4 Discussion

The etiology of most autoimmune diseases (AIDs) is unsolved, despite extensive studies. The tremendous effects on the quality of life of those afflicted, the global economic losses due to autoimmune diseases, as well as rising incidences of many AIDs, are motivating the need to develop curative therapies and appropriate measures to lower these incidences. Therapies have currently been focusing on symptoms; these therapies are used although they can be connected with severe or even fatal side effects: rheumatoid arthritis patients who were treated with TNF-antagonists showed a higher overall tumor risk compared with the general population (Geborek et al., 2005, Askling et al., 2005, Askling et al., 2005a), and in cases with poor prognosis for the patients affected by, for example MS, scleroderma or SLE, stem cell transplantation is performed as ultimate therapy (Mancardi et al. 2005, Hellings et al, 2004, Tyndall and Matucci-Cerinic, 2003, Gratwohl et al., 2001, Jayne et al., 2004).

An understanding of the etiology is the basis for the development of curative therapies. A major requirement for this goal is the clarification of whether infectious agents are involved in the etiopathogenesis of AID and if so, the identification of the respective microorganism(s). These agents could either be persisting, or they could have triggered an autoimmune disease before being cleared by the host's immune system. If persisting viruses are involved in the etiology of a slow disease, elimination of the virus from the host might be accomplished, and in consequence, the remission of disease. In the case of viruses triggering a disease prior to being eliminated, prevention of infections with the respective virus could lead to a lower incidence.

Epidemiological data compiled over the last decades suggest multifactorial origins for many AIDs. Geographical, familial, as well as clusters in time of different slowly progressing diseases have been analyzed. As a result, the data suggests that most AIDs seem to involve genetic predisposition, familial as well as ethnic; some seem to be monogenic while others have a multigenic background. The involvement of factors other than genetic is shown by the 25% – 60% concordance of AIDs among monozygotic (MZ) twins, depending on the respective disease (Fujinami, 2001). While genetic predispositions and other known risk factors such as age and gender can not be influenced, environmental factors could be. Diffuse influences and intakes are comprised under the umbrella of 'environmental agents', which include nutrition, sunlight as well as other climatic factors, intoxications from smoking, organic solvents or industrial toxins, heavy metals, socioeconomic status, pets, drugs and many others. Infectious agents, subdivided into bacteria, viruses and fungi, are one of the additional groups of potential causes to be considered in the etiology. A wide variety of viruses – pathogenic and apathogenic – are known to infect plants, animals, and humans. Manifold clinical pictures and disease mechanisms have been disclosed so far, and among these are disease outcomes which resemble those of slowly progressing or autoimmune diseases. Retroviruses constitute one

of the virus families being implicated in AIDs due to several findings. The known human retroviruses can cause diseases with clinical pictures similar to AIDs. Disease in animal models for AIDs can be triggered by animal retroviruses. Antibodies reacting with retrovirus epitopes have been detected in the blood of AID patients who were not infected with HIV or HTLV. Furthermore, besides exogenous human or animal retroviruses, a considerable part of the human genome consists of HERV sequences. These sequences could act as viral agents but are genetic components at the same time. The search for new retroviruses as well as the coexistence of exogenous and endogenous retroviruses renders necessary the use of different experimental approaches. Two general approaches have to be followed: The involvement of already proposed viruses has to be examined by using the available information. On the other hand, generic methods need to be applied in order to detect unknown zoonotically transmitted or mutated viruses.

There is only one catch-all virus tool, electron microscopy. Viruses can be detected and even categorized on the basis of morphological features by EM. Apart from the generic approach, a major advantage of EM is that many different materials such as blood cells, cell culture, or tissue material can be analyzed. The major disadvantage of EM, however, is the relatively high detection limit of about 10^5 to 10^6 particles/mL (Gentile and Gelderblom, 2005, Biel and Gelderblom, 1999). Furthermore, in the case of slowly progressing diseases, the infection may have taken place long before clinical symptoms become apparent and virus load may be low due to the host's immune response. The virus may persist in a compartment which is not accessible. The infectious agent could even be cleared from the host completely, although this is hardly conceivable for retroviruses which integrate in the host's genome. The analytical potential of EM is therefore limited, and persisting virus infections with a low virus load or previous infections will not be detectable through this method. These limitations could account for the inconsistent results obtained with EM. Some publications indicate detection of retrovirus-like particles in samples from patients with different AIDs, although these results are conflicting. More contradictory results have been obtained for various AIDs with generic molecular biological methods, e.g. epitope mapping. This substantiates the need for highly sensitive and at the same time retrovirus generic tools. One aim of this work was the development of broad reacting detection systems for retroviruses.

4.1 Development of detection methods for retroviruses

Molecular biological methods need a target, either genes or proteins of the microbe itself or its footprints, such as antibodies against the virus persistently produced by the host. Defining such a target requires information beforehand. The tools which are currently available for tracing infectious agents or their footprints aim at different targets and thus need different kinds of information. Methods for detecting virus proteins or antibodies against these proteins include, for example, Western blot and ELISA, PCR or RT-PCR. To a certain extent these methods can be used as generic tools. Virus proteins and antibodies

directed against a specific virus can be cross-reactive and reveal the presence of a virus, even if the assay used is not specifically designed for this virus. Key elements of genetic information can be conserved even among different virus families (e.g. the *pol* gene) and can be picked up by generic PCR. Therefore not only known and typified viruses can be detected by these means, but there is also a chance that related viruses will be tracked down. However, proof of absence can not be obtained. Zoonotically transmitted, rare, or new viruses might go undetected unless they do not share features with known viruses. This shortcoming can be balanced by introducing subtractive methods. The advantage of subtractive methods is that no specific information about the target is required. The disadvantage is the need for proper controls and well controlled study cohorts. The acquisition of confirmed negative controls is virtually impossible for multifactorial and slow diseases. If the AID in question requires a specific genetic predisposition for disease development, individuals lacking this specific condition could be infected with the triggering agent but never develop clinical symptoms. Thus actually infected individuals could be accidentally selected as negative controls and, as a consequence, the infection could not be detected using the subtractive approach. On the other hand, any known genetic predisposition could be used as an advantage with subtractive methods. If a genetic condition adding to a specific disease is known, for example, HLA-B27 for spondylitis, healthy individuals having the same genetic predisposition could be used as a control group. This approach could facilitate the detection of the factor(s) which determine the difference between health and autoimmune disease.

Also, an asymptomatic individual who subsequently develops the disease could be selected as a negative control, especially if the slow onset and progression of these diseases is taken into account. To overcome this problem, samples from negative controls could be stored until the healthy state is confirmed later on. Establishing a healthy donor bank over the years would certainly prove a very valuable source; however, it would require major efforts, in the financial aspect as well as in the logistics of follow-up checking on the development of the health status over years, maybe even decades. This task could not be achieved within a work planned for only a few years.

Another major problem for subtractive approaches is posed by the diverse nature of many AIDs. It is uncertain that all patients suffering from similar clinical pictures, even from the same disease, share the same etiopathogenesis. For example, many different ways leading to demyelination can be imagined, all of which would end up in similar clinical pictures. The rheumatoid disorders analyzed all show varying clinical outcomes. These different clinical pictures could mirror individual etiologies for single patients (e.g. regarding genetic predisposition or environmental factors) but could also reflect completely different etiologies. If patients with similar clinical pictures are grouped together, it would be impossible to detect a causative agent which is present in only a subset of them by subtractive methods. Thus, it is necessary to compile samples from patients showing very

similar disease patterns, stages of disease, sharing the same gender and receiving the same treatment.

These complications apply in principle to all of the subtractive methods used, and become even more complex if more than one cofactor is involved in the etiopathogenesis of the respective disease.

Subtractive methods like the differential display (DD) were developed to detect differences in expression patterns, e.g. of different tissues, or of diseased compared to healthy individuals (Liang and Pardee, 1992). The DD can basically be used as general screening method for infections, including retrovirus infections. However, analyzing retrovirus producing cell culture supernatants in comparison to non-infected supernatants showed that the original DD approach was too expensive, extremely labor intensive and lengthy to be used as a general screening tool. The introduction of primer binding site (PBS)-specific primers and in the following of staggered PBS-specific primers in this work simplifies the procedure, as the number of PCR products to be analyzed is reduced considerably (Uhlenhaut et al., 2001). In case of unknown viruses or zoonotically transmitted viruses, this panel of primers can be widened accordingly. For example, the walleye dermal sarcoma virus (WDSV) uses $tRNA^{His}$ and the snakehead fish retrovirus (SnRV) uses $tRNA^{Arg1,2}$ as primer (Telesnitsky and Goff, 1997). If all tRNAs used by animal retroviruses are included in the screening, eight different reverse primers have to be used in combination with eight random primers.

In conclusion, the DD was successfully adapted as a retrovirus-specific screening method; however, when this method was applied to RNA extracted from whole blood of patients and asymptomatic controls, a tremendous increase of PCR products was observed with the transfer from cell culture supernatant to whole blood samples. Many PCR products with exactly the same lengths were observed. This was reflected by the need to clone PCR products prior to sequencing, although the PAGE separates bands which differ only for one nucleotide in length. The frequent occurrence of PCR products of the same length but of different origin renders the DD approach on clinical samples impossible, since optical differentiation due to the banding pattern itself could not be accomplished.

This increase of PCR products observed with the change from cell culture to whole blood could be due to different reasons. While cell cultures consist in general of one cell type only, blood samples contain many different cell types. In addition, the comparison of individuals is much more complex than the comparison of infected and non-infected cells. While each cell culture represents a single tissue, different individuals vary in many more respects which can be mirrored in the detected expression patterns.

The differential display RT-PCR was built up as an effective screening tool for cell culture supernatants; however, it was shown to be inapplicable as a screening tool for clinical samples. The established assay system should be used to acquire sequence information from confirmed retrovirus positive samples rather than as a screening tool.

Consequently, another retrovirus generic screening method was needed. This method should allow the evaluation of single samples, as well as the screening of larger cohorts. Confirmed negative controls and specific information, such as, for example, sequence information, should not be a prerequisite. All this describes a generic but non-subtractive method. A retrovirus generic assay fulfilling these criteria has been published: The presence of retrovirus particles can be proved by the reverse transcriptase activity assay (Pyra et al., 1994). Commercially available tests (Cavidi) gave conflicting results and tested even negative with cell culture supernatants with high viral loads (HIV) in our laboratory (S. Duwe, personal communication). Since the results were neither reproducible nor reliable, it was decided to adapt and optimize the RT assay published by Pyra et al. as in-house assay.

One of the key enzymes of retroviruses is the reverse transcriptase, which makes it a prime target for antiretroviral therapy, as well as for virus detection. Although the reverse transcription is often described as a 'unique' retrovirus feature, several other enzymes – cellular as well as heat stable enzymes used for PCR - show RT activity to different extents depending on the assay conditions (Newton and Graham, 2001, Myers and Gelfand, 1991).

The assay was described to be extremely sensitive, but only Mg^{2+} was used as bivalent cation (Pyra et al., 1994, Boni et al., 1996, Andre et al., 2000, Voisset et al., 2001, Sears et al., 1999, Lovatt et al., 1999). One of the published assays used Mn^{2+} as well; however, Mn^{2+} and Mg^{2+} were used in the same buffer and no difference in RT activity was detected for Mg^{2+} buffer in comparison to a combined Mg^{2+}/Mn^{2+} buffer (Yamamoto et al., 1996). The differentiation of Mg^{2+} and Mn^{2+} preference with this approach is impossible. Quite the contrary, it was described that trace amounts of Mn^{2+} inhibited HIV-1 RT in vitro in the presence of the preferred Mg^{2+} cation (Bolton et al., 2002). Thus, the combinations of both ions in one buffer could lead to false negative results or too low activities. The reverse transcriptase activity assay modified in this investigation was optimized and evaluated independently for both, Mg^{2+} and Mn^{2+}, using retrovirus producing cell cultures. This new feature not only broadens the detection range of the assay, it can also be used to characterize the detected reverse transcriptase activity in terms of ion preference. This characterization can point to the type of virus which is detected. For example, gammaretroviruses were described to prefer Mn^{2+}, while lentiviruses are known to prefer Mg^{2+} (Avidan et al., 2003, Telesnitsky and Goff, 1997).

As a conclusion from the results obtained for human as well as for animal retroviruses in this work, it is sufficient to use the Mg^{2+} buffer and if any RT activity of unknown origin is detected, the ion specificity can be cleared by a comparative RT assay using Mg^{2+} and Mn^{2+} buffers in parallel. Since no background signals are observed for the Mg^{2+} based assay, it shows a higher sensitivity than the Mn^{2+} assay. This modus operandi renders the assay faster and cheaper without losing sensitivity.

The published assays included filtration of samples (Khan and Sears, 2001, Sears et al., 1999, Boni et al., 1996, Lovatt et al., 1999). Viruses are defined as filterable agents (2nd

Koch's postulate) and thus filtration should have no diminishing effect on the RT activity. Nevertheless, a strong decrease of RT activity specific for HIV and HIV-positive plasma was observed after filtration. The typical pore size used for filtration is either 0.22 µm or 0.45 µm; the antibody-mediated aggregation of HIV particles (which are approximately 100 nm in diameter) could easily render the virus not filterable. The same effect could happen in the plasma of patients infected with another retrovirus and thus it was crucial to overcome this obstacle. The antibody-stripping procedure developed included stringent and acidic conditions which led to a subsequent loss of assay sensitivity. Hence the antibody-stripping procedure was not introduced in the routine protocol of the reverse transcriptase activity assay (RTA). Instead of a removal of antibodies, the filtration was exchanged by centrifugation through a sucrose cushion. This modification is an important improvement of the assay. But it is conceivable to optimize this antibody-stripping procedure for future assays or develop other means to separate potentially existing antibodies from virus particles. For example, antibodies could be attached to columns and in the following adhering viruses could be washed out.

But aggregation is not the only problem which could be posed by antibodies directed against a virus. Any infection with an unknown retrovirus could result in the production of antibodies, which could also include RT-specific antibodies, as was shown for HIV (Odawara et al., 1996). These antibodies could inhibit the enzymatic activity of the RT directly and thus result in a lower signal or even in a false negative result for the RTA. However, data obtained with HIV plasma showed that this effect is rather small compared to the effect caused by filtration. Therefore the effect of RT-specific antibodies can be outweighed by the sensitivity of the RTA.

Leadoff examinations of human plasma (citrate plasma obtained from blood bank samples and EDTA plasma as clinical samples) showed that a clarification step was necessary, since the real-time PCR data of the RTA could not be analyzed due to interference of plasma components with the assay. Although intact cells were separated from the plasma by centrifugation, the plasma still contained electrolytes, nutrients, metabolites, vitamins, trace elements, hormones, proteins, and debris of cells which were disrupted in the time between sampling and centrifugation. As shown, the RTA is susceptible to various interferences caused by cellular enzymes or other compounds, e.g., ions, detergents, proteins, etc. A reduction of interfering components was achieved by a 10-fold dilution in Tris prior to centrifugation. The dilution of cell-free citrate plasma samples eliminated the interferences completely. This procedure lowers the sensitivity of the assay and has to be taken into account. Besides, it was demonstrated that EDTA plasma gives a background signal due to contaminant cellular enzymes which are released from cells until these are separated from the plasma. Apart from some limitations, it was conclusively shown that the RTA, as it was performed, is a suitable assay to evaluate the presence of reverse transcriptase activity in freshly collected, cell-free plasma.

Background activity or false positive results have been described as major problems for reverse transcriptase activity assays: Intrinsic RT activity was observed for various components (Pyra et al., 1994, Lugert et al., 1996), DNA polymerase and RNase inhibitors were described to be associated with DNA related to the MS2 bacteriophage (Kothapalli et al., 2003), cellular enzymes displayed RT activity, and DNA polymerases showed a template switch from DNA to RNA in the presence of Mn^{2+} (Newton and Graham, 2001). All components which were to be used for buffer composition were tested for interference with the assay performance. Some of them did show RT activity or enhanced Mn^{2+} related background activity; these components were not included in the RTA buffer system. Furthermore, a DNA trap, RNase digestion of the RNA template prior to PCR, and the UDG protocol were introduced. The combination of these countermeasures led to a reliable assay with no background signals for Mg^{2+} and only minor signals for Mn^{2+} buffer. Several controls were introduced: (1) a serial dilution of a standardized, commercially available reverse transcriptase was used as positive control and allowed the quantification of the detected RT activity; (2) RT-negative controls proved that there were no background signals; and (3) the comparison of three different dilutions of one sample in the same assay allowed the detection of any inhibitory factors or contaminations of the samples, as the 10-fold dilutions were mirrored in the ct values obtained by real-time PCR. Factors inhibiting the reverse transcriptase activity would also be detected by the use of different dilutions in parallel. These comprehensive control mechanisms allow the reliable detection of single virus particles.

The high sensitivity was achieved by changing the RTA-cDNA conditions compared to those used for RT-PCR. The limiting factor for cDNA synthesis in RT-PCR is the RNA template while the reverse transcriptase is added in surplus. However, the limiting factor for cDNA used for RTA is the enzyme activity. Reverse transcription is rather slow compared with DNA polymerases. It was suggested that RT adds about one nucleotide per second to a growing chain in vitro, although this rate may be higher in vivo. However, the relatively long time required to generate HIV DNA (roughly four hours from infection to the first appearance of a completed 9 kb DNA) suggests that the in vitro rates are approximately correct (Telesnitsky and Goff, 1997). For maximum sensitivity, in this investigation the time for cDNA synthesis was extended to five hours.

The assay was evaluated comprehensively, using cell culture supernatants and plasma. Human as well as animal viruses were analyzed by RTA. Inter and intra assay variations were also determined to be sound. The sensitivity of the assay was conclusively demonstrated by analysis of HIV-positive plasma in comparison to HIV-specific assays. Furthermore, it was shown that the RTA could also be used to determine the virus load of a given sample over a broad range.

Comparison with other RT activity assays shows that, while the freshly prepared buffers provide reproducible results with the RTA, other assays could give varying results or lose sensitivity in the course of time (e.g., if radioactive labeling is included, and the marker

shows different activities due to decay, Yamamoto et al., 1996). The comparison to a commercially available assay (Cavidi) also showed the capacity of the optimized in-house RTA. The commercially available reverse transcriptase activity assay consists of five different tests (www.cavidi.com). These tests comprise a test specifically designed for lentiviruses (Mg^{2+}), one test designed for Type C retroviruses (Mn^{2+}), and three highly sensitive tests, either specific for Mn^{2+}- or Mg^{2+}-dependent viruses. In order to detect all reverse transcriptase activity, combinations of these tests would have to be used. This approach would need more material and render the assay more complex and expensive than the RTA developed in this investigation. The effective range of the Mg^{2+} test as provided by the supplier is 2.4×10^4 up to 2.4×10^8 particles for the lentivirus test, and 5.9×10^3 up to 5.3×10^7 virus particles for the highly sensitive lentivirus kit. The supplier gives the sensitivity for the Mg^{2+} based assay as per pg/mL. In order to allow a direct comparison to the effective range of the RTA, the protein concentration was converted to virus particles by assuming that one RT molecule has a molecular weight of 51 kD (HIV reverse transcriptase) and 100 RT molecules are incorporated within one virion. The effective range for the Mn^{2+} based Cavidi assay is given in mU/mL by the supplier, the range for the C-type-specific assay is given as 0.02 mU/mL to 125 mU/mL and 0.6 to 4600 µU/mL for the highly sensitive Mn^{2+} assay. However, the effective range of the Mg^{2+} based RTA is few virus particles up to 1.5×10^9 particles per mL cell culture supernatant. The effective range of the Mn^{2+} based RTA was determined from 0.02 mU/mL up to 1.4×10^5 mU per mL.

This comparison shows that the sensitivity of the Mg^{2+} based RTA is higher and the range is broader than for the combination of the two commercially available Mg^{2+} tests. The Mn^{2+} based RTA is as sensitive as the commercially available test, but has a broader range. Furthermore, it was demonstrated that two tests (Mg^{2+} and Mn^{2+}) in parallel are sufficient for the RTA, while the commercially available tests also depend on the virus load.

Since the reference enzyme which is used as an internal standard, the RNA template and the DNA used as DNA trap are standardized and commercially available, the RTA allows the use in independent laboratories and direct comparison of the results. The basic re-evaluation of assay components and performance demonstrated the reliability, stability, and sensitivity of the RTA in comparison to virus-specific as well as to other RT activity assays. The RTA proved to be a suitable screening tool for the generic detection of retroviruses, and was used in further investigations for the detection of RT activity in clinical samples from AID patients.

4.2 Application of the specific test system

Certain retroviruses have been implied in the etiology of autoimmune diseases although many reports have been conflicting. Some of these hypotheses were reviewed using (RT-)PCR as specific approach. This involves exogenous (HTLV) as well as endogenous retroviruses. The different types of virus rendered two different approaches necessary: On

the one hand, all samples were tested for HTLV, looking for a yes-or-no answer, while on the other hand different expression levels for HERVs were determined and compared to healthy controls.

The clinical picture of the HTLV-1 associated myelopathy/tropical spastic paraparesis (HAM/TSP) resembles MS and other neurological diseases, such as disseminated encephalomyelopathy, Devic's syndrome, chronic myalgic encephalomyelitis, or nervous systemic lupus (Poser, 1994). All of these diseases have to be considered for differential diagnosis. More than 60 different neurological diseases have been linked to HTLV-1 infection (Gessain et al., 1985, Rodgers-Johnson et al., 1985, Roman and Osame, 1988, Zaninovic, 2004). Conflicting studies describe antibodies against this retrovirus for different AIDs, e.g. SLE (Nelson et al., 1994, Lipka et al., 1996) and scleroderma (Morozov, in press). Although this infection is rarely observed in Germany and the sporadic cases are usually related to migrants from endemic areas, the analysis of all samples for HTLV infection is advised.

More recently, the expression of HERV sequences received attention due to several potential roles, including the contribution to genomic plasticity through reverse transcription, the regulation of adjacent genes, and the involvement in physiological and pathological processes relevant in particular to reproduction and autoimmunity (Lower et al., 1996, Taruscio and Mantovani, 1998, Obermayer-Straub and Manns, 2001).

In theory, HERVs can be reactivated and infectious virus particles derived from endogenous sequences can be detected by RTA. HERVs are not only potentially infectious agents, they are also genetic elements at the same time; most of them are present as multicopy gene families. Therefore the mere detection of HERV sequences is not sufficient to estimate the pathogenic potential. Several HERV sequences have gained function in the course of evolutionary time and their expression is now indispensable for the host. Hence, the expression has not only to be detected, but also to be quantified and compared to expression levels in control groups. Some of these endogenous elements play a role in developmental processes, or their expression could change due to immunosenescence, which confirms the significance of age matched control groups. The average age of the healthy adult control group examined for this work was 32 years of age (mean and median); this is 16 years younger than spondylitis patients, 12 years younger than SLE patients and 25 years younger than the average scleroderma patient. The considerably younger age of the control group could limit the conclusions drawn from the different expression patterns, but controls which were more closely matching, were not available. Furthermore, expression of HERV sequences could differ depending on the gender. Gender-specific expression could be linked to hormone responsive elements within the LTR regions of HERV sequences and thus could be responsible for different expression patterns, different clinical pictures, and different prevalence rates for most AIDs in males and females.

Since most HERV families are multicopy families within the human genome, any DNA contamination of the RNA sample would render analysis of expression patterns impossible. In order to assure absence of DNA, whole blood was collected in an RNA protection buffer. It was impossible to recover DNA from this buffer. Furthermore, a DNase digestion was included in the process of RNA extraction. Following total RNA extraction, mRNA was prepared via the polyA tail to eliminate contaminating traces of DNA. RT-negative controls were performed for every sample. Additionally, a real-time PCR which distinguishes between cDNA and genomic DNA was established and performed for every sample in duplicate. These measures assured the DNA-free nature of the samples and thus that mRNA expression levels were comparable.

Another important aspect regards the varying half lives of mRNAs. The half life of eukaryotic mRNAs can be several hours but can also be as less than 20 minutes (Raghavan et al., 2002). In order to analyze the expression pattern at the time of sampling, all blood samples were collected in an RNA protection buffer which freezes the RNA pattern at the time of sampling (PAX system).

The direct comparison of different expression patterns can only be carried out for samples containing the same amount of cells or nucleic acids. Some AIDs include anemia in their clinical pictures (for example, SLE). Thus the expression patterns could not be linked to a certain volume of whole blood but were related to an independently expressed house keeping gene. The house keeping gene PCR was also used to determine a threshold for samples with too low RNA concentrations. All samples below the cut off were excluded from analysis since these samples would have resulted in high calculative correction factors due to low RNA content and thus would have distorted the actual expression levels. By comparison of the expression levels of the house keeping gene for all groups tested, it was shown that the expression was independent of age, sex or health status; thus the chosen gene was suitable as reference.

The assays optimized, adapted, and established as described above do contribute to the pool of tools which can be used to determine whether retroviruses represent pieces of the etiology puzzle of certain autoimmune diseases.

Retroviruses were the first viruses linked to the development of cancer. In 1908 Ellermann and Bang showed that a virus induced leukemia in chickens and in 1910 Rous showed that the Rous sarcoma virus (RSV) could induce tumors in poultry (Ellermann and Bang 1908, Rous, 1910). Since then, many more cancerous diseases have been linked to retrovirus infections. The human T cell lymphotropic virus can cause a rare form of leukemia, the adult T cell leukemia (ATL), in humans (Yoshida et al., 1982, Seiki et al., 1983). The well known cancerous potential of retroviruses can also be used to transform cells into permanent cell lines, and the expression of structural genes of HERVs has been described in several cancer cell lines, indicating that they may have pathological roles (Armbruester et al., 2002). Four melanoma derived cell lines, one teratocarcinoma cell line, and a human

leukemia cell line were tested for reverse transcriptase activity as cell lines with a cancerous origin.

In summary, the cell culture supernatant from melanoma cell lines showed no Mn^{2+}-dependent RT activity and one of them showed a rather low Mg^{2+}-dependent RT activity (Mewo). The negative results obtained with the RTA showed that no virus particles with functional RT enzymes are produced in the cell lines established from melanomas, with the possible exception of Mewo cells. The absence of retroviruses specifically expressed in the tested melanoma cell lines could indicate that replicating retroviruses were not involved in this cancer development. But it is also imaginable that a retrovirus induced a melanoma and the transformation of the infected cells into permanent cells, and its expression was then lost in the course of time. Furthermore, the transformation of a cell line does not require a 'complete' and infectious retrovirus; the insertion or (re)activation of a transactivating sequence at a susceptible gene locus could be sufficient. Another possible mechanism could be the interference of certain retrovirus gene products with the cell cycle or other mechanisms (e.g. DNA repair). If expression of specific HERV gene products but not of HERV particles triggered melanoma development, antibodies against this specific virus protein or expression of the sequence could be detected, but no reverse transcriptase activity.

The teratocarcinoma cell line GH expressed different mRNA species of HERV-K (Tonjes et al., 1996). Although the expression of mRNA does not indicate the presence of retrovirus particles conclusively, the results obtained with the RTA revealed a low virus load with an Mn^{2+} preference. This result needs to be confirmed by independently repeated RTAs. Supernatants of the melanoma and teratocarcinoma cell lines were only obtained once, and it has been described that the expression of retrovirus particles varies with the time of harvesting (Christensen et al., 1999). Furthermore, the results were obtained with a single sample which had been stored for more than two years at -70°C. Therefore the findings should be analyzed further; larger volumes of supernatant should be collected and subjected to a PEG precipitation in order to determine whether the enzymatic activity is particle-associated and to perform electron microscopy.

RT activity of unknown origin was described for the human leukemia cell line Kasumi-1, while it tested negative for HIV-1, HIV-2, HTLV-1 and HTLV-2. The RT activity inherent in this cell line had not been previously quantified. The determined Mg^{2+}-dependent RT activity was equivalent to approx. 240 retrovirus particles per mL; this value is probably too low, since it could be shown that the assumed Kasumi-1 RT slightly preferred Mn^{2+} to Mg^{2+} and the virus load was determined using a Mg^{2+}-preferring RT (M-MLV). The detected RT activity was particle-associated, since it was precipitable by PEG. The Mn^{2+} preference was described as a characteristic feature of the gammaretroviruses, some of which are linked to leukemia development (e.g., MuLV, FeLV, GALV, Burmeister, 2001). The Kasumi-1 cell line should be analyzed further in order to determine the source of RT activity. Since no negative control is available (i.e. non-infected cells from the same

individual), a generic retrovirus PCR approach could be used to detect retrovirus sequences (Burmeister et al., 2001). A larger volume of cell culture supernatant could be subjected to PEG precipitation in order to yield a concentration of more than 10^6 particles per mL. Based on the RTA results, 4000 mL could be sufficient. This would allow the use of electron microscopy and perhaps a categorization of virus particles according to morphological characteristics as well as providing starting material for a molecular characterization.

In conclusion the melanoma cell lines could not be linked to expression of retrovirus particles. The leukemia and possibly the teratocarcinoma cell line as well showed genuine RT activity that needs to be characterized further.

While human endogenous retrovirus genomes are mostly truncated and non-functional, several animal endogenous retroviruses are expressed as infectious viruses. Examples of retroviruses which exist as exogenous and endogenous virus in parallel are known for different virus families: JSRV in sheep (betaretrovirus, Coffin et al., 1983), MMTV and murine leukemia viruses (betaretrovirus, Griffiths, 2001, Boeke and Stoye, 1997), AVL (alpharetrovirus, Crittenden et al., 1979), FeLV (gammaretrovirus, Mikkelsen and Pedersen 2000, Boeke and Stoye, 1997), SMRV in NWM (Schochetman et al., 1977), and PERVs (Wilson et al., 1998, Patience et al., 1997a). Especially the expression of PERVs poses a potential risk, since their natural hosts – pigs – are proposed for use in the xenotransplantation of solid organs. Two subtypes of PERVs, PERV-A and PERV-B, have been shown to infect human cells in vitro, while this could not be shown for PERV-C (Patience et al., 1997a, Specke et al., 2001). These findings are of major importance for the virus safety of xenotransplants, particularly since the recipients are immunosuppressed. However, porcine liver cells have been used for some time as xenotransplants. The bioartifical liver is one form of extra corporal therapy for hepatic failure, using pig hepatocytes as its source of hepatic function. This therapy includes the possibility of zoonotic infection; known or unknown pathogens of pig origin could infect the human recipient. Retrospective studies on hundreds of patients who have received pig xenotransplants have shown that in none of these cases has an infection with PERVs been described (Fujita et al., 2003, Irgang et al., 2003, Magre et al., 2003).

Two porcine kidney cell lines (PK15 and PS cells) showed high RT activity with Mg^{2+} as well as with Mn^{2+} buffer, with Mn^{2+} being clearly preferred. Since PERVs are gammaretroviruses, this was expected and has been described previously (Avidan et al., 2003).

This study showed that the RTA is able to detect different human, animal, and potentially new retroviruses, and can also be used as a sensitive monitoring tool for virus safety.

The RTA was also used to examine the specific enzymatic activities of different reverse transcriptases from one virus, HIV. It was analyzed whether mutations that confer resistance to RT inhibitors would alter the RT activity. Four different HIV isolates with drug resistance associated RT-mutations were analyzed in comparison to wild type RT.

The RTA results corroborated previous findings (Duwe, 2003, Uhlenhaut et al., 2003). The detected infectivity of the RTs with drug resistance associated mutations compared to the wild type indicated that these viruses were still replication competent and infectious, but showing a reduced fitness. All four samples with drug resistance associated mutations had similar p24 values (~ 20% compared to the wild type virus). Three of the four samples showed 10-fold lower RT activity compared to the wild type RT, while one sample with higher infectivity showed 57% RT activity when compared to the wild type. These findings indicate that the viral fitness depends on the mutation pattern of the complete virus genome. While some mutations lead to diminished fitness, others could abrogate this effect.

In conclusion, it was shown that the RTA can, in principle, be used to compare the fitness of different reverse transcriptases. The proposed use of the RTA for HIV analysis does not include the routine testing of samples for a new HIV infection. The established systems (ELISA, Western blot, and RT-PCR) are sensitive, standardized, and well established for the common HIV subtypes (e.g., HIV-1, subtype B in Germany). In rare cases, detection of an infection with uncommon HIVs, for example HIV-1 O or recombinant HIVs, could pose difficulties. The RTA could be a suitable tool for these cases, since variations of subtypes do not interfere with assay performance, while detection of p24 antigen capture assay and Western blots could be affected (Marozsan et al., 2004). But the established and commercially available tests have the further advantage that the potentially infectious plasma is either inactivated (ELISA, Western blot) or infectivity is destroyed in the process of nucleic acid extraction. The RTA uses potentially infectious samples until the cDNA buffer is added; the infectivity is even concentrated by ultracentrifugation, which prolongs the time of handling potentially infectious material. Furthermore, the mere detection of RT activity in a sample obtained from an individual who belongs to a risk group would imply but not prove an HIV infection, since the assay is generic for the retrovirus family.

Currently 16% of the newly transmitted HIV infections in Germany occur with viruses carrying at least one resistance associated mutation (www.rki.de). The testing for resistant HIV is important in order to choose the appropriate therapy options, and should be performed prior to any therapy. However, the testing is time-consuming and expensive. Testing of HIV reverse transcriptases in the presence and absence of RT inhibitors could be used as quick or complementary tests for resistance associated mutations in the reverse transcriptase. In order to establish this option, it has to be verified whether the RT inhibitors interfere with the assay performance. Furthermore, the viral load has to be determined otherwise (e.g. by RT-PCR) in order to compare RT activity in relation to virus particles instead of U per mL. The analysis of RT activity per mL can lead to misinterpretation of the actual viral fitness. The diminished fitness of these viruses can be overcome by other mutations or by more RT molecules packaged into each virion (Soriano and de Mendoza, 2002). However, it was described that the RT and RNA levels in plasma of HIV infected individuals correlate well, especially during seroconversion. But it was

also noticed that RT and RNA ratios are not constant and may vary in different individuals during both the early and the late stages of HIV infection (Garcia Lerma et al., 1998).

4.3 Investigations of MS cell lines and clinical samples

In the following, the results for plasma samples obtained from patients with different autoimmune diseases (MS, scleroderma, SLE and spondylitis) will be discussed.

4.3.1 MS – cell lines

Besides many other viruses, retroviruses have been implicated in the etiopathogenesis of MS and their potential role had been investigated extensively (Munch et al., 1995, Haahr et al., 1994, Tuke et al., 2004, Shirazian et al., 1993, Ferrante et al., 1997, Kam-Hansen et al., 1989, Perron et al., 1997, Blond et al., 1999, Komurian-Pradel et al., 1999, Serra et al., 2001, Christensen et al., 2000). It was shown that HERV-H particles derived from cell cultures of MS patients were transmissible, albeit at a very low level (Christensen et al., 2002).

Conflicting results were obtained with electron microscopy: detection of retrovirus particles by S. Haahr, and negative results for the same cell line by H. Gelderblom (Haahr et al., 1994, Christensen et al., 1999, H. Gelderblom, personal communication); however, these results could be due to low viral loads. Hans Gelderblom described ‘unsual’ structures observed in the MS cell line MS1533; however, these structures were too large to be retrovirus virions (~ 500 nm in diameter, while retroviruses are about 100 nm in diameter) and they incorporated no virus core (H. Gelderblom, personal communication). Two of the cell lines described by Christensen et al. (MS1533, MS1845) were cultivated and supernatant was analyzed.

None of the two cell lines showed Mn^{2+} related RT activity and only minor Mg^{2+} related RT activity in repeated assays. In order to analyze whether a low virus titer in the supernatant was given, larger volumes of supernatants were collected and subjected to a polyethylene glycol (PEG) precipitation. The PEG pellets (100-fold concentration of the original supernatant) showed RT activity enhanced by a factor of 20 for MS1533 and by a factor of 55 for MS1845 supernatant. Due to the very low overall RT activity, these results can be due to inter assay variances. Retrovirus expression could not be chemically induced with butyrate in the cell lines.

From these results it was concluded that there was no significant retroviral load present in the analyzed cell culture supernatants of cell lines from MS patients.

The RT activities described by Christensen et al. ranged over 13 $\log_{10}$ for MS1533 and over 2 $\log_{10}$ for MS1845 (Christensen et al., 1999). The RT activity given by Christensen et al. was determined using the PERT protocol (Pyra et al., 1994); however, RT activity was not quantified by real-time PCR but by ELISA. Thus, the findings of Christensen are not directly comparable to the findings obtained in this work. The differences observed could not only be attributed to different ways of quantification of RT units, especially

regarding the broad range of RT activity of MS1533 cells. The extremely broad range of RT activity, especially for MS1533 cells, raises questions regarding the reliability of the assay used and the source of the detected RT activity. It is hardly conceivable that the detected RT activity within a given cell line can vary over 13 $\log_{10}$ without major changes. These changes could include an unnoticed infection with another retrovirus. The analyzed cell lines were described as 'long term cell cultures from blood cells' and all of the cell lines which were tested were also producing EBV particles. The expression of EBV could be responsible for change in expression pattern of HERVs. The conflicting results obtained with the RTA and by Christensen et al. could also be linked to the different assay conditions: It was shown that the unexplained RT activity detected in a non-retrovirus but EBV infected cell line disappeared when a surrogate DNA was employed in the RT activity assay (Voisset et al., 2001). It is also conceivable that the MS cell cultures tested lost their ability to express retrovirus particles in the course of time.

The EBV infection can explain the long-term survival of the respective cell cultures. On the other hand, the presence of EBV is not surprising, given its high prevalence: Approximately 95% of adults are infected on an average; 15% to 20% of individuals secrete EBV (Gärtner and Müller-Lantsch, 2002). Thus, an EBV infection is likely for the majority of the Danish MS patients. Furthermore, two different hypotheses link EBV and MS ('dual infection hypothesis', Munch et al., 1997, Haahr et al., 1994; 'polio hypothesis', Hernan et al., 2001). The expression of EBV in all MS cell lines could indeed be linked to disease development, and that the EBV infection of the Danish samples reflects a causative role in MS development as suggested (Munch et al., 1997). Recently, studies described a temporal relationship between the time of EBV infection and susceptibility to MS (Levin et al., 2005, Alotaibi et al., 2004). If involvement of EBV could be confirmed, a vaccination against EBV could lower the MS incidence.

A strong dependence on the time of harvesting of the cell culture supernatant was described by Christensen et al.; the first samples were only tested after more than 40 days of cell cultivation. A timely relation between time of cultivation or time of harvesting and the level of RT activity was not observed in repeated tests with the RTA.

Moreover, the data obtained with cell culture supernatants do not mirror the in vivo situation of patients. Cell cultures represent only one type of cells that are immortalized. The 'artificial' conditions for cell cultures influence the expression of various gene products, including HERV expression (Brorson et al., 2002). Therefore the findings obtained with cell cultures are of limited informational value.

For the time being, the conflicting results (obtained for the same cell cultures, with EM as well as with RTA) remain unexplained. One way to explain these discrepancies would be an exchange of supernatants between the laboratories and independent testing.

4.3.2 MS – clinical samples

In recent years, specific expression of two different HERV families – HERV-H and HERV-W – was described not only in long term cell cultures but also in blood and tissue samples of MS patients (Christensen et al., 1998, Garson et al., 1998, Serra et al., 2001). The expression of these HERV sequences was investigated for the first time three different groups of German MS patients (early onset MS, EOMS), children and adolescents affected by active MS, and adult MS patients). These expression levels were compared to those of age matched healthy controls, to neurologically diseased children and adolescents, and to three different groups of autoimmune disease patients (scleroderma, SLE, and spondylitis; tab. 11).

Tab 11: Compilation of PCR results

Group	**HERV-H**			**MSRV**			**HERV-W**		
	Range	Median	Negative (%)	Range	Median	Negative (%)	Range	Median	Negative (%)
Healthy children/adolescents (n=26)	2-419	19	0	0-101	8	4	0-360	52	31
Neurologically diseased children/adolescents (n=18)	5-214	30	0	2-218	14	0	0-1112	32	33
EOMS patients (n=59)	1-697	36	0	3-45	10	7	0-1911	22	27
Patients with active EOMS (n=17)	9-126	71	0	0-18	13	0	0-248	26	23
Healthy adults (n=26)	22-1547	74	0	0-138	11	15	0-129	25	39
MS patients (n=21)	1-81	16	5	0-18	1	19	0-18	7	33
Scleroderma patients (n=29)	2-438	70	0	0-13	2	21	0-193	0	66
SLE patients (n=19)	6-491	40	0	0-14	2	11	0-1503	132	21
Spondylitis patients (n=7)	16-1849	72	0	0-4	0	86	0	0	100

Range: copy numbers per 10^5 copies of the house keeping gene used as reference (L13); Median: median copy numbers per 10^5 copies of L13; Negative: % individuals who tested completely negative for expression of the respective sequence.

The statistically significant differences were observed for the comparison of different groups. However, the range of expression for individuals within each group was rather broad, depending on the analyzed sequence.

Strikingly, statistically significant differences were determined for the expression levels of healthy children and adolescents in comparison to healthy adults. A statistically significant higher expression for HERV-H was observed for healthy adults in comparison to healthy children and adolescents. In contrast to the findings for HERV-H expression, the age dependent expression of MSRV was significantly higher in children and adolescents compared to adults. The expression of HERV-H and MSRV sequences could therefore depend on the age or developmental stage of the single individual. Due to the observed age effect, the samples of adults and of children/adolescents were analyzed independently.

Analysis of the expression levels detected in blood samples from children and adolescents either with EOMS, with active EOMS, children and adolescents with neurological symptoms or diseases, and healthy controls showed no significant differences. However, significant differences were detected for the adult MS patients when compared to healthy adults. Expression of HERV-H was statistically significantly lower in MS patients compared to healthy controls (P value <0.0001). The same applied to the expression of MSRV (P value: 0.0475), while the expression of HERV-W did not vary depending on the health status.

These findings for children and adolescents as well as those for adult MS patients are in contrast to the findings in France, Italy and Denmark, with the limitation that these studies examined adult patients only. These cohorts were all described to specifically express either HERV-H or HERV-W related sequences or even virus particles. In all studies expression was considerably lower or not detectable in control groups.

HERV-H expression was described in cell-free plasma from 24 of 33 Danish MS patients, but could not be demonstrated in 29 plasma samples from patients with other diseases nor in 20 plasma samples from healthy controls. It was suggested that MS is associated with replication of otherwise quiescent endogenous retroviruses (Christensen et al., 2000). In contrast to these findings, the expression level of MS patients in this study was either the same as for healthy individuals (in the group of children and adolescents) or was significantly lower for adult MS patients compared to healthy controls. 5% of the MS patients did not show any detectable HERV-H expression, while expression was detected in all of the samples from healthy controls (tab. 11).

The described multiple sclerosis associated retrovirus (MSRV, Perron et al., 1989) belongs to the HERV-W family (Blond et al., 1999), and its expression was described specifically in samples from MS patients in France and Italy (Perron et al., 1997, Blond et al., 1999, Komurian-Pradel et al., 1999). The results were obtained in independent studies, using different methods and various samples, such as cell culture, whole blood, and CSF. MSRV sequences were detected in controls without MS (7%), in MS patients (53%), but in 100% of patients who were untreated at the time of sampling (Garson et al., 1998). Specific reverse transcriptase activity was detected in 12 out of 21 cultures from patients with MS (Perron et al., 1991). Retroviral particles were produced by cell cultures from patients with MS (Perron et al., 1997, Blond et al., 1999, Komurian-Pradel et al., 1999). A 100%

correlation between evidence of extracellular MSRV in the plasma and the presence of MS was described, with 12% of healthy blood donors harboring the virus (Serra et al., 2001). In contrast to these findings, plasma samples from German MS patients analyzed in this study showed a significantly lower expression for MSRV and no significant differences for HERV-W when compared to healthy adults. Some MS patients were completely negative for MSRV or HERV-W expression (19% and 33%, respectively); this was within the same range as determined for the control group (15% and 39%, respectively). All of the four different groups of children and adolescents included individuals who tested negative for HERV-W expression (tab. 11). MSRV expression was only detected in a part of the cohort of healthy children and adolescents, and in those affected by quiescent EOMS. But all individuals in the group of neurologically diseased and those affected by active MS showed detectable expression of MSRV, although not statistically significantly higher when compared to the healthy controls (tab. 11). This could be indicative for elevated expression in patients with acute MS or other ongoing neurological diseases in contrast to those with quiescent EOMS status, as suggested (Johnston et al., 2001).

While in France not only blood but also CSF and tissue were analyzed, the Italian groups analyzed blood in comparison to CSF (Perron et al., 1997, Tuke et al., 1997). The presence or absence of MSRV within the CSF was even used as a parameter for the prediction of disease progression (Sotgiu et al., 2002). The different materials and methods which were used for the different studies could account for some diversity of the results. However, the major differences which were observed could not be linked to technical reasons. It would be conceivable to obtain such difference when different materials or compartments are examined, but other studies also examined and detected HERV expression in the plasma of MS patients. Therefore other factors have to account for the observed differences. These comprise environmental and genetic factors. However, taking the geographical proximity into account, it is rather unlikely that environmental factors would account for the observed differences. Also it is hardly conceivable that ethnic predisposition could be the reason for different expression patterns observed in Denmark, France, or Italy. However, the genetic predisposition could be different for the extraordinary MS cluster on the island of Sardinia. In fact, several striking anomalies regarding MS clusters as exceptions from the global distribution have been repeatedly observed in temporal as well as in geographical respects. Sardinia is a high risk area for MS; the incidence and prevalence rates are among the highest in the world (Cocco et al., 2004, Dolei et al., 2002). The incidence rate is up to four times higher compared to continental Italy (Dyment et al., 2004). Moreover, MS incidence on the island has increased in the last four decades by almost 3-fold (Dolei et al., 2002). Sardinians have otherwise rare HLA phenotypes and red blood cells/protein polymorphisms which are fundamentally different from the rest of the Europeans (Dyment et al., 2004). However, there is a possible explanation for the rising MS incidence. It could be founded in the substantially improved general health on the island since the late 1940s; malaria, endemic on the island from the time of Herodotus, has been eradicated, major

vaccination campaigns (against poliomyelitis, pertussis, and diphtheria) were started in the 1960s, and improved economic conditions defeated tuberculosis (Dyment et al., 2004, Cocco et al., 2004). These changes may have been relevant environmental factors contributing to the elevated and still rising MS prevalence, especially considering the protective effect of Mycobacterium tuberculosis against experimental autoimmune encephalomyelitis (EAE), while malaria offers protection against AID (Cocco et al., 2004).
Another MS cluster was observed on the isolated Faroe Islands. MS was not observed there until 1943. The so-called Faroe-epidemic comprises 24 cases of MS developing on the island in the period from 1943 to 1960. The Faroe Islands were occupied from 1940 to 1945 by English soldiers who lived in close contact with the local population. A similar increase was also found in Iceland during 1945 to 1954 following the occupation by the United Kingdom, the United States, and Canada in the period from 1940 to 1945. It was hypothesized that these epidemics could be linked to the fact that soldiers frequently transmit EBV to native populations.
Clusters like these were alleged when it was hypothesized that at least certain subtypes of MS were triggered by vaccination, EBV infection or changing hygienic conditions. Moreover, the incidence of these MS cases could decrease again once the triggering agent has been removed, but currently MS is globally on the rise.
Although the expression of HERV sequences in samples from German MS patients, especially from children and adolescents, was not higher when compared to controls, different subgroups were analyzed in order to determine whether other disease-specific expression patterns could be detected. These analyses were not performed for the adult groups, since almost all patients with AID were female and medications varied widely. In contrast, the analysis was feasible for the different groups of children and adolescents since they were well characterized and allowed further analysis of subgroups. The expression of HERV-H, HERV-W, and MSRV sequences in relation to health status was analyzed according to gender and medication.
The gender-specific expression showed no significant differences for healthy individuals for HERV-H and HERV-W, but significantly higher expression for MSRV in females compared to males (P value: 0.0155). This finding could indicate not only an age dependent but also a gender dependent expression of MSRV. However, the developmental stage of the children and adolescents ranged from pre-puberty to post-puberty. Major changes in hormone status occur during this time in life, and as previously shown, HERV expression could be influenced by hormones (Ono et al., 1987). To evaluate whether MSRV expression is indeed gender related, matched controls of adults should be analyzed. The gender-related analysis of the adult control group employed in this study showed no gender-specific expression for any of the tested HERV sequences. However, these data are not statistically significant since only four individual of the control group were males.
Strikingly, the analysis of males affected by active MS at the time of sampling showed significantly higher expression for HERV-H (P value: 0.0075), MSRV (P value: 0.0110),

and HERV-W (P value: 0.0225) compared to healthy males. Thus, all three tested HERV sequences were expressed at a significantly higher level in patients with active MS compared to controls, but only in male patients and not in females. Besides the fact that MS affects more females than males, the different MS subtypes are also not equally distributed between the genders. Thus, elevated expression levels could be due to other types of MS that predominantly affect males. However, it is not clear whether this disease and gender-specific effect was a trigger for the active MS or was induced by the disease itself, e.g., by inflammatory cytokines being released. The difference observed for females and males could be related to gender-specific variations of immune responses. Since the group of young males affected by active MS at the time of sampling comprised only six patients, the obtained data is of limited informational value. Analysis of a larger cohort and also of adult and male MS patients affected by active as well as quiescent MS would be of great interest.

The EOMS patients were either therapy-naïve or were treated with Rebif. Statistical analysis showed no significant differences in expression of HERV-H, MSRV, or HERV-W sequences for treated and non-treated individuals. Also no statistically significant differences were observed between EOMS patients and patients with active MS at the time of sampling. This indicates that Rebif treatment has no effect on the expression level of the examined retrovirus sequences. This is again in contrast to findings by other groups: MSRV sequences were detected in controls without MS (7%), in MS patients (53%), but in 100% of patients who were untreated at the time of sampling (Garson et al., 1998).

Follow-up samples were obtained from several patients. Blood samples from patients were obtained within a short time frame and were analyzed for inter and intra assay variances. It was shown that only minor differences were observed. A limiting factor for the assessment of assay variances was, however, that all three patients showed relatively low copy numbers (below 30 for HERV-H, up to 10 copies for MSRV, and none of the patients showed any expression of HERV-W). Blood samples from six patients were analyzed over a longer period of time (five months up to 16 months). In general, HERV-H was stable over time. The MSRV expression was declining as a general trend, while the HERV-W expression was relatively stable over time. In sum, the samples obtained within a time frame of more than 15 months showed an increased or stable expression for HERV-H and no detectable expression of HERV-W and MSRV for the second sample. These findings correspond to the general finding that HERV-H expression is higher in adults than in children and adolescents. MSRV was expressed at significantly higher level in younger individuals, as shown by the comparison of healthy children/adolescents to adults. Due to the small number of patients tested and lack of control follow-up samples, these findings are not significant. However, they are still interesting in combination with the statistically significant findings regarding the expression levels of healthy children and adolescents in comparison to healthy adults.

Blood samples from two patients were obtained in a therapy-naïve state and three days after beginning treatment with Rebif. No immediate effect of Rebif was observed. This finding corresponds to the general analysis of therapy-naïve patients and patients under Rebif therapy. The analysis of two follow-up samples from patients under Rebif therapy that were obtained within a time frame of more than twelve months showed inconsistent findings. Two samples obtained within a time frame of more than twelve months showed no significant changes in the expression patterns. Three consecutive samples were obtained from another patient who was also treated with Rebif. This patient showed a steady decline for MS and an increase for the second sample followed by a strong decrease for the third sample. This patient had no changes in his EOMS course; he is the only patient who was treated with Rebif and with a long term follow-up from whom three samples were obtained. Thus, it is impossible to analyze whether the course of expression is unusual. However, variations like this were not observed for long-term follow-up analysis of non-treated EOMS patients.

Follow-up samples were obtained from two patients who had different EOMS status over time. Both patients showed the same expression patterns; while all tested HERV sequences were expressed on a relatively low level within the blood samples obtained during quiescent EOMS phases, HERV-H and HERV-W expression was considerably higher in samples obtained in the active state of the disease. Expression of MSRV did not show major changes. Thus, expression of HERV-H and HERV-W might be linked to the state of EOMS or active EOMS. Although these findings are of no statistical significance, since samples with different EOMS status were obtained for two patients only, they are in corroboration of the findings that significantly elevated expression for HERV sequences was observed for male patients during the active phase of the disease. But the results obtained for one of the long term follow-ups under Rebif therapy showed that variations are possible. Nevertheless, the observed similar expression patterns indicate the need for further analysis of these conditions and potentially related expression patterns. However, even if active EOMS is associated with elevated expression of HERV-H and HERV-W, the question remains whether the active state is triggered by the expression of the respective sequence or whether the expression is enhanced by inflammation. It would also be necessary to analyze expression patterns in adults regarding active and non-active state of MS. This analysis needs to be done separately for the different types of MS and for females and males.

4.4 Scleroderma

Scleroderma has been previously linked to retrovirus infections, although no final proof could be obtained (Maul et al., 1989, Hishikawa et al., 1997).

The absence of detectable RT activity in the peripheral blood of scleroderma patients indicates that exogenous retrovirus infection or endogenous retrovirus replication is not

involved in the etiology of scleroderma of German patients, although it could be possible that retroviruses were persisting and replicating in other compartments.

The analysis of HERV expression patterns in comparison to healthy controls showed no difference for HERV-H and MSRV sequences, as shown in tab. 11. Although not statistically significant (P value: 0.0774), the expression of HERV-W*gag* was lower in scleroderma patients when compared to healthy controls. The percentage of healthy individuals who tested negative for HERV-W expression was 39%, compared to 66% of scleroderma patients (tab. 11). The results obtained for HTLV, HERV-H, MSRV and HERV-W showed that neither HTLV nor any of the endogenous sequences tested could be linked to scleroderma by analysis of peripheral blood. Previous studies described the involvement of endogenous retrovirus sequences, including antibodies to HERV-E Gag protein which were described in 33% of patients with mixed connective tissue disease while no unaffected control subject had detectable levels of these antibodies (Talal et al., 1990b, Hishikawa et al., 1997). This work's findings do not contradict the previous findings but add further information to the etiology puzzle of scleroderma. The tested sequences can be excluded as causative agents for scleroderma patients in Germany with the limitation of peripheral blood being analyzed.

4.5 SLE

Previous studies testing for cross-reactive antibodies against retroviruses suggested that ERV-9, HERV-H, ERV-3, HTLV, and HIV could be involved in the etiology of SLE (Bengtsson et al., 1996, Li et al., 1996, Nelson et al., 1994, Ono et al., 1987). The described reactions with HTLV and HIV antigens did not relate to infections with these viruses, but indicated antibodies which were cross-reacting with HIV and HTLV epitopes. The analyses of SLE samples for reverse transcriptase activity showed no elevated RT activity when compared to plasma from healthy adults. In accordance, all samples also tested negative for HTLV infection, indicating that no retrovirus particles were present in the peripheral blood of German SLE patients.

The analysis of expression levels of three different HERV sequences showed a significantly lower expression of HERV-H when compared to healthy controls (P value: 0.0149; tab. 11). The expression of MSRV was not significantly different from that of healthy controls, while the expression of HERV-W*gag* was significantly higher in SLE patients when compared to healthy controls (P value: 0.0014). Antibodies against ERV-9 (which is related to but distinct from HERV-W) and HERV-H epitopes have been previously described in samples from SLE patients (Bengtsson et al., 1996). Although the expression of HERV-H*pol* was not higher when compared to healthy controls, other segments of HERV-H could be expressed and induce antibodies. The elevated expression of HERV-W*gag* when compared to healthy controls could either play a role in the etiology of SLE or be an epiphenomenon. Even if the HERV-W expression is not involved in the etiopathogenesis of SLE, it could constitute a disease marker. Since only one of 19 patients

with SLE was male, it was not analyzed whether the expression could be gender specific. A gender related analysis for the group of healthy adults showed no gender-specific expression; however, this result is statistically not sound since only four male individuals were included in the control group.

4.6 Spondylitis ankylosans

Epidemiological and molecular biological studies imply a multifactorial origin for spondylitis ankylosans which includes a genetic and a bacterial component. Spondylitis has a defined genetic predisposition with a relatively high concordance rate for monozygotic twins of ~75% (HLA-B27; Sieper and Braun, 2002, Sieper et al., 2002, Zhang et al., 2004). This implies the involvement of cofactors in the etiopathogenesis. Bacterial infections have been mainly suggested as inducing agents (Sieper et al., 2002, Duchmann et al., 1999, Ebringer et al., 1978, Maki-Ikola et al., 1991). There are also indications that different etiologies could be involved in disease development, since 10% of those affected lack the specific genetic predisposition and at least 23 subtypes have been identified.

The differential display analysis was performed for two SA samples. The sequences which were recovered in comparison to healthy controls did not reveal unknown or known virus or retrovirus sequences. However, due to the complexity of the DD approach for plasma samples, not all potentially different sequences were recovered and analyzed. All spondylitis samples were analyzed for RT activity; none of them showed elevated activity compared to healthy individuals. Thus exogenous retrovirus infection was not be detected in any of the peripheral blood samples which were tested. Hence, the findings achieved with generic screening methods substantiate the absence of retroviruses as causative agents for autoimmune disease in patients with SA.

The comparison of the expression patterns of three different HERV sequences revealed some statistically significant differences between SA patients and healthy controls. As compiled in tab. 11, the HERV-H expression patterns of spondylitis patients did not differ from those of healthy controls. However, the results obtained for the MSRV/HERV-W related sequences showed statistically significant lower expression for the spondylitis patients. The lower expression is reflected in lower medians but also in a lower percentage of individuals with a detectable expression. While 15% of healthy adults were negative for MSRV expression, 86% of the spondylitis patients were negative, too. Among the healthy adults, 39% were negative for HERV-W, while none of the spondylitis patients showed any detectable expression.

These findings are based on the analysis of only seven spondylitis patients and need to be substantiated further by examining larger groups which allow age and gender dependent analysis. These analyses have to be performed for age and gender matched controls as well. A gender-specific analysis is necessary since men are afflicted two to three times more often than women. Furthermore, the expression of HERV sequences in tissue samples needs to be analyzed in comparison to healthy controls.

The absence or lower expression of HERV-W sequences in the peripheral blood of diseased individuals compared to healthy controls could indicate that HERV-W has a biological function for the host and lack of it could induce disease. Otherwise, the lower or lack of expression could also be an epiphenomenon caused by the disease. The specific HERV-W expression pattern of spondylitis patients may even constitute a disease marker.

4.7 Perspectives

The composition of the etiopathogenesis of autoimmune diseases (AIDs) could be seen as puzzle with an unknown number of pieces of very different natures, which interact in unknown ways and on different levels, resulting in very complex multidimensional diseases. Therefore the clinical pictures of autoimmune diseases seem to vary much more than those of acute diseases.

Many attempts have been performed to identify an etiological pathogen, careful interpretation of clinical and experimental findings is required and appropriate controls are crucial. Negative results do not prove absence of an infectious agent; it could just be the wrong compartment being analyzed, unsuitable controls, a new virus with new features which is therefore not detectable, or low virus load. Negative findings could also be obtained if the infectious agent was eliminated from the host, since cleared virus infections can not be detected by RTA or RT-PCR with hindsight. Conversely, the detection of retrovirus is not compelling for an involvement in disease development. Cross-reactive antibodies to retrovirus antigens are frequently detected in non-infected individuals, for example, in pregnant women or in individuals affected by allergies. The expression of HERV sequences can be detected in many tissues and might be influenced by various factors. The expression could, indeed, be involved in the etiology of AID, it could be an epiphenomenon, it could be triggered by co-factors, it could be gender-or age-related, or it could be a contamination. A prominent example for a misapprehension of the involvement of a retrovirus in AID is the discovery of the so-called human retrovirus 5 (HRV-5). The putative fragment of a new human retrovirus was identified during an investigation into the potential role of retroviruses in Sjogren's syndrome. The 932 bp retrovirus sequence was related to type B and type D retroviruses; it was detected in individuals with and without Sjogren's syndrome. It was concluded that the sequence represented an infectiously acquired genome, thus a new exogenous human retrovirus, and it was named HRV-5 (Griffiths et al., 1997). It was shown that HRV-5 was not lymphotropic or involved in lymphoma (Rigby et al., 1998). However, it was linked to rheumatic disorders. It was detected in 50% of synovial samples of arthritic joints; it was also found in 10% of blood samples of patients with RA and SLE, while it was only detected in 1 out of 200 autopsy samples from non-rheumatic patients (Brand et al., 1999, Griffiths et al., 1999). A study of whole blood samples from patients with hematological malignancies showed a statistically significant correlation of HRV-5 and B-cell non-Hodgkin's lymphoma (Murovska et al., 2000, Kozireva et al., 2001). Yet in 2002 it was published that HRV-5 DNA was possibly a

laboratory contamination and that HRV-5 is not integrated into human DNA. Subsequent surveys of other species revealed that HRV-5 is present in the genomic DNA of the European rabbit and belongs to an endogenous retrovirus family; it was renamed rabbit endogenous retrovirus H (RERV-H; Griffiths et al., 2002).

The results of this study have to be evaluated in the light of the limitations described above. Peripheral blood was used, because other samples, such as CSF or tissue samples, were not available. Since the studied diseases are all slowly progressive by nature and the patients were mostly in a stable condition, sampling of CSF or even tissue as well as the acquisition of corresponding material from healthy individuals was ethically not justifiable. Several assays were adapted, optimized and established, and their potential was carefully evaluated. These methods were not only usable to analyze samples from AID patients, but can also be added as sensitive tools to the pool of molecular biological methods. The retrovirus-specific DDRT-PCR can be used to detect retroviruses in infected cell culture supernatants. The optimized reverse transcriptase activity assay is capable of detecting minute amounts of reverse transcriptase activity in cell culture supernatants and plasma from retrovirus-infected humans. Facing these results and the shown features of the adapted and optimized RT assay, sampling and testing of cell free plasma should be aspired to. Further investigations could comprise CSF as well. However, adaptation of the RTA to the testing of solid organs seems hardly possible. In order to analyze tissue samples, the tissue would have to be disintegrated, which would release large amounts of cellular enzymes, thus creating an enormous background.

Furthermore, other RNA templates could be compared to MS2 RNA, although so far all tested RTs were able to use MS2 RNA as template. However, different template preferences have been described and for particular analyses other RNA templates might be more sensitive. Therefore future assays could evaluate other RNA templates, such as BMV RNA or synthetic oligos. It would be interesting to find out whether this could be done within one reaction. The real-time PCR could be performed using CYBR green, which detects PCR products in general in contrast to the sequence-specific probes. If PCR products were detected, further analyses could be performed in order to determine which template would be preferred.

It was shown that the RTA can be used as a tool in virus safety studies, for example, in the screening of porcine cell lines or cell lines used for vaccine production. Furthermore, the suitability of the RTA for testing of enzymatic activity of different HIV-RTs with and without resistance associated mutations was shown. It should be analyzed whether the RTA could be performed in presence of RT inhibitors. If this was the case, the RTA could also be used to screen for resistance associated mutations in HIV-positive plasma. Samples from patients affected by other autoimmune diseases or with leukemias could be tested for the presence of retrovirus particles. The detected RT activity in two cell lines of cancerous origin needs to be characterized further.

While virus particles can be traced, the effect of expressed endogenous virus sequences is harder to assess. Each individual could have a specific expression pattern which could be influenced by external factors or which could trigger diseases. It is easily conceivable that a dysregulation of normally expressed or quiescent retroviral sequences could result in severe or even fatal consequences. HERV expression can be up or down regulated by various agents (addendum 5.8). Moreover, an infection with a helper virus could reactivate truncated HERVs. The age of HERV sequences in the human genome and the consequential grade of adaptation to the human host has led to several symbiotic relationships. One prominent example is syncytin, which is derived from the HERV-W Env protein. However, the expression of syncytin was recently linked to demyelination (Antony et al., 2004).

The complete sequencing of the human genome (Lander et al., 2001) allows the screening for endogenous retrovirus sequences in the human genome; as a result a multitude of publications regarding new HERVs or the intra genomic spread of HERVs is available. However, the expression of HERV sequences *in vivo* is not well analyzed yet.

One striking observation made in this study was the age dependent expression of HERV sequences. It would be of major interest to assess this effect further; however, in order to do so, long term analysis of consecutive samples from children and adults, healthy and diseased, would be necessary. It would be interesting to analyze the different expression patterns for AIDs patients with different ages at onset of disease in comparison to age-matched controls. The onset of disease for most AID is seen post puberty and in the later years of life. This age dependent accumulation could be linked to a kind of immunosenescence, or simply the statistically occurring accumulation of factors necessary for onset of disease. But it could be linked to age-dependent expression of specific sequences. Since the first description of MS more than 150 years ago up until twenty years ago, MS was diagnosed in adults only. Now roughly 5% of all MS cases are diagnosed in patients younger than 16 years of age. Obviously this development could be linked to changed environmental factors, which could of course include a newly emerged infectious agent. But it could as well be the consequence of changed living conditions, such as improved sanitary conditions, newly developed vaccinations, or combinations of these factors. The appearance of EOMS seems to be linked to environmental factors since it has only been observed in recent decades, and this time frame is too short to be due to genetic changes.

The expression of HERVs is often examined using cell cultures derived from certain tissues. It is questionable whether expression patterns from permanent cell lines which were derived from, for example, a liver or a carcinoma, and the actual expression in vivo, i.e., in the respective (healthy) human organs are directly comparable. Although it is well known that certain HERV sequences are expressed in the testis or play an important role in placenta formation, general gender-specific analyses of HERV expression still need to be done. This is even more important in the light of the fact that HERVs can be activated by

hormone responsive elements in the LTR, and on the other hand that up to 85 % of all AID patients world-wide are females. Also lacking is solid analysis of ethnic-specific expression of HERV sequences; this has been done for HERV-K (Mamedov et al., 2004) on a small scale, but not for other HERVs. Besides gender and ethnic related expression patterns, expression depending on the developmental stage needs to be analyzed further. If HERVs could be linked to a disease, another important point would be to determine whether the HERV expression is, so to speak, the chicken or the egg. HERVs could trigger expression of genes which then could lead to autoimmunity, or autoimmune reactions could produce e.g. cytokines which stimulate expression of HERV sequences as a side effect. However, all these analyses represent again only pieces of the larger puzzle which constitutes the picture of the etiopathogenesis of each multifactorial disease.

In general, this study provides some insights on the role of retroviruses for the etiopathogenesis of different autoimmune diseases. While the involvement of exogenous retroviruses seems unlikely, the general results of this work point to the expression of endogenous sequences. Although significant differences were observed, it is still unclear whether these HERVs exert any pathogenic role in AIDs. It is possible that the expression is only an epiphenomenon, but even then, it may constitute a diagnostic marker, as implicated for males affected by active MS. Significantly different expression patterns were also detected for spondylitis and SLE patients. These findings can be used as starting points for further analyses.