

AN INFECTIVE ENDOGENOUS RETROVIRUS AND ITS ASSOCIATION WITH DEMYELINATING DISEASES AND OTHER DISEASES

5 FIELD OF INVENTION

The invention relates to the field of diagnosing and treating diseases associated with endogenous retroviruses. In particular, there is provided evidence that a retrovirus that is found in a majority of human patients suffering from demyelinating diseases such as
10 multiple sclerosis is transmissible to human cells including mononuclear blood cells, and to non-human cells. Specifically, a specific splice variant comprising the *env* region and a part of the *pol* region is present in retroviral mRNA isolated from blood cells of MS patients and patients suffering from autoimmune diseases and malignancies, but not in mRNA isolated blood cells from healthy subjects. These findings provide the potential for
15 novel approaches to diagnosing and treating such diseases.

TECHNICAL BACKGROUND AND PRIOR ART

20 Multiple sclerosis (MS) is a demyelinating inflammatory disease affecting the central nervous system. The aetiology of the disease remains uncertain. Epidemiological studies indicate that environmental agents contribute to the development of MS, the agents presumably being one or more viruses (Martyn, 1991; Christensen et al., 1994). This may also be indicated by the occurrence of putative MS clusters (Haahr et al., 1997).

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Following the observation of retrovirus-like particles in short-term cultured lymphocytes from MS patients, spontaneously formed cell lines from peripheral blood mononuclear cells from several MS patients have been established (Haahr et al., 1991). These cell lines produce type C retrovirus-like particles in addition to occasional expression of
30 mature EBV particles (Sommerlund et al., 1993; Munch et al., 1995). These retrovirus particles possess reverse transcriptase (RT) activity (Christensen et al., 1997; Christensen et al., 1999) and share a few antigenic determinants with HTLV-1, but are distinct from the known exogenous retroviruses at the antigenic level (Christensen et al., 1997).

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Endogenous retroviruses that are primarily transmitted vertically are found ubiquitously in the mammalian genome and constitute several percent of the genome. It has been suggested that endogenous retroviruses may play a role in autoimmune diseases (Hohenadl et al., 1979; Krieg et al., 1992; Dalglish, 1997). Only once before has a
5 human endogenous retrovirus, HTDV/HERV-K, been clearly demonstrated as particles with possible pathological connotation - in teratocarcinoma cell lines (Tönjes et al., 1996; Boller et al., 1993). It has not yet been determined whether HTDV/HERV-K occurs as particles in patient plasma.

- 10 The presence of variants of the human endogenous retrovirus HERV-H produced by MS cell lines has been reported in WO 99/53103. HERV-H sequences with high homology to the HERV-H variant RGH-2 were also found specifically at particle level in cell-free plasma from 24 out of 33 MS patients but could neither be demonstrated in 29 plasma samples from patients with autoimmune or other diseases nor in 20 plasma samples from
15 healthy controls. It was found that nucleic acid binding proteins analogous to the nucleocapsid protein, Gag NC, of other retroviruses are found in the above retrovirus particles.

- Additionally, WO 99/53103 discloses methods of diagnosing demyelinating disease
20 including MS, subgrouping patients suffering from such disease and/or monitoring the stage of activity of demyelinating disease including MS based on the detection, in a retroviral particle or an encapsidated virion-like particle, of the RGH virus-derived SEQ ID NO:1 disclosed in the above patent application, or a subsequence or variant thereof. Diagnostic agents comprising probes capable of hybridising to such sequences,
25 subsequences or variants, antibodies reacting with epitopes or proteins or peptides encoded by the sequences, subsequences or variants and/or such epitopes for such methods are also disclosed in WO 99/53103.

- However, up till now there has been no evidence that could indicate that otherwise
30 quiescent endogenous retroviruses occurring in the genome of MS patients may enter a replicative stage and thereby be transmitted to cells of healthy human subjects and to cells of patients suffering from a disease associated with the presence of endogenous retroviruses.

The findings described herein are highly suggestive of the potential infectivity of endogenous retroviruses. Based on these findings, it is suggested that MS and possibly other diseases are associated with replication and transmissibility of otherwise quiescent endogenous retroviruses. This could be a phenomenon parallel to the association of HTDV/HERV-K with male germ cell tumours (Boller et al., 1997; Tönjes et al., 1996) and in accordance with the recent report of expression of another putatively endogenous retrovirus in MS (HERV-W/ERV-9) (Perron et al.; Garson et al.; Komurian-Pradel et al.) and of expression of a superantigen and of sequences in plasma, encoded by a HERV-K-variant (IDDMK1,222) in type 1 diabetes (Conrad et al.).

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RGH viruses belong to the RTVL-H/HERV-H (priming of transcription with tRNA^{His}) family of endogenous retroviruses. This family of type-C like retroviruses is related to the onco-retroviruses human T-cell leukemia virus (HTLV-1/-2), to bovine leucosis virus (BLV), and to ERV-9. A genomic RTVL-H clone which potentially encodes functional proteins has been described (Wilkinson et al.) and most interestingly, a HERV-H element with an intact open *env* reading frame (and highly homologous to RGH) has been described recently (Lindeskog et al., 1999; Blond et al., 1999). HERV-H/RTVL-H RNA is found in normal lymphocytes, placental tissue and some neoplasias (Medstrand et al., 1992; Mager et al., 1987; Kelleher et al., 1996; Löwer et al., 1993; Johansen et al., 1989), but RGH particles as such have not been reported previously, nor has their potential transmissibility. RGH is also separate from the RTVL-H viruses according to the classification based on LTR-types (Goodchild et al., 1993) as the RGH-2 LTR region contains three class 1 repeats rather than two (LTR type 1) or one (LTR type 1a and 2). Two RGH clones have been described: the published RGH-1 clone is 4869 bp long and encompasses a partial *pol* and an *env*-3'LTR region and thus lacks *gag*, whereas the published RGH-2 clone is 8715 bp long and includes the complete coding potential: 5'LTR *gag-pol-env* 3'LTR. The majority of cDNA clones isolated from MS patient material are closely related to the full-length clone RGH-2 as they contain *gag* sequences. RGH-like sequences were reported present in about 100 copies/haploid genome (Hirose et al., 1993).

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Using *gag*-primed PCR on a chromosomal DNA panel (Coriell Cell Repositories, USA) followed by sequencing, we have so far confirmed the presence of RGH-like sequences on chromosomes 6 and X in the human genome. An updated search in the human genome databases demonstrated RGH-2-homologous copies on chromosomes 2, 3, 5, 7, 10, 11, 14, 16, 19, X and Y.

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The retrovirus particles disclosed in WO 99/53103 possess reverse transcriptase activity and share a few antigenic determinants with HTLV-1, but are distinct from the known exogenous retroviruses at the antigenic level. They are associated with sequence variants of
5 the human endogenous retrovirus HERV-H family, and amplicons obtained by RNA PCR have >80% homology at the nucleic acid level to the HERV-H clone RGH-2 (Hirose et al., 1993) and to the HERV-H clone with intact open Env reading frame described in Lindeskog et al., 1999. The retroviral particles are also described in: Christensen et al., 1997; Christensen et al., 1999; Møller-Larsen et al., 1998; Christensen et al., 1998; Christensen
10 at al., 2000.

Evidence is presented herein to the effect that endogenous retroviruses belonging to the RTVL-H/HERV-H family including RGH2 retroviruses appear to be transmissible to human blood cells. Additionally, it is demonstrated herein that not only are such endogenous
15 retroviruses transmissible to other human cells than blood cells, but the viruses are also able to infect cells from other species, i.e. they are capable of acting as exogenous retroviruses in relation to non-human species.

As disclosed in WO 99/53103 and discussed above, the HERV-H RGH2 sequences present
20 in virus particles produced in MS cell lines or isolated from the blood of MS patients show >80% homology to the RGH-2 sequence and the HERV-H Env reading frame. As it will be described below, blood cells from MS patients, but not from healthy controls, contain an aberrantly spliced HERV-H *env* mRNA which may represent a genetic marker of cells producing or able to produce the infectious HERV-H RGH2 retrovirus particles.

25 Furthermore, a comparison of a number of such aberrantly spliced mRNA molecules from different individuals provides indications that the genetic information encoding the mRNA may originate at a single endogenous HERV-H locus or at a small number of such loci.

30 The implications of the above highly unexpected findings on which the present invention are based include that it may become possible to control demyelinating diseases and other diseases by designing appropriate effective antiviral or immunological treatment regimens for such diseases. Additionally, the findings provide the basis for providing improved diagnostic or prognostic methods.

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SUMMARY OF THE INVENTION

Accordingly, the invention pertains in a first aspect to a human endogenous retrovirus
5 belonging to the RTVL-H/HERV-H family in isolated form or in retroviral particle form that
is capable of infecting a mononuclear cell and of replicating therein.

In a further aspect the invention provides a method of detecting *intra-species* infectivity
(transmissibility) of such a retrovirus, the method comprising the steps of isolating the
10 retrovirus from a patient suffering from a demyelinating disease such as multiple sclerosis,
a patient suffering from an autoimmune disease, a patient suffering from diabetes or a
patient suffering from a malignancy, contacting said isolated virus with a culture of
mononuclear cells derived from a human subject not suffering from any of the above
diseases, and detecting infection of at least a proportion of the mononuclear cells with the
15 retrovirus.

In a still further aspect, there is provided a method of detecting *inter-species* infectivity
(transmissibility) of the retrovirus as defined above, the method comprising the steps of
isolating the retrovirus from a patient as also defined above, contacting said isolated
20 viruses with a culture of mononuclear cells derived from a non-human individual, and
detecting infection of said cells with the retrovirus.

Further objectives of the invention are to provide:

25 a culture of mononuclear cells derived from a human subject selected from the group
consisting of a multiple sclerosis (MS) patient, a patient suffering from an autoimmune
disease, a patient suffering from diabetes and a patient suffering from a malignancy, said
cells are capable of expressing a spliced HERV-H RGH retrovirus *env* mRNA sequence
comprising a region from the protease encoding (*pol*) region of said retrovirus;

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a method of diagnosing a disease selected from the group consisting of multiple sclerosis,
an autoimmune disease, diabetes and a malignancy, the method comprising isolating
mononuclear blood cells from a patient suspected of suffering from any of said diseases
and detecting in said cells a spliced HERV-H RGH retrovirus *env* mRNA sequence
35 comprising a region from the protease encoding (*pol*) region of said retrovirus, including a

mRNA sequence comprising a region from the protease encoding region that is selected from the group consisting of SEQ ID NO:44; SEQ ID NO:45; SEQ ID NO:46; SEQ ID NO:47; SEQ ID NO:48; SEQ ID NO:49; and SEQ ID NO:50 as defined herein, and variants and subsequences hereof;

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a method of diagnosing a disease associated with the retrovirus according to the invention, the method comprising the steps of isolating the retrovirus from a human subject suffering from any of the above diseases and testing its infectivity according to the above methods;

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a method of treating a patient suffering from a disease, the clinical manifestations of which are associated with the presence of the retrovirus according to the invention, the method comprising administering to the patient an effective amount of a pharmaceutical agent that is capable of at least partially preventing the infection of cells in the patient with the

15 retrovirus, adverse immunological responses to the infection and/or the migration of the retrovirus to cells in the patient that can be infected by the retrovirus;

a method of treating a patient suffering from a disease, the clinical manifestations of which are associated with cells transformed (immortalised) by infection with the retrovirus

20 according to the invention, the method comprising administering to the patient an effective amount of a pharmaceutical agent that is capable of at least partially eliminating such transformed (immortalised) cells;

an antiviral agent for use as a medicament in the control of a disease, the clinical

25 manifestations of which are associated with the presence of the retrovirus according to the invention;

use of an antiviral agent in the manufacturing of a medicament for controlling a disease associated with the retrovirus according to the invention;

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a method of controlling a disease, the clinical manifestations of which are associated with immunological responses to the retrovirus of the invention, the method comprising administering to the patient an effective amount of a pharmaceutically active compound that is capable of at least partially inhibiting said immunological responses; and

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a pharmaceutical composition comprising a pharmaceutically active compound that is capable of at least partially inhibiting in a human subject suffering from any of the above diseases an immunological response to the retrovirus according to the invention.

5 In other aspects the invention relates to :

a pharmaceutical composition comprising a pharmaceutically active compound that is capable of at least partially preventing the infection of cells in a patient with the retrovirus according to the invention and/or the migration of the retrovirus to cells in the patient that
10 can be infected by the retrovirus;

a pharmaceutical composition comprising a pharmaceutically active compound that is capable of at least partially eliminating in a patient cells transformed (immortalised) by infection with the retrovirus according to the invention; and
15 the use of an allelically occurring spliced HERV-H RGH retrovirus *env* mRNA sequence comprising a region from the protease (*pol*) region of said RGH retrovirus as a marker for the occurrence or the state of a disease selected from the group consisting of a demyelinating disease including multiple sclerosis, an autoimmune disease, diabetes and
20 a malignancy.

DETAILED DISCLOSURE OF THE INVENTION

25 It has been found previously that the presence of particles of endogenous retroviruses in blood cell cultures derived from MS patients may be associated with the disease, as similar virus particles cannot be found in healthy human subjects. The present invention is i.a. based on the unexpected finding that certain human endogenous retroviruses belonging to the RTVL-H/HERV-H family are capable of being transmitted to (infect)
30 mononuclear cells and of replicating therein. As it will be outlined in the following, this unexpected and surprising finding renders it likely that such infective retroviruses play a role in the development of demyelinating diseases, in particular multiple sclerosis, and possibly also in the development of autoimmune diseases such as e.g. diabetes, and malignancies. Accordingly, these achievements of the inventors imply that novel
35 therapeutic approaches to the treatment of such diseases can be designed and

additionally, the present findings can be the basis for improved diagnostic and prognostic methods.

It is thus one objective of the invention to provide a human endogenous retrovirus
5 belonging to the RTVL-H/HERV-H family in isolated form or in retroviral particle form that is capable of infecting a mononuclear cell and of replicating therein. In one specific embodiment such a retrovirus is a retrovirus that encodes a spliced HERV-H RGH retrovirus *env* mRNA sequence comprising a region from the protease (*pol*) region of said RGH retrovirus. Such a retrovirus includes a retrovirus wherein the spliced mRNA
10 sequence comprises a region from the protease encoding region that is selected from the group consisting of SEQ ID NO:44; SEQ ID NO:45; SEQ ID NO:46; SEQ ID NO:47; SEQ ID NO:48; SEQ ID NO:49; and SEQ ID NO:50 as defined herein, and variants and subsequences hereof.

15 By the term "subsequence" is meant a sequence which comprises at least 10, 15, 20, 30, 40 or 50 nucleotides of any of the above specific sequences. By the term "variant" is meant a sequence which is not identical to any of these specific sequences, but which has a sequence identity of at least 80%, 85%, 90%, 95%, 98%, 99% or 99.5% with any of these sequences. These two terms may be combined, i.e. the invention comprises
20 detection of a variant of any of SEQ ID NO:44; SEQ ID NO:45; SEQ ID NO:46; SEQ ID NO:47; SEQ ID NO:48; SEQ ID NO:49; and SEQ ID NO:50 which has a sequence identity of at least 80% with a subsequence of any of the sequences of at least 10, 15, 20, 30, 40 or 50 nucleotides. It will be appreciated that the person of skill in the art will know how to make subsequences and variants of the specific sequences, e.g. by deletion, substitution
25 and insertion of one or more nucleotides.

As used herein, the term "sequence identity" indicates that a given sequence is compared with a similar part of any of the above specific sequences. The best possible alignment of the sequences is made and then the degree of identity is calculated. A computer program
30 such as GCG, Wisconsin Package, Version 9.1, Genetics Computer Group (GCG), 575 Science Drive, Madison, Wisconsin, USA 53711 using the defaults proposed in the program Bestfit with respect to gap weight (50), length weight (3), average match 10,000, average mismatch (-9,000) may be useful in this respect.

All of such spliced mRNA sequences that were isolated had a size of about 950 bp and were spliced *env* mRNA sequences containing the same splice donor site in the leader region downstream from the PBS sequence and a splice acceptor site in the part of the *pol* that encodes the integrase. In these 950 bp fragments a region of 104 bp from the protease encoding region was inserted between the splice donor site and the splice acceptor site. The spliced mRNA sequences were detected in retroviruses isolated from MS patients and from a patient suffering from diabetes, whereas they could not be detected in healthy subjects. It was found that the 950 bp spliced sequences shared 87-95% identity with the RGH2 leader/integrase and *env* region and 98-100% identity with each other and with a previously published sequence isolated from a human T-cell leukaemia cell line. An interesting finding was that, when 10 clones isolated from 3 MS patients and 1 patient with diabetes mellitus and representing 7 variants of the 104 bp protease encoding region of the 950 bp spliced mRNA sequences were aligned using one of the variants as the reference, it was found that one variant having, relative to the reference sequence, two similar substitutions could be isolated from 3 out the 4 source patients. This finding strongly indicates that the presence of these specific substitutions are markers of RGH alleles at the same genetic locus in the human genome or markers of closely related RGH genes at different genetic loci.

As it will be appreciated, these findings imply that the presence of the 950 bp sequence in itself may be a marker for the presence of MS or other diseases and the fact that specific variants of the sequence appear to occur in an allelic manner suggests that such specific sequences and/or retroviruses comprising such sequences may be particularly interesting disease marker candidates.

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A retrovirus according to the invention can e.g. be isolated from a patient suffering from a demyelinating disease such as multiple sclerosis (MS), an autoimmune disease, diabetes mellitus or a malignancy including leukaemia, e.g. from blood cells, blood plasma or a cell culture derived from peripheral blood of said human subject. In this connection, it was found that blood cell cultures derived from such patients constantly comprised a significant proportion of a specific type of T-cells which were characterised according to CD antigens on their surface as being CD3+, CD4- and CD8- T-cells. The proportion of such T-cells in the blood cell cultures producing the retrovirus according to the invention typically is in the range of 1-20% of mononuclear cells, e.g. at least 2% including the range of 2-15%.

Retroviruses according to the invention include retroviruses obtainable by ultracentrifugation of a cell culture supernatant or a cell-free plasma sample in Optiprep™ density gradient medium followed by recovery of the fraction containing intact retroviral particles. Protocols for purification of retroviral particles from cell culture supernatants or cell-free plasma samples using ultracentrifugation on an Optiprep gradient are provided in the accompanying Examples.

Retroviruses according to the invention include human endogenous retroviruses of the RTVL-H/HERV-H family in isolated form. The term "isolated form" includes, but is not necessarily limited to, retrovirus purified from patient serum or a cell culture supernatant, for example using the protocols supplied in the accompanying examples.

The retrovirus according to invention is, as it is demonstrated in the following examples, capable of infecting a blood cell derived from a human subject not suffering from MS, an autoimmune disease, diabetes or a malignancy, and of replicating therein. Additionally, it has been found that the retrovirus is also capable of infecting non-blood human cells such as e.g. liver cells.

However, besides being capable of *intra-generic* transmission, the retrovirus is also capable of infecting a non-human cell, i.e. the retrovirus shows *inter-generic* infectivity. As it is demonstrated in the examples, the retrovirus can be transmitted to cells derived from bats, rabbits and rodents.

In specific embodiments, the infective endogenous retrovirus according to the invention is an RGH virus including a retrovirus comprising a nucleic acid sequence that is at least 80% identical to the RGH-2 virus sequence published by Hirose et al., Virology, 1993, 192:52-61.

In a further aspect the invention provides a method of detecting *intra-species* infectivity (transmissibility) of the endogenous retrovirus according to the invention. The method comprises that the isolated retrovirus is contacted with a culture of mononuclear cells such as blood cells derived from a human subject not suffering from MS, an autoimmune disease, diabetes or a malignancy, and detecting infection of said cells with the retrovirus.

The detection of the infection can be carried out in several ways. Thus, in one way of

detecting infectivity, the infection is detected by the appearance or increase of RT activity in the infected culture using methods for detection of RT activity which are known in the art including the very sensitive assays for detecting RT activity generally referred to as RT-PCR and Product Enhanced Reverse Transcriptase (PERT) assay, or by an assay
5 based on co-cultivating of cells suspected of harbouring the infective retrovirus e.g. cell cultures from peripheral blood from an MS patient with a cell line harbouring a non-transmissible retroviral construct followed by an assay for rescue of the infecting retrovirus into indicator cells such as it is described in details in the following examples.

- 10 In specific embodiments, the infection of the culture of mononuclear cells is detected by the appearance in cells of said culture of a spliced HERV-H RGH retrovirus *env* mRNA sequence comprising a region from the protease (*pol*) region of said RGH retrovirus, including a region from the protease encoding region that is selected from the group consisting of SEQ ID NO:44; SEQ ID NO:45; SEQ ID NO:46; SEQ ID NO:47; SEQ ID
15 NO:48; SEQ ID NO:49; and SEQ ID NO:50 as defined herein, and variants and subsequences hereof.

In addition to the above assays, infection of cell cultures by the retroviruses of the invention, can be detected by the occurrence of clonal growth of the infected cells.

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- It is also an objective of the invention to provide a method of detecting *inter-species* infectivity (transmissibility) of the endogenous retrovirus according to the invention. This method comprises the steps of isolating the retrovirus from a human subject suffering from a demyelinating disease, an autoimmune disease, diabetes or a malignancy,
25 followed by contacting the isolated virus with a culture of mononuclear cells including blood cells derived from a non-human individual, and detecting infection of said cells with the retrovirus using any of the above methods for detection of infection of a cell with the retrovirus. In this method, the mononuclear recipient cells may be derived from any non-human animal such as a rodent, a rabbit or a bat.

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In certain preferred embodiments of the above methods for detecting infection with the retroviruses, the recipient mononuclear cells being contacted with the retrovirus are in the form of a culture of mononuclear cells that are pre-stimulated with a mononuclear cell stimulating compound including PHA.

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A further objective of the invention is to provide a culture of mononuclear cells derived from a human subject selected from the group consisting of a multiple sclerosis (MS) patient, a patient suffering from an autoimmune disease, a patient suffering from diabetes and a patient suffering from a malignancy, said cells are capable of expressing a spliced
5 HERV-H RGH retrovirus *env* mRNA sequence comprising a region from the protease encoding (*pol*) region of said retrovirus. In specific embodiments hereof, the culture comprises cells wherein the spliced mRNA sequence being expressed comprises a region from the protease encoding region that is selected from the group consisting of
SEQ ID NO:44; SEQ ID NO:45; SEQ ID NO:46; SEQ ID NO:47; SEQ ID NO:48; SEQ ID
10 NO:49; and SEQ ID NO:50 as defined herein, and variants and subsequences hereof. Such cultures include cultures comprising a proportion of CD3+, CD4- and CD8- T-cells. The proportion of such T-cells in the mononuclear cell cultures producing the retrovirus according to the invention is typically in the range of 1-20% of mononuclear cells, e.g. at least 2% including the range of 2-15%.

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One important objective of the invention is to provide a method of diagnosing a disease selected from the group consisting of multiple sclerosis, an autoimmune disease, diabetes and a malignancy. This method comprises as a first step the isolation of mononuclear blood cells from a patient suspected of suffering from any of such diseases followed by
20 detecting in these cells the expression of a spliced HERV-H RGH retrovirus *env* mRNA sequence comprising a region from the protease encoding (*pol*) region of said retrovirus including such a sequence that comprises a region from the protease encoding region that is selected from the group consisting of SEQ ID NO:44; SEQ ID NO:45; SEQ ID NO:46; SEQ ID NO:47; SEQ ID NO:48; SEQ ID NO:49; and SEQ ID NO:50 as defined
25 herein, and variants and subsequences hereof.

In WO 99/53103 specific RGH virus-encoded peptide antigens are disclosed that can be used as reagents in serologically testing human subjects for the presence of antibodies directed against such antigens. When using such antigens as reagents in testing MS
30 patient and control subjects (C) in an ELISA assay it was found in WO 99/53103 that the specific antigens gave rise to an MS:C ER ratio in the range of 0.8 to 1.5. It is within the scope of this invention to apply antigenic components of the retrovirus according to the invention in a similar manner as diagnostic reagents. As an example, the specific novel peptide: LSDLSQISHLDSFSSNTKNPAQF gave rise to an MS:C ER ratio as high as 1.6
35 (8 MS sera vs. 8 control sera).

In a further aspect, the invention relates to a method of diagnosing a disease associated with the retrovirus of the invention, the method comprising the steps of isolating the retrovirus from a human subject suspected of suffering of a demyelinating disease
5 including multiple sclerosis, an autoimmune disease, diabetes or a malignancy, and testing its infectivity according to any of the methods defined above and explained in details in the following examples.

Based upon the finding that endogenous retroviruses that are associated with diseases as
10 mentioned above are infective for human cells, methods of controlling such diseases are contemplated. Accordingly, in one aspect, the invention provides a method of treating a patient suffering from a disease, the clinical manifestations of which are associated with the presence of the retrovirus according to the invention which method comprises administering to the patient an effective amount of a pharmaceutical agent that is capable
15 of at least partially preventing the infection of cells in the patient with the retrovirus and/or the migration of the retrovirus to cells in the patient that can be infected by the retrovirus. In the present context, the term "migration" includes the targeting of the retrovirus to a susceptible cell in the body by the transfer of a cell harbouring the infective retrovirus to the susceptible target cell whereby the retrovirus may infect the susceptible target cell by
20 cell-to-cell contact.

Any compound that is capable of interfering with at least one step in the infection of new cells in the body, including compounds that, at least partially, eliminates the retrovirus-carrying immortalised cells, preferably in a selective manner, compounds that prevent or
25 inhibit migration of such cells, compounds that interfere with the cell-to-cell contact or which interfere with the transmission of the retrovirus into the target cells (infection), can be used in the treatment methods of the invention. The compounds which can be used in such methods include antiviral agents, antibodies, anti-sense nucleic acids, nucleic acid binding proteins and ribozymes.

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It is conceivable that the clinical manifestations of diseases associated with the endogenous virus according to the invention, such as demyelinating diseases including multiple sclerosis, autoimmune diseases, diabetes and malignancies are associated with immunological responses to the retrovirus. Such an immune response may be "direct", i.e.

directed against an antigen on the infected cell or "indirect", i.e. the immune response is directed against other cell antigens.

Accordingly, the invention pertains in another aspect to a method of controlling the above
5 diseases which comprises administering to a patient suspected of suffering from such a disease an effective amount of a pharmaceutically active compound that is capable of at least partially inhibiting such immunological responses. In this connection, useful compounds include compounds that inhibit cells of the immune system that are activated to generate the immunological responses. In a specific embodiment, the pharmaceutically
10 active compound that is capable of at least partially inhibiting immunological responses to the retroviral infection is a compound that is directed against an immunological response elicited specifically by a retrovirus expressing a spliced HERV-H RGH retrovirus *env* mRNA sequence comprising a region from the protease encoding (*pol*) region of said retrovirus including such a sequence that comprises a region from the protease encoding
15 region that is selected from the group consisting of SEQ ID NO:44; SEQ ID NO:45; SEQ ID NO:46; SEQ ID NO:47; SEQ ID NO:48; SEQ ID NO:49; and SEQ ID NO:50 as defined herein, and variants and subsequences hereof. In yet other embodiments, the pharmaceutically active compound is a compound directed against inhibiting the biological activity of activated T-cells including CD3+, CD4- and CD8- T-cells or against eliminating
20 such T-cells.

In other aspects there are provided an antiviral agent for use as a medicament in the control of a disease, the clinical manifestations of which are associated with the presence of the retrovirus according to the invention and use of an antiviral agent in the
25 manufacturing of a medicament for controlling a disease associated with such a retrovirus such as a demyelinating disease, a malignancy, diabetes and an autoimmune disease. In the present context, the expression "antiviral agent" should be understood in its broadest sense as indicating any compound that prevents or inhibits the infectivity and replication of the retroviruses and/or the pathological manifestations of the infection including
30 adverse immunological manifestations, optionally resulting in an autoimmune state of the patient. The use of currently used antiviral agents is contemplated but it is conceivable that antiviral agents that selectively inhibits or eliminates the retrovirus according to the invention can be developed or selected from known antiviral agents. It is within the scope of the invention to use antibodies, anti-sense nucleic acids and ribozymes as antiviral
35 agents.

It is another objective of the invention to provide a pharmaceutical composition comprising a pharmaceutically active compound that is capable of at least partially inhibiting in a human subject as defined in claim 4 an immunological response to the retrovirus

5 according to the invention, including a composition wherein the pharmaceutically active compound is directed against an immunological response elicited specifically by a retrovirus according to the invention including a retrovirus expressing a spliced HERV-H RGH retrovirus *env* mRNA sequence as defined hereinabove and a composition wherein the pharmaceutically active compound is a compound directed against inhibiting the

10 biological activity of activated T-cells including CD3+, CD4- and CD8- T-cells or against eliminating such T-cells.

There are also provided a pharmaceutical composition comprising a pharmaceutically active compound that is capable of at least partially preventing the infection of cells in a

15 patient with the retrovirus according to the invention and/or the migration of the retrovirus to cells in the patient that can be infected by the retrovirus and a pharmaceutical composition comprising a pharmaceutically active compound that is capable of at least partially eliminating in a patient cells transformed (immortalised) by infection with the retrovirus.

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Compounds having potential antiviral activity may be identified with the aid of a screening assay based on the production of retroviral particles from a culture of mononuclear cells. Compounds that inhibit production of retrovirus from cells which normally (i.e. in the absence of the compound) produce retroviral particles would be scored as having

25 potential anti-retroviral activity. A typical assay may be carried out using a B-lymphoblastoid MS derived cell line established in culture. The cells are dispensed into the wells of a standard microtiter plate and produce virus particles in the cell culture medium. Virus particles may be detected by identifying their structure in Optiprep gradients prepared by ultracentrifugation. Candidate compounds are then added and the

30 production of viral particles in the presence of the test compound analysed by suitable means. For example, production of intact viral particles may be analysed by ultracentrifugation on an Optiprep gradient. Typically, the effect of the candidate compound will be tested at several different concentrations. Inhibition of virus production is scored as an indication that the candidate compound has potential antiviral activity.

35 Compounds identified as having potential antiviral activity using such a screening assay

may be useful as antiviral agents or as lead compounds in the development of antiviral agents with pharmaceutical potential.

- In a further variation, screening assays for compounds having potential antiviral activity
- 5 may be based on an infectivity assay. A typical assay may comprise exposing target cells susceptible to infection with a virus according to the invention, for example cultured PHA stimulating lymphocytes from healthy control subjects, to sample containing a retrovirus according to the invention in the presence or absence of a candidate compound. The sample containing the retrovirus may be, for example, a cell supernatant from a cell line
- 10 which produces the retroviral particles (e.g. B-lymphoblastoid MS derived cell lines, as discussed above) or a cell-free plasma sample containing retrovirus. Candidate compounds which prevent or substantially reduce the infection of the lymphocytes by retroviral particles present in the cell supernatants are scored as having potential antiviral activity. Such compounds may again be useful as antiviral agents or as lead compounds
- 15 in the development of antiviral agents with pharmaceutical potential. An infectivity assay may be based on the biochemical tests for indicating retroviral activation in PHA stimulated lymphocytes already established and described in the accompanying Examples.
- 20 Cultures of MS derived cell lines producing infectious retroviral particles may also be used to identify compounds that prevent the synthesis of viral polypeptides, including those polypeptides that may act as a superantigen in the body, thereby triggering an autoimmune response. In a typical screen an immunoassay may be set up as a screening test using the cell lines themselves, or cell supernatants, indicating the
- 25 production of the viral specific epitopes in the MS derived cell lines.

- In an important aspect the invention relates to the use of an allelically occurring spliced HERV-H RGH retrovirus *env* mRNA sequence comprising a region from the protease (*pol*) region of said RGH retrovirus as a marker for the occurrence or the state of a
- 30 disease selected from the group consisting of a demyelinating disease including multiple sclerosis, an autoimmune disease, diabetes and a malignancy. In this connection, the expression "allelically occurring" indicates the presence of RGH alleles at the same genetic locus in the human genome or markers of closely related RGH genes at different genetic loci. In specific embodiments such use is based on mRNA sequences comprising
- 35 a region from the protease encoding region that is selected from the group consisting of

SEQ ID NO:44; SEQ ID NO:45; SEQ ID NO:46; SEQ ID NO:47; SEQ ID NO:48; SEQ ID NO:49; and SEQ ID NO:50 as defined herein, and variants and subsequences hereof.

The invention will now be described in further details in the following non-limiting
5 examples and in the figures wherein:

Fig.1 shows examples of RGH sequences in particulate form from MS cell lines. RNA templates were from Optiprep-gradient purified retroviral particles from the MS cell lines MS1533, MS1845 and MS 1851. The corresponding sequences from RGH-2 (Hirose
10 et al., 1993) are included in the alignment (above: RGH-2₁₅₈₉₋₁₈₇₂ (SEQ ID NO:1); below: RGH-2₂₁₄₁₋₂₃₁₃ (SEQ ID NO:2) . The MS1533/MS1845 sequences (above) (SEQ ID NO:3/SEQ ID NO:4) were obtained with primer-set I; whereas the MS1533/MS1851 sequences (below) (SEQ ID NO:5/SEQ ID NO:6) were obtained with primer-set II. Clones appear to be true variants of RGH-2 (the full-length RGH clone), as the variation is too
15 great to be attributed to the Taq error-rate. ... indicates gaps introduced to optimise alignment;

Fig. 2 shows, as examples of RGH sequences in particulate form from MS patient plasma, *env* primed RT-PCR derived sequences from cell-free, filtered, ultracentrifuged plasma
20 samples from the MS patients 2094 (SEQ ID NO:9) and 2095 (SEQ ID NO:10). The corresponding sequences from RGH-1 (SEQ ID NO:7) and RGH-2 (SEQ ID NO:8) (Medsrand et al., 1992) are included in the alignment. Clones appear to be true variants of RGH-2 (the full-length RGH clone), as the variation is too great to be attributed to the Taq error-rate. indicates gaps introduced to optimise alignment;

25

Fig. 3 illustrates South-Western analyses of ssDNA binding proteins in retroviral particles isolated from MS cell lines. Retroviral pellets obtained by ultracentrifugation of growth media from the indicated MS cell lines were analysed for the presence of ssDNA binding proteins by comparative South-Western analysis. The M_r marker is shown to the left. Left
30 arrow: 35 kD protein also present in unconditioned medium with human serum (HS).]: triple ssDNA binding bands. Lane 1: human serum, 2: MS1533, 3: MS1851, 4: B95-8(EBV⁺), 5: C91-PL(HTLV-1⁺), 6: FLK(BLV⁺), 7:MS1845, 8: C8166-(HTLV-1 Δ *gag*). The gels were run under conditions ensuring the best resolution in the low M_r range, thus cross-reactivities for the higher M_r proteins (> 40 kD) are poorly resolved. The three low
35 molecular weight bands (M_r 10-20 kD) in the MS cell lines are also found with the positive

control retroviruses HTLV-1 and BLV, but the MS band pattern exhibits slight differences in size. The cell line C8166 contains three mutated HTLV-1 genomes and it is known that no expression occurs from the *gag* and *env* regions (Bhat et al., 1993). In the lane with the EBV-producing line B95-8 only two bands are present in the low M_r region. The middle band of the three is absent, clearly demonstrating that this cannot be an EBV-related
 5 band. The primary reason for using B95-8 was to confirm that the middle of the low M_r ssDNA bands of the MS cell lines is not of EBV origin, but it should be kept in mind that B95-8 contains some transmissible SMRV-H type D retrovirus-like sequences (Sun et al., 1995). The HTLV-1 *tax* gene product $p40^{Tax}$ is a trans-regulatory protein containing other
 10 zinc-finger motifs. Tax-related bands would not be expected in this assay as $p40^{Tax}$ is rarely packaged in virions;

Fig. 4 are translations of the $CX_2CX_4HX_4C$ corresponding *gag* sequence regions.

Sequences (upstream of the protease domain) from HTLV-1 (SEQ ID NO:11) and RTVL-
 15 H (SEQ ID NO:12) (Mager et al., 1987) and RGH-2 (SEQ ID NO:13) (Hirose et al., 1993) (translated) and examples of translated *gag*-primed RT-PCR derived sequences from Optiprep-purified retrovirus particles from the MS1533 donor (D) (SEQ ID NO:14) and an infected recipient (R) (SEQ ID NO:15/SEQ ID NO:16). * indicates termination codons, indicates gaps introduced to optimise alignment. Note the high proline content. The motif
 20 $CX_2CX_4HX_4C....CX_3HX_4C$ is conserved in both donor and recipient clones;

Fig. 5 illustrates a Product Enhanced Reverse Transcriptase (PERT) assay indicating cell-free transmission of purified retroviral particles from long-term cell cultures from peripheral blood from MS patients using PHA-stimulated lymphocytes from healthy human
 25 individuals as recipient. The results are indicated as OD (PERT/ 10^6 cells)

Fig. 6 illustrates another PERT assay indicating cell-free transmission of purified retroviral particles from long-term cell cultures from peripheral blood from MS patients using PHA-stimulated lymphocytes from healthy human individuals as recipient. The results are
 30 indicated as OD (PERT/ 10^6 cells); and

Fig. 7 illustrates a PERT assay indicating cell-free transmission of purified retroviral particles from long-term cell cultures from peripheral blood from MS patients using PHA-stimulated lymphocytes from rabbit as recipient. The results are indicated as OD
 35 (PERT/ 10^6 cells).

EXAMPLES 1-4

Materials and methods5 Cells

All blood samples were obtained with informed consent. Heparinised blood was subjected to Ficoll-Isopaque density gradient centrifugation. The establishment of long-term cultured, spontaneously transformed cell lines from isolated mononuclear cells was
10 performed as described previously (Munch et al., 1995).

FLK-BLV (Foetal Lamb Kidney-Bovine Leucosis Virus)(BLV⁺) was obtained from The National Veterinary Serum Laboratory, Copenhagen) and two HTLV-1 infected T-cell lines C91-PL (HTLV-1⁺) and C8166 (HTLV-1Δ gag) were obtained from the MRC AIDS
15 Reagent Project, (London). The EBV-producing cell line B95-8 was obtained from ATCC. The cell lines were supplemented with foetal calf serum instead of human serum and were grown in a separate laboratory.

Plasma

20

All blood samples were obtained with informed consent. The samples were from healthy volunteers or patients from the Neurology Dept., Aarhus University Hospital; the Dermatology Dept., Marselisborg Hospital, Aarhus; the MS Hospital in Ry; or the Dept. of Internal Medicine, Middelfart Hospital (all in Denmark). Samples were drawn in the
25 respective clinics and delivered by hand immediately after drawing. All samples were processed in our laboratory. Cell-free and debris-free plasma samples were obtained from 40 ml citrate blood samples (about 10 ml plasma). After an initial centrifugation, the plasma was aspirated and subjected to another centrifugation at 4°C, 30 min., 1000 x g to pellet remaining cellular debris. Following aspiration, plasma samples were treated as
30 described below.

Particle RNA purification

Retroviral RNA was purified from cell culture supernatants and from plasma samples:
35 1.3 l suspension cultures were centrifuged at 4°C, 30 min. at 2500 x g. The cell-free

supernatant was aspirated, underlayered with a cushion of 4 ml 50% Optiprep in NaCl/Hepes (Nycomed) in 60 ml tubes and ultracentrifuged at 4°C, 2 hrs. at 45.000 x g. Optiprep is an iodinated, non-ionic density gradient medium (Nycomed Pharma, Norway) which, in contrast to sucrose, maintains intact retroviral particles (Møller-Larsen et al.,
5 1998).

The cushion and proximal overlaying retrovirus-containing medium is mixed (5-6 ml), filtered through a 0.45 µm filter (Acrodisc 32, Gelman Sciences) to remove cellular debris, adjusted to 20% Optiprep and centrifuged in 11.2 ml Optiseal (Beckmann) tubes in a
10 Beckmann NVTi rotor at 364.000 x g for 3.5 hrs. 400 - 500 µl fractions (12 drops) were collected after centrifugation. RT activity was measured for each fraction and poly-A RNA was purified by lysing the 5-6 fractions with major RT activity directly in 5 volumes of lysis-binding buffer (DynaI, Norway), adding 100 µl beads/sample (mRNA Direct kit, Dynal, Norway) and following the manufacturer's instruction.

15

Cell-free plasma samples were layered on 50% Optiprep, ultracentrifuged and filtered as described above. The retrovirus/Optiprep mixture was directly lysed in 5 volumes of lysis-binding buffer (DynaI, Norway) and poly-A RNA was purified on 50 µl beads/sample using the mRNA-Direct kit (DynaI, Norway) as above.

20

If not used directly, the RNA-coupled beads were stored in 80% EtOH at -80°C. Before use, each RNA sample was treated with amplification grade DNase (Life Technologies) according to the manufacturer's instructions. After cooling on ice the DNase was removed by exchanging the buffer containing the RNA-complexed paramagnetic beads with DEPC-
25 treated ddH₂O.

Product enhanced reverse transcriptase (PERT) assay

The ultrasensitive RT assays (PERT (Product Enhanced Reverse Transcriptase)) were
30 performed directly on 5 µl gradient fraction in 5 µl buffer A, essentially as described previously (Christensen et al., 1999; Lugert et al., 1996; Silver et al., 1993; Pyra et al., 1994).

35

RT-PCR, cloning and sequencing

RT-PCR was performed using the GeneAmp RNA PCR kit (Perkin Elmer) according to the manufacturer's suggestions, except that 1 μ of Taq polymerase was used per
5 reaction. First strand synthesis was primed with random hexamers, in conjunction with the bead-oligo-dT complexed RNA-template. In some experiments, we ensured the integrity of the RNA template by cDNA synthesis, specifically primed with the downstream primer; in this case, control cDNA synthesis primed with the corresponding upstream primer was negative.

10

Primer sets for PCR: initially, we used various retroviral consensus primers at low stringency conditions. The results lead us to assay for endogenous retrovirus sequences, following which we obtained positive results with the HERV-H/RTVL-H *gag* consensus primers of the endogenous retrovirus panel (Medstrand et al., 1992); the primers were
15 kindly donated by P. Medstrand and J. Blomberg:

5'-CTTTTATTACCCAATCTGCTCCCGAYAT-3' (SEQ ID NO:17)/5'-
TTAGTGGTGGACAGTCTCTTTTCCARTG-3' (SEQ ID NO:18).

20 We then developed the RGH-specific primer sets for the *gag* (I,II) and immediate upstream *env* regions:

I: 5'-CGTTTACATATCACTCCCTTCCTAGTCTCTGT-3' (SEQ ID NO:19)/5'-
GCATTAACCTTGACTATGTCTTTAGCTCCAG-3' (SEQ ID NO:20) (50°C) and/or

25

II: 5'-ATTTTATTACCCAATCTGCTCCAAACAT-3' (SEQ ID NO:21)/ 5'-
AGGTGAGTTGAACAGTCTGATTTTAA-3' (SEQ ID NO:22) (50°C);

5'-GATCCTCCCCACTGGGTTCAACATT-3' (SEQ ID NO:23)/ 5'-

30 GGAAGTATTGGAGGGTGCCCTGCC-3' (SEQ ID NO:24) (60°C).

All reactions were run in a Perkin Elmer DNA Thermocycler. Conditions for first strand synthesis: 5 min. at room temperature, 45 min. at 42°C and 5 min. at 95°C followed by cooling to 4°C. PCR: one cycle 2 min. at 95°C was followed by 45/50 cycles with
35 annealing temperatures as indicated in brackets above: 1 min. at 94°C, 2 min. annealing,

3 min. at 72°C with 10 s extension. This was followed by a final extension 7 min. at 72°C. In each assay, water controls corresponding to each step were included together with duplicates of each reaction without RT to ensure that no DNA-templates were amplified. PCR products were analysed by agarose gel electrophoresis, and each product was
5 cloned in pUC using the SureClone Ligation kit (Pharmacia) and sequenced using the ABI Prism kit (Applied Biosystems) using an automatic sequenator (ABI 377).

South-Western analyses

10

60 ml suspension cultures were subjected to initial centrifugation as described above; viral particles were pelleted by ultracentrifugation at 70.000 x g at 4°C for 1 hr (SW41 rotor/Beckmann), resuspended in 20 µl Tris-HCl pH 8, 0.1% Na-azide and kept at 4°C until use. Protein concentration for each sample was determined by a colorimetric assay
15 (Bio-Rad protein assay).

Before SDS polyacrylamide gel electrophoresis (SDS-PAGE) half of each sample was subjected to immunoprecipitation to remove any co-purified EBV particles. 1/10 volume of a solution containing 2,5 M NaCl, 50 mM Na-borate pH 9 and 10 µg/ml of each of the
20 antibodies 9243 and 9247 (anti-EBV membrane and capsid, Dupont) was added to each sample and the mixture was incubated at room temperature 15 min. At this point 1 ml protein A Sepharose suspension (6MB, Pharmacia) in 100 mM Tris-HCl pH 8 was added. After 10 min incubation at room temperature the mixture was centrifuged at 12.000 x g at 4°C for 5 min to precipitate protein A sepharose-antibody-EBV complexes. The
25 supernatant was subjected to lysis by adding 1/3 volume of lysis-buffer: 3% NP-40, 0.3% SDS, 3% Triton-X-100, 450 mM NaCl and 150 mM Tris-HCl pH 7.5. Equal amounts of protein from each lysed viral sample were subjected to SDS-PAGE on 4-20% NOVEX gels. A prestained Mr marker (Sigma) was co-electrophoresed on each gel. Positive controls were BLV and HTLV-1. Other controls were culture medium and B95-8. All
30 samples for SDS-PAGE were prepared by ultra-centrifugation. After electrophoresis the gels were electroblotted onto nitrocellulose filters (Millipore) in 62,5 mM Tris-HCl pH 8.3, 13,5 mM Glycin, 5% EtOH and 0.1% SDS. Electrophoresis and subsequent blotting was performed in a NOVEX Excell assembly. ssDNA probes were radioactively labelled with a Random Primed DNA Labelling Kit (Boehringer) and α-32P-dATP (Amersham) according
35 to the manufacturer's recommendations. The template was *EcoRI/BamHI* digested λDNA

(Boehringer). The probes were heat-denatured at 90°C 10 min before use. After blotting, the nitrocellulose filters were equilibrated in a buffer containing 10 mM Tris-HCl pH 7.0, 1 mM EDTA, 1 X Denhardts buffer, 50 mM NaCl, and incubated with radioactively labelled probe in the same buffer for 1 hr at room temperature. The filters were washed
5 extensively in the same buffer, air dried and exposed to Fuji X-ray film (Santax) at -80°C.

Transmission

Lymphocyte samples isolated by Ficoll-Isopaque density gradient centrifugation of citrate
10 blood from healthy volunteers were seeded and cultured as described previously (Munch et al., 1995) (10^7 cells/aliquot). Two μ l PHA (Difco)/10 ml was added. After 2 days purified retroviral particles from an MS cell line, pelleted by ultracentrifugation, suspended in TNE and filtered was added (particles from 60 ml culture/aliquot), this representing a cell-free infection. The suspensions were cultured and examined in the microscope twice weekly
15 and supernatant samples for PERT (Christensen et al., 1999) were collected regularly. Transmission of the retrovirus was considered successful when RT activity is observed concomitantly with the major appearance of clonal growth; this happens usually after about a month. Continuous clonal growth refers to an increased growth rate as illustrated by the need for feeding and subculturing 3 times a week contrasting feeding once a week
20 without subculturing. This is accompanied by the occurrence of many cell-clusters (clones). The corresponding controls die out. These controls were parallel PHA-stimulated as well as untreated cultures of the recipient lymphocyte preparations for each transmission assay. Absence of contaminating cells from the donor culture was confirmed by chromosomal analysis (the transfer is from male donor to female recipient or vice
25 versa). Retroviral particles from the supernatant of the established transmission cultures were concentrated and characterised by RT-PCR as described above.

EXAMPLE 1

Identification of RGH sequences in retroviral particles produced by MS-derived cell lines

5

B-Lymphoblastoid cell lines were established from venous blood from MS patients (Table 1.1). The cell lines arose spontaneously after long-term cultivation. All cell lines were tested for mycoplasma contamination and were found negative by repeated analyses.

- 10 Particles were purified from cell culture supernatants by ultracentrifugation in Optiprep gradients. Their retroviral origin was confirmed by negative staining electron microscopy (EM) on each gradient fraction and by the sensitive PERT (Product Enhanced Reverse Transcriptase) assays (Christensen et al., 1999; Lugert et al., 1996; Silver et al., 1993; Pyra et al., 1994) also performed on each gradient fraction. The presence of RNA in the
- 15 particles was demonstrated by chasing the culture with [5-³H]-uridine (Amersham) for 24 hrs before harvest, followed by TCA precipitation of each gradient fraction, and counting in a β -counter. PERT activity, presence of retroviral particles in EM and RNA content co-located in the gradients (Møller-Larsen et al., 1998).
- 20 Using RT-PCR on retroviral RNA templates isolated from cell culture supernatants with subsequent sequencing of the cloned amplicons, we initially identified several *gag* and *env* fragments with high homology to the human endogenous retrovirus HERV-H variant RGH-2 (Hirose et al., 1993) (Fig. 1) in the retroviral particles produced by the cell lines MS1533, MS1844, MS1845 and MS1851. The level of homology to RGH was in the range
- 25 of 80-95%. The packaging of RGH RNA at particle level is clearly demonstrated in the material from cell lines by the co-migration in Optiprep gradients of retrovirus particles, morphologically identifiable by EM, and RT activity. Wistar rats immunised with purified retroviral particles exhibit a specific serological response towards potentially immunogenic synthetic peptides translated from the HERV-H sequences but it remains, however, to be
- 30 assessed whether all the retroviral proteins in the particles are encoded by HERV-H. The virion proteins may also be encoded by other retroviral sequences in the genome as heterologous RNA co-packaging in virions (Linial, 1990) is a well-known phenomenon.

Furthermore, we tested MS cell line particle RNAs, purified by our standard procedure,

35 using the recently reported ERV-9-MS related nested primer set ST1-1/-2 and RT-PCR

conditions (Perron et al., 1997). The results were negative.

Table 1.1. B-Lymphoblastoid cell lines were established from venous blood from MS patients

5

The cell lines were established as part of an extensive culturing series and arose after more than 2 months of cultivation. They are continuously growing. No external EBV source was involved. Their origin was confirmed by RFLP mapping and by HLA-DO β typing (Christensen et al., 1997).

10

Type	No. of patients	Sex	Age, years	Diagnosis	Duration, years
RGH positive by RT-PCR	1	F ^b	24	PP ^d (active ^a)	2
Multiple sclerosis cell line	1	F	40	Benign MS	11
n=4	1	M ^c	30	Chronic prog. Myelopathy	7
	1	M	33	RR ^e (active)	2
Multiple sclerosis plasma	6	F	16-50	RR (2 active)	1-11
n=24	2	M	50-52		9-15
	5	F	34-56	SP ^f (2 active)	4-20
	7	M	40-72	(2 active)	9->20
	4	F	33-47	PP (3 active)	3-7
RGH negative by RT-PCR	1	F	35	RR	10
Multiple sclerosis plasma	1	M	30		1
n=9	1	F	34	SP	15
	3	M	40-50		9-15
	2	F	31-36	PP	2-5
	1	M	36		6
Healthy control plasma	13	F	24-53		
n=20	7	M	27-34		
Patient control plasma	3	F	56-62	Diabetes mellitus	
n=29	5	M	50-63		
	1	F	42	Prurigo nodularis hide	
	1	M	68	Hairy cell leukaemia	
	2	F	35-61	Colitis ulcerosa	
	2	M	31-32		
	1	F	52	SLE ^g	
	1	F	55	Arteritis temporalis	
	1	F	53	Rheumatoid arthritis	
	4	M	47-61		
	1	F	63	Thyreotoxicosis	
	1	M	55		
	1	M	47	Sarcoidosis	
	1	M	50	Collagenosis	
	1	F	58	Polymyositis	
	1	F	76	Myelomatosis	
	1	M	58	Leukemia/lymphoma	
	1	F	72	Polymyalgia rheumatoides	

^aAll MS patients had clinically definite MS. Chronic progressive MS with no stationary

15 phase, and exacerbations in relapsing-remitting MS was defined as active MS. ^bfemale,

^cmale, ^dprimary progressive, ^erelapsing-remitting, ^fsecondary progressive, ^gsystemic lupus erythematosus.

EXAMPLE 2

5 HERV-H sequences specifically present in plasma from MS patients

To determine if RGH-homologous sequences at particle level could also be associated with MS *in vivo*, we subsequently performed RT-PCR analyses on clinical specimens, i.e. cell-free, filtered, ultracentrifuged plasma samples from MS patients, from patients with
10 autoimmune diseases, and from healthy controls. RNA extracted from particles isolated from plasma was assayed by RT-PCR with subsequent sequencing of the cloned amplicons. The results of the plasma RT-PCR and sequencing analyses are presented in Table 1.1. Examples of MS patient sequences are shown in Fig. 2 (SEQ ID NOS:9-10). Expression of RGH sequences at particle level was specific for MS (24 out of 33 cell-free
15 plasma samples from MS patients). These sequences were absent in all of 29 cell-free plasma samples from patients with autoimmune diseases and in all of 20 cell-free plasma samples from healthy controls.

Different RGH sequence variants were thus found both in the virus particles from cell line
20 supernatants and in the particulate fraction of plasma from different MS patients. It is presently unknown how many of the genomic copies are transcribed or if complementation occurs between the molecules.

EXAMPLE 3

**Detection of HTLV/BLV Gag NC protein equivalents in retroviruses from MS cell
5 lines by South-Western analyses**

To determine functional characteristics of proteins isolated from the particles, we searched for the Gag nucleocapsid (NC) protein, which contains zinc-finger domain(s) with the characteristic CX₂CX₄HX₄C motifs, involved in the interaction between NC and
10 RNA (or ssDNA). This motif is known to be present as one or two copies among retroviruses (Mager et al., 1987). Interestingly, this motif which occurs twice, but is imperfect in both motifs in RTVL-H (Mager et al., 1987) also occurs twice, but with one perfect copy in RGH. Two of the gag primer sets used in our RT-PCR assay span this motif.

15

Using the method outlined by Morozov et al., 1992, where electrophoretically separated proteins from particles are incubated with ssDNA under renaturing conditions we have demonstrated that a nucleic acid binding protein, equivalent to the Gag NC proteins of HTLV and BLV, is also present in the retrovirus from 3 MS cell lines assayed (Fig. 3).
20 Several data indicate, that the middle of the three ssDNA binding bands present in the 10-20 kD range corresponds to the retroviral nucleocapsid protein: the band-patterns for the MS cell lines (lanes 2, 3 and 7), HTLV-1 (lane 5) and BLV (lane 6) are similar; and the lack of a corresponding band in the lane with the EBV-producer B95-8 (lane 4) excludes that the middle band is of EBV-origin. Furthermore, when the same filter is used in a
25 Western blot with HTLV-1+ serum, the p15 band corresponds exactly to the middle-band of the three ssDNA-binding bands of HTLV-1 (not shown).

If EBV is removed by immunoprecipitation from the dissolved pellets prior to electrophoresis the band pattern observed in all retrovirus-infected cell lines were identical
30 to the pattern found for non-precipitated material (not shown).

The origin of the other two bands is not quite clear. The absence in C8166 (lane 8) of similar bands indicates that these bands may also have a retroviral origin. The origin of the 60 kD band in C8166 is not known.

35

EXAMPLE 4

Transmissibility of retroviruses produced by MS cell lines

5 In this Example the transmissibility of the retrovirus produced by the MS cell lines was investigated. As cell-free type-C-like retroviruses (HTLV-1) are capable of infection, albeit with low efficiency (DeRossi et al., 1985), purified particles from MS cell lines were used to infect cultured, PHA-stimulated lymphocytes from healthy human individuals. Concomitant occurrence of RT-activity and continuous clonal growth in 8 out of 23
10 experiments in which the retrovirus was attempted transmitted to PHA-stimulated lymphocytes from healthy human individuals was interpreted as an indication of transmission, substantiated by the lack of activity and growth in the respective control cultures. Continuous clonal growth refers to a highly increased growth rate accompanied by the occurrence of many free-floating clusters (clones) of cells together with free single
15 blast-like cells. The corresponding controls die out.

In these experiments, we have demonstrated the presence of RT activity and clones in 8 out of 23 independent infections using MS1533, MS1844 or MS1874. All corresponding controls remained untransformed and without RT activity. RGH sequences have been
20 demonstrated in gradient-purified retroviral particles produced from three of the independent putatively transformed cell cultures (two with MS1533, one with MS1844) more than one month after infection. RGH sequences from donor and an infected recipient are shown in Fig. 4. The differences between presented donor and recipient sequences do not exclude transmission as the RGH variants constitute a population of
25 molecules (at least in the donor) rather than a single sequence. Interestingly, the CX₂CX₄HX₄C motif is better conserved in the isolated clones from both donor and recipient than in RGH-2. The absence of transformation of any of the PHA-stimulated control cultures and the particle production in the infected recipients are indicative of transmission.

30

Discussion of Examples 1-4

In the above examples a specific association between MS and the endogenous retrovirus family HERV-H has been demonstrated. The RGH-homologous variants were
35 demonstrated at particle level in supernatants from 6 MS-derived cell lines. We have also

demonstrated RGH in particulate form specifically in the plasma from 24 out of 33 MS patients.

Indications that this endogenous retrovirus may be transmissible is demonstrated by the
5 cell-free transmission of purified virions to lymphocytes from healthy individuals. The
activation of endogenous sequences already present in the recipient can not be ruled out,
but we consider it doubtful that such an activation could be due to EBV. EBV is unlikely to
co-purify with the retrovirus, and spontaneous EBV transformation of the healthy
lymphocytes seems implausible as i) by no means all healthy lymphocyte samples are
10 activated/infected, ii) the cells are cultured for a short time (4-8 weeks), iii) we observe
clonal growth and RT activity concomitantly, and iv) the controls all die out.

Previously, we have demonstrated a correlation between MS disease activity and the
presence of cross-reacting antibodies towards an HTLV-1-derived Env polypeptide. This
serological study included 48 MS patients, 19 patients with other neurological diseases
15 and 57 healthy controls. Serologic cross-reactivity towards the HTLV-1-derived Env
polypeptide was found in more than 60% of the MS sera (Christensen et al., 1999). We
have also used PERT-assays on fresh plasma from MS patients and controls and showed
that 7 of 17 MS patient samples had measurable RT activity whereas all 21 controls were
negative (Christensen et al., 1999). A recent study demonstrated the absence of
20 serological reactivity towards HTDV/HERV-K Gag, an MMTV/type-D like retrovirus in MS
patient sera (Boller et al., 1997).

Recently, it was reported that ERV-9/type-C-related (MSRV) sequences are present in
plasma samples from 5 MS patients (Perron et al., 1997) and in serum from 9 out of 17
25 MS patients vs 3 out of 44 controls (Garson et al., 1998). As the published MSRV
consensus sequence (2304 bp) (Perron et al., 1997) is about 90% homologous to several
human genomic sequences, i.e. AC000064 and U85196, MSRV may be exogenous but is
most likely an endogenous ERV-9 variant. Alternatively, MSRV could belong to the
recently described HERV-W family (Blond et al., 1999). Even though we could not
30 demonstrate such sequences in particles from the MS cell lines, the fact that HERV-
H/RGH-2 and MSRV/ERV-9/HERV-W belong to the same subsection of the type-C
oncovirinae may indicate that we are looking at two aspects of the same phenomenon.

Retroviral association has been reported recently for several autoimmune diseases: in
35 addition to the previously mentioned implication of a HERV-K variant in insulin dependent

diabetes mellitus (IDDM) (Conrad et al., 1997), in Sjögren's syndrome, *pol* sequences from a putative exogenous type-D-like retrovirus were found in salivary glands (Griffith et al., 1997); in another study, the *pol* sequences from a putative exogenous type-D-like retrovirus were found in lymph nodes (Yamano et al., 1997); in a systemic lupus erythematosus study seroreactivity towards short, type-C-like endogenous retroviral Env peptides and a HTLV-1-derived Gag peptide was described (Bengtsson et al., 1996). Finally, in rheumatoid arthritis, differential expression of multiple endogenous retroviral *pol* sequences in synovial fluid cells where RTVL-H sequences were absent, has been reported (Nakagawa et al., 1997).

10

It is suggested that MS is associated with production of otherwise replicatively quiescent endogenous retroviruses. Whether this represent a causal factor by eliciting an autoimmune response is not fully substantiated yet. However, in the study presented here, the presence of HERV-H/RGH sequences at particle level is clearly MS specific.

15 This could indicate a direct and specific implication in the disease process, also because all plasma samples from MS patients with active disease were HERV-H/RGH positive. In the case of MS, the epidemiology of the disease implies that if replication of endogenous retroviruses is a causal factor, the activation mechanism(s) is/are likely to be of external origin. Suggestive possibilities are interactions with an as yet unidentified exogenous

20 retrovirus and/or, as we and others have previously suggested, a member of the herpes virus group. Interactions through RT with non-retroviral RNA viruses (Klennerman et al., 1997) presents another possible role for expressed endogenous retroviruses.

EXAMPLES 5-8

25

Introduction

In the following examples it is demonstrated further that retroviral particles associated with sequence variants of the human endogenous retrovirus HERV-H/RGH and produced by

30 spontaneously formed long-term cell cultures from peripheral blood mononuclear cells from several MS patients, can be transmitted to other, i.e. non-blood human cells and non-human cells. It is also shown that these long-term cell cultures consist of a mixed population of mainly B cells, and a smaller fraction of CD3+, CD4-, CD8- T cells.

EXAMPLE 5

Intra-species transmission of RGH/HERV-H retrovirus particles

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Intra-species transmission was demonstrated by co-cultivation of long-term cell cultures from peripheral blood from MS patients with a retroviral vector construct-harboring cell line followed by assays for reverse transcriptase (RT) activity and assays for rescue of the retroviral vector-construct in indicator cells. Briefly outlined, the principles behind this co-cultivation assay are as follows: Transmission of retroviruses is to some extent dependent on cell-to-cell contact; i.e. cell-free transmission is possible, but occurs at a low frequency (de Rossi et al., 1985) (see Example 6).

Co-cultivation of LacZ-donor cells with retrovirus producing cells allows cell-to-cell contact in the initial stage of the assay described below. LacZ-donor cells harbour a retroviral vector-construct with the LacZ marker-gene. This construct is not transmissible in itself, but can be mobilised *in trans*. The LacZ-donor cells are co-cultivated with cells that produce retroviral particles. If the LacZ-donor cells are productively infected with retrovirus, a proportion of the subsequently produced infectious retroviral particles will contain the (mobilised) construct. These retroviral particles are produced into the co-cultivation supernatant. Upon addition of this supernatant (with retroviral particles but completely depleted for cells by filtration) to indicator cells that do not harbour the construct, (cell-free) retroviral infection of these indicator cells will transduce the retroviral vector-construct to the indicator cells. When the indicator cells are fixed and stained, blue cells will only occur if the gene-product (β -galactosidase) of the LacZ marker gene is present, and the LacZ marker gene will only be present if a retroviral infection has transduced the LacZ marker gene from the LacZ-donor cells through an infectious retroviral particle to the indicator cells. The main principles of the assay are described in Porter et al., 1998.

Monitoring of transmission is performed by assaying for blue indicator cells and/or increase/lack of decrease in RT-activity. PERT assays measured via OD values show that RT activity for the produced retroviral particles in the supernatant without *de novo* particle production decreases about 3-fold after 3 days at 37°C, reflecting degradation.

The results described below are interpreted as follows:

No blue cells and decrease in RT activity: no transmission;

No blue cells and increase/lack of decrease in RT activity: possible transmission as there
5 is *de novo* production of retroviral particles; construct may not be mobilised;

Blue cells and decrease in RT activity: possible transmission as construct is mobilised;
retroviral production may be low;

10 Blue cells and increase/lack of decrease in RT activity: transmission.

The retrovirus producing long-term MS cell cultures are subcultured three times a week at a density of 0.5×10^6 cells/ml and cultivated in RPMI 1640 (Whittaker) supplemented with 200 i.u./ml penicillin (Leo), 0.2 mg/ml streptomycin (Rosco), 290 mg/ml glutamine (Sigma), 10
15 mM HEPES (Bioproduct) and 10% heat inactivated human serum (serum-pool obtained from the blood bank at Skejby Hospital, Denmark) in Costar bottles.

The indicator cells are the human rhabdomyosarcoma cell line TE671, and the LacZ-donor cells are TELacZ-cells. TELacZ is a clone of TE671 harbouring the MFGnlslacZ retroviral
20 vector (Takeuchi et al., 1994). TE671 and TELacZ are subcultured once weekly at a density of 0.5×10^6 cells/ml and cultivated in D-MEM (Dulbecco's modified Eagle's medium, Gibco, Life Technologies) supplemented with 5% FCS (foetal calf serum, Gibco, Life Technologies) and supplemented with 200 i.u./ml penicillin (Leo), 0.2 mg/ml streptomycin (Rosco) and 145 mg/ml glutamine (Sigma) in Costar bottles.

25

For a standard transmission assay, 6-well trays (Falcon 1146, non-tissue culture coated) are coated with human fibronectin (Sigma F-0895) at 10 µg/ml in PBS (standard phosphate buffered saline: 150 mM NaCl, 150 mM Na-phosphate, pH 7.4) for 2 hrs at room temperature. The fibronectin is aspirated and the wells are blocked with 2% BSA (FAF,
30 Boehringer Mannheim) in PBS for 30 min. Then the wells are washed in culture medium, and TE671 or TELacZ are seeded at 3×10^4 cells/2ml medium/well and incubated at 37°C in a CO₂ cabinet. Fibronectin facilitate the possibility of transmission through co-localization of virus particles and target cells (Williams, 1999)

After 3 days, the medium is aspirated and the retrovirus producing MS cells are added (in their own growth medium but with 5% HS only) at 3×10^5 cells/2ml medium/ well. After addition of the MS cells, the trays are centrifuged at $185 \times g$ at room temperature for 10 min. and the trays are incubated again at 37°C in a CO_2 cabinet. A typical experiment include the

5 following duplicate cell combinations:

TElacZ + MS cells $\pm$ fibronectin $\pm$ wash; TELacZ + mock-infection $\pm$ fibronectin $\pm$ wash ; TE671 + mock-infection $\pm$ fibronectin $\pm$ wash. The controls without fibronectin are trays blocked with BSA, or Costar bottles. After 5 days of co-cultivation, half of the wells are

10 washed gently 3 times with PBS and all wells are supplemented with fresh medium (with 5% HS) up to 5-6 ml. This washing step removes most MS cells, but not the MS cells associated with the well walls or with the indicator cells. After further 3 days of co-cultivation, a 1 ml sample is taken from each well and prepared for RT-assay. The remaining culture from each well is aspirated and filtered through a $0.45 \mu\text{m}$ filter (Acrodisc, Gelman Science) (saturated

15 with PBS). This cell-free supernatant is plated onto TE671 indicator cells in Nunclon gridded culture dishes (Nunc 169558A), 1-2 dish(es)/well. The indicator cells are seeded at 2.5×10^5 cells/5ml medium/dish the day before, and incubated at 37°C in a CO_2 cabinet. 3.5 ml medium is aspirated from the dish before the addition of the filtered, cell free supernatant. The dishes are then incubated at 37°C in a CO_2 cabinet for 4-5 days. The supernatants are

20 aspirated from the dishes and a 1 ml sample is taken from each dish and prepared for RT-assay. The cells are fixed by addition of 5 ml/dish of 0.05% glutaraldehyde in PBS. The dishes are incubated for 15 min at room temperature and then washed twice in PBS. The cells are then histochemically stained for β -galactosidase expression by the following procedure: 2 ml stain solution is added to each dish and they are wrapped air- and light-tight

25 and incubated at 37°C for 24 hrs. The stain solution is aspirated, and the dishes are washed twice with 5 ml PBS. 2 ml ddH_2O is added and the entire dishes are scored for blue cells by microscopy (Leitz Wetzlar microscope). The stain-solution is prepared immediately before use and consists of a 1:40 dilution of 40mg/ml X-Gal (5-bromo-4-chloro-3-indolyl- α -D-galacto-pyranoside; Sigma) in DMSO in 5 mM K-ferro cyanide, 5 mM K-ferri cyanide, 2 mM

30 MgCl_2 , pH 7.

A typical time-chart for the assay can be outlined as follows (if all of LacZ-donor cells, retrovirus producing cells and indicator cells are in culture on day 1):

35 Day 1: Fibronectin coating of 6-well trays and seeding of TE621 and/or TELac;

Day 3: Addition of retrovirus producing MS cells for co-cultivation;

Day 8: Wash of wells;

5

Day 10: Seeding of indicator cells in gridded dishes;

Day 11: Filtration and transfer of co-cultivation supernatant to indicator cells; RT samples;

10 Day 15: Aspiration of supernatant from indicator cells; fixing and staining of cells; RT samples;

Day 16: Scoring.

15 Scoring can be performed both as positives/negatives and using multiplicity of infection (MOI). MOI can be determined from the proportion of uninfected cells (P) using the formula $MOI = -(\log P)/0.4343$: the number of viruses leading to successful transduction events (n_v) as a fraction of the number of target cells (n_c) is the multiplicity of infection ($MOI = n_v/n_c$). The probability of any cell for infection by one of the viruses is $1/n_c$,
 20 therefore the probability of any cell being uninfected (P) is $[1 - (1/n_c)]^{n_v}$. Thus, $\log P = n_v \cdot \log [1 - (1/n_c)]$. If $n_c > 3 \times 10^3$, $n_c \cdot \log [1 - (1/n_c)] = -0.4343$. MOI corrects for the non-linearity of a transmission assay with a simple positive-negative scoring.

Alternatively, the specific activity of β -galactosidase can also be determined using the following quantitative photometric assay (Felgner et al., 1994):

25

Cells are washed in PBS and lysed in 350 μ l of 250 mM Tris-HCl (pH 8.0), 0.1% Triton X-100, and the lysates are stored at -80°C for at least 1 h. To determine amounts of β -galactosidase, 50 μ l of lysates and dilutions are combined with 50 μ l of PBS, 0.5% BSA and 150 μ l 60 mM Na_2HPO_4 (pH 8.0), 1 mM MgSO_4 , 10 mM KCl, 50 mM β -
 30 mercaptoethanol, 0.1% chlorophenol red galactopyranoside (Boehringer). After light-protected incubation at 37°C , absorption at 578 nm is measured and converted to pg of β -galactosidase using a standard curve obtained with purified enzyme (Sigma). These values are standardised against the total amount of protein in each lysate. A 10- μ l volume of lysate is combined with 150 μ l of ddH₂O and 40 μ l of Bio-Rad protein assay
 35 reagent. Absorption at 595 nm is converted to μ g protein using a BSA standard curve.

Specific activity is expressed as pg β -galactosidase/ μ g protein. Statistical comparisons could be performed by Student's *t* test.

Assays for reverse transcriptase (RT) activity: RT is a retrovirus specific enzyme, which is present in retroviral particles. Monitoring of the RT activity thus constitutes a method for monitoring *de novo* production of retroviral particles. As the number of retroviral particles is very low, highly sensitive, PCR-based RT assays are necessary for measuring directly on small volumes of supernatant. The general assays and the RT profiles of the MS long-term cell cultures have been described in Christensen et al., 1999.

10

The ultrasensitive RT assays (RT-PCR or Product Enhanced Reverse Transcriptase (PERT)) are based on the conversion of an exogenous RNA template to amplifiable DNA by the sample-contained RT. The assays are performed essentially as described by Pyra et al., 1994:

15

1 ml aliquots of cell culture supernatant is obtained by aspiration. The samples are passed through a 0.2 μ m filter (Sartorius Minisart) and centrifuged at 4°C 63.000 x *g* for 100 min (Sigma 3K-30) or 100.000 x *g* 1 hr in a SW 55 (Beckmann). Supernatants are aspirated and pellets suspended in 5 μ l buffer A/ml aliquot of supernatant (buffer A: 50 mM KCl, 25 mM Tris-HCl pH 7.5, 5 mM DTT, 0.25 mM EDTA, 0.025% Triton X-100; 50% Glycerol).

20

Samples must be taken and processed on the same day to prevent loss of RT activity. Samples in buffer A are stored at -70°C.

The initial steps in the assay must be performed under RNase-free conditions. Template (MS2 RNA, Boehringer Mannheim) and primer (RT-1: 5'-CATAGGTCAAACCTCCTAGGAATG-3' (SEQ ID NO:25) are annealed before each assay by adding 0.28 pmol MS2 RNA to 9 pmol in a volume of 1.4 μ l in an RNase-free 0.5 ml Eppendorf-tube. RNA/primer is heated 95°C 5 min, incubated 37°C 30 min and cooled at 4°C (5 min), spun briefly and kept on ice during the next steps. 23.6 μ l of a mixture containing 60 mM KCl, 60 mM Tris-HCl pH 8.3, 9.5 mM MgCl₂, 12 mM DTT, 0.45% Triton X-100, 1 mM dATP, 1 mM dCTP, 1 mM dGTP, 1 mM dTTP and 0.15 μ g BSA/ μ l is added together with 0.6 μ l RNase inhibitor (40 u/ μ l; Boehringer Mannheim).

30

Then 3 μ l resuspended pellet in buffer A is added and the mixture is covered with mineral oil for PCR, incubated at 37°C 5 hrs; 95°C 7 min and cooled at 4°C (5 min). PCR is

35

performed on the RT reaction by adding 75 µl PCR buffer through the oil. (1.5 µl RT-1 (14 pmol), 1 µl dil RT-2 (25 pmol; RT-2: 5'-TCCTGCTCAACTTCCTGTCGAG-3' (SEQ ID NO:26), 1 µl boiled RNase A (10 mg/ml, Boehringer Mannheim), 1 µl Taq (Perkin Elmer, 5 u/µl), 2.8 µl 1 M KCl, 7.5 µl 100 mM Tris-HCl pH 8.3 and 60.2 µl ddH₂O). The mixture is
5 incubated 30 min at 37°C, followed by 25 cycles of 94°C 30 sec, 55°C 100 sec., 72°C 110 sec.; cooled at 4°C 5 min all in a Perkin Elmer Cetus 480 Thermocycler.

PERT assays can be performed with two variants of detection: either by hybridisation of amplified products with hapten conjugated oligonucleotides followed by ELISA, essentially
10 as described or by adjustment to the TaqMan technology (Perkin Elmer Cetus). In the latter case an oligonucleotide labelled with two fluorescence dyes, (fluorescein at the 5' end (reporter) and rhodamine at the 3' end (quencher)) binding to the amplified MS2 sequence region between forward and reverse primers, was used as a probe. During PCR, the annealed probe is hydrolysed by the 5' nuclease activity of Taq polymerase,
15 thereby enabling the differential measurement of emitted fluorescence in a spectrophotometer.

Each sample is analysed in triplicate or duplicate and "blind" assay controls are included in all experiments. Positive controls are cloned RT enzymes (Life Technology or Boehringer).
20 ELISA detection is performed as follows: NUNC 96-well titer plates are coated with 50 µl Avidin-solution 50 µg/µl in coating-buffer (200 mM NaCl, 100 mM Tris-HCl pH 7.4), wrapped light-tight and incubated at room temperature overnight. The plates are washed 3 times with 200 mM NaCl, 100 mM Tris-HCl pH 7.4, 0.3% Tween 20, blocked with 200 µl blocking-buffer in each well for 30 min at room temperature (200 mM NaCl, 100 mM Tris-HCl pH 7.4, 0.05% Tween 20, 20% FCS; washed 3 times and are ready for hybridisation:
25 25 µl of PCR-reaction are added to a 0.5 ml Eppendorf-tube with 10 µl hybridising-mix (0.625 µl RT-3Bio (10 pmol, 5'-TTAATGTCTTTA-GCGAGACGC-3' (SEQ ID NO:27), 0.4 µl 500 mM EDTA pH 8, 0.375 µl 1 M KCl, 0.15 µl RT-5Dig (10 pmol, 5'-ATGGCTATCGCTGTAGGTAGC-3' (SEQ ID NO:28), 1.0 µl 100 mM Tris-HCl pH 8.3, 7.45
30 µl ddH₂O), incubated 95°C 5 min, 45°C 30 min, cooled at 4°C (5 min) and 35 µl hybridisation-mix is added to each coated well, incubated 30 min at room temperature and the wells are washed 5 times.

Then 50 µl Fab anti-digoxigenin (Boehringer Mannheim, dil. 625 x in TBS/0.5mM EDTA
35 pH 8) is added to each well, incubated 1 hr at room temperature, washed 5 times and 50

μ l substrate (*p*-nitrophenyl phosphate) is added in substrate-buffer (Boehringer Mannheim), incubated 20 min, and OD₄₀₅ is measured in an ELISA-reader.

Table 5.1. Examples of transmissibility assayed by PERT-assays (OD) and β -galactosidase

5 induction

					Experiment	
Indicator Cell	Construct	Donor	Wash	Fibronectin	PERT dish/tray	Blue Cells
TE671	lac Z	MS1946	+	+	3.67	+
TE671	lac Z	MS1946	-	+	0.34	-
TE671	lac Z	MS1946	+	-	3.09	+
TE671	lacZ	MS1874	+	+	4.25	+
TE671	lacZ	MS1874	-	+	0.35	-
TE671	lacZ	MS1845	+	+	1.25	+
TE671	lacZ	MS1845	-	+	0.55	-
TE671	lac Z	MS1851	+	+	1.89	+
TE671	lac Z	MS1851	-	+	0.53	-
TE671	lac Z	MS1851	-	-	0.47	-
TE671	lacZ	MS1533	+	+	1.43	+
TE671	lacZ	MS1533	-	+	0.67	-
TE671	lac Z	C91-PL	+	+	0.18	+
TE671	lac Z	C91-PL	-	+	0.25	+
TE671	lac Z	None	+	+	<1	-
TE671	lac Z	None	-	+	<1	-
TE671	none	None	+	+	<1	-
TE671	none	None	-	+	<1	-
Positive, cloned RT control vs. Negative control					19.6	na

Nd: not done; na: not applicable; <1: numbers below cut-off-value; Fibronectin: cells
 10 cultured in fibronectin coated trays; wash: wells washed at day 8. MS1533, MS1845,
 MS1851, MS1874, MS1946 are long-term cell cultures from MS patients; C91-PL is a
 human cell line producing HTLV-1; Relative RT values around 1 and above indicates *de*

novo particle production; MOI values for the MS cell cultures are typically about 10^{-6} to 10^{-5} , for C91-PL they are around 10^{-3} .

EXAMPLE 6

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***Intra-species* transmission of RGH/HERV-H retrovirus particles**

In this Example *intra-species* transmission was demonstrated by cell-free transmission of purified retroviral particles from long-term cell cultures from peripheral blood from MS
10 patients to stimulated or non-stimulated peripheral blood lymphocytes from healthy individuals followed by assays for reverse transcriptase (RT) activity and characterisation of the transformed recipient cultures. Briefly outlined, the principles behind this cell-free transmission assay are as follows: Transmission of retroviruses is to some extent dependent on cell-to-cell contact; i.e. cell-free transmission is possible, but it occurs at a
15 low frequency (de Rossi et al., 1985; Fan et al., 1992).

Purified retroviral particles from long-term cell cultures from peripheral blood from MS patients are attempted transmitted to cultured, PHA-stimulated or non-stimulated lymphocytes from healthy individuals. Concomitant occurrence of RT-activity and
20 continuous clonal growth in about one third of the experiments in which the retrovirus was attempted transmitted to such lymphocytes from healthy individuals was interpreted as indication of transmission, substantiated by the lack of activity and growth in the respective control cultures.

25 Continuous clonal growth refers to a highly increased growth rate as illustrated by the need for feeding and subculturing 3 times a week contrasting feeding once a week without subculturing, accompanied by the occurrence of many free-floating clusters (clones) of cells together with free single blast-like cells. Transmission of the retrovirus is not always successful. Around one third of the recipient cultures will start to grow continuously as
30 described above. Absence of contaminating cells from the donor culture is confirmed by chromosomal analysis of sex chromosomes (the transfer is from male donor to female or vice versa) The corresponding controls eventually die out.

For chromosomal analysis, the cells are prepared for standard analysis: cells in growth
35 phase are treated with colcemide (100 μ l to 10 ml culture, incubated for 30 min at

37°C) for arrest of mitosis in the metaphase, then treated with hypotonic KCl (10 ml 0.56% to 10 ml culture for 5 min) to spread the chromosomes, followed by fixation (in methanol:glacial acetic acid 3:1) and quinacrine banding (Q-banding): highly AT-rich DNA, such as poly(dA)-poly(dT), markedly enhance quinacrine fluorescence while GC
5 containing DNA quench fluorescence. (Dr. U. Friedrich, personal comm, see also Comings et al., 1975) before visualisation in the microscope.

As an example, the presence of RT activity and clones was demonstrated in about one third of independent infections using 4 different retrovirus producing MS cell cultures. All
10 corresponding controls remained untransformed and without RT activity. HERV-H/RGH sequence variants were demonstrated in gradient-purified retroviral particles produced from the putatively transformed cell cultures more than one month after infection.

The set-up for an assay was as follows: lymphocytes were isolated from 50 ml citrate
15 blood samples from healthy volunteers by Ficoll-Isopaque (Amersham) density gradient centrifugation (900 x g for 30 min at room temperature), the cells were seeded and cultured as described in Example 5 (10^7 cells/aliquot). Six bottles were prepared of each, to three of which 2 µl PHA (Difco)/10 ml was added. After 2 days, purified retroviral particles from the supernatant from a long-term cell culture from an MS patient (100 ml
20 culture supernatant/aliquot) was added to 4 of the bottles, this representing a cell-free infection.

The MS cells were cultured as described in Example 5. They have a fluctuating and rather low yet continuous production of retrovirus particles. Retroviral particles from the cell
25 cultures are released into the supernatants which were collected for retrovirus preparation and purification. Twenty-four hours before harvest of the supernatants, the cells were diluted with one third fresh medium to obtain optimal growth conditions and thereby optimal virus production. 400-500 ml of cell-suspension: $1.2 - 1.4 \times 10^6$ per ml was used for one round of virus purification: cells and cell debris were removed from the
30 supernatants by centrifugation at 1000 x g for 30 min at 4°C. The supernatant was transferred to 60 ml Sorvall centrifuge tubes in volumes leaving sufficient room for underlying 4 ml of 50% OptiPrep cushion solution (OptiPrep is the trade name for Iodixanol, an iodinated, nonionic density gradient medium from Nycomed Pharma, Oslo, Norway). OptiPrep leaves the retroviral particles intact, as opposed to sucrose. The tubes
35 are centrifuged in a Sorvall rotor A641 at 45.000 x g for 2 h at 4°C and the supernatant

removed from the tubes by aspiration, leaving a volume of 4-5 ml of the medium in immediate proximity of the cushion. The cushion and the overlying medium are mixed and the volume of the mixture is measured for final regulation of the concentration of the gradient which is optimal at 20% of OptiPrep. For dilution a buffered saline solution is
5 used (0.8 % NaCl, 10 mM HEPES-NaOH, pH 7.4). The mixture is filtered through 0.45 µm (Acrodisc, Gelman Science) filters and transferred to 11.2 ml Ultraclear Tubes (Beckmann) and run in a near vertical rotor (Beckmann NVT 65) at 364.000 x g at 4°C for 3.5 h. Following centrifugation, the fractions were harvested in 0.5 ml aliquots by puncture of the bottom of the tubes. Fractions 2 - 5 were collected for use in the transmission
10 assays. These fractions were pooled, diluted in buffered saline and pelleted by a final run in an SW41 rotor at 150.000 x g for 90 min at 4°C.

The pellets were resuspended in 100 µl TNE buffer (50 M Tris pH 7.5, 100 mM NaCl, 1 mM EDTA). Usually, the pooled fractions were centrifuged in 4 tubes, resulting in 400 µl
15 of virus suspension which was added in 100 µl aliquots to 4 bottles prepared as described above. The suspensions were cultured and examined in an upside-down microscope (Leitz Wetzlar microscope) twice weekly and supernatant samples for PERT (see Example 5) were taken regularly. Transmission of the retrovirus is judged successful when RT activity is observed concomitantly with the major appearance of clonal growth;
20 this happens usually after about one month. The corresponding controls eventually die out. These controls were parallel PHA-stimulated as well as untreated cultures of the recipient lymphocyte preparations for each transmission assay. Assays for reverse transcriptase (RT) activity were performed as described above.

25 Retroviral particles from the supernatant of the established transmission cultures were purified by OptiPrep gradient ultracentrifugation as also described above and characterised by RT-PCR as follows: RT activity was measured for each gradient fraction and poly-A RNA was purified by lysing the 5-6 fractions with major RT activity directly in 5-10 volumes of lysis-binding buffer (Dynal, Norway), adding 100 µl beads/sample (mRNA
30 Direct kit, Dynal, Norway) and following the manufacturer's instruction. If not used directly, the RNA-coupled beads were stored in 80% EtOH at -80°C. Before use, each RNA sample was treated with amplification grade DNase (Life Technologies) according to the manufacturer's instructions. After cooling on ice the DNase was removed by exchanging the buffer containing the RNA-complexed
35 paramagnetic beads with DEPC-ddH₂O.

RNA PCR (RT-PCR) was performed using the GeneAmp RNA PCR kit (Perkin Elmer) according to the manufacturer's suggestions, except that 1u of Taq polymerase was used pr. reaction.

- 5 First strand synthesis was primed with random hexamers, in conjunction with the bead-oligo-dT complexed RNA-template. In some experiments the integrity of the RNA template was confirmed by cDNA synthesis, specifically primed with the downstream primer; in this case, control cDNA synthesis primed with the corresponding upstream primer was negative.

10

Primer sets for PCR: HERV-H/RGH-specific primer sets for the *gag* (I,II) and immediate upstream *env* regions:

I: 5'-d(CGTTTACATATCACTCCCTTCCTAGTCTCTGT)-3' (SEQ ID NO:29)/

- 15 5'-d(GCATTAACTTGACTATGTCTTTAGCTCCAG)-3' (SEQ ID NO:30) (50°C) and/or

II: 5'-d(ATTTTATTACCCAATCTGCTCCAAACAT)-3' (SEQ ID NO:31)/

5'-d(AGGTGAGTTGAACAGTCTGATTTTAA)-3' (SEQ ID NO:32) (50°C);

- 20 5'-d(GATCCTCCCCACTGGGTTCAACCATT)-3' (SEQ ID NO:33)/5'-
d(GGAAGTATTGGAGGGTGCCCTGCC)-3' (SEQ ID NO:34) (60°C).

- All reactions were run in a Perkin Elmer DNA Thermocycler 480. Conditions for first strand synthesis: 5 min. at room temperature, 45 min. at 42°C and 5 min. at 95°C followed by
25 cooling to 4°C. PCR: one cycle 2 min. at 95°C was followed by 45/50 cycles with annealing temperatures as indicated in brackets above: 1 min. at 94°C, 2 min. annealing, 3 min. at 72°C with 10 sec extension. This was followed by a final extension 7 min. at 72°C. In each assay, water controls corresponding to each step were included together with duplicates of each reaction without RT to ensure that no DNA-templates were
30 amplified. PCR products were analysed by agarose gel electrophoresis, and each product was cloned in pUC using the SureClone Ligation kit (Pharmacia) and sequenced using the ABI Prism kit (Applied Biosystems) using an automatic sequencer (ABI 377). Figures 5 and 6 are examples of the development of RT activity in two independent assays.

35

The below Table 6.1 shows translation of HERV-H/RGH related sequences isolated from retroviral particles from donor and an infected recipient.

Table 6.1. Translation of HERV-H/RGH related sequences isolated from retroviral

5 particles from donor and an infected recipient

	PKKPPPNQPCFRCGKAGHWSRDCTQPRPPPGPCPLCQDPTHWKRDCPR	HTLV-1
	PPEPPPPGACYKCQKSGHWAKE*PQPRIPPKPCPICVGP.HWKSDCPT	RTVL-H
10	PPEPSPPGACYKCQKSGHWAKECPQTRIPPKLYPISVGP.H*KSDCST	RGH-2
	PSDPPPPASCFKCLKSGHWTKECPQPGIPPKDSPICAGP.HWKKDCPP	1533 D
	PPDPPPPGACYTCQKSGHWAKECPQRGIPPKPRPICVGP.H*KSDCST	3.10 R
	PPDPPPPGACYTCWKSGHWAKECP*PGIPPKPRPICVGP.H*KSDCST	3.03 R

15

(HTLV-1: SEQ ID NO:35; RTVL-H: SEQ ID NO:36; RGH-2: SEQ ID NO:37; 1533 D: SEQ ID NO:38; 3.10 R: SEQ ID NO:39; 3.03 R: SEQ ID NO:40)

The differences between presented donor and recipient sequences do not exclude

20 transmission as the HERV-H/ RGH variants constitute a population of molecules (at least in the donor) rather than a single sequence. The absence of transformation of any of the PHA-stimulated control cultures and the particle production in the infected recipients are also indicative of transmission.

25 EXAMPLE 7

Inter-species transmission of RGH/HERV-H retrovirus particles

Inter-species transmission of RGH/HERV-H retrovirus particles was demonstrated by

30 cultivation of PHA-stimulated or non-stimulated cells from non-human species with purified retroviral particles from long-term cell cultures from peripheral blood from MS patients followed by assays for reverse transcriptase (RT) activity.

Details about the retroviral particles, details about PERT assays and details about the long-

35 term MS cell cultures are as in Examples 5 and 6. In this example, the principles for inter-

species transmission of retrovirus are outlined together with details of the the experimental set-up which are different from that described in the above Examples 5 and 6.

Endogenous retroviruses are defined as being either xenotropic (i.e. do not infect cells of
5 host, but are frequently able to infect other species), ecotropic (confined to host species, but may infect somatic cells within the species) or amphotropic (able to replicate in host and in distantly related species). Thus, endogenous retroviruses *per se* may or may not be infectious and, if infectious, may or may not be able to cross the species border.
Many endogenous retroviruses are known to have spread to other species through
10 horizontal transfer (Todaro, 1975) .

Thus the putatively infectious HERV-H/RGH associated retroviral particles from the long-term MS cell cultures might also be able to infect cells from other species. To avoid any interference from genomic retroviral copies, the obvious choice of species for inter-species
15 transmission assays are species, that are evolutionarily distinct from the host species of the endogenous retrovirus (human in this case), i.e species that diversified from the human/primate line before the endogenous retrovirus entered the germ line. Almost all HERVs entered the primate lineage around or after the divergence of Old World and New World monkeys (see Anderssen et al., 1997). Accordingly, rodent cells or virus-permissive
20 rodent cell lines appear to be an obvious possibility.

In contrast to the results obtained in assays for transmission to human cells, transmission attempts across the species barrier to cells (e.g. rabbit cells or other rodent cells or bat cells) in the set-up described below does not lead to continuous clonal growth (highly increased
25 growth rate accompanied by the occurrence of many free-floating clusters of cells together with free single blast-like cells). Rather, a distinct, but transient peak in RT activity as measured by PERT assays is observed and is interpreted as indicative of transmission. Both putatively infected cells and uninfected controls eventually die out.

30 Cell-free transmission of retroviral particles was assayed with rabbit lymphocytes: rabbit lymphocytes were isolated from 50 ml heparinised blood samples by Ficoll-Isopaque (Amersham) density gradient centrifugation (900 x g for 30 min at room temperature), the cells were seeded and cultured as described in Example 5 (5×10^6 cells/aliquot).
Alternatively, the culture medium was supplemented with heat inactivated rabbit serum
35 rather than human serum.

4-5 bottles were prepared of each, to which 2 µl PHA(Difco)/10 ml was added. After 2 days purified retroviral particles from the supernatant from a long-term cell culture from an MS patient, (100 ml culture supernatant/aliquot) was added to 3-4 of the bottles, this
5 representing a cell-free infection. Alternatively, filtered supernatant was added directly to the cells. The MS cells were cultured as described in Example 5.

The putatively infected rabbit cells were cultured and monitored essentially as outlined in Example 6. The cells were examined in the upside-down microscope (Leitz Wetzlar
10 microscope) twice weekly, and supernatant samples for PERT were taken regularly. Transmission of the retrovirus was considered possible, when RT activity is observed, usually developing a transient peak in RT activity over time. The control cells remain RT negative. An example is shown in Fig. 7.

15 In another application of this assay approach, similar experiments were set up with freshly prepared murine spleen macrophages or with a virus-permissive bat lung cell line (CCL 88). In yet another application, transduction of the LacZ-construct (or a similar construct) as described in Example 5 would enable scoring of transmission by both RT-assays and by assays for induction of β-galactosidase activity.

20

EXAMPLE 8

Analyses by flow cytometry of long-term cell cultures from peripheral blood from 25 MS patients: characterisation of surface markers

Different types of white blood cells can be characterised (and are often classified) by distinctive proteins on the cell surface: CD antigens (Cluster of Differentiation). Each CD antigen can bind monoclonal antibodies specific for that surface protein. By linking a
30 fluorescent dye to the different antibodies it is possible to identify the different cells by the fluorescence they emit as they flow through a laser beam: so-called FACS analysis (fluorescence-activated cell sorting).

The long-term cell cultures from peripheral blood from MS patients were further
35 characterised using this technique. These cultures were originally defined as "MS cell

lines" and, using immunocytochemistry, shown to be B-lymphoblastoid in nature as outlined in Sommerlund et al., 1993. The more sensitive flow cytometric analyses described below have shown that, whereas the majority of cells in the culture are B cells (CD19+), a small fraction of the cells are actually T cells (CD3+). Thus, the cell cultures
5 constitute a mixed population of cell-types that are co-existing. It is thus possible that an interaction within the population is necessary for the active production of retroviral particles; alternatively, either the B cells or the T cells produce the retrovirus.

The cells were characterised as follows:

- 10 Cells from the long-term cell cultures from peripheral blood from MS patients were counted in trypan blue (standard procedure for viability). 1×10^6 cells were placed in a V-shaped centrifuge tube and washed once in 10 ml RPMI 1640 (Whittaker)(centrifugation $400 \times g$ for 10 min at room temperature) and washed once in RPMI 1640 (Whittaker) supplemented with 2% foetal calf serum (FCS, Gibco, Life Sciences). The cells were
15 resuspended and transferred to FACS-tubes (Struers-KeboLab 107 102-520), 500,000 cells/tube. The cells were spun down at $400 \times g$ for 5 min at room temperature and the supernatant was discarded. Initially, a panel of different antibodies were tested: anti-CD3 (the cell receptor complex, T cells), anti-CD4 (accessory molecule in MHC II associated antigen recognition, subgroup of T cells, monocytes, macrophages), anti-CD8 (accessory
20 molecule in MHC I associated antigen recognition, subgroup of T cells), anti CD-10 (common acute lymphoblastic leukaemia antigen (CALLA), haematopoietic stem cells), anti-CD14 (LPS complex receptor, B cells, monocytes, macrophages) anti-CD19 (regulation of proliferation, B cells) anti-CD45 (leukocyte common antigen), anti-CD56 (cell adhesion molecule, NK cells), and anti-HLA-DR (tissue type) (all antibodies from
25 Becton Dickinson). It is possible to use more than one marker per tube when different fluorescent dyes are conjugated to the different antibodies.

20 μ l of each antibody was added to the cells and the mixtures were incubated in the dark for 15 min. at room temperature, washed twice in PBS pH 7.4 (centrifugation as above).

- 30 Then 500 μ l 1% formaldehyde in PBS was added while vortexing in a safety cabinet. The cells were kept refrigerated in the dark until reading in a flow cytometer (at the Immune-Haematologic Laboratory at Aarhus County Hospital, Aarhus Denmark).

For the continuous testing the following antibodies were found relevant and important:

- 35 anti-CD3, anti-CD4, anti-CD8, anti-CD19.

The results were as follows: Most of the cells were identified as being CD 19+ cells, i.e. they are B-cells. A small fraction of all MS cell-cultures appeared to be CD3+ cells, i.e. are CD4- and CD8-. Examples of the precise percentages of the latter category are shown in
5 the below table:

Table 8.1. Proportion of CD3+ cells in long-term cell cultures from peripheral blood from MS patients

Cell culture	% CD3+ T cells
MS 1533	4.6 %
MS 1845	7.4 %
MS 1847	5.2 %
MS 1859	5.2 %
MS 1851	5.4 %
MS 1874	14.6 %
MS 1946	4.4 %

10

Most T-cells in peripheral blood are either CD4+ or CD8+. The finding of CD3+ cells without these markers is an interesting observation. For further characterisation of the CD3+ fraction, separation of the two cell types, and an extended panel of antibodies will
15 be necessary.

EXAMPLE 9

**Expression of HERV-H (RGH) mRNA splicing variants in mononuclear cells from
20 MS patients.**

1. Introduction and summary

In this Example it is reported that spliced HERV-H mRNA sequence variants are
25 expressed *in vivo* in cellular RNA from mononuclear cells from MS patients. The sequence variants were found specifically in cells from some of the blood samples from

MS patients and autoimmune patients. The sequence variants were not found in mononuclear cells from healthy controls.

The endogenous HERV-H virus family consists of about 1000 defective members containing large deletions and stop codons, whereas 100 copies are approximately full length, potentially encoding functional proteins (Mager and Freeman 1987, Hirose *et al.*, 1993, Wilkinson *et al.*, 1993). One *env* sequence with an open reading frame has been isolated and transcribed *in vitro* (Lindeskog and Blomberg, 1999). Expression of HERV-H RNA *in vivo* has not been very well characterised though there is accumulating evidence that transcription occurs in normal as well as in malignant cells (Hirose *et al.*, 1993, Lindeskog *et al.*, 1993, Lindeskog and Blomberg, 1997). Splicing of HERV-H subgenomic transcripts has been described, revealing a complex pattern of multiply spliced mRNA species (Wilkinson *et al.*, 1990, Lindeskog and Blomberg 1997).

Spliced HERV-H *env* mRNA sequence variants were demonstrated by performing RT-PCR analysis on clinical specimens: blood samples from MS patients, patients with different autoimmune diseases and healthy controls. Mononuclear cells were isolated, total cellular RNA were extracted and assayed by RT-PCR. Amplicons were cloned and sequenced.

2. Experimental

All blood samples were obtained with informed consent from either healthy volunteers or from patients at the Neurology Dept., Aarhus University Hospital; the Dermatology Dept., Marselisborg Hospital, Aarhus; the MS Hospital in Ry; or the Dept. of Internal Medicine, Middelfart Hospital (all in Denmark). Blood samples were drawn at the respective clinics into VT-100SCPDA Venoject tubes containing 1.4 ml of CPD-adenine solution (Meda, Denmark) and delivered by hand immediately after drawing.

All samples were processed immediately in the inventors' laboratory. Mononuclear cells were isolated by centrifugation as described in Example 6, washed two times in PBS (phosphate buffered saline, 150mM NaCl, 150 mM Na-phosphate, pH 7.4) and suspended in Trizol (TRIzol™ Reagent, LifeTechnologies), 10⁷ cells /ml. Extraction of cellular RNA was performed according to the manufacturer's instructions. If not used directly, the RNA pellets were stored in 80% EtOH (in DEPC-treated H₂O) at -80°C. For RT-PCR analyses the RNA pellet was washed two times in 80% EtOH, air dried and suspended in DEPC-treated H₂O,

50 μ l per 10^7 cells. 7 μ l were used for RT-PCR analyses. RNA aliquots were stored at -80°C.

- RT-PCR was performed using the SuperScript System (SuperScript™ Preamplification System for First Strand cDNA Synthesis, Life Technologies). Before cDNA synthesis the RNA was treated with DNaseI (Deoxyribonuclease I, Amplification Grade, Life Technologies) according to the manufacturer's instructions, including Ribonuclease Inhibitor (Life Technologies), 0.01 U/ μ l, at relevant steps. All reactions were performed in a Perkin Elmer DNA Thermocycler model 2400.
- 10 Following inactivation of DNaseI by treatment with EDTA at 65°C, the RNA was annealed, 70°C 10 min, to a primer designed from the RGH2 sequence (Hirose et al., 1993):

(i) 5'-GGGAATTAGTGGAATAACTCTTTTTTGTG-3' (SEQ ID NO: 41) (T_m = 59.8°C)

- 15 cDNA synthesis was performed using the conditions prescribed in the protocol and RNA was digested with RNaseH (Ribonuclease H, Life Technologies) before purification of the cDNA using a QIAquick PCR purification kit (QIAGEN) according to the manufacturer's instructions. The cDNA was eluted in the EB buffer 2 x 25 μ l and stored as aliquots at -80°C, unless used directly as template for PCR.

20

PCR was performed in a Perkin Elmer DNA Thermocycler model 2400 using 50 μ l reactions containing: 15 mM Tris-HCl, pH 8.0, 50 mM KCl, 2.5 mM $MgCl_2$, 200 μ M dNTP, 0.5 μ M of each primer, 2.5 U AmpliTaq Gold polymerase (Perkin-Elmer) and 5 μ l cDNA. Conditions for PCR were: 10 min 95°C 1 cycle, 94°C 20 sec, 59°C 20 sec, 72°C 30 sec 50 cycles and 72°C

25 2 min 1 cycle.

Primers used for PCR amplification were designed from the assumed primer binding site (PBS) in the RGH2 sequence (Hirose et al., 1993) and from an HERV-H sequence containing an open *env* reading frame (Lindeskog et al., 1999):

30

(ii) 5'-CATGAAATTTGGTGCCTTGACTCGGAT-3' (SEQ ID NO:42) (T_m = 63.8°C)

(iii) 5'-GAGATAGCTGGGGAGAGGTAGAGGATGA-3' (SEQ ID NO:43) (T_m = 61.3°C)

- 35 PCR products were analysed by agarose gel electrophoresis. Relevant bands were cut out,

cloned in the pCR[®]2.1 vector (Original TA Cloning[®] kit, Invitrogen) and sequenced in both directions using the Thermo Sequenase fluorescent labelled primer cycle sequencing kit (Amersham Life Science). Sequence reactions were analysed on the ALFexpress DNA automatic sequencer (Pharmacia Biotech) and sequence analyses were performed using
5 fasta and blast programs (Wisconsin Package, Version 9.1, Genetics Computer Group (GCG)).

Using the above primer (i) for cDNA synthesis and the above primers (ii) and (iii) for PCR amplification, variations in band patterns were observed when comparing samples from MS
10 patients, autoimmune patients and healthy controls. PCR fragments of 850 bp (+/- a few bp) were amplified in samples from 20 MS patients, 3 patients with autoimmune diseases (AU), 1 patient with leukaemia and 10 healthy controls. An additional fragment of 950 bp (+/- a few bp) was amplified in 14 of 37 MS patients, 3/3 AU patients, 1/15 autoimmune controls, 2/14 healthy controls and 1 leukaemia patient.

15

Sequence analysis of the 850 bp fragments and the 950 bp fragments revealed that all were spliced *env* mRNA sequences containing the same splice donor site in the leader region downstream from the PBS sequence and a splice acceptor site in the part of *pol* encoding the integrase. In the 850 bp fragments the two sites were joined directly, whereas in the 950
20 bp fragments an additional region of 104 bp from the protease encoding region were inserted between the two sites, resulting in a second alternatively spliced *env* mRNA.

In the present experiments, primers for PCR were derived from the RTVLH2 (PBS) and RGH2 (*env*) sequences (Mager and Freeman 1987, Hirose et al., 1993) , probably
25 explaining why alternatively spliced *env* mRNA sequences were also seen in healthy samples. It is our experience that PCR primers derived from the RGH2 sequence generally detects a broader group of HERV-H sequence variants and do not discriminate between samples from MS patients, other autoimmune patients or healthy controls (results not shown).

30

The 850 bp sequences shared 90-95 % homology with the RGH2 leader/integrase and *env* region and 93-97% homology with each other. Most of the sequences contain 100 bp of an open *env* reading frame (5' of the *env* PCR primer).

35 The 950 bp sequences shared 87-95% homology with the RGH2 sequence and 98-100%

homology with each other and with a previously published sequence isolated from a human T-cell leukaemia cell line (GenBank accession number u88896, Lindeskog and Blomberg, 1997). Two sequences with a similar splicing structure were isolated from two healthy blood donors (Genbank accession numbers u88897, u88898, Lindeskog and Blomberg, 1997).

- 5 These sequences are 91% and 93% homologous with the cell line sequence, u88896, and 83% and 90% when only the protease regions are compared.

An alignment of sequence variants containing the 104 bp protease encoding region are shown in Table 9.1. The sequences represent 10 clones (7 variants) isolated from 3 MS
10 patients (MS1, MS2 and MS3) and 1 patient with diabetes mellitus (AU1). The sequence variants contain single base changes: substitutions and deletions, as compared to the clone MS1-1. This sequence was found in only one of the MS patients and was identical to the sequence isolated from a T-cell leukaemia cell line (u88896, Lindeskog and Blomberg, 1997).

15

In Table 9.2, sequences of the inserted protease region is compared to the u88896 sequence (see above) with deviations highlighted. The material is the same as in Table 9.1, i.e. 10 independent clonings of the 950 bp fragment from 4 individuals. The region analyzed in Table 9.2 starts at the splice donor site (position 1) and
20 ends at the splice acceptor site (position 104) in the u88896 sequence (reference sequence). This region is designated PSR in the following.

The single PSR sequence cloned from patient MS1 is identical to the reference sequence, while the single PSR sequence from patient MS2 shows two
25 substitutions at positions 82 and 97. These two substitutions are shared by three individuals (MS2, MS3, and AU1). This may indicate that positions 82 and 97 are markers of RGH alleles at the same genetic locus in the human genome or markers of closely related RGH genes at different genetic loci. Similarly, the single-base deletion at PSR position 53 which is shared by two individuals (MS2
30 and AU1) may represent a further such marker.

The single-base changes at position 13 in one PSR-region from patient MS2 and at position 75 in two PSR-regions from AU1 may represent additional genetic markers as described above or may be attributed to errors introduced into
35 proviral DNA during reverse transcription.

Table 9.1. Alignment of sequence variants from the protease encoding region in the alternatively spliced *env* mRNA from three MS patients and one patient with an autoimmune disease: diabetes mellitus.

5

The sequences represent 10 clones: MS1-1, MS2-1, MS2-2 (=AU1-4), MS2-3, MS2-4 (=MS3-1, AU1-3), AU1-1, AU1-2. The splice sites are shown with arrows. Single bp deletions or substitutions relative to MS1-1 are indicated by bold letters. Lower case = unsure base, ~ = not determined, K=G/T, Y=C/T, N=A/C/G/T.

10

		1				50
	MS2-1	CACCTATGAC	CTCAGGTCCT	CAGA.CGANC	CAGCCCAAGA	AACATCTCAC
	MS2-4	CACCTATGAC	CTCAGGTCCT	CAGA.CG.AC	CAGCCCAAGA	AACATCTCAC
	MS2-2	~~~~~	~TCAGGTCCT	CAGA.CG.Ac	CAGCCcAAGA	AACATCTCAC
15	AU1-1	CACCTATGAC	cTCAGGTCCT	CAGA.CG.AC	CAGCCCAAGA	AACATCTCAC
	MS1-1	~~~~~	~~~~~	~~~~~	~~~~~	~~~~~
	MS2-3	CACCTATGAC	CTCAGGTCCT	CAGA.CcGAC	CAGCCCAAGA	AACATCTCAC
	AU1-2	CACCTATGAC	CTCAGGTCCT	CAGACcGAAC	CAGCCCAAGA	AACATCTCAC
20		51	▶			100
	MS2-1	CAATTTCAAA	TCCGATCTTC	TCGGCCTAGC	GGCTGAAGAC	TGACGCTGCC
	MS2-4	CAATTTCAAA	TCCGATCTTC	TCGGCTTAGC	GGCTGAAGAC	TGACGCTGCC
	MS2-2	CAATTTCAAA	TCCGATCTTC	TCGGCTTAGC	GGCTGAAGAC	TGACGCTGCC
	AU1-1	CAATTTCAAA	TCCGATCTTC	TCGGCTTAGC	GGCTGAAGAC	TGACGCTGCC
25	MS1-1	~~~~~	~~~~~	~CGGCTTAGC	GGCTGAAGAC	TGACGCTGCC
	MS2-3	CAATTTCAAA	TCCGATCTTC	TCGGCTTAGC	GGCTGAAGAC	TGACGCTGCC
	AU1-2	CAATTTCAAA	TCCGATCTTC	TCGGCTTAGC	GGCTGAAGAC	TGACGCTGCC
30		101				150
	MS2-1	CGATCGCCTC	GGAAGCCCCC	TAGACCATCA	CGGATGCCGA	GCTTTGGGTA
	MS2-4	CGATCGCCTC	GGAAGCCCCC	TAGACCATCA	CGGATGCCGA	GCTTTGGGTA
	MS2-2	CGATCGCCTC	GGAAG.CCCC	TAGACCATCA	CGGATGCCGA	GCTTTGGGTA
	AU1-1	CGATCGCCTC	GGAAG.CCCC	TAGACCATCA	CGGATGCTGA	GCTTTGGGTA
	MS1-1	CGATCGCCTC	GGAAGCCCCC	TAGACCATCA	CGGATGCCGA	GCTTCGGGTA
35	MS2-3	CGATCGCCTC	GGAAG.CCCC	TAGACCATCA	CGGATGCCGA	GCTTTGGGTA
	AU1-2	CGATCGCCTC	GGAAGCCCCC	TAGACCATCA	CGGATGCTGA	GCTtKGGgTA
40		151	◀			200
	MS2-1	ACTCTCACAG	TGGAAGATCC	CCAGCCATAT	GAAGACAACC	TAGCTGGACG
	MS2-4	ACTCTCACAG	TGGAAGATCC	CCAGCCATAT	GAAGACAACC	TAGCTGGACG
	MS2-2	ACTCTCACAG	TGGAAGATCC	CCAGCCATAT	GAAGACAACC	TAActGGACG
	AU1-1	ACTCTCACAG	TGGAAGATCC	CCAGCCATAT	GAAGACAACC	TAGCTGGACG
	MS1-1	ACTCTCACAA	TGGAAGATCC	CCAGCCATAT	GAAGACAACC	TAGCTGGACG
	MS2-3	ACTCTCACAG	TGGAAGAT.C	CCAGCCATAT	GAAGACAACC	TAGCTGGACG
45	AU1-2	AYTCTCACAG	TGGAAGAT.C	CCAGCCATAT	GAAGACAACC	TAGCTGGACG

(MS2-1: SEQ ID NO:44; MS2-4: SEQ ID NO:45; MS2-2: SEQ ID NO:46; AU1-1: SEQ ID NO:47; MS1-1: SEQ ID NO:48; MS2-3: SEQ ID NO:49; AU1-2: SEQ ID NO:50)

Table 9.2. Alignment of 7 sequence variants (see Table 9.1) from the protease region in the alternatively spliced *env* mRNA from three MS patients, one patient with diabetes mellitus and the sequence isolated from a human T-cell leukemia cell line, u88896 (Blomberg and Lindeskog, 1997). Only variant positions are shown. X = not sequenced.

5	10	20	30	40	50	60	70	80	90	100
	u88896	GATCTTCTCGGCTTAGCGGCTGAAGACTGACGCTGCCCCGATCGCCCTCGGAAGCCCCCTAGACCATCACGGATGCCGAGCTTCGGGTAACTCTCACAATGGAAGA								
10	MS1-1	XXXXXXXXXX								
	MS2-1							T		G
	MS2-2				-			T		G
	MS2-3				-			T		G
15	MS3-1							T		G
	AU1-1							T		G
	AU1-2				-			T		G
	AU1-3							T		G
20	AU1-4							T		G

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20

CLAIMS

1. A human endogenous retrovirus belonging to the RTVL-H/HERV-H family in isolated form or in retroviral particle form that is capable of infecting a mononuclear cell and of
5 replicating therein.
2. A retrovirus according to claim 1 which is obtainable by ultracentrifugation of a cell culture supernatant or cell-free plasma sample in Optiprep™ density gradient medium followed by recovery of the fraction containing retroviral particles .
- 10 3. A retrovirus according to claim 1 or claim 2 that encodes a spliced HERV-H RGH retrovirus *env* mRNA sequence comprising a region from the protease (*pol*) region of said RGH retrovirus.
- 15 4. A retrovirus according to claim 3 wherein the spliced mRNA sequence comprises a region from the protease encoding region that is selected from the group consisting of SEQ ID NO:44; SEQ ID NO:45; SEQ ID NO:46; SEQ ID NO:47; SEQ ID NO:48; SEQ ID NO:49; and SEQ ID NO:50 as defined herein, and variants and subsequences hereof.
- 20 5. A retrovirus according to any of claims 1-4 that is isolatable from a human subject selected from the group consisting of a multiple sclerosis (MS) patient, a patient suffering from an autoimmune disease, a patient suffering from diabetes and a patient suffering from a malignancy.
- 25 6. A retrovirus according to claim 5 that is isolatable from a cell culture derived from peripheral blood of said human subject.
7. A retrovirus according to claim 6 that is isolatable from a cell culture derived from peripheral blood of an MS patient, said cell culture comprising at least 2% CD3+ T-cells.
- 30 8. A retrovirus according to any of claims 1-7 that is capable of infecting a blood cell derived from a human subject not suffering from MS, an autoimmune disease, diabetes or a malignancy, and of replicating therein.
- 35 9. A retrovirus according to claim 1-8 that is capable of infecting a non-blood human cell.

10. A retrovirus according to any of claims 1-9 that is capable of infecting a non-human cell.
- 5 11. A retrovirus according to claim 10 that is capable of infecting a cell derived from an animal selected from the group consisting of a bat, a rabbit and a rodent.
12. A retrovirus according to any of claims 1-11 which is an RGH virus.
- 10 13. A retrovirus according to any of claims 1-12 that comprises a nucleic acid sequence that is at least 80% identical to the RGH-2 virus sequence published by Hirose et al., Virology, 1993, **192**:52-61.
14. A method of detecting *intra-species* infectivity (transmissibility) of a retrovirus
- 15 according to any of claims 1-13, the method comprising the steps of isolating the retrovirus from a patient as defined in claim 5, contacting said isolated virus with a culture of mononuclear cells derived from a human subject not suffering from MS, an autoimmune disease, diabetes or a malignancy, and detecting infection of said cells with the retrovirus.
- 20 15. A method according to claim 14 wherein the infection of the culture of mononuclear cells is detected by the appearance of RT activity in the infected culture or by an assay for rescue of the infecting retrovirus in indicator cells as described hereinbefore.
- 25 16. A method according to claim 14 or 15 wherein the infection of the culture of mononuclear cells is detected by the appearance in cells of said culture of a spliced HERV-H RGH retrovirus *env* mRNA sequence comprising a region from the protease (*pol*) region of said RGH retrovirus, including a region from the protease encoding region that is selected from the group consisting of SEQ ID NO:44; SEQ ID NO:45; SEQ ID
- 30 NO:46; SEQ ID NO:47; SEQ ID NO:48; SEQ ID NO:49; and SEQ ID NO:50 as defined herein, and variants and subsequences hereof.
17. A method of detecting *inter-species* infectivity (transmissibility) of a retrovirus according to any of claims 1-13, the method comprising the steps of isolating the
- 35 retrovirus from a human subject as defined in claim 5, contacting said isolated viruses

with a culture of mononuclear cells derived from a non-human individual, and detecting infection of said cells with the retrovirus.

18. A method according to claim 17 wherein the infection of the culture of mononuclear
5 cells is detected by the appearance of RT activity in the infected culture or by an assay for rescue of the infecting retrovirus in indicator cells as described hereinbefore.

19. A method according to claim 17 or 18 wherein the infection of the culture of mononuclear cells is detected by the appearance in cells of said culture of a spliced
10 HERV-H RGH retrovirus *env* mRNA sequence comprising a region from the protease (*pol*) region of said RGH retrovirus, including a region from the protease encoding region that is selected from the group consisting of SEQ ID NO:44; SEQ ID NO:45; SEQ ID NO:46; SEQ ID NO:47; SEQ ID NO:48; SEQ ID NO:49; and SEQ ID NO:50 as defined herein, and variants and subsequences hereof.

15

20. A method according to any of claims 14-19 wherein the culture of mononuclear cells being contacted is pre-stimulated with a mononuclear cell stimulating compound including PHA.

20 21. A method according to any of claims 14-20 wherein the mononuclear cells are blood cells.

22. A culture of mononuclear cells derived from a human subject selected from the group consisting of a multiple sclerosis (MS) patient, a patient suffering from an autoimmune
25 disease, a patient suffering from diabetes and a patient suffering from a malignancy, said cells are capable of expressing a spliced HERV-H RGH retrovirus *env* mRNA sequence comprising a region from the protease encoding (*pol*) region of said retrovirus.

23. A culture according to claim 22 wherein the spliced mRNA sequence comprises a
30 region from the protease encoding region that is selected from the group consisting of SEQ ID NO:44; SEQ ID NO:45; SEQ ID NO:46; SEQ ID NO:47; SEQ ID NO:48; SEQ ID NO:49; and SEQ ID NO:50 as defined herein, and variants and subsequences hereof.

24. A culture according to claim 22 or 23 which comprises at least 2% CD3+ T-cells.

35

25. A method of diagnosing a disease selected from the group consisting of multiple sclerosis, an autoimmune disease, diabetes and a malignancy, the method comprising isolating mononuclear blood cells from a patient suspected of suffering from any of said diseases and detecting in said cells a mRNA sequence as defined in claims 22 or 23 or a
5 polypeptide transcribed from said mRNA sequence.

26. A method of diagnosing a disease associated with the retrovirus according to any of claims 1-13, the method comprising the steps of isolating the retrovirus from a human subject as defined in claim 5 and testing its infectivity according to the method of any of
10 claims 14-20.

27. A method according to claim 26 wherein the disease being diagnosed is multiple sclerosis.

15 28. A method of treating a patient suffering from a disease, the clinical manifestations of which are associated with the presence of the retrovirus according to any of claims 1-13, the method comprising administering to the patient an effective amount of a pharmaceutical agent that is capable of at least partially preventing the infection of cells in the patient with the retrovirus and/or the migration of the retrovirus to cells in the patient
20 that can be infected by the retrovirus.

29. A method of treating a patient suffering from a disease, the clinical manifestations of which are associated with cells transformed (immortalised) by infection with the retrovirus according to any of claims 1-13, the method comprising administering to the patient an
25 effective amount of a pharmaceutical agent that is capable of at least partially eliminating such transformed (immortalised) cells.

30. A method according to claim 28 or 29 wherein the disease is a disease selected from the group consisting of a demyelinating disease including multiple sclerosis, an
30 autoimmune disease, diabetes and a malignancy.

31. An antiviral agent for use as a medicament in the control of a disease, the clinical manifestations of which are associated with the presence of the retrovirus according to any of claims 1-13.

32. Use of an antiviral agent in the manufacturing of a medicament for controlling a disease associated with the retrovirus according to any of claims 1-13.
33. Use according to claim 32 wherein the disease is selected from the group consisting
5 of a demyelinating disease, a malignancy, diabetes and an autoimmune disease.
34. Use according to claims 32 or 33 wherein the antiviral agent is an agent that selectively inhibits or eliminates the retrovirus according to any of claims 1-13.
- 10 35. Use according to claim 34 wherein the agent is selected from the group consisting of an antibody, an anti-sense nucleic acid and a ribozyme.
36. A method of controlling a disease, the clinical manifestations of which are associated with immunological responses to the retrovirus according to any of claims 1-13, the
15 method comprising administering to the patient an effective amount of a pharmaceutically active compound that is capable of at least partially inhibiting said immunological responses.
37. A method according to claim 36 wherein the pharmaceutically active compound is
20 directed against an immunological response elicited specifically by the retrovirus according to claim 3 or claim 4.
38. A method according to claim 37 wherein the pharmaceutically active compound is directed against inhibiting the biological activity of activated T-cells including CD3+, CD4-
25 and CD8- T-cells or against eliminating such T-cells.
39. A pharmaceutical composition comprising a pharmaceutically active compound that is capable of at least partially inhibiting in a human subject as defined in claim 5 an immunological response to the retrovirus according to any of claims 1-13.
30
40. A composition according to claim 39 wherein the pharmaceutically active compound is directed against an immunological response elicited specifically by the retrovirus according to any of claims 1-13.

41. A composition according to claim 39 or 40 wherein the pharmaceutically active compound is a compound directed against inhibiting the biological activity of activated T-cells including CD3+, CD4- and CD8- T-cells or against eliminating such T-cells.
- 5 42. A pharmaceutical composition comprising a pharmaceutically active compound that is capable of at least partially preventing the infection of cells in a patient with the retrovirus according to any of claims 1-13, and/or the migration of the retrovirus to cells in the patient that can be infected by the retrovirus.
- 10 43. A pharmaceutical composition comprising a pharmaceutically active compound that is capable of at least partially eliminating in a patient cells transformed (immortalised) by infection with the retrovirus according to any of claims 1-13.
44. Use of an allelically occurring spliced HERV-H RGH retrovirus *env* mRNA sequence
- 15 comprising a region from the protease (*pol*) region of said RGH retrovirus as a marker for the occurrence or the state of a disease selected from the group consisting of a demyelinating disease including multiple sclerosis, an autoimmune disease, diabetes and a malignancy.
- 20 45. Use according to claim 44 wherein the mRNA sequence comprises a region from the protease encoding region that is selected from the group consisting of SEQ ID NO:44; SEQ ID NO:45; SEQ ID NO:46; SEQ ID NO:47; SEQ ID NO:48; SEQ ID NO:49; and SEQ ID NO:50 as defined herein, and variants and subsequences hereof.
- 25 46. A method for identifying compounds capable of inhibiting the production of a retrovirus according to any of claims 1-13 from a cell, the method comprising exposing cells capable of producing a retrovirus according to any of claims 1-13 to a candidate compound and comparing the amount of virus produced by the cells in the presence of the compound with the amount of virus produced by the cells in the absence of the compound, and
- 30 thereby identifying compounds capable of inhibiting the production of virus from said cells.
47. A method for identifying compounds capable of preventing infection of a target cell with a retrovirus according to any of claims 1-13, which method comprises exposing target cells susceptible to infection with a retrovirus according to any of claims 1-13 to a sample
- 35 containing the said virus in the presence of a candidate compound, determining the extent

of infection of the target cells in the presence of the compound and thereby identifying compounds capable of preventing infection of the target cell with the virus.

SEQUENCE LISTING

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WITH DEMYELINATING DISEASES AND OTHER DISEASES

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 <213> RETROVIRUS

<400> 38
 Pro Ser Asp Pro Pro Pro Pro Ala Ser Cys Phe Lys Cys Leu Lys Ser
 1 5 10 15
 Gly His Trp Thr Lys Glu Cys Pro Gln Pro Gly Ile Pro Pro Lys Asp
 20 25 30
 Ser Pro Ile Cys Ala Gly Pro His Trp Lys Lys Asp Cys Pro Pro
 35 40 45

<210> 39
 <211> 47
 <212> PRT
 <213> RETROVIRUS

<220>
 <221> SITE

10

<222> (41)

<223> Xaa represents stop codon

<400> 39

Pro	Pro	Asp	Pro	Pro	Pro	Pro	Gly	Ala	Cys	Tyr	Thr	Cys	Gln	Lys	Ser
1				5					10					15	

Gly	His	Trp	Ala	Lys	Glu	Cys	Pro	Gln	Arg	Gly	Ile	Pro	Pro	Lys	Pro
			20					25					30		

Arg	Pro	Ile	Cys	Val	Gly	Pro	His	Xaa	Lys	Ser	Asp	Cys	Ser	Thr
		35					40					45		

<210> 40

<211> 47

<212> PRT

<213> RETROVIRUS

<220>

<221> SITE

<222> (25)

<223> Xaa represents stop codon

<220>

<221> SITE

<222> (41)

<223> Xaa represents stop codon

<400> 40

Pro	Pro	Asp	Pro	Pro	Pro	Pro	Gly	Ala	Cys	Tyr	Thr	Cys	Trp	Lys	Ser
1				5					10					15	

Gly	His	Trp	Ala	Lys	Glu	Cys	Pro	Xaa	Pro	Gly	Ile	Pro	Pro	Lys	Pro
			20					25					30		

Arg	Pro	Ile	Cys	Val	Gly	Pro	His	Xaa	Lys	Ser	Asp	Cys	Ser	Thr
		35					40					45		

<210> 41

<211> 30

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Primer

<400> 41

gggaattagt ggaataactc ttttttggtg

30

<210> 42

<211> 27

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Primer

<400> 42

catgaaattt ggtgccttga ctcggat

27

<210> 43
 <211> 28
 <212> DNA
 <213> Artificial Sequence

<220>
 <223> Description of Artificial Sequence: Primer

<400> 43
 gagatagctg gggagaggta gaggatga 28

<210> 44
 <211> 199
 <212> DNA
 <213> RETROVIRUS

<400> 44
 cacctatgac ctcaggctct cagacgancc agcccaagaa acatctcacc aatttcaaatt 60
 ccgatcttct cggcctagcg gctgaagact gacgctgccc gatcgctcgg gaagccccct 120
 agaccatcac ggatgccgag ctttgggtaa ctctcacagt ggaagatccc cagccatatg 180
 aagacaacct agctggacg 199

<210> 45
 <211> 198
 <212> DNA
 <213> RETROVIRUS

<400> 45
 cacctatgac ctcaggctct cagacgacca gcccaagaaa catctcacca atttcaaattc 60
 cgatcttctc ggcttagcgg ctgaagactg acgctgcccg atcgctcggg aagcccccta 120
 gaccatcacg gatgccgagc tttgggtaac tctcacagtg gaagatcccc agccatatga 180
 agacaacctc gctggacg 198

<210> 46
 <211> 186
 <212> DNA
 <213> RETROVIRUS

<400> 46
 tcaggctctc agacgaccag cccaagaaac atctcaccaa tttcaaattcc gatcttctcg 60
 gcttagcggc tgaagactga cgctgcccga tcgctcggga agcccctaga ccatcacgga 120
 tgcgagcctt tgggtaactc tcacagtgga agatccccag ccatatgaag acaacctaac 180
 tggacg 186

<210> 47
 <211> 197
 <212> DNA
 <213> RETROVIRUS

<400> 47
 cacctatgac ctcaggctct cagacgacca gcccaagaaa catctcacca atttcaaattc 60
 cgatcttctc ggcttagcgg ctgaagactg acgctgcccg atcgctcggg aagcccctag 120
 accatcacgg atgctgagct ttgggtaact ctacacagtg aagatcccca gccatatgaa 180
 gacaacctag ctggacg 197

<210> 48
 <211> 129
 <212> DNA
 <213> RETROVIRUS

<400> 48

cggcttagcg gctgaagact gacgctgccc gatcgccctcg gaagccccct agaccatcac 60
ggatgccgag cttcgggtaa ctctcacaat ggaagatccc cagccatatg aagacaacct 120
agctggacg 129

<210> 49

<211> 197

<212> DNA

<213> RETROVIRUS

<400> 49

cacctatgac ctcaggtcct cagaccgacc agcccaagaa acatctcacc aatttcaaata 60
ccgatcttct cggcttagcg gctgaagact gacgctgccc gatcgccctcg gaagccccta 120
gaccatcacg gatgccgagc ttggggtaac tctcacagtg gaagatccca gccatatgaa 180
gacaacctag ctggacg 197

<210> 50

<211> 199

<212> DNA

<213> RETROVIRUS

<400> 50

cacctatgac ctcaggtcct cagaccgaac cagcccaaga aacatctcac caatttcaaaa 60
tccgatcttc tcggcttagc ggctgaagac tgacgctgcc cgatcgccctc ggaagcccc 120
tagaccatca cggatgctga gcttkgggta aytctcacag tggaagatcc cagccatatg 180
aagacaacct agctggacg 199