controls which were treated in the same way as the patient samples were averaged; blood bank samples were excluded. RT activity is given as 45-ct value; 3.3 ct value is equivalent to 1 $\log_{10}$ RT activity.

3.1.2.4.2.3 RT activity in plasma of scleroderma patients

Antibodies to retroviral Gag protein were described in samples from scleroderma or systemic sclerosis patients (Hishikawa et al., 1997). In general, three to four times more women than men develop the disease. Plasma from of 32 female scleroderma patients was obtained and tested for particle-associated reverse transcriptase activity (fig. 39).

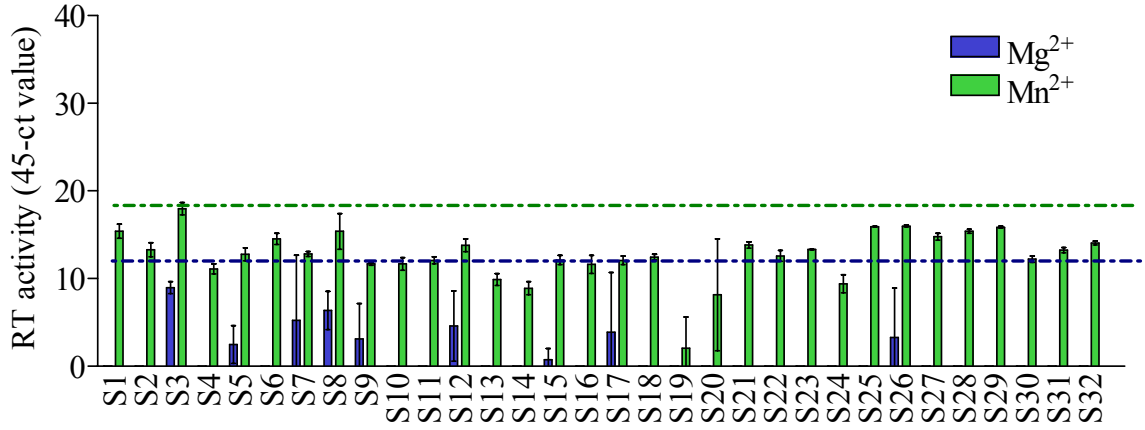

Fig. 39: Reverse transcriptase activity in plasma from scleroderma patients. 32 plasma samples were tested in triplicate, using two different buffers for cDNA synthesis (Mg^{2+} and Mn^{2+}) and in three dilutions each (non-diluted, 1:10, and 1:100 diluted). The data shown represents the mean RT activity per 7.5 µL of plasma. The horizontal lines indicate the mean RT activity obtained with plasma from healthy donors. Only ct values of controls which were treated in the same way as the patient samples were averaged; blood bank controls were excluded. RT activity is given as 45-ct value; 3.3 ct value corresponds to approximately 1 $\log_{10}$ RT activity.

None of the scleroderma samples showed elevated RT activity when compared to the average reverse transcriptase activity detected for the group of healthy controls. Of the

patient samples, 22 of 32 were completely negative for Mg^{2+}, while none of the controls was. The signals obtained with Mn^{2+} were also considerably lower compared to samples from healthy controls.

3.1.2.4.3 Results of the analysis of RT activity detected in plasma samples

None of the samples from autoimmune disease patients affected by spondylitis ankylosans, scleroderma, or SLE showed any indication of retroviral particles circulating in their peripheral blood (tab. 10).

Tab. 10: Compilation of RT activity detected in plasma samples

Group	n	RT activity Mg^{2+} (45-ct value/SD)	RT activity Mn^{2+} (45-ct value/SD)
Blood bank plasma	10	0 +/- 0	11.7 +/- 1.33
Healthy donors	6	12.54 +/- 2.92	19.11 +/- 3.49
Ankylosans spondylitis	6	2.41 +/- 3.93	17.11 +/- 1.75
SLE	19	3.22 +/- 6.52	16.30 +/- 2.69
Scleroderma	32	1.17 +/- 2.86	12.68 +/- 3.12

The RT activity detected in asymptomatic individuals depends strongly on the sampling procedure. While no RT activity was detected in the blood bank samples, the samples stored for two hours prior to centrifugation (‘healthy donors’) showed relatively high RT activity due to cellular enzymes which were released in the meantime. The samples obtained from spondylitis, SLE, and scleroderma patients were stored at 4°C until transportation; separation of cells and plasma was performed as quickly as possible. None of the diseased groups showed higher RT activity than the healthy donor group.

3.2 Specific detection of expressed retroviral sequences

3.2.1 Expression of an exogenous retroviral sequence: HTLV

The human T-cell leukemia virus (HTLV)-1 is associated with the development of a rare form of leukemia – the adult T cell leukemia (ATL) – and the neurological disease HAM/TSP, a disease which resembles MS. So far, there is no proof for diseases other than ATL and HAM/TSP associated with HTLV. However, expression of the HTLV regulatory tax sequence has been described for different autoimmune diseases; antibodies against this protein have also been depicted in samples from patients with slowly progressing, autoimmune or neurological diseases (e.g., scleroderma, V. Morozov, personal communication). Therefore all samples were tested for proviral HTLV-1 and HTLV-2 DNA, and expression of these exogenous retroviruses as well, by an HTLV*tax*-specific real-time PCR assay (fig. 40).

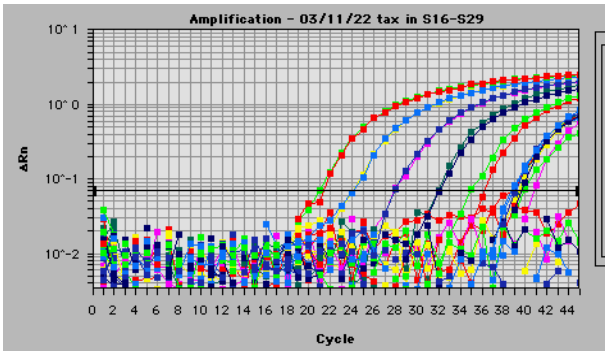

Fig. 40: HTLV*tax*-specific real-time PCR. Plasmid standards ranging from 1 × 10^6 to 5 copies were used as positive controls and for quantification. The tax-specific PCR detects HTLV-1 as well as HTLV-2. All samples were tested and PCR was performed using either DNA and/or cDNA.

DNA and cDNA of adult controls, scleroderma, SLE, and spondylitis ankylosans patients were analyzed for proviral load and expression of HTLV*tax* sequence. Samples from children and adolescents were made available as whole blood in RNA stabilization reagent only, no DNA was obtained. Thus it was impossible to screen these samples for proviral load, but cDNA was analyzed for HTLV expression.

All samples – DNA and cDNA – were negative for tax DNA sequence and/or expression of tax mRNA. HTLV could therefore be excluded as an etiological agent for the patients analyzed in this study.

3.2.2 Expression of endogenous retroviral sequences

Multiple sclerosis is one of the best-studies autoimmune diseases. In the course of time, many viruses have been proposed as playing a role in the etiology of MS, but sound proof could never be obtained. In recent years publications especially from Denmark, France and Italy indicated enhanced expression of endogenous retrovirus particles – detected at different levels – in samples or cell cultures derived from MS patients. Depending on the geographical origin of samples, expression of endogenous retroviral sequences was described in tissue, plasma and CSF of MS patients to different extents. Specific expression of HERV-H-related sequences was detected by PCR in cell cultures derived from Danish MS patients (Christensen et al., 1998), HERV-W-related MSRV sequences were detected in 53% of MS patients and 7% of controls in France (Garson et al., 1998), as well as in 100% of MS patients and 12% of controls on the island of Sardinia (Serra et al., 2001). An incubation period of decades had been postulated for multiple sclerosis, the onset of disease is usually seen in early adulthood. Until recently, MS in children and adolescents was considered impossible. Within the last years it has been recognized that young individuals may be affected by this autoimmune disease as well. Early onset of MS (EOMS) is defined by an age of less than 16 years at the first MS attack and is observed in about 5% of the total MS population (Duquette et al., 1987, Sindern et al., 1992, Ghezzi et al., 1997, Liguori et al., 2000, Boiko et al., 2002, Simone et al., 2002). Following the hypothesis of an initial retrovirus infection triggering MS with a prolonged incubation time of decades and an extremely low persisting viral load (e.g. molecular mimicry as mechanism for disease induction), the possibility has been proposed that higher viral loads could be found in younger MS patients. Therefore not only the specific expression of

endogenous retrovirus sequences described elsewhere, but also different expression levels of adults and children/adolescents affected by MS were analyzed. Samples from children and adolescents with EOMS who were all in remission for the last six months or with active MS who were seen 24 to 48 hours after the onset of an acute episode were analyzed for expression of endogenous retroviral sequences. In parallel, samples from adult MS patients and samples from adult individuals affected by other autoimmune diseases – spondylitis ankylosans, scleroderma and systemic lupus erythematosus – were analyzed. Samples from healthy (asymptomatic) adults, children and adolescents were used as controls. Since most neurological autoimmune diseases have a relatively late onset, samples from children with non-autoimmune-related neurological symptoms were used as controls. Control groups included children/adolescents with non-inflammatory neurological diseases (e.g. MELAS, Turner syndrome, migraine, vitiligo, brain tumor) and children/adolescents with inflammatory diseases of the CNS other than MS (e.g. encephalitis, optic neuritis, meningitis). Blood samples from MS patients were collected with the PAX system (Qiagen) and directly diluted in PAX buffer in order to freeze the RNA expression profile of the samples. Thus no plasma was available for the RTA, and these samples could only be analyzed for expression of retroviral sequences using specific quantitative PCR assays. All samples analyzed for expression of three different endogenous retroviral sequences based on the publications of HERV-H and HERV-W-related sequences and independent quantitative real-time PCR assays were established.

Although (probably) human endogenous retrovirus sequences have lost infectivity and replication competence because their proviral genomes are truncated and non-functional, some retroviral genes have acquired host-functions in the course of time and are now expressed at certain points in time or in specific tissues. Aberrant expression of HERV-sequences may have severe or even fatal consequences for the host. (Lower et al, 1996, Patzke et al., 2002). To differentiate between regular and aberrant expression, the samples from autoimmune disease patients – children and adolescents with MS and active MS, adults with MS, scleroderma, SLE or spondylitis ankylosans – were quantitatively analyzed in comparison to samples from healthy (asymptomatic) children/adolescents and adults and children and adolescents with neurological symptoms. Expression was analyzed according to the health status and age for all samples. The samples from children and adolescents were also analyzed according to gender and medication. These data were not available for the group of adult MS patients.

3.2.2.1 Standardization and quality assessment

Since most endogenous retrovirus families are present as multicopies within the human genome, minute amounts of genomic DNA would have interfered with the assay quality and resulted in false positive findings. All cDNA syntheses were carried out in parallel with an RT-negative control using aliquots of the identical reaction mix, using the same amount of RNA but without RT. These controls were tested in every PCR assay in parallel

to the cDNA. All cDNA samples and RT-negative controls were diluted 1:10 prior to PCR in order to minimize pipetting errors for small volumes. Volumes of 3 to 20 µL of the respective dilutions were applied to the PCR assays. Since the absence of contaminant DNA is crucial for this assay, an additional control was introduced. A real-time PCR assay was established to distinguish genomic DNA and cDNA. The assay was based on the gene coding for porphobilinogen deaminase (PBGD); the cDNA was subjected to two different PCR assays using the same probe but different primers, one assay being specific for unspliced genomic DNA and the other for spliced cDNA. All samples were tested with both PBGD assays, and samples with detectable genomic DNA were excluded. All PCRs were carried out in duplicate. Acquired data for the respective retroviral sequences were standardized on the expression of a house keeping gene, a ribosomal structural protein (L13; fig. 41).

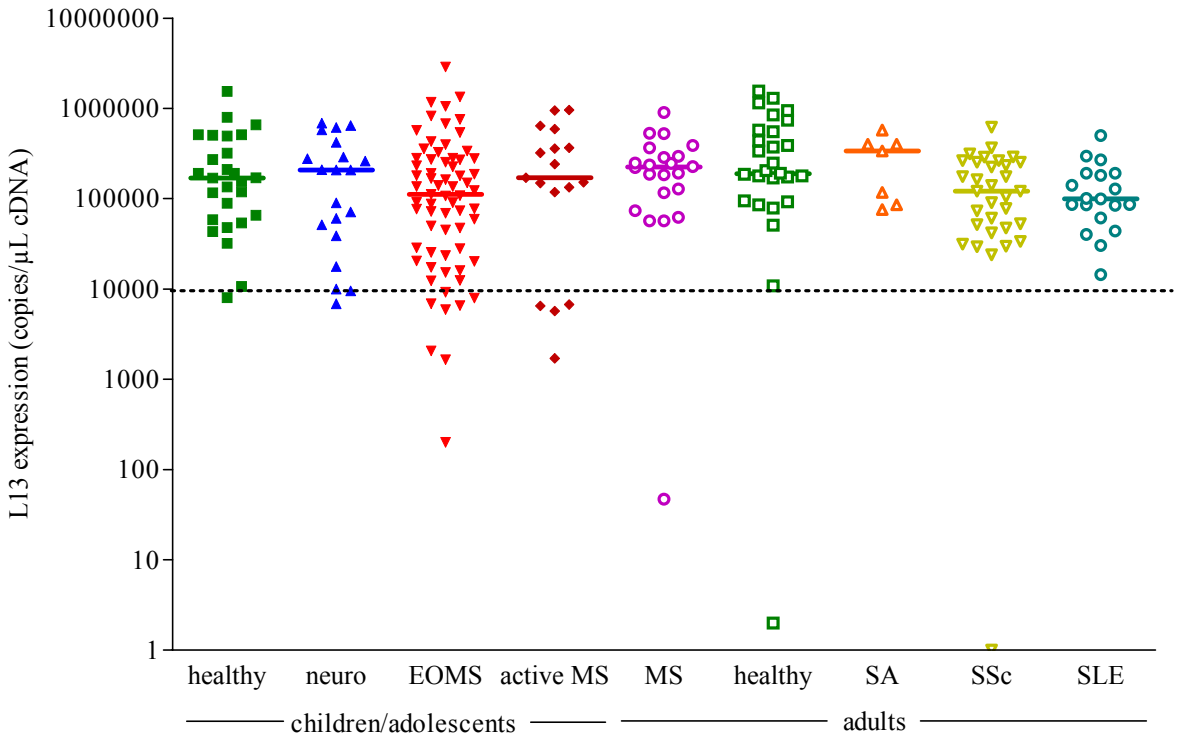

Fig. 41: Compilation of L13 values for all samples. The cDNA syntheses were performed using mRNA and random hexamer primers. Given values are related to 1 µL of cDNA. Neuro: samples from individuals with neurological symptoms, SA: spondylitis ankylosans, SSc: scleroderma, SLE: systemic lupus erythematosus. The horizontal dashed line shows the cut-off defined to exclude samples with low RNA content; small horizontal lines indicate median values.

All following data regarding expression of retrovirus sequences are based on 10^5 copies of the L13 house keeping gene. This standardization allows the direct comparison of the samples, regardless of the volume of whole blood or number of blood cells used for nucleic acid extraction or the final RNA or mRNA concentration. The L13 values varied about two $\log_{10}$ for the compared individuals, with no statistically significant differences for any

group. Samples with less than 10,000 copies L13 per µL cDNA, indicating a low RNA concentration, were excluded.

3.2.2.2 Expression of HERV-H

Expression of HERV-H-related sequences was described repeatedly for plasma, CSF and autopsy material from MS patients or in cell free supernatant of cell cultures derived from MS patients (Christensen et al., 2000). To substantiate or disprove expression of HERV-H sequences and production of endogenous retroviral particles in German MS patients, especially in children and adolescents, a quantitative real-time PCR assay based on the *pol*-region of HERV-H was established. Expression of *pol*-genes is mandatory for replication-competent viruses, approximately 20-100 RT polymerase molecules are packed into one virion (Maudru and Peden, 1997, Andrews, et al., 2000, Telesnitsky and Goff, 1997); expression should thus be detectable. Since efficient enzymatic activity is crucial for the virus, the *pol*-region is usually highly conserved for members of one retrovirus family, and is therefore a proper target for generic PCR assays. Expression was compared to samples from healthy controls and children/adolescents with neurological symptoms, MS, spondylitis ankylosans, SLE and scleroderma patients (fig. 42).

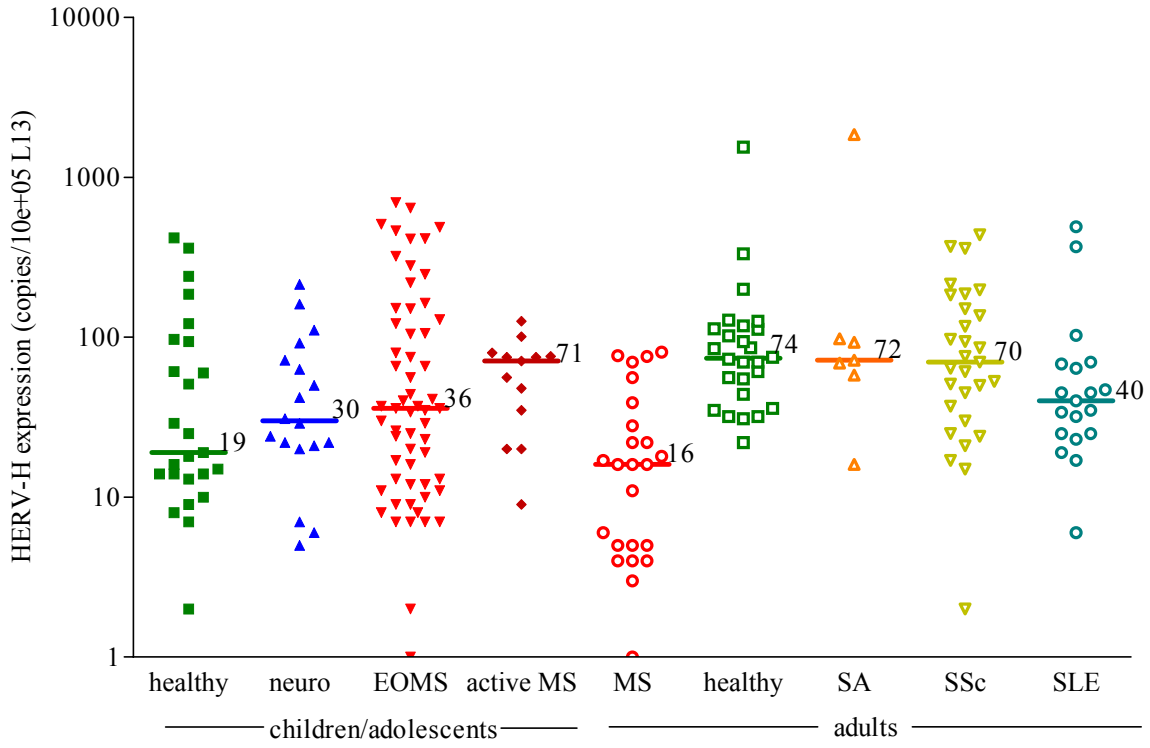

Fig. 42: Expression of HERV-H*pol* sequences. Samples from healthy individuals – children/adolescents (n=26) and adults (n=26) – were compared to samples from children/adolescents with neurological symptoms (n=18), from children and adolescents with EOMS (n=59) and active EOMS (n=17), from adults with MS (n=21) and from adults affected by different autoimmune diseases: spondylitis ankylosans (n=7), scleroderma (n=29), and SLE (n=19). Neuro: samples from individuals with neurological symptoms, SA: spondylitis

ankylosans, SSc: scleroderma, SLE: systemic lupus erythematosus. Figures and horizontal lines indicate median values.

Expression of the HERV-H*pol* sequence was detected in all samples, in healthy controls as well as individuals affected by autoimmune disease, but to different extents. The expression in healthy children/adolescents ranged from 2 to 419 copies (median 19). Neurologically diseased young individuals exhibited 5 to 214 copies (median 30). Children/adolescents with EOMS showed expression ranging from 1 to 697 copies (median 36), and the group of young individuals with active EOMS expressed 9 to 126 copies (median 71). Expression in healthy adults ranged from 22 to 1547 (median 74). Adults with MS exhibited 1 to 81 copies (median 16). The highest expression was detected for a spondylitis ankylosans patient with 1849 copies. The expression for all spondylitis ankylosans patients ranged from 16 to 1849 copies (median 72). Scleroderma patients showed an expression of 2 to 438 copies (median 70). For SLE patients, expression of 6 to 491 copies was detected (median 40).
In contrast to the findings of Christensen et al, HERV-H expression was also detected in controls. Furthermore, healthy adults showed significantly higher expression compared to adult MS patients (Mann Whitney test, P value: < 0.0001). In contrast, no significant differences were observed when comparing expression levels of the different groups of children and adolescents. The expression in SLE patients was also significantly lower when compared to healthy controls (P value: 0.0149). No significant differences regarding expression were detected in samples from spondylitis or scleroderma patients. Interestingly, statistical analyses showed a significantly higher expression in healthy adults than in healthy children and adolescents (P value: 0.0061).

3.2.2.3 Expression of MSRV

The multiple sclerosis associated retrovirus (MSRV) was detected as particles produced by cell cultures derived from French and Italian patients with multiple sclerosis (Perron et al., 1997, Blond et al., 1999, Komurian-Pradel et al., 1999). In order to determine the expression of MSRV in German MS patients, especially in children and adolescents, an MSRV*env*-specific quantitative real-time PCR was established. Samples from children and adolescents with MS and active MS were compared to those from adults with MS, children and adolescents affected by neurological diseases, from adults with other autoimmune diseases (spondylitis ankylosans, scleroderma and SLE), as well as samples from healthy control groups (fig. 43).

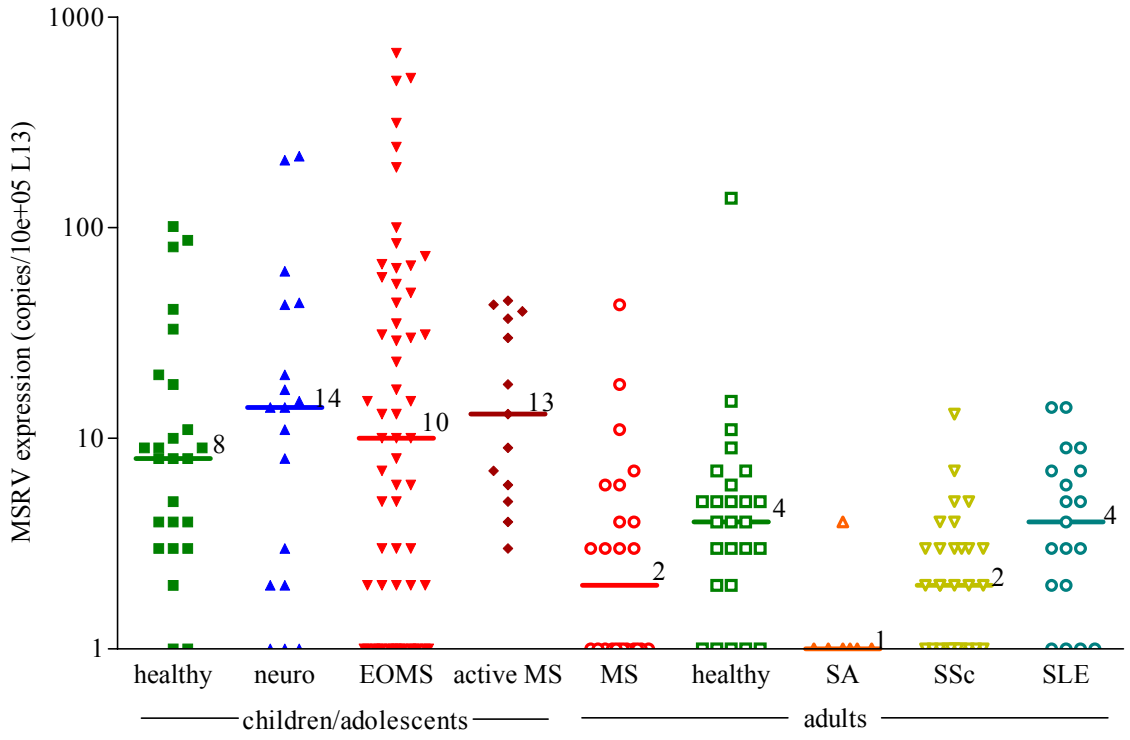

Fig. 43: Expression of MSRV*env* sequences. Samples from healthy individuals – children/adolescents (n=26) and adults (n=26) – were compared to samples from children/adolescents with neurological symptoms (n=18), from children and adolescents with EOMS (n=59) and active EOMS (n=17), from adults with MS (n=21) and from adults affected by different autoimmune diseases: spondylitis ankylosans (n=7), scleroderma (n=29), and SLE (n=19). Neuro: samples from individuals with neurological symptoms, SA: spondylitis ankylosans, SSc: scleroderma, SLE: systemic lupus erythematosus. Figures and horizontal lines indicate median values.

The expression level for healthy children/adolescents ranged from 0 to 101 copies (median 8). Neurologically symptomatic children/adolescents exhibited expression of 0 to 218 copies (median 14). EOMS affected children/adolescents expressed 0 to 672 copies (median 10). The young individuals with active EOMS generally had a slightly lower expression of 3 to 45 copies (median 13), though no sample was completely negative. Adults with MS exhibited a lower expression of 0 to 18 copies (median 1) compared to healthy adults (expression ranging from 0 to 138 copies, median 4). All adult groups, including three groups of non-MS autoimmune disease patients, showed relatively low expression. Only one of seven spondylitis ankylosans patients showed any expression, expression for scleroderma patients ranged from 0 to 13 (median 2) and from 0 to 14 (median 4) for SLE patients.

Statistical analysis (Mann Whitney test) showed a significantly higher expression in healthy children and adolescents than in adults (P value: 0.018). Adult MS patients showed a significantly lower expression compared to healthy adults (P value: 0.0475). The expression level for scleroderma and spondylitis ankylosans patients was also significantly lower than for healthy adults (P value: 0.0119 and 0.0184, respectively). The expression of

MSRV*env* detected in samples from SLE patients did not differ significantly from the data obtained from healthy controls. Expression levels detected in samples from young individuals regardless of health status showed no statistically significant variations. In contrast to the findings in France and Italy, patients affected by MS showed no elevated expression of MSRV as indicated by the quantification of MSRV*env* levels. In contrast to the findings in Sardinia, some children and adolescents with MS, as well as adults with MS, had no detectable expression of MSRV while all samples from children and adolescents with active MS showed detectable expression. On average, diseased children and adolescents exhibited the same expression levels as the control groups, while adult MS patients showed a significantly lower expression compared to younger patients with MS. This difference between children/adolescents and adults may be linked to an age-related expression independent of disease.

3.2.2.4 Expression of HERV-W

Recently, HERV-W has been one of the most extensively studied HERVs, following the detection of HERV-W-related MSRV (Perron et al., 1997, Blond et al., 1999, Komurian-Pradel et al., 1999). Transcriptional activation of HERV-W has been related to schizophrenia (Karlsson et al., 2001). The human genome contains at least 70 copies of HERV-W-related *gag* sequences (Nelson et al., 2004). Therefore a quantitative real-time PCR assay was set-up to detect expression of *gag*-sequences of the HERV-W family (fig. 44).

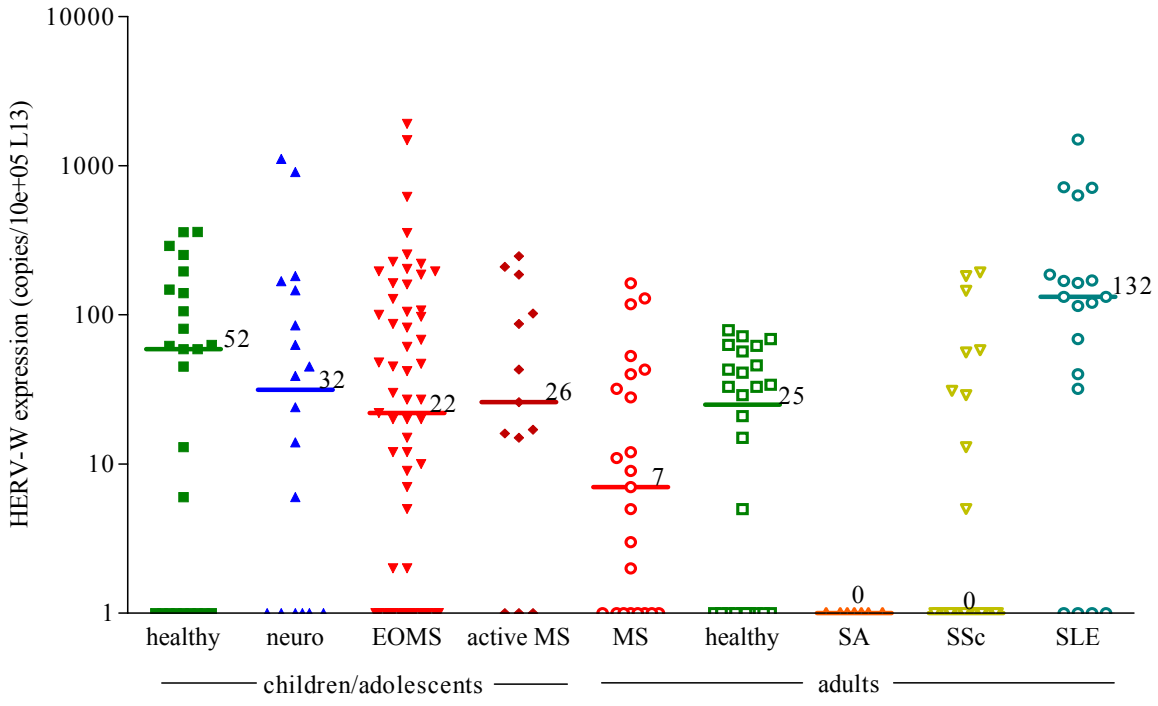

Fig. 44: Expression of HERV-W*gag* sequences. Samples from healthy individuals – children/adolescents (n=26) and adults (n=26) – were compared to samples from children/adolescents with neurological symptoms

(n=18), from children and adolescents with EOMS (n=59) and active EOMS (n=17), from adults with MS (n=21) and samples from adults affected by different autoimmune diseases: spondylitis ankylosans (n=7), scleroderma (n=29), and SLE (n=19). Neuro: samples from individuals with neurological symptoms, SA: spondylitis ankylosans, SSc: scleroderma, SLE: systemic lupus erythematosus. Figures and horizontal lines indicate median values.

Expression of HERV-W*gag* sequences was detected in all groups but the spondylitis ankylosans patients, conversely, each group included individuals who did not express HERV-W*gag*.

The expression level for healthy children/adolescents ranged from 0 to 360 copies (median 52). Neurologically symptomatic children/adolescents exhibited expression of 0 to 1112 copies (median 32). EOMS affected children/adolescents expressed 0 to 1911 copies (median 22). The young individuals with active EOMS showed expression of 0 to 248 copies (median 26). Adults with MS exhibited a lower expression of 0 to 18 copies (median 1) compared to healthy adults (expression ranging from 0 to 129 copies, median 7). None of seven spondylitis ankylosans patients showed any expression, while expression for scleroderma patients ranged from 0 to 193 (median 0) and from 0 to 1503 (median 132) for SLE patients.

Statistical analysis (Mann Whitney test) showed no significant differences in expression for MS affected individuals compared to healthy donors. There was also no significant difference for healthy individuals of different ages. However, SLE patients showed a significantly higher expression compared to healthy individuals (P value: 0.0014). The findings regarding MS patients also do not correspond to the publications from France and Denmark. In groups of MS affected patients, several patients tested negative for expression of this HERV-W sequence; each group of MS patients showed – though not statistically significant – lower expression than healthy controls.

3.2.2.5 Gender-specific differences in HERV sequence expression

Autoimmune diseases generally affect more females than males; roughly 85% of patients are females. The ratio for MS is two men for every three women affected by the disease. Since almost all patients with SLE, scleroderma or spondylitis ankylosans were female, the comparison of expression rates over gender was carried out for EOMS patients only. The gender ratio for EOMS was 36 females : 23 males and 7 females : 6 males affected by active EOMS at the time of sampling (fig. 45a, b, and c).

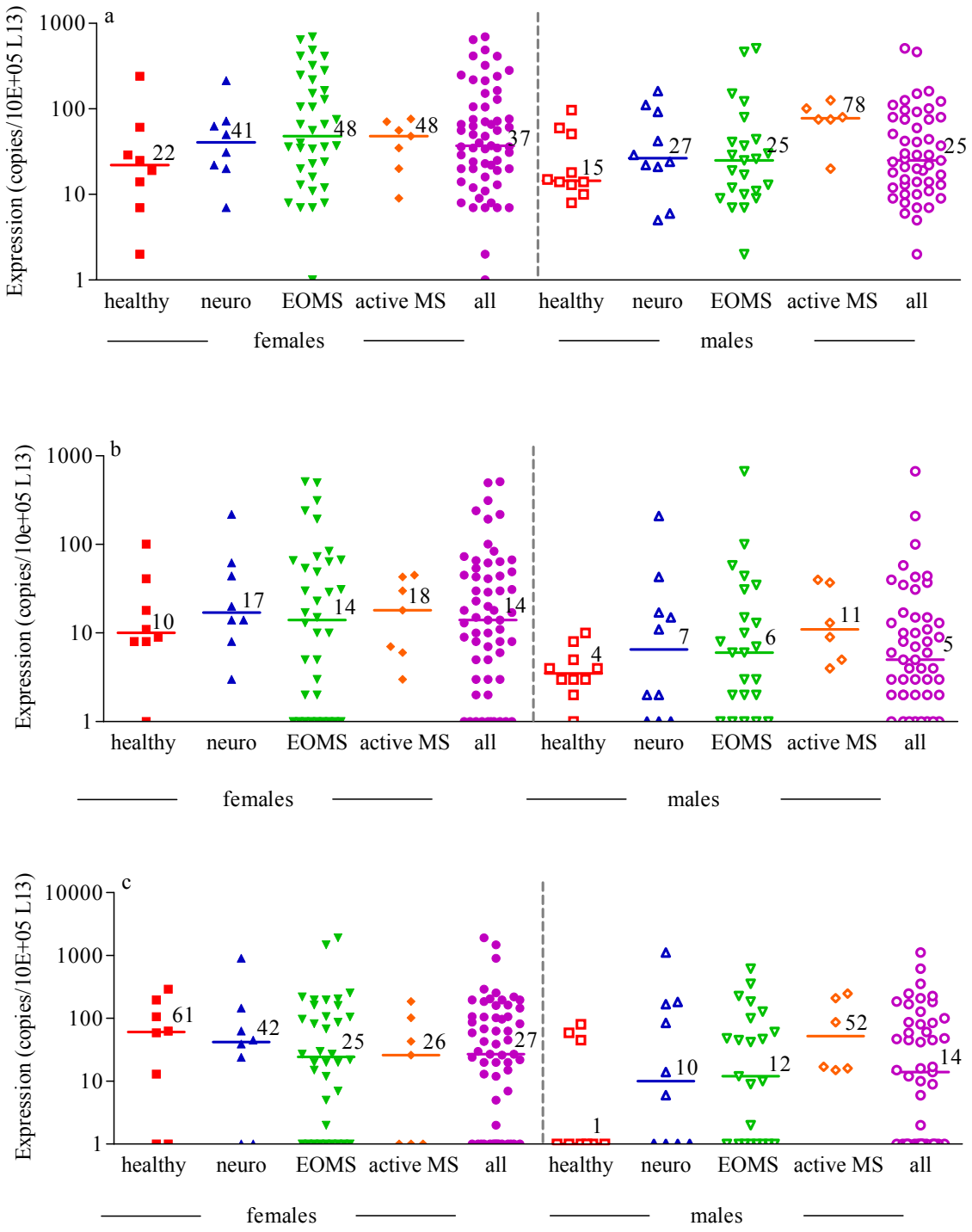

Fig. 45: Gender related HERV expression. The expression in samples from healthy females (n=8) was compared to expression in healthy males (n=10). Samples from female patients affected by neurological diseases (n=8) were compared to those from males affected by similar disease patterns (n=10). Expression for female EOMS patients (n=36) and female patients with active EOMS (n=7) was compared to expression for males affected by EOMS (n=23) and active EOMS (n=6), respectively. The data for all samples from females and males were compiled and the expression level was compared. Neuro: samples from patients with neurological symptoms. Figures and horizontal lines indicate median values; a: HERV-H, b: MSRV, c: HERV-W expression.

HERV-H (fig. 45a): healthy female children/adolescents (n=8, representing 44% of healthy individuals) showed a median expression of 22, ranging from 2 to 241, while healthy males in this group (n=10, representing 56% of healthy individuals) showed 8 to 97, with a median expression of 15 copies. All female children/adolescents (n=59, representing 55%; including healthy individuals, individuals with neurological symptoms, patients with EOMS and with active EOMS) expressed 1 to 697 copies, with a median of 37; compilation of all males (n=49, representing 45%) resulted in 2 to 510 copies, median 25.
Statistical analysis of EOMS samples and the compilation of all EOMS affected (Mann Whitney test) showed no significant differences in expression of HERV-H*pol* for female and male children and adolescents, regardless of their health status. However, separate analysis of healthy males and male children/adolescents with active EOMS showed a significantly higher expression in the EOMS group (P value: 0.0075).
MSRV (fig. 45b): healthy female children/adolescents (n=8, representing 44% of the healthy individuals) expressed 1 to 101 (median 10), while males (n=10, representing 56%) expressed 1 to 10 copies (median 4). All female children/adolescents (n=59, representing 55%, including healthy individuals, as well as children/adolescents with neurological symptoms, patients with EOMS and active EOMS) expressed 1 to 512 copies (median: 14). The corresponding male group (n= 49, representing 45%) expressed 1 to 672 copies (median 5).
Statistical analysis showed a significantly lower expression in healthy males than in females (P value: 0.0155) as well as for the comparison of all males and females (P value: 0.0207) regardless of health status. Separate analysis of healthy males and males affected by active EOMS showed a significantly higher expression of MSRV in the patient group (P value: 0.0110). Neurologically diseased or EOMS-affected males and females exhibited no significant differences for expression of MSRV*env*.
HERV-W (fig. 45c): healthy female children/adolescents (n=8, representing 44% of the healthy individuals) expressed 0 to 291 copies (median 61), while males (n=10, representing 56%) expressed 0 to 81 copies (median 1). All female children/adolescents (n=59, representing 55%, including healthy individuals, as well as children/adolescents with neurological symptoms, patients with EOMS and active EOMS) expressed 0 to 1482 copies (median 27). The corresponding male group (n= 49, representing 45%) expressed 0 to 1112 copies (median 14).
Statistical analysis showed a significantly lower expression in healthy males than in females (P value: 0.0434). EOMS patients or neurologically diseased males and females exhibited no significant differences in expression of HERV-W*gag* when compared to healthy controls of the respective gender. Male patients with active MS showed a significantly higher expression compared to healthy males (P value: 0.0225).

3.2.2.6 Effect of therapy on HERV expression

Some children and adolescents with EOMS and active EOMS were under Rebif-therapy at the time of sampling. This interferon-derivate is applied in order to delay or suppress active EOMS.

Samples from treated and non-treated individuals affected by EOMS were analyzed as a whole and according to EOMS/active EOMS to determine possible effects of medication on the expression of endogenous retroviral sequences (fig. 46a, b, c).

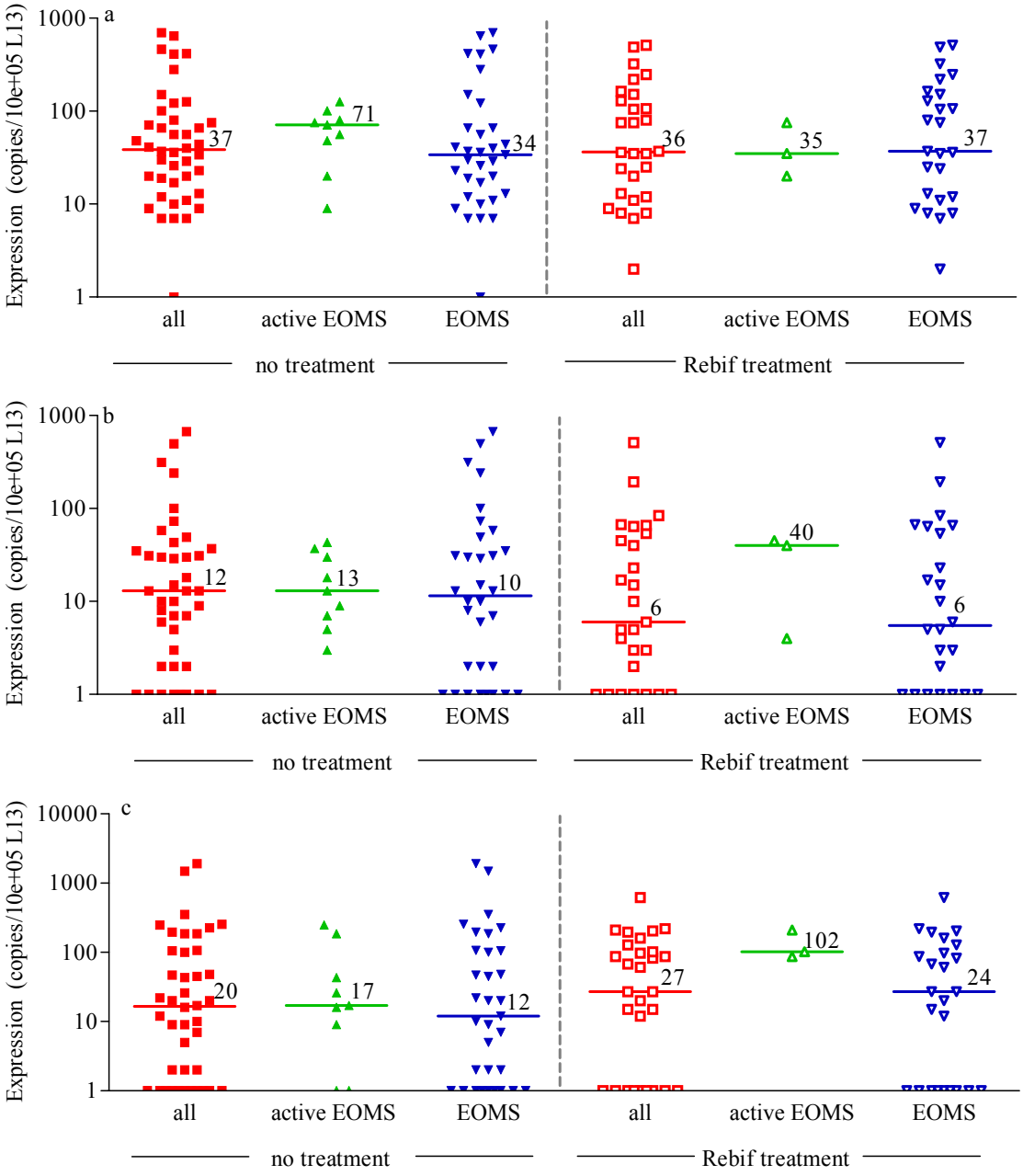

Fig. 46: HERV expression in correlation to Rebif therapy. The expression of HERV-H*pol* in samples from therapy-naïve patients was compared to expression in samples from patients treated with Rebif. Compilations of all EOMS patients (non-treated n=42, treated n=27) were compared as well as patients with EOMS (non-treated n=33, treated n=24) and active EOMS (non-treated n=9, treated n=3). Figures and horizontal lines indicate median values; a: HERV-H, b: MSRV, c: HERV-W.

A possible effect of Rebif therapy on the expression levels of MSRV*env* was analyzed. Expression of samples obtained from children and adolescents treated with Rebif were compared to samples from therapy-naïve individuals. Compilations of all EOMS-affected individuals, as well as data from patients with active EOMS and EOMS were compared.
The expression of HERV-H*pol* for Rebif-treated individuals ranged from 2 to 488, median 36 (n=27). Five individuals (19%) expressed fewer than 10 copies, 13 individuals (48%) expressed 10 to 100 copies and 9 individuals (33%) expressed more than 100 copies. The data obtained for individuals without treatment (n=42) ranged from 1 to 644, median 37 copies. Six therapy-naïve individuals (14%) expressed fewer than 10 copies, 26 individuals (62%) expressed 10 to 100 copies and 10 individuals (24%) expressed more than 100 copies (fig. 46a).
The expression of MSRV in blood samples from patients under Rebif treatment (n=27) ranged from 1 to 512 copies (median 6). 14 individuals (52%) expressed fewer than 10 copies, 11 individuals (41%) expressed 10 to 100 copies and 2 individuals (7%) expressed more than 100 copies. The expression within the therapy-naive group (n=42) ranged from 0 to 672 copies (median 12). 19 individuals (45%) expressed fewer than 10 copies, another 18 individuals (43%) expressed 10 to 100 copies and 5 individuals (12%) expressed more than 100 copies (fig. 46b).
The HERV-W*gag* expression levels in patients under Rebif treatment (n=27) ranged from 0 to 220 (median 27). Nine individuals (33%) expressed fewer than 10 copies, 11 individuals (41%) expressed 1 to 100 copies and 7 individuals (26%) expressed more than 100 copies. The expression within the therapy naive group (n=42) ranged from 0 to 1911 copies (median 20). 18 individuals (43%) expressed fewer than 10 copies, 12 individuals (29%) expressed 10 to 100 copies, 10 individuals (24%) expressed 100 to 1000 copies and 2 individuals (5%) expressed more than 1000 copies. Treatment did not have a significant effect on the expression level of HERV-W*gag* in EOMS patients (fig.46 c).
Statistical analysis (Mann Whitney test) showed no significant variations of expression for treated and non-treated individuals. In addition, no statistically significant differences in relation to treatment were seen when EOMS and active EOMS were compared separately.

3.2.2.7 Expression of endogenous sequences as diagnostic marker

Even for samples and patient collectives with prominent expression of HERV-sequences, as described in Denmark, France and Italy, a pathogenic role for the respective endogenous retroviruses could not be proved. Although the expression detected may be an epiphenomenon, it could constitute a diagnostic marker. This hypothesis was examined by analyzing follow-up samples from children and adolescents with EOMS and active EOMS. Expression of all previously described endogenous sequences were assayed either in samples that had been collected from individuals within a short time frame or as follow-up samples over a period of 16 months and expression pattern under Rebif therapy was

examined. Samples from two individuals with recurrent active EOMS were analyzed. Expression was assayed for all previously described endogenous sequences.

3.2.2.7.1 Inter and intra assay variances for individual patients

A prerequisite for analysis of expression of endogenous sequences as diagnostic markers is the assessment of variation of expression levels over time in different samples from single individuals. The inter and intra assay variances were analyzed using samples from three randomly selected therapy- naïve non-active EOMS patients; sampling was performed within the same day up to three days (fig. 47a-c).

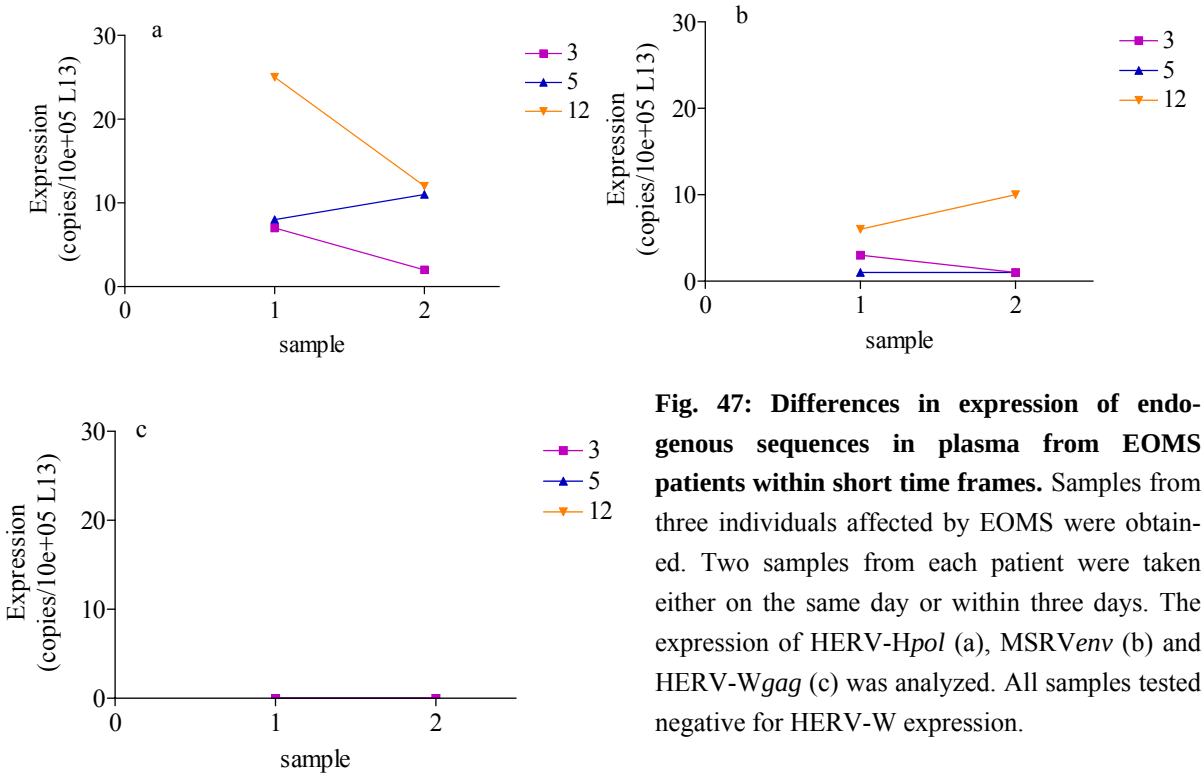

Fig. 47: Differences in expression of endogenous sequences in plasma from EOMS patients within short time frames. Samples from three individuals affected by EOMS were obtained. Two samples from each patient were taken either on the same day or within three days. The expression of HERV-H*pol* (a), MSRV*env* (b) and HERV-W*gag* (c) was analyzed. All samples tested negative for HERV-W expression.

HERV-H expression varied slightly for the individual samples while all three patients showed generally low expression. MSRV expression also showed no variability; two patients expressed one to three copies. For patient 12, expression at the two time points was 12 and 25 copies, respectively. None of the three patients expressed HERV-W.
The values obtained reflect the variances for individuals within a short time frame and were considered to be stable. Thus, inter and intra assay variances were shown to be small.

3.2.2.7.2 Differences in expression of endogenous sequences for follow-up samples from therapy-naïve EOMS patients

Variations of expression of endogenous sequences in samples from therapy-naïve EOMS patients were analyzed over a period of 16 months. Follow-up samples could be obtained for six patients, sampling was performed irregularly (fig. 48a-c).

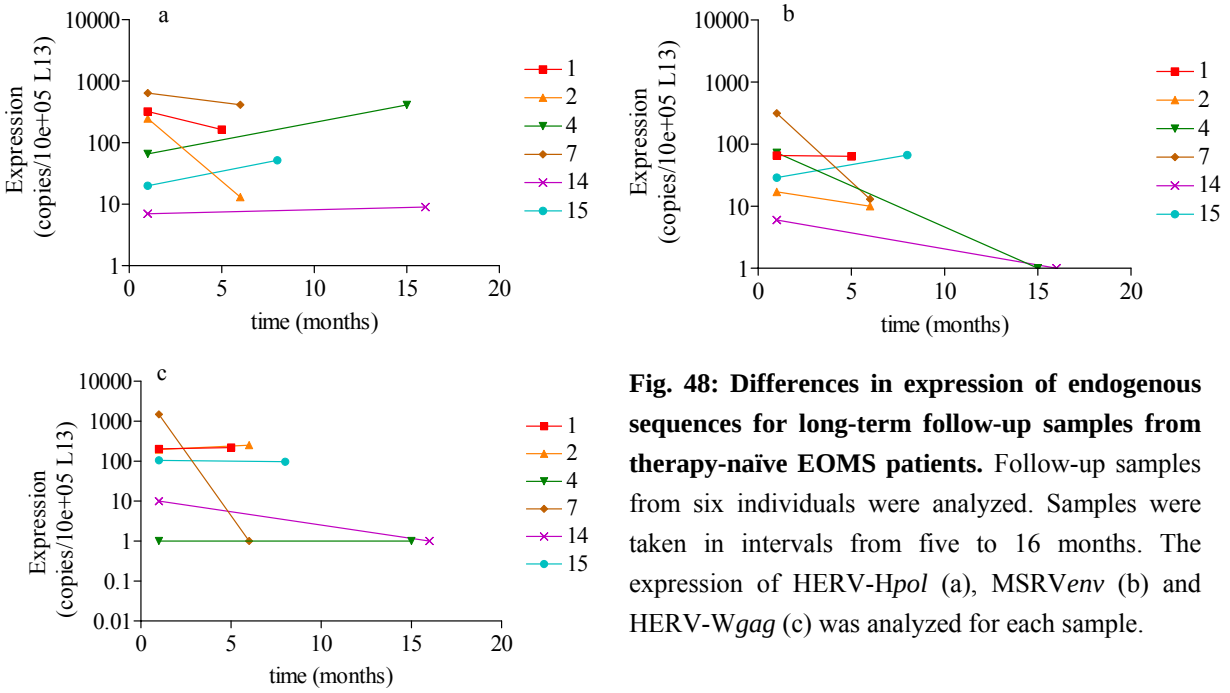

Fig. 48: Differences in expression of endogenous sequences for long-term follow-up samples from therapy-naïve EOMS patients. Follow-up samples from six individuals were analyzed. Samples were taken in intervals from five to 16 months. The expression of HERV-H*pol* (a), MSRV*env* (b) and HERV-W*gag* (c) was analyzed for each sample.

The HERV-H expression levels varied substantially for individual patients (fig.47a). Four individuals showed variations of up to 2-fold (samples 1, 7, 14 and 15), while one sample showed a 6 -fold increase of expression (sample 4) over time and another sample exhibited a 20-fold decrease of HERV-H expression (sample 2).

MSRV*env* expression in samples from three patients showed less than 3-fold variation or fewer than 10 copies for all analyzed samples in the course of time (patients 1, 2, and 14). One sample showed a more than 2-fold increase after eight months (patient 15), while two individuals showed a strong decrease in expression (patients 4 and 7). None of the individuals followed for more than ten months exhibited any detectable MSRV expression (fig. 48b).

HERV-W*gag* expression showed limited variability (fig. 47c). One individual did not express HERV-W*gag* at all (patient 3); three patients followed for up to eight months showed a relatively stable expression (patients 1, 2 and 15), one patient followed for more than ten months showed no detectable HERV-W*gag* expression for the last sample (patient 14). One patient showed expression of more than 1000 copies in the first sample and no detectable expression six months later (sample 7).

The analysis showed stable expression for all sequences for patients 1, 7 and 14. Patient 2 showed a decrease in HERV-H expression (248 to 13 copies), while expression of the two HERV-W-related sequences remained quite stable. Patient 4 showed a strong increase for HERV-H (66 to 412), a strong decrease for MSRV (73 to 0) and no expression of HERV-W in both samples. Patient 15 showed a 2-fold decrease for HERV-H and MSRV, while HERV-W remained stable.

3.2.2.7.3 Differences in expression of endogenous sequences for Rebif-treated individuals

3.2.2.7.3.1 Immediate reaction on Rebif therapy

Samples from two EOMS patients were obtained; the first sample was obtained for therapy naïve status, the second after three days of Rebif treatment. The samples were analyzed for HERV expression (fig. 49a-c).

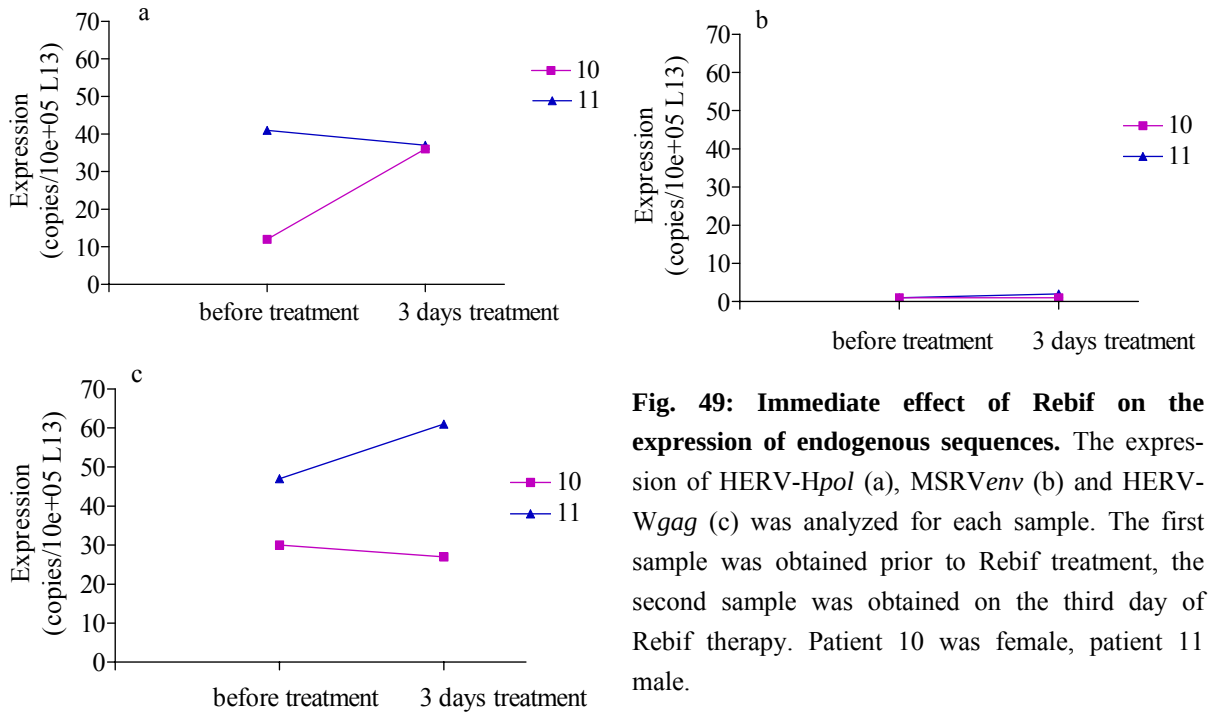

Fig. 49: Immediate effect of Rebif on the expression of endogenous sequences. The expression of HERV-H*pol* (a), MSRV*env* (b) and HERV-W*gag* (c) was analyzed for each sample. The first sample was obtained prior to Rebif treatment, the second sample was obtained on the third day of Rebif therapy. Patient 10 was female, patient 11 male.

Neither of the two patients showed a significant change in the expression pattern of endogenous sequences. The highest variability is a 3-fold increase of HERV-H expression for patient 10 (12 and 36 copies, respectively). These findings indicate no immediate effect on the expression pattern of the analyzed sequences.

3.2.2.7.3.2 Expression of endogenous sequences under long term treatment

The differences of expression for all previously tested endogenous sequences were analyzed for samples from two non-active EOMS patients under Rebif-therapy over a period of 12 or 13 months, respectively (fig. 50a-c).

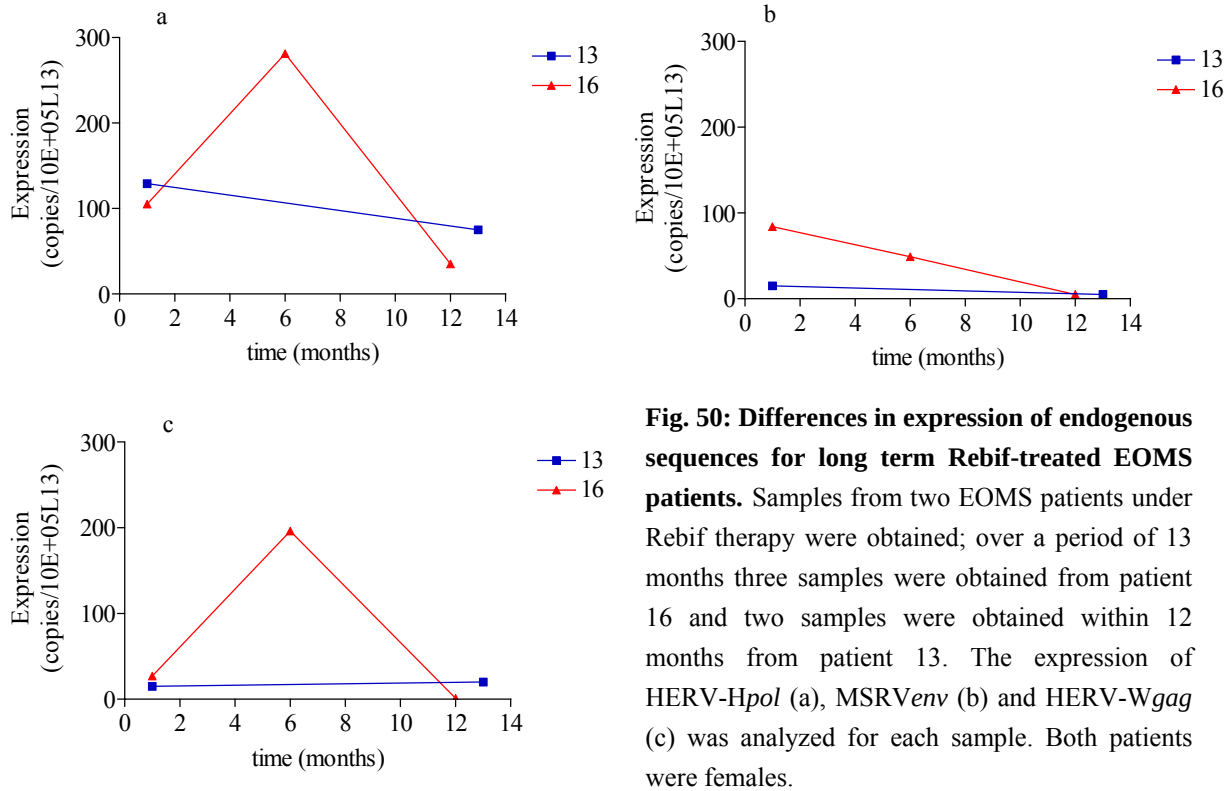

Fig. 50: Differences in expression of endogenous sequences for long term Rebif-treated EOMS patients. Samples from two EOMS patients under Rebif therapy were obtained; over a period of 13 months three samples were obtained from patient 16 and two samples were obtained within 12 months from patient 13. The expression of HERV-H*pol* (a), MSRV*env* (b) and HERV-W*gag* (c) was analyzed for each sample. Both patients were females.

Two samples could be obtained within one year from the first patient (13). She exhibited no major changes in expression for any of the endogenous sequences tested. HERV-H expression decreased from 129 to 75, MSRV expression decreased from 15 to five and HERV-W expression increased from 15 to 20 copies in these two samples.

Three samples could be obtained from patient 16. Substantial changes in expression levels for all endogenous sequences were detected. HERV-H and HERV-W showed similar expression patterns. The first sample showed 105 copies for HERV-H and 27 for HERV-W, after six months a strong increase of expression was detected for both sequences: 281 copies for HERV-H and 196 copies for HERV-W. The sample taken twelve months after the first sample showed strong decrease; 35 HERV-H copies were expressed and HERV-W was not detectable. The HERV-W related MSRV showed a different expression pattern. While the first sample showed an expression of 84 copies, the second sample showed a 2-fold decrease and the third sample taken again six month later showed only minor expression of five copies.

Comparison of expression for the two patients showed strong similarities for HERV-H and HERV-W for the first sample and the sample taken 12 or 13 months later. The two patients have a different expression level for MSRV for the first sample, but both showed a decline of expression and both end up with five copies after 12 and 13 months. Since no intermediate sample could be obtained for patient 13 within the year of follow-up, no information regarding his expression pattern in-between was available.

3.2.2.7.4 Differences in expression of endogenous sequences for different states of MS

The expression of endogenous sequences was compared for two individuals with recurrent active EOMS. Samples were taken for non-active and active EOMS status from both individuals, and changes in expression pattern were analyzed for all previously tested endogenous sequences (fig. 51a and b). Both patients were males.

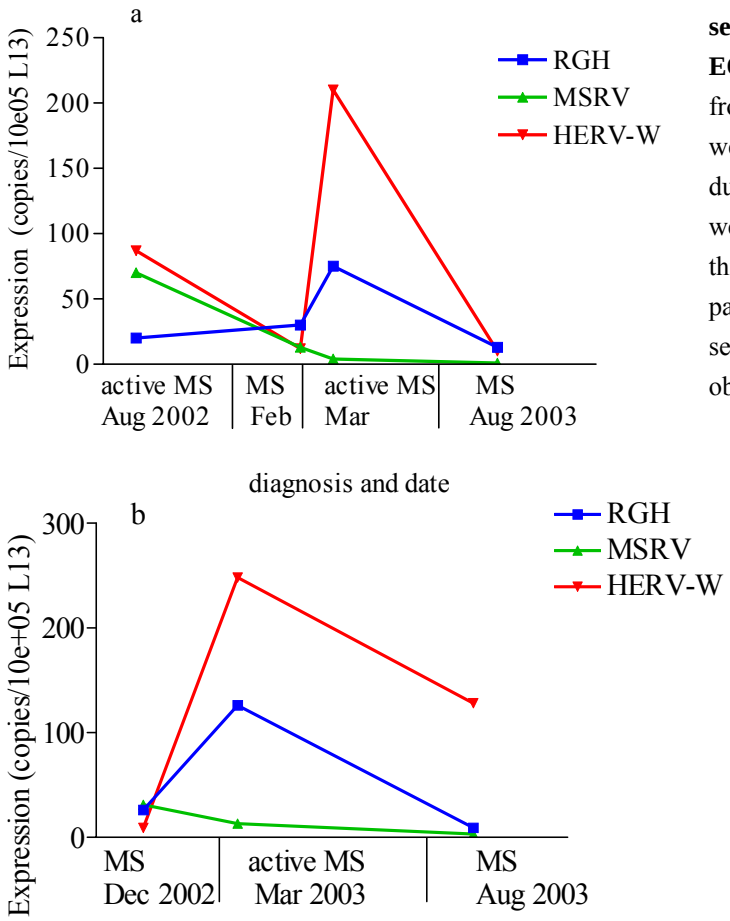

Fig. 51: Expression of endogenous sequences for active EOMS and EOMS. (a) Four consecutive samples from a patient with recurrent EOMS were obtained. Information regarding the duration of the active phases of disease were not available. The first and the third sample were obtained while the patient was affected by active MS. The second and the fourth sample were obtained during quiescent states of MS.

(b) Three samples from a Rebif-treated EOMS patient were obtained. The first sample was obtained with a non-active MS status in Dec 2002. In March 2003 the patient had active EOMS; the third sample was obtained five months later with a non-active EOMS status. Information regarding the duration of the active phases of disease was not available.

The first sample was obtained from the patient with active EOMS in August 2002 (fig. 51a). HERV-H was expressed on a low level with 20 copies, while the two HERV-W related sequences were expressed (70 or 87 copies respectively). The next sample was obtained in February 2003 when the patient had a non-active EOMS status. The expression of HERV-H was about the same as six months before, while expression of HERV-W*gag* and MSRV*env* was decreased to 12 and 13 copies, respectively. In March 2003 a sample during a recurrent active EOMS phase was obtained. MSRV expression was even lower than for the non-active phase of the disease, while expression of HERV-H and HERV-W*gag* was substantially increased. The last sample was taken in August 2003; the disease status was non-active EOMS at that time. MSRV expression could not be detected and expression of HERV-H was roughly 6-fold lower than for the active phase five months before. The expression of HERV-W*gag* decreased from 210 to ten copies.

The first sample obtained from another patient with a non-active MS status was obtained in December 2002 and showed low expression for all three endogenous sequences (fig. 51b). A strong increase was observed for HERV-H and HERV-W for the active-MS status, while MSRV exhibited only few copies. The last sample was taken in August 2003 with a non-active EOMS status again. The elevated expression of HERV-H and HERV-W that was previously seen for the active phase of the disease was decreased again; HERV-H showed a decrease from 126 to 9 copies and HERV-W from 248 to 128 copies. MSRV showed no substantial change in the course of time, but a slow decrease from 31 to 13 to 3 copies for the last sample.

Both individuals affected by recurrent EOMS showed a similar pattern for the expression of all three analyzed sequences. HERV-H*pol* and HERV-W*gag* showed a strong increase for the active phase and a decrease for the preceding and following non-active phases, while MSRV*env* did not follow this profile but slowly decreased over time.