MOLECULAR TECHNIQUES FOR THE DETECTION AND CHARACTERISATION OF A NOVEL RETROVIRUS ASSOCIATED WITH MULTIPLE SCLEROSIS

BY

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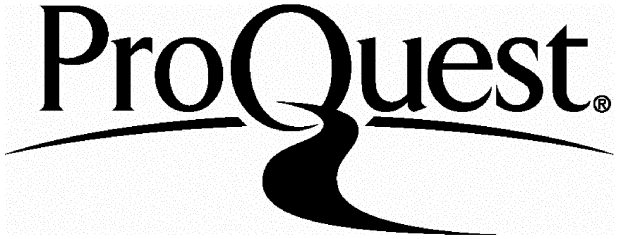

Abstract

Multiple sclerosis (MS) is thought to be an autoimmune disease precipitated in genetically susceptible individuals by environmental factors. Recent attention has focused on the possible involvement of retroviruses in its aetiology. Initial experiments performed to detect the human retrovirus HTLV, in lymphocytes from 12 patients with MS, proved negative.

In an attempt to identify a putative novel human retrovirus, a polymerase chain reaction (PCR) technique was developed which was capable of detecting a very diverse range of retroviruses including HIV, HTLV, MPMV and MMLV. This 'Pan-Retrovirus' PCR employed semi-nested, degenerate primers complementary to the two most highly conserved motifs of the *pol* gene. Using this technique a novel retroviral sequence, designated MSRV c-*pol*, was detected in the serum from a patient with a 12 year history of MS. This sequence was also present in retroviral particles which had been isolated from MS patient derived tissue cultures in France. MSRV is related to the endogenous retrovirus ERV-9, however it remains uncertain whether MSRV itself is an exogenous or endogenous retrovirus.

By combining the 'Pan-Retrovirus' PCR with a hybridisation-based detection assay, MSRV c-*pol* RNA was detected in serum from 24 of 40 (60%) patients with MS but not in 30 controls; and in cerebrospinal fluid from 5 of 10 patients with MS but not in 10 other neurological disease controls. An MSRV specific RT-PCR assay was also developed. This detected virion-associated MSRV *pol* RNA in serum from 9 of 17 (53%) patients with clinically active MS (in all 6 of those not undergoing immunosuppressive treatment), compared with only 3 of 44 (7%) controls.

A novel human retroviral sequence has been identified, and an association demonstrated between the presence of MSRV-RNA and MS. Further work will be required to determine the significance of MSRV in the aetiopathogenesis of this common neurological disease.

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Abbreviations

1°	Primary
2°	Secondary
A	adenine
ALSV	Avian Leukosis-Sarcoma Virus
Amp	Ampicillin
AMV	Avian Myeloblastosis Virus
APC	Antigen Presenting Cell
Arg	arginine
BAEV	Baboon Endogenous Virus
BLV	Bovine Leukaemia Virus
bp	base pairs
BSA	Bovine Serum Albumin
C	cytosine
CA	capsid
CaMV	Cauliflower Mosaic Virus
CAT	Chloramphenicol Acetyltransferase
cfu	colony forming units
CNS	Central Nervous System
CPM	Counts Per Minute
CSF	Cerebro Spinal Fluid
Dept.	Department
DMSO	dimethyl sulfoxide
DNA	DeoxyriboNucleic Acid
DNase	Deoxyribonuclease
DPM	Decays Per Minute
*Dra*1	*Deinococcus radiophilius* 1
DTT	dithiothreitol
EAE	experimental allergic encephalitis
EDSS	expanded disability status scores
EDTA	disodium ethylenediaminetetra-acetate
EIAV	Equine Infectious Anaemia Virus
ELOSA	enzyme linked oligo-sorbent assay
EM	electron microscopy
EMBL	European Molecular Biology Laboratory
env	envelope
ERV	endogenous retrovirus
F-MuLV	Friend-Murine Leukaemia Virus
FCS	foetal calf serum
G	guanine
gag	group specific antigen
GaLV	Gibbon Ape Leukaemia Virus
*Hae*III	*Haemophilus aegyptius* III
HBV	Hepatitis B Virus
HCV	hepatitis C virus
HEPA	high efficiency particulate air filter
HIV1	Human Immunodeficiency Virus type 1
HIV2	Human Immunodeficiency Virus type 2
HMSRV	Human Multiple Sclerosis associated Retrovirus

HSERV-9 (ERV-9),	Human Sequence of Endogenous Retrovirus 9
hsps	heat shock proteins
HSRV	human spuma retrovirus
HTLV-I	Human T-cell Leukaemia Viruse type I
HTLV-II	Human T-cell Leukaemia Viruse type II
HUMER41	Human Endogenous Retroviral sequence clone 41
I	inosine
IAP	Intracisternal A-type Particle
IDDM	insulin-dependent diabetes mellitus
IFN	interferon
Ig	immunoglobulin
IPTG	Isopropylthio-β-D-galactoside
Jr.	junior
kbp	kilo base pairs
kDa	kilo Daltons
LB	Luria-Bertani
LINES	long interspersed sequences.
LTR	long terminal repeat
Lys	lysine
M	molar
MAG	myelin associated glycoprotein
MALDI-TOF	matrix assisted laser desorption/ ionisation time of flight
MBP	myelin basic protein
MHC	major histocompatability complex
min	minute
MLV	murine leukaemia virus
MMLV	Moloney Murine Leukaemia Virus
MMTV	Mouse Mammary Tumour Virus
MOI	Multiplicity Of Infection
MoMLV	Moloney-Murine Leukaemia Virus
MP	methylprednisilone
MPMV	Mason-Pfizer monkey virus
MRI	Magnetic Resonance Imaging
mRNA	messenger RNA
MS	Multiple Sclerosis
MSRV	Multiple Sclerosis associated RetroVirus
MW	molecular weight
NC	nucleoprotein
NEN	New England Nuclear
nm	nanometer
OD	optical density
OPD	o-phenylenediamine
ORF	open reading frame
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PCP	*Pneumocystis carnii* pneumonia
PCR	polymerase chain reaction
Pfu	*Pyrococcus furiosus*
PLP	Proteolipid Protein

pol	polymerase
Pwo	*Pyrococcus woesei*
RA	rheumatoid arthritis
RNA	ribonucleic acid
RNase	ribonuclease
RPMI	Roswell Park Memorial Institute
RR	relapsing remitting
RSV	Rous sarcoma virus
RT	reverse transcriptase
RT-PCR	combined reverse transcriptase and polymerase chain reaction
RTVL-H	Retrovirus-Like Human endogenous sequence
S	Svedberg unit
SD	standard deviation
secs.	seconds
SFV	Simian Foamy Virus
SFVcpz	chimpanzee simian foamy virus
SINES	short interspersed sequences.
SIV1	Simian Immunodeficiency Virus type 1
SIV_{mac}	simian immunodeficiency virus macaque
SMRV-H	Simian Monkey Retrovirus H
SNAP™	simple nucleic acid preparation
SRV-2	Simian Retrovirus type 2
*Ssp*1	*Sphaerotilus natans*1
STLV-I	Simian T-cell lymphotropic virus I
STLV-II	Simian T-cell lymphotropic virus II
T	thymine
Taq	*Thermus aquaticus*
TCA	trichloroacetic acid
TcR	T cell receptor
TE	Tris EDTA
Tm	melt temperature
Tth	*Thermus thermophilus*
U	uracil
UCLMS	University College London Medical School
USB	United States Biochemical
VEP	visual evoked potential
uv	ultraviolet
v/v	volume/volume
VISNA	Visna virus
w/v	weight/volume
X-gal	5-Bromo-4-chloro-3-indolyl-β-D-galactoside
xg	times gravity

PUBLICATIONS ARISING FROM THIS THESIS

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1 INTRODUCTION

1.1 Retroelements

In order to understand the role of the retroviruses, both exogenous and endogenous, in both the pathogenic and non-pathogenic context, it is necessary to place them within the setting of retroelements as a whole. The unifying feature of retroelements is that they have been generated by reverse transcription. This group of entities forms a continuum from purely repetitive sequences in genomic deoxyribonucleic acid (DNA) such as short interspersed sequences (SINES), through long interspersed sequences (LINES) and pseudogenes, to complex fully functional retroviruses.

Earliest pre-cellular life is thought to have been ribonucleic acid (RNA) based, initially with self replicating RNAs and then later with RNA genomes and their encoded proteins (Weiner, 1987). The transition to DNA as the genetic material required the evolution of reverse transcriptase (RT). The evolution of the retroelement, and hence RT, is considered as one of the key initial stages in the evolution of life. It may well be the case that the retrotransposons (described below), represent molecular fossils of this evolution from RNA based life to DNA based. The ubiquitous nature of retroelements across kingdoms is consistent with this hypothesis.

The simplest retroelements are highly repetitive non-coding sequences of DNA e.g. *Alu* family and SINES. Then come the pseudogenes which have been generated by reverse transcription and integration of cellular RNAs and hence have coding capability. Pseudogenes that have acquired an upstream promoter sequence and can be actively transcribed are termed retrogenes. Pseudogenes and other repetitive non-long terminal repeat (LTR) sequences e.g. SINES and *Alu* sequences are thought to have been generated by LINE encoded RT (Hutchison III *et al.* 1989; Jenson *et al.* 1991) rather than by misdirected retroviral co-packaging (Dornburg and Temin, 1990; Levine *et al.* 1990).

Retroelements are found in organisms from Eubacteria to Eukaryotes. Retroelements which encode their own RT (retrotransposons) are phylogenetically related and computer alignment of all these sequences has allowed phylogenies to be constructed (Doolittle *et al.* 1989; Xiong and Eickbush, 1990). From these phylogenetic trees one can attempt to reconstruct the history of events leading to the formation of these entities. There are also homologous proteins in some plant and animal DNA viruses (Toh *et al.* 1985), and their reverse transcriptase is similar to sequences found in the introns of some fungal mitochondria (Michel and Lang, 1985).

Retrotransposons can be phylogenetically divided into two main groups based upon their reverse transcriptase (RT) sequences (Xiong and Eickbush, 1990). Firstly, there is the branch containing the LINE like sequences, or non-LTR retrotransposon group, which includes the Group II introns, the *Neurospora crassa* Mauriceville

mitochondrial plasmid, RT-like sequences of *Chlamydomonas reinhardtii* (Boer and Gray, 1988), multicopy single stranded DNA (msDNA) found in bacteria, and telomerases (Eickbush, 1997). Secondly, there is the branch containing the LTR retrotransposons, both the Copia/Ty1 group and Gypsy group of transposable elements, and also the true retroviruses as well as the hepadnaviruses and caulimoviruses (both DNA viruses which do not possess LTRs) (Xiong and Eickbush, 1990).

LINES represent the next level of complexity being retroposons or retrotransposons of the non-LTR type. Retroposons encode the RT which is responsible for their own retrotransposition. They also possess an internal GC rich promoter and a partially characterised protein, ORF1. Retrotransposons (of the LTR type) are distinguished by the possession of a capsid gene, the equivalent of the retroviral *gag*, and LTRs which contain the enhancer/promoter sequences responsible for their own expression.

The most complex type of retroelement is the retrovirus which contains at least one extra gene, the envelope gene (*env*), in addition to the genes present in retrotransposons. The *env* gene would appear to be the final acquisition necessary for the formation of a fully infectious viral particle. Retroviruses can be divided into two types, exogenous and endogenous, according to their mode of transmission. In general, endogenous retroviruses can be considered to be the molecular fossils of ancient viral infections of the germ cell line which have remained quiescent. The fact that the majority of endogenous retroviral sequences are defective to a greater or

lesser degree supports this theory. However, an alternative and perhaps equally valid view, is that exogenous retroviruses represent a currently activated form of an endogenous retrovirus (Lower *et al.* 1996). By entering the germ cell line endogenous retroviruses have escaped from the selection pressure for maintaining the integrity of their open reading frames (ORFs). Their new mode of transmission results in a lack of necessity for formation of viral particles.

Recombinatorial exchange between retroelements, as between retroviruses, would seem to be an extremely rare event, as evidenced by the concordance between the RT based phylogeny and the genome structure of the element (Xiong and Eickbush, 1990). LTR containing retrotransposons fall on the LTR branch and non LTR containing retrotransposons fall on the non LTR branch of their RT based phylogenetic tree. Similarly, there is no evidence for genomic exchange between members of the different groups of retroelements present in the same species. However, there is evidence for limited genomic exchange within the retroviruses, (typically within *env*), giving rise to new retroviruses (McClure *et al.* 1988). The acquisition of new genes and functional abilities during the evolution of retroelements is also thought to have been due to recombination.

Retrotransposons are present in the genomes of organisms as evolutionarily diverse as animals, plants, protozoans and fungi (Garfinkel 1992). Closely related retrotransposons may be present in organisms which are evolutionarily extremely distant. However, the viruses are contained within individual taxa, the

caulimoviruses are restricted to plants, and the hepadnaviruses and retroviruses to vertebrates. It is not possible to simply superimpose the deduced evolutionary histories of the retroelements on that of the host organisms.

The interpretation of such phylogenies in terms of the evolutionary relatedness of the various sequences is fraught with difficulty. Although a phylogeny gives an accurate representation of the current degree of homology of the various entities, the implied inference of past temporal ancestries may well be misleading.

The high rates of divergent evolution observed for exogenous retroviruses, in particular those that are not highly cell associated such as HIV-1 and HIV-2, and the overall phylogeny and species distribution of retroviral sequences would appear to be incompatible. If retroviruses diverged at the rate currently observed for HIV then their common ancestor would only date back 10,000 years or so (Doolittle *et al.* 1989). The species diversity of retroviral infections alone, (not even accounting for the endogenous retroviruses), from fish and foul to mammals, would argue against this. The most likely explanation for this apparent contradiction would be that exogenous retroviruses and their associated high rate of genetic change, represent a very short period in the evolutionary history of any given retrovirus. The limited number of exogenous retroviruses in comparison to endogenous retroviral sequences would appear to support this assertion.

Cross species transfer of retroviruses would also be a feature of this model of retroviral evolution. HIV may be a classic example in this sense, representing a recent zoonosis. All the major sub types of HIV 1 (A-G) are thought to have originated from a single zoonotic transmission event within the last 50 years or so (Weiss, 1998). Allowing for such zoonoses it has been estimated that retroviruses evolved after vertebrate evolution and perhaps subsequent to mammalian evolution (Doolittle *et al.* 1989). The time period at which the evolution of individual retroviruses took place can similarly be estimated. Human endogenous retrovirus (HERV) evolution can also be traced, and the majority of retrotransposition events and the most significant expansion seems to date back to early primate evolution (Haltmeier *et al.* 1995). Endogenous retroviruses can be used like any other genetic marker to date the history of their evolution and incorporation into the genome (section 1.1.1.10.2) (Arvidsson *et al.* 1995; Svensson *et al.* 1996; Andersson *et al.* 1998; Svensson and Andersson, 1997; Svensson *et al.* 1995; Widegren *et al.* 1996). The majority of HERVs demonstrate sequence homology to murine or primate retroviruses (Rasmussen *et al.* 1993) and only a few are related to the exogenous human retroviruses, HIV (Tonjes *et al.* 1996) (Shih *et al.* 1989) (Perl *et al.* 1989), HTLV (Perl *et al.* 1989; Shih *et al.* 1989) and human foamy virus (Cordonnier *et al.* 1995).

It is interesting to note that the most variable region of the retroviral genome is the *env* gene and this applies to both the endogenous and exogenous forms. Endogenous retroviruses with defective *env* genes may be able to use in *trans* the gene products of other retroviruses and hence maintain their ability to transmit via viral

pseudotypes (Lusso *et al.* 1990; Hu and Temin, 1990a; Hu and Temin, 1990b). The most defective endogenous retroviruses may be considered to be the lone LTR elements. These are commonly found at high copy levels in the genome and are thought to be the result of recombination between retroviral LTRs with excision of the internal sequence.

1.1.1 Retroviridae

1.1.1.1 History

Retroviruses were first isolated at the beginning of the century during studies of the aetiology of infectious diseases. Equine infectious anaemia, which is now known to be due to a mammalian C-type retrovirus, was one of the first diseases shown to have a viral aetiology by the filterable nature of the causative agent (Vallee and Carre 1904). In 1911 Peyton Rous isolated Rous sarcoma virus (RSV) as the cause of a spontaneous chicken sarcoma (Rous 1911). The virus was observed under EM in 1947 by Claude and colleagues (Claude *et al* 1947) but the morphology of the viral particle was not determined until thin section methods were developed (Gaylord 1955).

1.1.1.2 Classification

The classification of retroviruses into a single family is warranted by the unique character of their genome and mode of replication, and by their common physical, biochemical and morphological properties. Nucleotide sequence analysis of their genomes has confirmed that retroviruses represent an individual family of viruses and has enabled further phylogenetic analysis.

Retroviruses are classified as a family within the taxon of reverse transcribing viruses, which also includes the *Caulimoviridae* and *Hepadnaviridae* families of viruses (Mayo and Pringle 1997) as detailed below.

Family *Retroviridae*

Genus Spumavirus

This includes Human spuma retrovirus (HSRV) and Simian spuma retrovirus

Genus unnamed, mammalian B-type retroviruses

Mouse mammary tumour virus (MMTV)

Genus unnamed, mammalian and reptilian C-type retroviruses

This includes many murine leukaemia viruses (MLV)-related e.g. Abelson, AKR (endogenous), Friend, Maloney murine leukaemia viruses (MMLV); many murine

sarcoma viruses (e.g. Harvey, Kirsten, Moloney murine sarcoma viruses); feline leukaemia virus; feline sarcoma viruses; gibbon ape leukaemia virus; woolly monkey sarcoma virus; porcine type C virus; guinea pig type C virus; and viper type C virus.

Genus unnamed, avian type C retroviruses

This includes Rous sarcoma virus (RSV); avian carcinoma viruses; avian sarcoma viruses; avian leukosis viruses; avian myeloblastosis viruses (AMV); avian reticuloendotheliosis viruses; and duck spleen necrosis virus.

Genus unnamed, type D retroviruses

This includes Mason-Pfizer monkey virus (MPMV); simian type D virus 1; Langur type D virus; squirrel monkey type D virus; and ovine pulmonary adenocarcinoma virus (Jaagsiekte).

Genus unnamed, HTLV/BLV viruses

This includes human T-cell lymphotropic viruses (HTLV-I and HTLV-II), simian T-cell lymphotropic viruses (STLV-I and STLV-II) and bovine leukaemia virus (BLV).

Genus Lentivirus

This includes human immunodeficiency viruses (HIV-1 and HIV-2), simian immunodeficiency viruses (SIV) (African green monkey, sooty mangabey, stump-tailed macaque, pig-tailed macaque, Rhesus, chimpanzee, and mandrill viruses); visna/maedi virus; caprine arthritis-encephalitis virus; equine infectious anaemia virus; feline immunodeficiency virus; and bovine immunodeficiency virus.

Alternatively, retroviruses can be divided into four groups according to their RT sequence based phylogenies; the MLV group (which includes the spumaviruses), the MMTV/RSV group, the HTLV/BLV group and the lentiviral group (Xiong and Eickbush, 1990).

Retroviruses can also be divided into two subgroups according to their mode of transmission. Exogenous retroviruses can be regarded as those where transmission occurs via an extracellular intact viral particle, whereas endogenous retroviruses are inherited in Mendelian fashion. The division of retroviruses into these two categories is not straightforward, since families of retroviruses exist with both exogenous and endogenous members. Also an endogenous retrovirus may be infectious in a different host context (Weiss, 1984).

Retroviruses are further classified on the basis of the range of host species they can infect. This is a reflection of the species distribution of the cellular receptor to which the virus binds. Xenotropic viruses are ones which are normally endogenous in their natural host species, where they do not usually replicate, but have a wide range of heterologous species which they can infect. Ecotropic viruses will only grow in cells of the species from which they were isolated. Amphotropic viruses are capable of both growing in cells of the species from which they were isolated and have a wide range of heterologous species which they can infect (Teich, 1984; Weiss, 1984).

The criteria used to classify retroviruses may also include genome structure as well as morphology, structure and disease associations (Sections 1.1.1.4 and 1.1.1.5).

1.1.1.3 Replication strategy

1.1.1.3.1 Virion-receptor interactions

The virus binds to a specific cell surface molecule which acts as a receptor. To date only a few retroviral receptors have been identified but they vary greatly in structure, function and tissue distribution. Their only common feature is that they are all cell surface proteins with transmembrane domains.

The principal HIV-1 receptor is CD4, which is part of the immunoglobulin superfamily and is involved in the interaction of helper T cells with antigen presenting cells. Recently two of the long searched for co-receptors for HIV-1 were identified and found to be chemokine receptors. The CXCR4 molecule is the major co-receptor for cellular entry by T-cell tropic HIV-1 strains and the CCR5 molecule is the major co-receptor for macrophage tropic strains (Doranz *et al.* 1997).

The receptor for the ecotropic murine leukaemia virus is Rec-1, the receptor for the rat amphotropic virus is Ram-1 and the receptor for the gibbon ape leukaemia virus is Glvr-1. All of these three proteins have the numerous transmembrane domains characteristic of membrane transport systems. Rec-1 is a transporter of basic amino acids (Kim *et al.* 1991; Wang *et al.* 1991) and Ram-1 and Glvr-1 are phosphate transporters (Johann *et al.* 1993; Kavanaugh *et al.* 1994). Glvr-1 also acts as a receptor for feline leukaemia virus subgroup B (Takeuchi *et al.* 1992) and simian

sarcoma virus (Tailor *et al.* 1993). It is interesting to note that these viruses are all related members of the mammalian C-type genus of retroviruses.

The bovine leukaemia virus receptor has a single transmembrane domain but it is unrelated to any of the other known retroviral receptors and its function remains to be determined (Ban *et al.* 1993).

The receptor for Rous sarcoma virus and subgroup A avian leukosis-sarcoma virus has been identified as the protein Tva. This resembles a single membrane spanning domain of the much more complex low density lipoprotein receptor (Bates *et al.* 1993; Young *et al.* 1993).

A putative cellular receptor for the feline immunodeficiency virus has been identified as the feline homologue of CD9. This identification was achieved by using a monoclonal antibody which blocks infection (Hosie *et al.* 1993; Willett *et al.* 1994). Initial reports of a similar approach identifying a putative cellular receptor for HTLV-I (Gavalchin *et al.* 1993) have not been confirmed. Recently a novel MMTV receptor has been identified which has no homology with any known membrane proteins (Golovkina *et al.* 1998b). Once a retrovirus has bound to a cell, the exact mechanism by which viral penetration occurs has not been fully elucidated.

Virus interference

The infection of a cell by a given retrovirus results in resistance to superinfection with that retrovirus and with any others which utilise the same receptor molecule. This phenomenon is termed interference and arises from the interaction of the retroviral Env protein with the receptor. Virion formation is not necessary for interference to occur, indicating that the Env protein produced by an infected cell can interact in this manner with the receptor from the same cell. The exact mechanism of this process is not known but it has been shown for Rec-1, the ecotropic MLV receptor, that the important step is the binding and continued blocking of the receptor by the Env protein. This blocking of the receptor does not interfere with the normal cellular functioning of the protein (Coffin 1996).

The viral glycoproteins encoded by the *env* genes of endogenous retroviral genomes are frequently expressed as host antigen at the cell surface. The expression of these endogenous retroviral *env* genes may thus protect the host organism against infection by a closely related pathogenic exogenous retrovirus by receptor interference (Lower *et al.* 1996). This subject is reviewed in further detail by Coffin (1996) and Weiss (1984).

1.1.1.3.2 Reverse transcription

Retroviruses replicate by means of their virally encoded reverse transcriptase (RT). This enzyme is packaged within the virion and is present at between 15 and 50 molecules (Pyra *et al.* 1994). Reverse transcription takes place within the core particle, whether this occurs entirely in the cytoplasm of the newly infected cell, or at least partially within that of the progenitor is uncertain. Extracellular viral particles may contain replicative intermediates which possess at least partial cDNA copies of the viral genome (Zhu and Cunningham, 1993; Lori *et al.* 1992; Trono, 1992). In addition, other retroviral RNAs (predominately those transcripts including a packaging signal, ψ) and even various transfer RNAs (tRNAs), 5 Svedberg units (S) rRNAs and cellular messenger RNAs (mRNAs) (e.g. globin) may be co-packaged within the virion (Coffin, 1996).

Infection of a cell by a retrovirus results in the synthesis of a cDNA copy of the viral genome by the viral RT, the RNA within the DNA:RNA hybrid is then degraded by the RNase H activity of the enzyme and the synthesis of the second strand of the DNA is initiated. The linear double stranded DNA copy is completed prior to integration into the host's genome. Circularised forms of the double stranded DNA are also formed, these may possess one or two LTRs (Farnet and Haseltine, 1991). Initially the two LTR form was thought to be the pre-integration form of the virus (Panganiban and Temin, 1984) but these DNA circles are now thought to be replication artefacts (Barbosa *et al.* 1994; Brown *et al.* 1989).

1.1.1.3.3 Nuclear transport of DNA

The precise mechanism of nuclear transport of the pre-integration complex of viral proteins and double stranded DNA has not been fully elucidated but the size of the complex would seem to preclude entry through the nuclear pores (Bowerman *et al.* 1989). This conclusion would appear to be supported by the observation that cell division and the preceding breakdown of the nuclear membrane are a prerequisite for integration (Roe *et al.* 1993) with only one of the daughter cells becoming infected (Hajihosseini *et al.* 1993). However this is not true for all retroviruses, lentiviruses seem to employ a separate mechanism not requiring mitosis (Lewis and Emerman, 1994).

1.1.1.3.4 Integration

Integration of the retroviral DNA genome into that of the host is generally considered to be a prerequisite for viral replication. The site of integration is precise with respect to the viral genome, but essentially random with respect to that of the host. Integration occurs by a strand transfer mechanism which has an absolute requirement for the viral integrase protein (IN) but may also normally include other proteins. There is a reported preference for transcriptionally active regions i.e. where the DNA within the chromatin structure is more accessible, generally these are non methylated regions (Rohdewohld *et al.* 1987; Scherdin *et al.* 1990).

The integrated provirus can become part of the inherited genetic material of the host genome if it infects germ line cells. All animal genomes are known to contain retroviral sequences. The majority of these sequences are quiescent, forming a substantial proportion of the repetitive genomic sequences. Frequently they are located in the methylated sections of the genome and consequently not usually expressed, although some endogenous retroviruses are capable of forming intact retroviral particles. Endogenous retroviruses can be activated by certain chemicals such as mutagens or carcinogens (Irons *et al.* 1987) e.g. the chemical carcinogens 5-iododeoxyuridine (IUdR) and 5-bromodeoxyuridine (BUdR) (Lowy *et al.* 1971, Stoye and Moroni, 1983) or by mitogens (Stoye and Moroni, 1985), by radiation with UV and X-rays (Weiss *et al.* 1971) and γ-rays, and by other mechanisms such as DNA-viruses or physiological processes (e.g. ageing) to express antigens or to form infectious virus particles.

1.1.1.3.5 Transcription

Once the DNA provirus has integrated into the host genome the expression of the virus is controlled by the enhancer and promoter elements of the 5' LTR. These are contained within the U3 region of the LTR and frequently have duplicated enhancer motif regions, as is also often found in cellular genes. Typical cellular transcription factor binding motifs are frequently found in these U3 regions e.g. AP-1, SP1, CCAAT and TATA boxes, and hormonally and tissue specific responsive elements may also be present (Majors, 1990). Transcription of the proviral DNA utilises the

ordinary cellular transcription machinery, the RNA being transcribed by the cellular RNA polymerase II. The full length RNA transcripts are consequently capped at their 5' terminus and cleaved and polyadenylated at the 3' end of R following the polyadenylation signal AAUAAA. These full length genomic RNAs then have a variety of subsequent fates, a proportion are destined to become new viral genomes and are transported from the nucleus to the cytoplasm and assembled into the nascent viral particles. The precise cellular location of this process depends on the type of virus (Section 1.1.1.5). Another fraction is also transported to the cytoplasm but functions as the polycistronic mRNA for the Gag and Pol proteins. Finally, some of the transcripts are spliced at various splice junctions to form the mRNAs for the Env proteins and any accessory regulatory proteins (e.g. Tax/Rex of HTLV, Tat/Rev of HIV) (Coffin, 1996).

1.1.1.3.6 Translation

The full length genomic RNA and the spliced viral mRNAs all maintain the same 5' LTR structure. This serves as the initiation site for translation, ribosomal subunits binding initially to the capping group and progressively scanning the RNA 5' to 3', with protein synthesis commencing at the *gag* AUG initiation codon. The *gag*, *pro*, and *pol* genes form a single translational unit. This is expressed in differing lengths to different extents. Hence Gag, Gag-Pro, and Gag-Pro-Pol proteins are synthesised on free polyribosomes from the same genomic length mRNAs. Only between one tenth and one twentieth of the amount of Pol as Gag is synthesised, which reflects

the enzymatic verses structural roles of these products. Most retroviruses produce either Gag or Gag-Pro rather than both. The precise mechanisms by which this proportionality is ensured differ between the retroviruses but all consist of a partial block to translational read-through. (Hatfield *et al.* 1992).

The first mechanism identified was the nonsense suppression which occurs in the murine leukaemia virus (MLV). The *gag* and *pro-pol* genes are in the same reading frame, but there is an amber stop codon (UAG) separating them. This provides a weak translational stop codon, which is occasionally read-through by the translational machinery of the cell by the mis-incorporation of a glutamine residue (CUG or CUA) (Yoshinaka *et al.* 1985). Also involved are a purine rich sequence immediately downstream of the UAG, a 49 bp sequence which forms a stem loop structure, and a sequence which base pairs with this loop to form a structure termed a pseudoknot. This pseudoknot is thought to impede the passage of the ribosome. (Feng *et al.* 1992).

The second mechanism is ribosomal frame-shifting, where for the Pro or Pol protein to be synthesised, the ribosome has to slip since the protein is coded for in a different reading frame. As with the nonsense suppression mechanism there is a stop codon, but this is overcome by frame-shifting rather than by simple mis-incorporation. A shift to the -1 frame requires, in all viruses studied, the stop codon and a specific (but unique) seven base A-U rich sequence at the frame-shift site, followed by a pseudoknot. The efficiency of ribosomal frameshifting is controlled so that the proportion of Gag and Pol proteins is constant. In avian leukosis-sarcoma virus

(ALSV) where only one ribosomal frame-shift takes place, the probability of it occurring is ~5% (Arad *et al.* 1995). In HTLV-II and MMTV where two ribosomal frameshifts are required the probability of the first occurring is ~25% and the second ~10% hence the ratio of Gag to Pol is ~2.5% (Hizi *et al.* 1987; Jacks *et al.* 1987; Mador *et al.* 1989). Spumaviruses require a +1 frame shift to occur but the mechanism behind this is not clear (Jacks *et al.* 1988; Kupiec *et al.* 1991).

The Env protein is synthesised from spliced viral RNA on polyribosomes of the rough endoplasmic reticulum (RER), in the same manner as ordinary cell surface proteins (Lake, 1981) . The Env proteins contain a signal peptide sequence at their amino terminus which directs their synthesis to the RER, and as with cellular proteins it is subsequently cleaved. The Env precursor proteins are anchored in the RER by their hydrophobic trans-membrane domain which is located toward the carboxy terminus of the protein. Deletion of this sequence results in the synthesis of a soluble truncated form of the protein which is exported and released from the cell (Perez *et al.* 1987). Glycosylation of the Env protein occurs shortly after translation. Subsequent modification of the carbohydrate and cleavage of the Env precursor protein into surface (SU) and trans-membrane (TM) subunits occurs in the Golgi apparatus. The surface (SU) and trans-membrane (TM) subunits remain associated with each other subsequent to their cleavage. This occurs at a conserved amino acid sequence which follows three or four basic amino acid residues (typically Arg/Lys-X-Lys-Arg) (Dong *et al.* 1992). Failure of this cleavage to occur results in the formation of non-infectious viral particles.

1.1.1.3.7 Virion assembly and budding

The details of retroviral particle assembly are poorly understood, but some general observations can be made. The Gag precursor protein associates with the retroviral genome via the carboxy-terminal nucleoprotein (NC) region and the packaging signal psi (ψ) located 3' of the 5' LTR. The middle capsid (CA) regions of the precursor proteins self aggregate, and the amino-terminal matrix (MA) region associates with the cell membrane. The same interactions of the polyproteins Gag-Pro and Gag-Pro-Pol brings these protein domains into the virion in the requisite proportion and location. The interaction with the cell membrane and acquisition of the viral envelope, occurs after particle formation where A-type particles are observed, (as is the case for the A-type, B-type and D-type retroviruses), but occurs simultaneously for C-type retroviruses (Wills and Craven, 1991).

Subsequent to assembly of the viral particles the domains of the polyprotein are cleaved by the protease to yield the individual proteins. This results in the formation of a virion capable of performing the reverse transcription of its genome. The Env proteins are incorporated into the envelope of the virion presumably by interactions with the matrix protein. The functional proteins during virion assembly are all polyproteins, Gag (MA-CA-NC), Gag-Pro, and Gag-Pro-Pol, but are individual separate proteins during the early stages of infection. Virion assembly requires the polyproteins, whilst the uncleaved precursor to RT shows no or very reduced reverse transcriptase activity. Although all of the major genes and proteins are required to produce infectious viral particles, only MA and CA are required to produce assembly

of viral particles. Discernible viral particles can even be produced by just the first 180 of the 701 amino acids of RSV Gag, hence there is an aggregation domain within the MA domain. These viral particles were much less dense than the authentic retrovirions but were still capable of budding and release from the plasma membrane (Weldon, Jr. and Wills, 1993).

1.1.1.4 Structure

Retrovirus particles have a common structure. Their genome is packaged within an icosahedral core or nucleoid which is formed by the capsid protein of the Gag polyprotein. This is in turn surrounded by the matrix (MA) protein fragment which directly underlies the envelope. The amino terminal residue of MA is usually myristilated. The matrix protein seems to determine the intracellular location of virion formation. Finally, the extracellular virion is contained within an outer envelope made of a unit membrane derived from the host cell membrane, but containing in addition the retrovirally derived envelope protein, which is often clearly visible as projecting spikes under electron microscopy (Teich, 1984).

Retroviruses are diploid single stranded RNA viruses and possess a genome of 60 to 70 Svedberg units composed of identical subunits 7 to 10 thousand bases in size. Each RNA molecule is effectively an mRNA, being the positive sense strand, having a 5' methylated cap structure and a poly A 3' tail approximately 200 bases in length. The structure of the RNA dimer has not been fully elucidated but the region of

contact has been localised to near the packaging signal (ψ) and the primer binding site (PBS). The reason for this diploidy is also unknown but it may be involved in replication or enable complementation of deleterious mutations. In addition to the viral genome, the nucleocapsid contains the virally encoded reverse transcriptase (RT), and base paired with the RNA genome, a host encoded specific tRNA molecule. The genomic structure of all retroviruses is essentially similar. The RNA genome of simple retroviruses consists of three major coding regions, *gag*, *pol* and *env* and possesses long terminal repeat (LTR) sequences at either end. The *gag* region encodes the capsid proteins, the *pol* region the protease, reverse transcriptase, RNase H and the integrase, and the *env* region the envelope glycoprotein. The LTRs of the RNA genome consist of identical direct terminal repeat (R) sequences with unique sequences internal to these at the 5' (U5) and 3' (U3) ends of the genome. Complex retroviruses have accessory genes which play a role in the regulation of the viral life cycle (Coffin, 1996).

The typical chemical composition of retrovirus particles is 60-70% protein, 30-40% lipid, 2-4% carbohydrate, ~1% RNA with trace amounts of DNA. Their physicochemical properties include a buoyant density between 1.16 and 1.18 g/ml in sucrose and 1.16 and 1.21 g/ml in caesium chloride, susceptibility to inactivation by organic solvents, detergents, formaldehyde and heat (56 °C for 30 minutes), but high resistance to inactivation by UV- and X-ray irradiation (Teich, 1984).

All retroviruses have an essentially similar general morphology but their detailed structure can vary and be used to subdivide them (Section 1.1.1.5). Typically they

are spherical enveloped virions (80-120 nm in diameter) possessing variable surface projections. Internal to the envelope is an icosohedral capsid containing a ribonucleoprotein complex within a core shell (Figure1).

1.1.1.5 Morphology

A-type

These particles were initially viewed as the immature forms of MMTV, but the term now applies to viruses which produce core particles that remain strictly intracellular. They are typically spherical, 60-90 nm diameter, with a hollow double walled appearance. The intracellular location of these particles has led to two subdivisions, intracisternal and intracytoplasmic. Both B-type and D-type retroviruses pass through stages of maturation where intracytoplasmic A-type particles can be observed. Intracisternal A type particles (IAPs) are also produced by certain endogenous retroviral sequences.

B-type

The mature extracellular intact viral particles of MMTV are the archetypal B-type retroviral particles. During the budding process at the cell membrane, toroidal (doughnut shaped) cores ~75 nm in diameter are evident. After budding, the electron dense core is eccentrically located within the 125-130 nm diameter enveloped particle.

C-type

Extracellular particles are 80-110 nm in diameter and have a centrally located core. Immature extracellular particles possess an electron-lucent core whereas mature particles are electron dense. Some C-type particles have discernible membrane spikes (although these are generally not as prominent as those of B-type particles), while others do not. Early intracytoplasmic precursor forms (e.g. A-type particles) are not observed. The first discernible feature of immature particles is an electron dense crescent shaped core which forms at the cell membrane prior to budding, viral assembly and budding occurring synchronously at the cell membrane.

D-type

MPMV is the archetype for the D-type retroviruses. Mature extracellular D-type particles are 100-120 nm in diameter, have an electron dense eccentrically located core and possess discernible membrane spikes although these are shorter than those of B-type particles. Early intracytoplasmic precursor forms, A-type particles, are observed. These are toroidal 60-95 nm in diameter and are most frequently observed near the plasma membrane.

Viruses which lie outside this strict morphological A-D type classification include both the lentiviruses and spumaviruses and the BLV/HTLV group. Lentiviruses resemble C-type viruses but have a distinctive truncated cone shaped nucleocapsid. Spumaviruses pass through an intracellular A-type particle stage to develop as mature particles with prominent envelope spikes. The BLV/HTLV group bud like

C-type viruses and possess a central nucleocapsid but have different envelope projections.

For further details on retroviral morphology see Weiss, (1984).

1.1.1.6 Disease association

1.1.1.6.1 Exogenous retroviruses in human disease

There are only four established exogenous human retroviruses associated with disease, these are the HTLVs I and II, and the HIVs 1 2. The classical disease associations of these viruses are, HTLV with adult T-cell leukaemia (ATL) and HIV with the Acquired Immune Deficiency Syndrome (AIDS). The origin and evolution of these four human exogenous retroviruses is now widely accepted to be the result of four separate zoonotic infections (Weiss, 1998). The human foamy virus (HFV) (also known as the human spumaretrovirus (HSRV)) has not been ascribed any definite aetiological role in disease (Heneine *et al.* 1998).

1.1.1.6.1.1 Human spumaretrovirus (HSRV)

Although the human spumaretrovirus (HSRV) was isolated from the lymphoblastoid cells of a patient with nasopharyngeal carcinoma, it has not been shown to have a definite aetiological role in any disease. Humans are now not thought to be natural hosts of foamy virus infections (Schweizer *et al.* 1995; Ali *et al.* 1996). HSRV would appear to be the result of a zoonotic infection, HSRV having high homology with the chimpanzee simian foamy virus (SFVcpz) (Herchenroder *et al.* 1995). Indeed there is evidence for ongoing zoonotic infections of SFVs into man (Schweizer *et al.* 1997; Heneine *et al.* 1998). It has long been established that both endogenous retroviruses (if activated) and non-pathogenic retroviral infections when transferred to a new host species can become lethal (e.g. SIV, African mangabeys to Asian macaques) but with the SFV zoonoses there is no evidence of disease or of secondary transmission to regular contacts or sexual partners (Schweizer *et al.* 1997; Heneine *et al.* 1998).

1.1.1.6.1.2 Human T-cell lymphotropic virus type I (HTLV-I)

Disease recognition

Adult T-cell leukaemia (ATL) was first described by Uchiyama and colleagues in 1977 (Uchiyama *et al.* 1977). Acute ATL is an aggressive disease which leads to death usually 3-6 months after diagnosis. The disease is characterised by peripheral lymph node enlargement, hepatomegaly, splenomegaly, skin lesions and hypercalcaemia. Variations in the clinical course of ATL have resulted in the need for sub-classification of the disease into five types, acute, chronic, smouldering, crisis and lymphoma (Takatsuki *et al.* 1985).

The term tropical spastic paraparesis (TSP) was first used by Roman and colleagues in 1985 to describe a chronic myelopathy of unknown aetiology (Roman *et al.* 1985). The disease itself had been recognised almost three decades prior to this as a chronic neurological disorder of Jamaican adults. It was sub-divided into a spastic and an ataxic group, the spastic group corresponded to TSP. In Japan HTLV-I associated myelopathy, was initially described as a new clinical entity (Osame *et al.* 1986) but this is now known to be the same as TSP.

Discovery of causative agent

HTLV-I was first detected in 1979 by van der Loo and colleagues (van der Loo *et al.* 1979). The virus was named by Poiesz and colleagues (Poiesz *et al.* 1980) who isolated it from cultured T-lymphocytes derived from the lymph nodes and peripheral blood of a 28 year old black American with a cutaneous T-cell lymphoma diagnosed as mycosis fungoides. This was the first isolation of a human retrovirus.

The link with ATL was confirmed by several studies. Miyoshi and colleagues (Miyoshi *et al.* 1981) demonstrated production of C-type virus particles in a cell line derived from co-cultivating leukaemic T-cells from a Japanese ATL patient. Hinuma and colleagues (Hinuma *et al.* 1981) showed that sera from 44 patients with ATL reacted with a cytoplasmic antigen from this cell line. Gallo and colleagues (Gallo *et al.* 1982) confirmed that this cell line was producing HTLV-I and Yoshikura and colleagues (Yoshikura *et al.* 1984) characterised an independent HTLV-I virus isolate from a Japanese ATL patient.

The involvement of HTLV-I in the aetiology of TSP was a serendipitous finding. Gessain and colleagues (Gessain *et al.* 1985) performed a large retrospective serological survey of haematological, infectious, and related diseases in Martinique. They found HTLV-I antibodies in two patients with TSP. This led them to undertake a systematic study of TSP patients which revealed that 59% of patients with no systemic symptoms were HTLV-I positive, and all TSP patients with systemic symptoms were HTLV-I positive. This finding was rapidly confirmed by

studies from other groups, Rodgers-Johnson and colleagues (Rodgers-Johnson *et al.* 1985) on patients from Jamaica and Colombia, and in the HTLV-I endemic island of Kyushu in Japan, where HTLV-I associated myelopathy, was originally described as a new clinical entity by Osame and colleagues (Osame *et al.* 1986), but was later considered to be identical to TSP.

1.1.1.6.1.3 Human T-cell lymphotropic virus type II (HTLV-II)

Discovery of virus and disease recognition

The discovery of HTLV-II was similarly serendipitous. Kalyanarman and colleagues were screening people with T-cell malignancies for HTLV-I by a variety of immunological assays. One of the patients "Mo" had a T-cell variant of relatively benign hairy cell leukaemia. "Mo" was HTLV-I p24 antibody positive and was shown to be infected with a new subtype of HTLV, termed HTLV-II. This was demonstrated by the clearly different immunological profile of the virus cultured from this patient (Kalyanaraman *et al.* 1982).

Although HTLV-II was first isolated from a leukaemic patient, and a second isolate has been obtained from a patient with atypical hairy-cell leukaemia, no disease association has been convincingly established. Recently HTLV-II has also been tentatively implicated in the aetiology of TSP (Murphy *et al.* 1997). The epidemiology of the two viral infections are quite distinct, HTLV-II being

predominantly associated with intravenous drug users. Regions where HTLV-II infections are quite prevalent have been described. A high frequency of HTLV-II infection amongst blood donors in New Mexico was reported (Hjelle *et al.* 1990). Initially this was linked to populations of American Indian and Hispanic ancestry (Hjelle *et al.* 1991) and subsequently extended to include populations of Amerindians from north Argentina (Biglione *et al.* 1993) and Venezuela (Echeverria de Perez *et al.* 1993) and also in pygmies (Goubau *et al.* 1992).

1.1.1.6.1.4 Human immunodeficiency virus type 1 (HIV-1)

Disease recognition

The next major event in the history of human retroviral infections was the description of the Acquired Immune Deficiency Syndrome (AIDS) and the subsequent discovery of its causative agent the HIV virus. AIDS was first reported in June 1981 in Los Angeles California (Anonymous, 1981a). Five young and apparently healthy gay men developed Pneumocystis carnii pneumonia (Pcp), a disease characteristic of severely immunocompromised patients (Walzer *et al.* 1974). In July 1981, 26 cases of an aggressive form of Kaposi's sarcoma, with 8 associated deaths, were reported in male homosexuals aged less than 50 (Anonymous, 1981b). Some of these Kaposi's sarcoma patients also had severe herpes simplex, pneumocystis or cytomegalovirus infection. Ten further cases of Pcp were reported

from California. It was concluded that there was a clustering of Pcp and other serious infectious diseases, comprising the Acquired Immune Deficiency Syndrome (AIDS), among male homosexuals in New York city and California (Anonymous 1981b).

Discovery of causative agent

HIV, the causative agent of AIDS was first isolated by Barre-Sinoussi and colleagues in 1983 from T lymphocytes of a patient at risk from AIDS, a male homosexual from Paris (Barre Sinoussi *et al.* 1983). Gallo and colleagues (Hahn *et al.* 1984) claimed to have independently isolated the virus. However their isolate has now been shown to be the viral isolate that they were sent by Barre-Sinoussi and colleagues. A large number of independent isolates of HIV-1 were quickly reported. IDAV-1 and IDAV-2 were isolated by the French group from a haemophiliac and from a contact of a patient with AIDS. Popovic and colleagues (Popovic *et al.* 1984) and Levy and colleagues (Levy *et al.* 1984) characterised isolates from patients with AIDS and persistent generalised lymphadenopathy (PGL).

1.1.1.6.1.5 Human immunodeficiency virus type 2 (HIV-2)

Disease recognition

Initially detected in healthy individuals, early studies on HIV-2 infected prostitutes in Senegal suggested that the virus was not associated with an AIDS like illness

(Barin *et al.* 1985; Kanki, 1987). However other reports described a clear association between HIV-2 infection, AIDS-related diseases and severe immunodeficiency (Clavel *et al.* 1986; Clavel *et al.* 1987; Brun Vezinet *et al.* 1987; Albert *et al.* 1989).

Discovery of causative agent

In 1985, Barin and colleagues identified antibodies to simian immunodeficiency virus macaque (SIV_{mac}) in healthy female prostitutes from Senegal (Barin *et al.* 1985). In 1986 an AIDS like illness was observed in two patients, one in the Cape Verde Islands and the other in Guinea Bissau. HIV was not detectable, but subsequent cultivation of peripheral blood mononuclear cells (PBMCs) yielded a novel retrovirus. This was shown to be antigenically related to HIV but distinct from it and more closely related to SIV (Clavel *et al.* 1986). This became the prototypic strain of HIV-2 designated HIV-2_{ROD} and all previous isolates of HIV became known as HIV-1.

1.1.1.6.1.6 Retroviruses and autoimmunity

The ability of retroviral infections to trigger autoimmune like conditions has long been established (Venables and Brookes, 1992). Among the lentiviruses the archetype is caprine arthritis-encephalomyelitis virus, which as its name suggests, causes both a rheumatoid condition and neurological disease (Cheevers and McGuire, 1988). Equine infectious anaemia virus (EIAV) causes lymphoproliferative disorders, glomerulonephritis and haemolytic anaemia

(Montelaro 1993; Teich *et al.* 1984). Infection of sensitive strains of mice with LP-BM5 murine leukaemia virus (MLV) leads to the murine acquired immune deficiency syndrome associated with the formation of autoantibodies and Sjogren's syndrome like disease (Suzuki *et al.* 1993). HIV-1 causes a range of autoimmune conditions in man, as well as the classical symptoms of AIDS. Autoimmune conditions, including Sjogren's syndrome-like, systemic lupus erythematosus-like, rheumatoid arthritis-like, and polymyositis-like have all been observed in HIV infected individuals (Morrow *et al.* 1991). In HTLV infected individuals an arthritis-like autoimmune condition, HTLV-I associated arthropathy, has also been observed (Nishioka *et al.* 1989; Kitajima *et al.* 1991).

Perhaps as a consequence of an underlying unifying mechanism, patients suffering from autoimmune diseases, who are not infected with either HIV or HTLV, frequently have raised antibody titres to retroviral proteins. Typically this heterologous antibody response is limited to Gag proteins but may include others (Walchner *et al.* 1997; Sauter *et al.* 1996). No known exogenous retrovirus has been demonstrated in these conditions but it has been postulated that as yet uncharacterised retroviruses may be involved (Krieg and Steinberg, 1990; Krieg *et al.* 1992; Jimenez *et al.* 1995; Blomberg *et al.* 1994; Bengtsson *et al.* 1996).

Possible mechanisms by which retroviruses may induce autoimmunity are discussed in Section 4.3.1.

1.1.1.6.1.7 Retroviruses and neurological disease

A similar situation exists for retroviral involvement in the aetiology of various neurological diseases as it does for the autoimmune conditions. There are retroviruses which are known to cause neurological disease and there are suggestions of retroviral involvement in a range of other neurological diseases. Some of the retroviruses that have been shown to cause neurological disease are discussed below.

A temperature sensitive mutant of Moloney MLV (*ts*1) induces neurological paralysis in mice (McCarter *et al.* 1977). The maedi (shortness of breath)/visna (wasting) virus causes inflammatory diseases in sheep, of the lung or central nervous system respectively (Haase, 1975; Petursson *et al.* 1976; Dawson, 1980). Both forms of disease involve infiltration of lymphocytes and macrophages to either the central nervous system or the septa dividing the alveoli of the lung. A congenitally transmitted C-type retrovirus found in feral Californian mice (Andrews and Gardner, 1974; Gardner *et al.* 1979) causes lymphoma and hind limb paralysis. These two diseases are not linked, and the paralysis is due to noninflammatory destruction of lower motor neurones in the spinal cord, reminiscent of poliomyelitis. Persistent viraemia is necessary for the disease to develop, and like the lymphoma, the onset of the disease is relatively late in life.

Both the lentiviral and HTLV group of characterised exogenous human retroviruses have been implicated in neurological conditions. Neurological disease is common in

HIV infection (Brew, 1993; Navia, 1997) and one of the conditions caused by HTLV is tropical spastic paraparesis TSP (Gessain *et al.* 1985; Rodgers Johnson *et al.* 1985; Osame *et al.* 1986). Neurological disease where retroviral aetiologies have been sought include motor neurone disease (MND) (Andrews *et al.* 1997) and multiple sclerosis (Karpas *et al.* 1986; Dalgleish *et al.* 1987; Schneider *et al.* 1987; Sandberg Wollheim *et al.* 1988).

1.1.1.6.1.8 Retroviruses and Multiple Sclerosis

The search for an aetiological agent in multiple sclerosis has, in recent years, focussed on retroviruses in particular. The reasons for this include the following three separate observations. i) Cross reactive antibody responses to various retroviral antigens have been demonstrated in patients with multiple sclerosis (Section 4.2.2; Banki *et al.* 1994; Shih *et al.* 1989; Rasmussen *et al.* 1996b; Shattles *et al.* 1992; Brookes *et al.* 1992; Rasmussen *et al.* 1995; Rasmussen *et al.* 1996a). ii) Retroviruses have been shown to cause degenerative neurological diseases (including demyelination) in a number of different species including mice, sheep, goats, and non-human primates (Sigurdsson *et al* 1957; Gardner, 1988). iii) The retroviruses that infect man, human immunodeficiency virus (HIV-1) and human T-cell leukaemia virus (HTLV-I), are both neurotropic. HIV-1 infection has been suggested to be the cause of an MS-like disease in some patients (Berger *et al.* 1989; Gray *et al.* 1991). Also, the clinical similarities between some cases of HTLV-I induced tropical spastic paraparesis (Gessain *et al.* 1985) and MS stimulated a

number of studies in which evidence of HTLV-I infection was sought in patients with MS. Although two reports (Reddy *et al.* 1989; Greenberg *et al.* 1989) initially claimed to have detected HTLV-I or HTLV-like sequences in patients with MS, the involvement of this retrovirus was eventually disproved (Ehrlich *et al.* 1991). However, research into the possible involvement of a novel retrovirus in MS has continued.

In 1989, Perron and colleagues in France isolated a novel retrovirus (LM7) from the leptomeningeal cells of a patient with MS (Perron *et al.* 1989). They subsequently isolated LM7-like viruses from the monocytes, B-lymphocytes and choroid plexus cells of several other patients with MS, and demonstrated a correlation between disease activity (i.e. relapse / exacerbation) and the production of virion-associated reverse transcriptase activity (Perron *et al.* 1991; Perron *et al.* 1991b; Perron *et al.* 1997). Similar findings were reported by Haahr's group in Denmark who described retrovirus particles and associated reverse transcriptase activity in MS patient-derived lymphocyte cultures (Haahr *et al.* 1991; Haahr *et al.* 1994).

Electron microscopy of LM7, concentrated by ultracentrifugation from tissue culture supernatant of the original MS patient-derived leptomeningeal cell line (Perron *et al.* 1991), revealed viral particles with a diameter of 100-120 nm (Figure 1). The location of the nucleoid in mature particles was markedly eccentric and the viral envelope bore numerous spike-like projections. Ultrathin sections of LM7 infected cells showed the virions budding through the plasma membrane and also into cytoplasmic vacuoles (Figure 1). Morphologically the particles were unlike any

previously described human retrovirus. Similar ultrastructural observations have subsequently been made on LM7 virions isolated from the choroid plexus cells and peripheral blood mononuclear cells (PBMC) of a number of other patients with MS (Perron *et al.* 1991b; Perron *et al.* 1997).

The buoyant density of the LM7 virions detected by electron microscopy is approximately 1.17g/ml as determined by sucrose density gradient ultracentrifugation (Perron *et al.* 1997). Reverse transcriptase activity peaks in sucrose gradient fractions of the same density, suggesting that the enzyme in culture supernatants is indeed virion-associated. Optimum conditions for the assay of LM7 reverse transcriptase include use of a poly-Cm/oligo-$dG_{12\text{-}18}$ template and 20 mM Mg^{++} at pH 8.2. These conditions are distinct from those required by either HIV or HTLV. Antigenically LM7 also appears to be distinct from other human retroviruses and Western blotting of purified LM7 proteins using antibodies from the sera of Patients with MS reveals bands of 90, 65, 60, 50, 45 and 15 kDa (Perron *et al.* 1992; Perron *et al.* 1997).

1.1.1.7 Endogenous retroviruses

1.1.1.7.1 Classification of human endogenous retroviruses

Since the majority of human endogenous retroviral sequences (HERVs) have homologies with animal retroviruses they have been classified accordingly (reviewed by Wilkinson *et al.* 1994). They are subdivided into Class I families, which have sequence homology with mammalian C-type retroviruses, and Class II families, which exhibit homology to mammalian B and D-type retroviruses. It is worthy of note that the human exogenous retroviruses do not have endogenous counterparts, although sequences with limited homology to HIV (Perl *et al.* 1989; Shih *et al.* 1989; Tonjes *et al.* 1996), HTLV (Perl *et al.* 1989; Shih *et al.* 1989) and human foamy virus (Cordonnier *et al.* 1995) have been found in the human genome.

Exogenous retroviruses have generally been named according to their host species, associated disease or discoverers. Taxonomy in HERV research has been more complex since disease associations have rarely been demonstrated, host species are widespread, (HERVs are usually also present in Old World monkeys), and different members of the same family have been identified and named by independent investigators, often with arbitrary laboratory names. An attempt at a systematic nomenclature based on the tRNA specificity of the primer binding site (PBS) has been made. The single letter amino acid code is used as a suffix to the acronym HERV (Larsson *et al.* 1989). This approach is limited by the conserved nature of the

PBS across retroviral families and its frequent mutation or deletion in endogenous retroviruses. All Class II elements so far identified being related to B or D-type retroviruses have a lysine PBS and would hence all be designated HERV-Ks using this system.

1.1.1.7.2 Endogenous retroviruses and genome plasticity

Retrotransposition is not a rare event (Heidmann and Heidmann, 1991) and if it occurs in germ cells, it can provide the genetic variation on which natural selection may act (Lower *et al.* 1996). Endogenous retroviruses and retroelements in general are thought to make a major contribution to the evolution of host organisms, by virtue of the increased genome plasticity that is conferred by these repetitive elements (Lower *et al.* 1996; Andersson *et al.* 1998). The correlation between the location of retroelements and the hypervariable regions of the genome supports this suggestion (Lower *et al.* 1996). They enable recombination and transposition as well as retrotransposition to take place. These mechanisms of gene rearrangement permit exon shuffling to occur, as well as introducing simple sequence variation and they occur more frequently than simple somatic mutations (Lower *et al.* 1996; Andersson *et al.* 1998). The increased error rate associated with RT as compared to cellular DNA polymerases also provides increased scope for the introduction of useful (as well as deleterious) mutations.

Retroelements can also be responsible for allelic variation within current populations (Mager and Goodchild, 1989) e.g. the complement and HLA loci (Zhu *et al.* 1994; Kambhu *et al.* 1990) and provide useful markers for evolutionary studies (Arvidsson *et al.* 1995; Svensson *et al.* 1995; Svensson *et al.* 1996; Widegren *et al.* 1996; Svensson and Andersson, 1997; Andersson *et al.* 1998). They are subject to evolution themselves, particularly of their regulatory sequences e.g. HERV-H LTRs (reviewed by Wilkinson *et al.* 1994; Nelson *et al.* 1996), which suggests that they may have a functional cellular role.

1.1.1.7.3 Endogenous retroviruses: a functional role?

Endogenous retroviral sequences comprise between 0.6% and 6% of the human (Seifarth *et al.* 1995; Lower *et al.* 1996) genome, while estimates of the total contribution of retroelements range from 5 to 10% (Temin, 1985; Baltimore, 1985; Leib Mosch *et al.* 1990; Larsson *et al.* 1989). The fact that such a relatively high proportion of the genetic composition of an organism is made up of endogenous retroviral sequences would suggest that they may perform a role in the survival of the host. Endogenous retroviral sequences are estimated to have entered the germ line several millions of years ago (Leib Mosch *et al.* 1990; Mager and Freeman, 1995). The expression of endogenous proviral genes may have a protective function for the host organism, for example preventing infection by a closely related pathogenic exogenous retrovirus. This may help explain their prevalence within the genome. This prevention of infection can occur by receptor interference (Section

1.1.1.3.1), or by superantigen mediated depletion of susceptible host cells (Lower *et al.* 1996).

This latter mechanism has been demonstrated as occurring in MMTV, where expression of the superantigen gene (SAG) within the endogenous MMTV LTR leads to elimination of the SAG responsive T-cell repertoire during the induction of self-tolerance (Acha Orbea *et al.* 1991; Acha Orbea and Palmer, 1991). The SAG gene product facilitates the infection of B and T cells and subsequently the mammary gland (Golovkina *et al.* 1998a). Hence expression of endogenous SAG protects against infection of cells by exogenous viruses which are of the same SAG subtype, however viruses which have a different SAG gene are still capable of infection (Golovkina *et al.* 1992).

MMTV may be regarded as a retrovirus which is undergoing the process of becoming endogenous, but currently exogenous subtypes exist which are not protected against. This hypothesis is consistent with the observation that feral mice differ in MMTV copy number, integration sites and subtypes of their endogenous proviruses (Imai *et al.* 1994).

LINES and HERVs are frequently expressed in tissues or cell lines with embryonic characteristics. The viral Env glycoprotein gp70 of mouse endogenous retroviruses is commonly expressed on cell surfaces and in secretions such as serum and seminal fluid (Elder *et al.* 1977). It has been suggested that these expressed viral glycoproteins could play a role in cell recognition events during development and

differentiation and in the potentiation of sperm for recognising and fertilising ova (Weiss, 1984). Viral glycoproteins related to ecotropic and xenotropic MLV are specifically expressed in B cell differentiation (Moroni and Schumann, 1977; Moroni *et al.* 1980) and HERVs are frequently expressed in normal PBMCs (Shih *et al.* 1989; Medstrand *et al.* 1992; Medstrand and Blomberg, 1993) and have been postulated to play a role during T-cell activation (Kelleher *et al.* 1996).

1.1.1.7.4 Endogenous retroviruses in disease

The extent of endogenous retrovirus (ERV) transcription and translation varies and can be tissue specific or differentiation dependent, suggesting cell specific transcriptional control (Rasmussen *et al.* 1993). Endogenous retroviral sequences may have the potential to cause disease by a variety of different mechanisms, which include the following.

1) Retrotransposition may cause insertional mutagenesis which results in a loss of normal gene function and consequent disease e.g. LINES, where the following germ line transpositions were detected (i) haemophilia A due to insertion into the coding region of factor VIII genes (Kazazian, Jr. *et al.* 1988; Dombroski *et al.* 1991) (ii) colon cancer due to disruption of the adenomatous polyposis coli gene (Miki *et al.* 1992) (iii) breast cancer due to insertion into the c-*myc* gene locus (Morse *et al.* 1988) (iv) insertion into exon 48 of the dystrophin gene (Holmes *et al.* 1994) and

(v) recently a retrotransposal insertion has been identified as the cause of Fukuyama-type congenital muscular dystrophy (Eberhart and Curran, 1998).

2) Promoter / enhancer elements within the LTR of endogenous retroviral sequences may effect the transcriptional regulation of adjacent cellular genes and their protein expression. Promoters act to increase the level of transcription from adjacent downstream sequences. Enhancers are capable of acting over far greater distances and also bidirectionally, being able to stimulate upstream as well as downstream genes. The overall extent to which ERVs are involved in the control of cellular genes is unknown. However there is evidence for cellular transcripts promoted by endogenous LTRs (Feuchter *et al.* 1992) and of retroviral insertion acting as an enhancer of a cellular gene 35 kbp downstream (Anson *et al.* 1992).

Read-through transcription from ERVs into the flanking cellular sequence can also occur (Herman and Coffin, 1986; Leslie *et al.* 1991) and after splicing, functional cellular mRNA may be produced (Leslie *et al.* 1991). The multicopy HERV RTVL-H (HERV-H) family is composed of approximately 1000 elements which are dispersed throughout the human genome (Kowalski and Mager, 1998). RTVL-H has been associated with read-through transcripts up to 17.5 kbp in length (Wilkinson *et al.* 1990) but these were not fully characterised. ERV-3 (HERV-R) exists as a single transcriptional unit in association with a genomic zinc-finger protein (related to Kruppel) (Kato *et al.* 1990). These transcripts are controlled by the upstream LTR. As stated earlier, retroviral integration often occurs in transcriptionally active regions of the host genome (Section 1.1.1.3.4). It is of

interest to note that some endogenous proviruses in mice localise to the genes encoding murine histocompatibility and lymphocyte differentiating antigens (Meruelo *et al.* 1983). Solitary LTRs are present in the human major histocompatibility complex (MHC) HLA-DQ region of some but not all haplotypes (Kambhu *et al.* 1990). The role of these elements in the control of the adjacent cellular genes is not clear.

3) Aberrant expression of endogenous retroviral sequences which are normally cryptic could occur. These ERVs may be induced by environmental (e.g. viral, chemical etc.) or endogenous (e.g. hormonal) factors which could lead to novel antigenic epitope expression and could subsequently give rise to autoimmunity. DNA viruses (e.g. herpes viruses) frequently encode proteins which act as transcriptional activators. Activation of HERV expression by transactivation of the HERV LTRs by these proteins has been postulated (Urnovitz and Murphy, 1996).

4) ERV gene products may be directly toxic, or could act as regulatory factors in *cis* or in *trans* on cellular genes which in turn could result in their aberrant expression. This could give rise to such phenomena as carcinogenesis, necrosis or autoimmunity (Lower *et al.* 1996).

5) ERV integration has recently been demonstrated to be capable of suppressing translation of an associated fusion transcript. This is the first example of the integration of an ERV affecting expression of a heterologous human gene at the translational level (Kowalski and Mager, 1998).

6) ERV proteins may act as superantigens to activate T cell subsets, some of which may be autoreactive and lead to autoimmunity (Conrad *et al.* 1997).

7) ERV sequences could provide the potential genetic pool and variation for the generation of a novel virus by recombination. This has been demonstrated in MMTV (Golovkina *et al.* 1994; Golovkina *et al.* 1997).

MMTV is a particularly interesting example of a B-type retrovirus having both endogenous and exogenous forms. The endogenous form shows hormonally induced expression. Since MMTV is known to be a cause of breast cancer in mice and is vertically transmitted via the milk, a great deal of research has been performed in trying to determine whether there is similar retroviral involvement in human breast cancer. Although a human B-type retrovirus has been isolated from a human mammary carcinoma cell line (Keydar *et al.* 1984), any pathogenic role of the virus in the disease has still to be established. The molecular characterisation of the genome of such particles has not generally been accomplished, so the hypothesis that they are the product of endogenous retroviruses has remained unsubstantiated. With the human mammary carcinoma cell line T47D it has been found that the particles both possess RT activity and contain RNA genomes, albeit derived from more than one endogenous sequence, (comprising both B & C type endogenous retroviral sequences (Seifarth *et al.* 1995)). One of the preferentially packaged endogenous retroviral sequences has recently been fully sequenced (Seifarth *et al.* 1998). This provides one of the best studied examples of human endogenous retroviral particles.

Retroviral-like particles have been demonstrated by electron microscopy in a wide variety of human diseases and tissues, but the aetiological significance of these observations remains unknown, as does the mechanism responsible for their appearance, as well as their identity in most cases. Human endogenous retroviruses have been identified by a variety of techniques and their sequences and expression studied. The majority of endogenous retroviral sequences are defective, containing multiple stop codons or deletions and insertions. The search for retroviruses has identified retroviral-like particles in a wide variety of different tissues. They have frequently been associated with the normal placenta (Johnson *et al.* 1990; Lyden *et al.* 1994), and normal foetus (Mondal and Hofschneider, 1982), cultured foetal fibroblasts (Panem *et al.* 1975; Mondal and Hofschneider, 1982) embryonal carcinoma cells (Lower *et al.* 1987), germ cell tumours (Boller *et al.* 1993), tumour cell lines and oocytes (Larsson *et al.* 1981). In the normal human and baboon placenta, C-type viral particles are easily observed (Kalter *et al.* 1973a; Kalter *et al.* 1973b). However, they are slightly abnormal in appearance, having no apparent space between the nucleocapsid and the envelope. The particles also lack infectivity for any of the cells and cell lines so far studied.

The existence of cross reactive retroviral antibody responses in patients with Sjogren's syndrome (SS) (Talal *et al.* 1990) led to the characterisation of a novel human endogenous retroviral sequence, human intracisternal A-type particle (HIAP) (Garry *et al.* 1990). HIAP-I antibody responses have been noted in a variety of autoimmune diseases. HIAP-I has been suggested to be the retroviral trigger of

Graves' disease in a proportion of patients (Jaspan *et al.* 1995; Jaspan *et al.* 1996). HRES1, an HTLV related endogenous retroviral sequence, which also has some homology with HIV-1 *gag*, was isolated from the DNA of a patient with type II cryoglobulinaemia by hybridisation with an HTLV-I *gag* probe (Perl *et al.* 1989). HRES1 reactive antibodies have been associated with SLE (Banki *et al.* 1992; Perl *et al.* 1995). However, the association of a disease with antibody reactivity to an endogenous retroviral protein of course does not prove causality. The antibody response could easily be a bystander effect of the pathology, giving rise to aberrant exposure of host proteins to the immune system.

1.1.1.8 Diagnosis and detection

The traditional methods for the detection of uncharacterised retroviruses are electron microscopy (EM) and RT assays, and for the diagnosis of retroviral infections serology, particularly Western blots. With the advent of the polymerase chain reaction (PCR) for DNA amplification, these techniques have been largely superseded by PCR based methods. Uncharacterised retroviruses can now be initially detected with approximately a million fold greater sensitivity by using PCR enhanced RT assays (Silver *et al.* 1993; Pyra *et al.* 1994; Heneine *et al.* 1995) and their genome characterised using other PCR-based methods e.g. degenerate primer PCR amplification and random amplification of cDNA ends (RACE) (Conrad *et al.* 1997). Other than tissue culture isolation, the 'gold standard' for diagnosis of known retroviral infections is no longer Western blot but PCR, be it for detecting virally

derived cDNA or proviral DNA (Tuke *et al.* 1992; Poiesz *et al.* 1997; Tedder *et al.* 1998) The theory of PCR is discussed in Section 2.5.1.

1.1.1.9 Prevention and treatment of infection

The prevention and treatment of retroviral infections has been an area of intensive research since the discovery of human retroviruses and their associated diseases, and in particular the recognition of the AIDS pandemic. This research has mainly concentrated along two avenues, that of prevention via vaccine development and of treatment via the use of antivirals.

Until recently retroviral infections have generally been considered to be incurable. This difficulty in treatment stems from problems associated with a viral infection in which the viral genome has integrated and effectively become part of the host's DNA. To date the most effective results have been obtained using cocktails of antiviral drugs, specifically RT inhibitors (generally nucleoside analogues) and protease inhibitors to arrest viral replication (Autran *et al.* 1997; Albrecht *et al.* 1998; Li *et al.* 1998; Ferrando *et al.* 1998; Sharland *et al.* 1998).

Prevention of transmission by preventing exposure to the virus has thus, until recently, been considered to be the most effective method of controlling retroviral infections. The major routes for transmission of the human retroviruses are vertical transmission, either during parturition or via breast feeding, and horizontal

transmission which is usually either parenteral or sexual (Anderson and May, 1988; Ueda *et al.* 1988; Parry *et al.* 1991; Figueroa, 1996; Takezaki *et al.* 1997).

Trials with vaccines (generally envelope derived) have proved less promising although recently (Graham *et al.* 1998), protective immune responses have been demonstrated against challenge with autologous viruses in primates (Goodkin *et al.* 1996; Joag *et al.* 1998).

1.2 MULTIPLE SCLEROSIS

Multiple sclerosis (MS) is the most common demyelinating disease of man. It is characterised by the occurrence of multiple, sclerotic plaques in the brain and spinal cord, the formation of which are separated temporally and spatially. It is thought to be an autoimmune condition precipitated in a genetically susceptible individual by environmental factors.

1.2.1 Epidemiology

1.2.1.1 Prevalence

The geographical distribution of MS cases can be described as occurring in three zones. The high prevalence regions include northern and central Europe, the northern USA, most of Canada, New Zealand and south-eastern Australia and are where there are more than 30 MS cases per 100,000 population. Rates as high as 300 per 100,000 population had been reported in the Orkney and Shetland islands but have subsequently declined (Kurtzke, 1993). The intermediate prevalence regions (most of Australia, southern USA, south-western Norway, far north of Scandinavia, much of the Mediterranean, Russia, South Africa's white population and possibly central South America) border these and have 5-29 cases per 100,000 population. The low prevalence regions have 0-4 MS cases per 100,000 population,

and include all other studied areas of Africa, Asia, the Caribbean, Mexico and probably northern South America (Kurtzke, 1993).

The high and medium prevalence regions are amongst predominantly white populations. There is a correlation with latitude, the prevalence being lower nearer the equator, which applies in the USA, Europe and the Mediterranean (Kurtzke, 1980) and Australia and New Zealand. Studies in the USA on war veterans (Page *et al.* 1993) demonstrated not only that latitude was important (accountable for 60 to 67% of variation in MS risk) but that there was a significant contribution to the risk factor from the population's ancestry (45 to 60%). This did not mean that genetic risk was the sole determinant, the local environment being an independent risk variable.

Studies in Europe have shown that there tends to be clustering of MS cases with 6 fold or greater differences between rates in the highest and lowest regions within each country. The incidence of MS shows an age specific distribution rising from zero in childhood to a peak at age 27 and then returning more slowly to zero by age 60. There is a sex ratio of 1.5 to 1, female to male, the reason for which is unknown but hormonal factors may be of influence. It has been suggested that the geographic distribution of MS is a reflection of the genetic and migrational history of the populations, MS originating in Scandinavia and spreading from there through Europe to the rest of the world (Kurtzke, 1993).

Migration studies have tended to show that the risk for developing MS is acquired before the age of 15. People who migrate before this age develop the risk factor of the area to which they migrate, whereas those who migrate after this age take with them the risk factor of the area from which they migrated (Kurtzke, 1993). This would seem to suggest that there is an environmental risk factor, possibly a virus, which is acquired before this age. Further evidence in support of this hypothesis is provided by the apparent epidemics of MS that have occurred in the Faroe Islands (Kurtzke *et al.* 1993), the Shetland and Orkney Islands (Martin, 1987) and Iceland (Kurtzke *et al.* 1982; Cook *et al.* 1980). These post war epidemics have been related to the stationing of allied troops on these islands during World War II.

The Faroe Islands are a group of 18 major volcanic islands situated in the North Atlantic 7° W 62° N between Scotland and Iceland, which were first settled by Norse Vikings in the ninth century. The Faroese are of Scandinavian ancestry, with some Celtic component, and are a fairly isolated population. In the Faroe Islands there have been four sequential epidemics of MS (Kurtzke *et al.* 1993) since the stationing there of British troops in World War II. Prior to their arrival in 1940 there were no recorded cases of MS within this population. Since then there have been 4 sequential epidemics of 20, 9, 6, and 7 cases respectively. The occurrence of successive epidemics is consistent with a transmissible infectious agent being the cause of MS within the Faroese population. This interpretation of the epidemiological data is however disputed by other authors (Sadovnick and Ebers, 1993).

1.2.1.2 Genetics

The distribution of MS world-wide and the ethnic variation observed within populations argue strongly for a genetic component to the disease. This is further supported by the concordance rates observed for the disease in familial and particularly twin studies. However, MS is clearly not a classical genetic disease with Mendelian inheritance. First degree relatives of Patients with MS appear to have an increased risk of developing MS, at about 3%, or 30-50 times that of the general population. About 20% of Patients with MS have an affected relative. The concordance rates observed for monozygotic and dizygotic twins are approximately 26% and 2-3% respectively (Compston and Sadovnick, 1992) or slightly higher 35% and 10% (Thorpe *et al.* 1994). Genetic susceptibility therefore at most accounts for only a third of the risk of developing MS. The genes involved have not been determined but associations with HLA class II DR2 antigens have been observed in certain populations (Section 1.2.5.1). Two genome wide screens have shown an association with 17q22-q24 (Kuokkanen *et al.* 1997; Sawcer *et al.* 1996). Genetic predispositions to MS are further discussed in Section 1.2.5.1.

1.2.2 Clinical manifestations.

Multiple sclerosis is a disease which has an extremely wide clinical spectrum. It can vary from being apparently symptomless, with diagnosis only being made after post-mortem, to being a cause of major disability and eventual mortality. The course of the disease is also variable, the classical form, which affects the majority of patients and accounts for ~85% of MS cases, being relapsing-remitting (RR) MS, where the disease progresses via a series of attacks. In each attack symptoms evolve over a few days or weeks then stabilise for a few weeks with gradual recovery over a period of a month or two.

Relapsing-remitting MS often leads to a progressive stage of the disease with gradual irreversible disability, this is secondary progressive MS. In primary progressive MS there is no initial recovery from the onset of the first episode of the disease, and a continuous progression in disability ensues. There may be rapid deterioration leading quickly to death. However less than 10% of Patients with MS are affected by this form of the disease and less than 4% die within 5 years of its onset. (Mc Alpine, 1972).

The final form of the disease is benign MS in which there is no further progression of the disease following the initial attack. The frequency of this form of MS is not fully determined since many cases are not diagnosed and estimates are based on autopsy studies, but it is thought to account for from between 5% and 33%

(Castaigne *et al.* 1981; Phadke and Best, 1983; Swingler and Compston, 1992). These patients do not go on to develop progressive disease but remain stable.

1.2.2.1 Presentation

The clinical presentation of MS is generally associated with the development of plaques in the white matter of the CNS. The most frequently involved sites and presenting symptoms are listed below.

Optic nerve - optic neuritis.

Spinal cord - motor/sensory limb disturbance, paraesthesia, numbness, weakness, clumsiness of limbs, bladder, bowel and sexual dysfunction.

Brain stem - vertigo, diplopia and oscillopia.

Cerebellum - ataxia.

1.2.2.2 Diagnosis

The diagnosis of clinically definite MS disease requires the demonstration of 2 or more separate lesions of the CNS in a patient who has had at least 2 episodes of neurological symptoms of the type observed in MS (Poser *et al.* 1983). This clinical diagnosis of MS requires that the lesions be separated in time and space and the exclusion of all other possible causes. In addition, laboratory investigation can enable clinically silent lesions to be detected which support an earlier diagnosis of MS than could otherwise have been achieved. Evidence of subclinical damage is

most commonly obtained from magnetic resonance imaging (MRI), cerebrospinal fluid (CSF) immunological disturbances and abnormal evoked potentials. On the basis of these observations and results MS is categorised into either definite or probable MS, each of which is split into 2 subgroups, clinically or laboratory supported.

MRI is the most useful laboratory investigation in MS diagnosis, capable of detecting CNS plaques in over 95% of clinically definite cases. An abnormal MRI typical of MS is however neither necessary nor sufficient for diagnosing MS in an individual, since similar pictures occur in a wide range of inflammatory disorders, as well as in normal people older than 50. However, its ability to detect the majority of clinically silent plaques makes it especially useful in diagnosis, since the appearance of a new lesion separate in time and space from the first can be identified. Thus a diagnosis of MS can be made prior to a second clinically apparent attack.

Raised levels of intrathecal IgG in the CSF or oligoclonal banding is observed in over 90% of clinically definite cases of MS, but again is not sufficient for diagnosing MS in an individual, since similar findings occur in a wide range of inflammatory disorders and central nervous system (CNS) infections.

Evoked potential responses can also be used to detect lesions in Patients with MS. Visual evoked potentials (VEP), brain stem and somatosensory evoked potentials are all commonly recorded. At least one of these is found to be abnormal in over 75% of clinically definite cases of MS. Again this is insufficient for diagnosing MS in an

individual, since similar findings occur in a wide range of other neurological disorders. VEP measurement is the most useful of these due to its sensitivity, the simplicity of the test, and the stability of any abnormality together with the frequency of visual pathway impairment. As is the case with MRI, VEP is particularly useful in the detection of clinically silent lesions in order for a diagnosis of MS to be made. Confirmation of VEP abnormality in patients who present with spinal cord or brain stem lesions demonstrates the spatial separation of lesions necessary for diagnosis of MS. The clinical manifestations of MS is reviewed in further detail by Prineas and McDonald (1997).

1.2.3 Pathology

1.2.3.1 Gross pathology

At autopsy the brain and spinal cord may appear normal, alternatively the leptomeninges appear thickened and subpial plaques are visible as grey patches on the surface of the spinal cord, brain stem, optic nerves and chiasm. Severe atrophy of the spinal cord is noted in ~50% of Patients with MS at autopsy. Loss of axons and dilatation of the lateral ventricles is common with severe hydrocephalus in between 5 and 10% of long standing MS cases and occasionally present in cases of quick primary progressive MS where only a few MS plaques may be present. The cranial nerves and spinal roots appear remarkably normal even in the presence of extensive demyelination and atrophy of the brain stem and spinal cord.

The sclerotic plaques from which MS derives its name are very discrete lesions of demyelination with a classical "punched hole" appearance within the surrounding tissue. The plaques visible on sectioning the brain are usually between 2 and 20mm in diameter and are distributed throughout the white and grey matter. Smaller lesions including microscopic ones, tend to be found in tissue immediately bordering larger visible plaques. Plaques are grey/off white in colour, which changes to pink or brown on exposure to air. This colour change is not observed where there are a large number of macrophages present in the lesion. Chronic active plaques which posses a concentration of macrophages round the edge of the lesion may thus appear

ringed by a continuous white line. Plaques may occur anywhere in the grey or white matter of the CNS but are more common in white matter. The most frequently affected regions are the optic nerves, the spinal cord and the periventricular regions of the brain.

The shape and boundaries of the plaques are reminiscent of ink blots and are normally independent of grey/white matter boundaries or the direction of fibre pathways, but almost all form around veins.

Plaques of various histological ages, resolving and quiescent are frequently found with newly forming or enlarging active lesions. Remyelinated lesions, or shadow plaques, which contain thinly myelinated axons, may be found alongside demyelinated ones.

1.2.3.2 Chronic active plaques

Within chronic active plaques there is a concomitant reduction in the population of oligodendroglia which is proportional to the demyelination at the centre of the plaque. At the edge of demyelinating chronic plaques the number of oligodendrocytes may be normal or even increased in number (Raine *et al.* 1981). This may possibly reflect an attempt at remyelination. These large numbers of oligodendrocytes at the periphery of expanding plaques may be either mature cells which survived the loss of their myelin forming processes or newly formed non

myelinated cells. Their possible function is to attempt to remyelinate neuronal processes, this remyelination occurs at the same time as the active demyelination and may also involve infiltrating Schwann cells from the periphery. This happens in ~50% of all MS cases, of small and large groups of demyelinated axons near spinal root entry zones. As the disease progresses there comes a point where oligodendrocyte numbers fall, as demyelination dominates, and reductions of between 30 and 40% have been described (Prineas *et al.* 1984a; Raine *et al.* 1981). Chronic demyelinated plaques would hence appear to be the end result of a war between demyelination and remyelination, which eventually the oligodendrocytes lose. Shadow plaques would represent the scarred battlefields of as yet unfinished wars, with their areas of thinly and sometimes inappropriately remyelinated axons. The characteristics of the disease, its classical relapsing remitting yet progressive course could easily be seen as a feature of this process.

The periphery of chronic active plaques is further characterised by the presence of macrophages which contain the breakdown products of myelin or are immunoreactive for myelin basic protein (MBP), myelin associated glycoprotein (MAG) and other myelin specific markers (Itoyama *et al.* 1980; Prineas *et al.* 1984b). It has been demonstrated that these macrophages do not actively strip axons but endocytose superficially loosened myelin lamellae via clathrin coated pits (Prineas *et al.* 1984a; Raine *et al.* 1981; Prineas and Connell, 1978). This being the major pathway for receptor mediated endocytosis, it can be assumed that specific receptor : ligand interaction is involved in the recognition and destruction of myelin by macrophages that is occurring in these lesions. Astrocyte involvement in the

breakdown of myelin sheaths has not been convincingly established (Lee *et al.* 1990).

1.2.3.3 Nascent plaques

The destruction of myelin within newly forming lesions (i.e. nascent plaques) is typically rapid, extensive and complete, although there is preservation of the neural architecture with axons and neurones remaining relatively intact. One of the primary features of an acute lesion in MS is the disruption that occurs to the blood brain barrier. This has been clearly demonstrated by MRI studies but the cause of the breakdown remains elusive. The results of this insufficiency are characterised by the perivascular cuffing that is characteristic of the disease. There is extensive evidence for inflammatory infiltration of the perivascular tissue, with lymphocytic cells usually present.

The foci of nascent demyelination are generally perivascular. The vascular relation of plaques can be fully understood only by assuming that an agent noxious to the myelin sheath arises from a blood vessel and then spreads into the surrounding tissue. This 'agent' is believed to be activated macrophages which have been primed by immuno-modulators generated either within the nascent plaque itself or within the Virchow Robin spaces surrounding the centrally located blood vessel. Hence an autoimmune process can be postulated to be involved in the progression of the disease via the exposure of a normally immuno-privileged site to the systemic

immune system. The site of lesion formation may be more related to the location of the Virchow Robin spaces and the corresponding spaces in the leptomeninges, than the blood vessels themselves. This is because these are where trafficking lymphocytes and antigen presenting cells are present and may therefore have a role in initiating T cell response (Esiri and Gay, 1990). The perivascular distribution of lesions in MS is very similar to that which occurs in lymphoproliferative disorders propagated in perivascular spaces.

Within active plaques the perivascular infiltration of leukocytes is predominantly of T cells and macrophages, with the occasional plasma cell. The lymphocytes and macrophages are generally found within the thin walls of the perivascular space and plasma cells externally located within the surrounding tissue. Low numbers of mast cells have also been observed adjacent to blood vessels in active and inactive plaques and may thus play a role in the process leading from inflammation and tissue destruction to autoimmunity. The walls of the veins within plaques are generally thickened with an increased perivascular space.

1.2.3.4 Inactive plaques

Chronic inactive plaques exhibit a sharp delineation from the surrounding brain tissue and have a hypocellular "punched hole" appearance. The lesions show astrocyte proliferation, (these cells being small and fibrous, possessing very little cytoplasm), with loss and/or shrinkage of axons and sparseness or absence of

oligodendroglia, (which are very small if present). Microglia and macrophages appear scattered throughout the lesion and occasionally have a 'foamy' appearance. Inactive plaques with few or no lipid containing macrophages lack inflammatory cells (i.e. lack the increased numbers of lymphocytes and plasma cells seen in active lesions). If lipid containing macrophages are present then more inflammatory cells are observed in the perivascular spaces. The plaques in primary progressive MS exhibit significantly less inflammation than those in secondary progressive MS. Neurones are usually preserved and appear normal in demyelinated tissue. Oligodendrocyte numbers may be raised in tissue immediately surrounding inactive chronic plaques.

1.2.4 Pathogenesis

The precise pathogenic mechanism in MS is unknown but it is thought to involve the interplay between autoimmune mechanisms, host genetic background and environmental factors (Compston and Sadovnick, 1992). Epidemiological studies have suggested that the environmental component of this complex pathogenesis may be viral, but as yet there is insufficient evidence to incriminate conclusively any of the many candidate viruses that have so far been proposed (Waksman, 1989; Kurtzke, 1993; Dalgleish, 1997). Animal models which closely resemble MS exist for both viral and autoimmune mechanisms (Macchi, 1987; Saeki *et al.* 1992; Tsunoda and Fujinami, 1996; Ewing and Bernard, 1998).

1.2.4.1 Viruses

The attempts to implicate a specific virus in the pathogenesis of MS have so far proven unsuccessful (Section 1.2.5.2.1). However, there are several possible mechanisms by which viruses could be involved. The demyelination observed could be a direct result of the infection of oligodendroglia causing cell death. Non specific tissue damage may result from the recruitment of inflammatory mediators to the site of infection. The viral infection could trigger an autoimmune response, which could occur by a variety of mechanisms. A virus protein may share epitopes with self antigens and induce the production of cross reactive cellular and humoral immune responses i.e. molecular mimicry. Viral antigens may be expressed at the surface of infected oligodendroglial cells or in myelin and induce an immune response which results in an attack on myelin as non-self. A viral infection may cause damage which results in the exposure of a normally immunoprivileged site (the CNS) to the immune system. Alternatively, if virally encoded superantigens are present, their expression could directly activate a broad polyclonal T cell subset (Skov and Baadsgaard, 1995) (as has recently been demonstrated in Type I Diabetes (Conrad *et al.* 1997) which may be responsible for the autoimmunity.

1.2.4.2 Autoimmunity

MS is generally considered to have an autoimmune component although there is no definite proof for this hypothesis; neither autoreactive T cells nor specific antibodies against the myelin sheath have been conclusively implicated. However, abnormalities in immunoregulatory lymphocytes, characteristic of autoimmune diseases such as rheumatoid arthritis (RA), have been demonstrated in MS (Martin *et al.* 1992). Experimental allergic encephalitis (EAE) provides an experimental animal model which argues strongly in favour of MS being autoimmune mediated. The pathology of EAE is similar to that found in MS (Kwon and Prineas, 1994). EAE can be induced by immunisation with whole myelin, purified myelin basic protein (MBP), proteolipid protein (PLP) or peptides from these proteins which are themselves encephalitogenic. It can also be induced by adoptive transfer of lymphocytes from affected animals or by T-cells clone specific for MBP or PLP. However, serum or oligodendrocyte preparations are not sufficient to transfer the disease.

The relationship between MS and EAE is strengthened by an early report of CNS lesions, characteristic of acute MS, developing in patients injected with preparations containing neural tissue (Shiraki and Otani, 1959).

Oligodendrocyte loss and demyelination both seem to occur simultaneously in MS, with neither being secondary to the loss of the other. However, oligodendrocytes

may survive even within areas of severe demyelination. Neither Class I nor Class II MHC molecules are expressed by oligodendrocytes or myelin and hence, antigen specific cytotoxic T-cells may not be involved in immune mediated demyelination (Sobel and Ames, 1988; Lee and Raine, 1989).

The exact mechanism of demyelination in EAE is also unknown. However, antibodies which recognise myelin surface peptides, activate complement and promote Fc or complement receptor mediated opsonic phagocytosis (Linington *et al.* 1988; Trotter *et al.* 1986), and myelinotoxic cytokines, proteases and other effector molecules released by activated macrophages, may be involved (Cammer *et al.* 1978; Brosnan *et al.* 1988).

Local mechanical disruption of the brain barrier is not thought to pre-dispose to the development of MS (Sibley *et al.* 1991). Many old inactive plaques demonstrate continuing disruption to the blood brain barrier. It would thus appear that leakage of plasma constituents into CNS tissue is not sufficient to trigger demyelination (Kwon and Prineas, 1994).

1.2.4.3 Immunopathology

The specific targets of the immunopathological process in MS are as yet unidentified, however demyelination, and the loss of oligodendroglial cells implies that myelin or antigens on oligodendroglia are the probable target. Active plaques contain T lymphocytes and macrophages (Prineas *et al.* 1984b; Li *et al.* 1996). The majority of T cells present express α/β T cell receptor (TcR) and some plaques have γ/δ TcR T cells (Wucherpfennig *et al.* 1992). $CD4^+$ and $CD8^+$ cells are both present (Traugott *et al.* 1982; Hauser *et al.* 1986). The proportion of $CD8^+$ cells present within the CNS is raised in MS in comparison to the proportion present in the peripheral blood (Woodroofe *et al.* 1986). T cells are perhaps responsible for the destruction of CNS cells which express heat shock proteins (hsps), a marker of cell stress or injury, via cytolysis. In fact, specifically γ/δ T cells are thought to participate in the immmunopathogenesis of MS through their interaction with hsps (Wucherpfennig *et al.* 1992; Battistini *et al.* 1995; Stinissen *et al.* 1995). Oligodendrocytes have been shown to express hsps on their surface (Selmaj *et al.* 1991a) and to induce the proliferation and expansion of γ/δ T cells, whilst γ/δ T cells are highly cytolytic to oligodendrocytes in vitro (Freedman *et al.* 1992). Although γ/δ T cell induced lysis of oligodendrocytes in MS would thus appear to involve hsps, recent evidence suggests that more than one ligand is involved (Freedman *et al.* 1997).

It is not agreed what the exact TcR repertoire of CNS T cells is in MS (Utz and McFarland, 1994). Cells which are primed specifically against myelin antigens are known to be present (Martin, 1997; Stinissen *et al.* 1997). Possible antigen presenting cells present in the CNS include microglial cells, endothelial cells and possibly astrocytes, all of which can be induced to express MHC antigens. Therefore, these may perhaps function by presenting myelin to other immune mediating cells leading to their stimulation. T cell stimulation can thus occur within the CNS itself or at cervical lymph nodes, this being the lymphatic site to which CNS antigens are transported. Alternatively, molecular mimicry, (exposure to an exogenous agent which shares antigenic determinants with self proteins e.g. an antigen of myelin) could result in autoimmunity.

In addition to antigen specific mechanisms activated T cells and microglia/macrophages can cause general damage to the surrounding tissue through the effect of released immunomodulatory cytokines e.g. T cells can release Il-2, γ-IFN and TNF-β (lymphotoxin) and macrophages or microglia can release TNF-α, proteases and complement components (Compston *et al.* 1989; Hofman *et al.* 1989; Selmaj *et al.* 1991b). Many of these induce the upregulation of adhesion molecules, which can result in a general increase in inflammation with the non specific recruitment of lymphocytes and macrophages to the site, and the subsequent activated immune cell / target cell interactions. B cells and immunoglobulins are found in plaques and although specific anti-myelin antibodies have not been conclusively implicated, both cellular and humoural immune responses are believed to be involved in the process of CNS damage in MS.

1.2.5 Aetiology

1.2.5.1 Genes

Although MS is clearly not a genetic disease in the classical sense, there is a genetic component to the disease. This is most clearly evidenced by the concordance rates for the disease occurring in monozygotic and dizygotic twins (Section 1.2.1.2). If MS has an autoimmune component, then the genes of the immune system may well be expected to have a role in its aetiology.

Antigen presentation to T cells occurs via the TcR and the MHC molecule of the antigen presenting cell (APC). A processed peptide fragment of the antigen which sits in the groove of the MHC molecule is presented. Antigen processing by the APC and its presentation by the MHC molecule determine whether a particular TcR is activated. Therefore the conformation of the MHC and TcR are central to the process and hence the sequence of genes of both the MHC and TcR might be expected to be critical in the immune response to a given peptide antigen. A recent Swedish study supports this theory of a functional role for the HLA class II molecules themselves in MS (Fogdell *et al.* 1997).

Within the white UK population an association between HLA class II DR2 antigens and MS has been demonstrated, especially the DRB1 1501, DQA1 0102 and DQB1 0602 haplotype which gives a 4 fold increase in risk of developing MS. However, although this haplotype is associated with the disease it makes only a modest contribution to the overall genetic susceptibility (Dyment *et al.* 1997). DR2 antigens have also been linked to increased expression of lymphotoxin and TNF-α (Zipp *et al.* 1995). This suggested immunogenetic component to the disease is however not universally applicable to all populations, with no definite association of DR2 antigens and MS demonstrable in Italy, India or East Asia, although it was in China (Barcellos *et al.* 1997).

The HLA system is thus insufficient to provide the explanation for genetic susceptibility, in fact not only do most people with these antigens never develop MS, but also not all Patients with MS possess these antigens. The maximum contribution of HLA genes to MS susceptibility has been estimated at only 10%. In a recent study of Caucasian and Chinese Patients with MS, Barcellos and colleagues suggested a non HLA disease susceptibility locus within the chromosome 19q13.2 region (Barcellos *et al.* 1997). Other genes which have been inconsistently reported as being either implicated or not in susceptibility to MS include the TcR genes V-α and V-β. TcRβ gene polymorphisms have been shown to be associated with MS (Epplen *et al.* 1997). The myelin basic protein (MBP) gene on chromosome 18q23 was tentatively linked with MS in Danes (Ibsen and Clausen, 1996), although other studies have shown no such linkage (Barcellos *et al.* 1997).

The role of a genetic component in the humoural immune response is unclear with reports of the role of immunoglobulin (Ig) variable gene segments in MS being contradictory. A recent study (Ligers *et al.* 1997) has also demonstrated that the IgH genes are unlikely to play a role in determining the genetic susceptibility to MS.

As discussed earlier (Section 1.2.1.2), two genome wide scans of MS in families from the United Kingdom and Finland have identified a common marker on 17q22-q24 (Kuokkanen *et al.* 1997; Sawcer *et al.* 1996). In conclusion, no single genetic locus has been identified which has a major influence on familial risk, but rather a number of genes with interacting effects are thought to be responsible for the genetic susceptibility to MS.

1.2.5.2 Environment

Almost every conceivable environmental factor has been considered in regard to a possible role in the aetiology of MS. These have included dietary components such as fatty acids and milk (Malosse *et al.* 1992), as well as climate (Norman, Jr. *et al.* 1983; Rimm, 1983; Laborde *et al.* 1988) and physical trauma (Bobowick *et al.* 1978; Sibley *et al.* 1991; Siva *et al.* 1993; Jellinek, 1994; Poser, 1994).

Vitamin D3 has recently been proposed as an environmental factor which can explain the distribution of MS (Hayes *et al.* 1997) cases, being nearly zero in equatorial regions and increasing dramatically with latitude in both hemispheres. It would also appear to explain two apparently anomalous findings, high prevalence of MS in Switzerland at low altitudes and low prevalence at high altitude, and in Norway high prevalence inland with lower prevalence along the coast.

The occurrence of apparent outbreaks of the disease however has favoured an infectious hypothesis. The variety of infectious agents which have been suggested to have a role in MS is as diverse as the other environmental factors considered and range from mycobacteria, spirochaetes, rickettsiae and mycoplasma to viruses (Kurtzke, 1993). None of the proposed agents have as yet been confirmed.

1.2.5.2.1 Viruses

Over the past half century more than twenty viruses have been proposed as the possible cause of MS (Waksman, 1989; Dalgleish, 1997). These include rubella virus, JC virus and several members of the Paramyxoviridae (e.g. paramyxovirus SV5, canine distemper virus, measles, mumps) and of the Herpesviridae (e.g. Marek's virus and the human herpes viruses HSV, EBV, VZV and HHV6). However, the evidence implicating these viruses remains inconclusive. Recently attention has focussed on retroviruses in particular. Among the principal reasons for this are that retroviruses have been demonstrated to cause both degenerative neurological diseases and autoimmune conditions reminiscent of MS. Research into the possible involvement of a retrovirus in MS has therefore continued, as discussed in Section 1.1.1.6.1.8.

1.2.6 Treatment

Although a large number of treatments for MS have been evaluated, unfortunately there is currently no cure. The majority of treatments which have been tested are immunosuppressive or immunomodulatory agents. This is based on the assumption that the underlying disease process is autoimmune.

1.2.6.1 Corticosteroids

This is the most common treatment for relapsing or progressive illness. Treatment is administered during the active phase of the disease but there is no conclusive proof of any major long term benefits. No reliable direct comparison of adrenocorticotropic hormone (ACTH) with oral corticosteroids has been made. Placebo controlled studies have demonstrated short term benefits; however, these have not been borne out in the long term (Becker *et al.* 1995b).

An acute optic neuritis study demonstrated a reduced progression to clinically definite MS in patients treated with intravenous methylprednisilone (MP) in comparison with oral prednisone and placebos (Beck *et al.* 1993).

1.2.6.2 Cyclophosphamide

Cyclophosphamide is an alkylating agent which has cytotoxic effects on lymphocytes. The beneficial effects of this treatment in MS are controversial and the side effects can be substantial; some studies indicate an arrest in disease progression in a subset of Patients with MS (Weiner *et al.* 1984; Becker *et al.* 1995a), others demonstrate no effect (Anonymous, 1991).

1.2.6.3 Copolymer 1

Copolymer 1 is a synthetic peptide formed from a random repeat of four amino acids from a section of MBP which is encephalitogenic in EAE. Copolymer 1 was found to be suppressive of EAE although it had been designed to mimic MBP. A large multi centre study in relapsing MS demonstrated a fall in relapse rate, comparable to that achieved with IFN β1b and the peptide was of modest benefit with respect to disability (Johnson *et al.* 1995). MRI results were not available but an earlier study on progressive MS patients (Bornstein *et al.* 1991) failed to show any significant clinical benefit.

1.2.6.4 Interferons

Interferons are a group of proteins which are part of the body's natural defence system and have antiviral, antitumour and immunoregulatory activity. There are two types of interferons, type I and type II, type I interferons comprise IFN α and IFN β. Type II interferon is comprised solely of interferon gamma (IFN γ). IFN α and β have many similarities, whereas IFN γ is significantly different. Panitch and Bever reviewed their use in MS (1993) and IFN β therapy has more recently been reviewed by Goodkin (1998). Both natural and recombinant IFN γ produce little or no beneficial effect in either relapsing remitting (RR) or progressive forms of MS. Gamma IFN has even been shown to cause an increase in the rate of relapse in Patients with MS.

A 3 year trial of recombinant IFN β by subcutaneous injection in RR MS patients demonstrated a significant reduction in relapse rates (Anonymous, 1993). Patients with MS treated with high or low doses of IFN β were compared with a placebo group. The relapse rate per year was 1.21 for the placebo group, 1.05 for the low dose group and 0.84 for the high dose group but there was no effect on progression of expanded disability status scores (EDSS). A slight reduction in plaque volume was observable by MRI in the treated group whereas an increase was observed with the control group, however the results for individual patients within each group were highly variable (Vamvakas *et al.* 1995). This study resulted in the first approved therapy for MS and showed that IFN β resulted in a reduction in new lesion

formation. In other studies IFN β1a given by injection has continued to be shown to produce a benefit in disability and reduction in relapse rates similar to these studies (Sandberg Wollheim *et al.* 1995; Cardy, 1997; Abdul Ahad *et al.* 1997; Anonymous, 1998a). Recently IFN β1b has been demonstrated as having a similarly beneficial effect on patients with secondary progressive MS (Anonymous, 1998b).

The possibility that IFN may be having a direct antiviral effect in the treatment of MS has also been considered (Medenica *et al.* 1994).

1.2.6.5 Cladribine

Cladribine, a specific antilymphocytic immunosuppressive has been shown to be of benefit in MS patients with respect to MRI, disability and CSF measurements in comparison to controls (Sipe *et al.* 1994). However severe marrow suppression can be produced by repeated doses (Beutler *et al.* 1994; Sipe *et al.* 1994).

1.2.6.6 Linomide

Linomide is a synthetic immunomodulator that increases natural killer cell activity and has been shown to cause improvement in disability and MRI scales in secondary progressive MS (Karussis *et al.*1995). In a subsequent study of patients with secondary progressive MS, linomide proved to be safe, well tolerated and tended to

inhibit the progression of the disease, especially preventing the appearance of new active lesions in the MRI scans. Two multicentre phase III trials are currently under way in the United States, Europe and Australia (Karussis *et al.* 1996).

1.2.6.7 γ-globulin

High dose IV γ-globulin has been used in small pilot studies, some but not all of which suggest that it has beneficial effect (Achiron *et al.* 1992; Achiron *et al.* 1996; Achiron *et al.* 1998). The mechanism of its action is unknown but the suggestion from animal experiments is that it promotes remyelination.

1.2.6.8 Cyclosporine

Cyclosporine is commonly associated with adverse side effects and consequently a high drop out rate amongst treated patients. Treatment itself has not been demonstrated to be beneficial in MS (Zhao *et al.* 1997).

1.2.6.9 Methotrexate

Low dose oral methotrexate has been shown to retard the development of upper limb dysfunction, but had no effect on EDSS in chronic progressive MS (Goodkin *et al.* 1995; Goodkin *et al.* 1996).

1.2.6.10 Plasma Exchange

Plasma exchange has been shown to be an effective treatment for diseases of the humoural immune system. MS has not been conclusively proven to be a disease of this type. Although initial studies reported beneficial effects (Khatri *et al.* 1991) a recent study failed to provide sufficient evidence of a significant beneficial effect to encourage a subsequent larger study (Sorensen *et al.* 1996). Nevertheless, a meta analysis showed that it significantly reduced the proportion of patients who experienced neurological decline after 12 months (Vamvakas *et al.* 1995).

1.2.6.11 Lymphoid irradiation

Total lymphoid irradiation resulted in a decrease in the progression rate of MS (Devereux *et al.* 1988) but also led to a number of deaths (Cook *et al.* 1989) and has consequently been generally discontinued as a treatment (Wiles *et al.* 1994; Rohowsky Kochan *et al.* 1997). However, it has recently been demonstrated to be of clinical benefit in combination with prednisone treatment (Cook *et al.* 1997).

AIMS OF THE PRESENT STUDY

The purpose of the experiments described within this thesis was to investigate the hypothesis of retroviral involvement in the aetiology of multiple sclerosis. The initial aim was to address the question of whether HTLV or a closely related virus was involved. Secondly, the aim was to develop a PCR based methodology which was capable of detecting and characterising any novel retrovirus which might be present in patients with multiple sclerosis. The approach that was used focussed on detecting cell free virus in order to avoid the problems associated with endogenous retroviral sequences present in the human genome.

Following the detection of a novel retroviral sequence in the serum of a patient with multiple sclerosis, the subsequent aim of the project was to establish whether this was the same retrovirus as that which had been cultured from patients with multiple sclerosis by Perron and colleagues in France. Having established that this was indeed the case, the final stage of the study was to determine whether multiple sclerosis was associated with the presence of this novel retroviral sequence in blood and cerebrospinal fluid.

2 MATERIALS AND METHODS

2.1 Materials

2.1.1 Patients, clinical samples, cells, viral cultures and plasmids

Diagnosis of multiple sclerosis was made according to the criteria of Poser (Poser *et al.* 1983) and supported by magnetic resonance imaging in most cases.

Dr. H. Perron obtained the choroid plexus cells used in this study from the Brain-Cell Library, Laboratoire R. Escourolles (Prof. J.J. Hauw), Hôpital de la Salpêtriére, Paris. Non-tumoural leptomeningeal cells from controls were obtained as previously described (Perron *et al.* 1992). Peripheral blood lymphocytes from MS and control patients, used by Perron and colleagues for obtaining the B-cell lines analysed in this study, were obtained from the Neurological Departments (Prof. Perret, Prof. Pellat, Dr. Mallaret), CHU de Grenoble, and from INSERM U 134 (Dr. E. Schuller), Hôpital de la Salpêtriére, Paris.

MPMV tissue culture supernatant was kindly provided by Dr. Myra McClure and Dr. Munaf Ali, Department of Genito-Urinary Medicine and Communicable Diseases, Saint Mary's Hospital Medical School, London.

Cell lines C81-66-45 (Hahn et al., 1983) and C1218M (Clapham et al., 1984) were obtained from Prof. Robin Weiss, Institute of Cancer Research, Royal Marsden Hospital, London.

Serum samples used in the "Pan-Retrovirus" ELOSA study (Section 3.4.2.1) were obtained from 40 MS patients (27 female, 13 male; average age 36.5 years) attending the Division of Clinical Neurology, University Hospital, Nottingham, UK and were provided by Prof. Blumhardt. Nineteen cases were classified as relapsing remitting and 21 as secondary progressive. All patients were in clinical remission at the time of blood sampling and none were on specific treatment. Clinical details of a further 10 MS patients who provided CSF samples and of 10 patients with other neurological diseases are given in Table 1. Control sera from 30 healthy volunteer UK blood donors were obtained from the North London Blood Transfusion Centre.

The 12 MS patient blood samples used for the DNA PCR studies (Sections 3.1.2 and 3.5.1) and for the detection of a novel *pol* sequence in serum from a patient with MS (Section 3.3.1) were provided by Dr. Alan Thompson, Institute of Neurology, National Hospital, Queen Square, London. Blood was also obtained from American blood donors 40413L, 40326L, 40349L, commercially available as HTLV-II standards, (Serologicals Inc. Florida 32522).

The serum samples and clinical information used in the Multiple Sclerosis associated Retrovirus (MSRV) specific RT-PCR study was obtained via H. Perron from

Professors Chazot, Broussolle, Vighetto and Mauguière (Dept. of Neurology, Hôpital P. Wertheimer, Lyon), Drs Boulliat and Lemaître (Dept. of Neurology, Centre Hospitalier de Bourg-en-Bresse) and Prof. Pellat and Dr Moreau (Dept. of Neurology, Hospitalier Universitaire de Grenoble)

Post mortem cryosections of white matter from normal brains and from plaques of patients with MS were provided by Dr. Jia Newcombe (MS Society Brain Bank, Institute of Neurology, National Hospital, Queen Square, London).

Normal healthy control PBMC DNA samples were provided by Jon N. Goulding (Department of Medicine. UCLMS, London).

The neonatal PBMC DNA samples and the HIV-1 plasmid pBH10 (Ratner, 1985) were provided by Dr. Steve Kaye, Department of Virology, UCLMS and the HTLV-I plasmid pMT2 (Hatanaka and Nam, 1989) was provided by Dr. Mark Boyd and Dr. Tom Schultz, Institute of Cancer Research, Royal Marsden Hospital, London.

2.1.2. General sample handling

Sterile pipette tips, reagents and tubes were used throughout. RNase, DNase and nucleic acid free aerosol-resistant pipette tips (ART™, Promega Ltd) were used for all procedures involving RNA or DNA.

2.1.2.1 Separation and storage of peripheral blood

For serum sample preparation whole venous blood was collected in Vacutainer™ (Becton-Dickinson) tubes. The clotted sample was centrifuged at 400xg for 10 minutes, the serum removed, frozen and stored at -20°C or -70°C.

For plasma and peripheral blood mononuclear cell (PBMC) isolation venous blood was collected in 10ml disodium ethylenediaminetetra-acetate (EDTA), citrate, or lithium heparin Vacutainer™ tubes. Upon receipt, either the PBMCs were immediately isolated or the blood cryopreserved in Glycigel (see below).

For immediate PBMC isolation whole blood samples were transferred to a 15ml centrifuge tube (Falcon™), centrifuged at 400xg for 10 minutes, and the plasma removed and stored at -70°C. An equivalent volume of sterile phosphate buffered saline (PBS) was added to the cells and briefly mixed by inversion. The cells were layered onto 7.5 ml of Ficoll-Paque (Pharmacia) and centrifuged for 30 minutes at 400xg. The separated lymphocytes were removed and washed in a total volume of

25ml PBMC wash (Appendix 1). This was centrifuged for 10 minutes at 400xg, the PBMC wash removed and the wash repeated. The washed and pelleted lymphocytes were resuspended in 2ml tissue culture freezing medium (Appendix 1). This was aliquoted into 2 cryotubes (Nunc™) and slowly frozen in tightly fitting polystyrene blocks at -70°C. Alternatively, the lymphocytes were re-pelleted by being centrifuged for 10 minutes at 400xg, the supernatant removed with a fine-tipped sterile pastette and the 'dry' cell pellet snap-frozen in liquid nitrogen. The snap-frozen cell pellet was stored at -70°C.

For cryopreservation of whole blood, 2ml of Glycigel (Kaye *et al.* 1991) was melted at 37°C, an equal volume of whole blood added and mixed thoroughly. This mixture was frozen and stored at -70°C until required.

2.1.3 Cell cultures, virus isolation and purification.

2.1.3.1 HTLV infected cell lines

C81-66-45 is a T-cell line which carries approximately three copies of integrated HTLV-I provirus per cell (Dr R. Jarrett, personal communication). C1218M cells are human umbilical cord T-cells transformed by HTLV-II. These cell lines had been maintained under 5% CO_2 at 37°C in RPMI 1640 medium supplemented with 10% foetal calf serum by Dr. Steve Kaye.

2.1.3.2 Mycoplasma screening of primary cultures

All cells cultured by Perron and colleagues were regularly screened for mycoplasma contamination with an ELISA mycoplasma-detection kit (Boehringer). No cell-extract nor supernatant used in any of these studies contained detectable mycoplasma. 2mM L-glutamine was added for B-cell lines and 6mM for leptomeningeal or choroid plexus cell cultures (Perron *et al.* 1989; Perron *et al.* 1991; Perron *et al.* 1992; Perron *et al.* 1997a; Perron *et al.* 1997b).

2.1.3.3 Monocyte cultures

Monocytes were cultured by Perron and colleagues as previously described (Perron *et al.* 1997a), the method is briefly outlined below. Venous blood samples were obtained in heparinised glass tubes and diluted 2 fold with RPMI 1640 medium, layered onto a Ficoll gradient and centrifuged for 20 minutes at 400xg. The PBMC layer was removed and washed twice with RPMI. The pelleted PBMCs were resuspended at a final concentration of $4x10^6$ cells/ml in RPMI 1640 supplemented with 15% human serum, 1% sodium pyruvate and 1% non-essential amino acids. 5ml of cell suspension was placed in electropositive culture flasks (Primaria, Becton-Dickinson) in a 5% CO_2 incubator at 37°C. The next day the culture medium was replaced with 5ml of fresh medium, (its composition was identical except the human serum content was reduced to 5%).

After 3 days the media were harvested, kept frozen at -80°C and medium subsequently replaced twice weekly.

2.1.3.4 EBV immortalised lines

EBV immortalised lines were obtained by Perron and colleagues as previously described (Perron *et al.* 1991), the method is briefly outlined below. Human lymphocytes were separated from heparinised blood diluted 1 in 2 with RPMI 1640 by Ficoll density gradient centrifugation. Cells were collected and washed twice in RPMI 1640 containing 200U/ml penicillin, 200mg/l streptomycin, 6mM L-glutamine, 1% sodium pyruvate, 1% non-essential amino acids. The cells were then resuspended at a density of $2x10^6$ cells/ml in RPMI 1640, supplemented with 20% heat-inactivated foetal calf serum. Cells were incubated at 37°C in 5% CO_2 humidified atmosphere and inoculated with 1ml (10^5 viral particles for 4 to $5x10^6$ lymphocytes) of filtered supernatant from the EBV B95-8 productive culture in the presence of 200µl (2µg CSA for 4 to $5x10^6$ total lymphocytes) of CSA (Sandoz) until the change of the medium, after which the medium was changed twice a week. Cultures were regularly screened for mycoplasma contamination using the Mycoplasma Detection Kit (Boehringer).

2.1.3.5 Leptomeningeal and choroid plexus cell cultures

These cells were cultured by Perron and colleagues as previously described (Perron *et al.* 1992; Perron *et al.* 1991; Perron *et al.* 1989) with acidic fibroblast growth factor (FGFa, Boehringer ref.1376462) at a final concentration of 10ng/ml and heparin at a final concentration of 50U/ml. The anti-interferon antibody used was a polyclonal horse anti α and β interferon (Boehringer, discontinued) with a final neutralising activity of 10U/ml.

2.1.3.6 Extracellular virion purification

All extracellular virion purification and sucrose density gradients were performed by Perron and colleagues as previously described (Perron *et al.* 1992; Perron *et al.* 1991; Perron *et al.* 1989). From each sucrose gradient 0.5-1ml fractions were collected from the top of the tubes, using a sterile tip for each fraction. 60µl was used to assay for RT activity, and the remainder mixed with an equal volume of buffer containing 4M guanidinium thiocyanate, 0.5% N-laurylsarcosine, 25mM EDTA, 0.2% ß-mercaptoethanol adjusted to pH 5.5 with acetic acid. These mixtures were stored frozen at -80°C until RNA extraction (Chomczynski and Sacchi, 1987).

2.1.3.7 Reverse transcriptase (RT) activity assay

RT-activity was measured by Perron and colleagues using 20mM Mg^{2+} and poly-Cm or polyC templates, in virion pellets or fractions from sucrose gradients as previously described and summarised below (Perron *et al.* 1989; Perron *et al.* 1991; Perron *et al.* 1992). Culture supernatant was thawed and cellular debris pelleted at 3000 rpm for 30 minutes and then centrifuged for 2 hours at 100,000xg. The pellet was resuspended in a volume of 0.05M Tris pH8.5 which resulted in a concentration factor of 200. 20µl was added to 5µl 0.5 M Tris, 0.04M DTT pH 8.2, 5µl 0.1M NaCl , 5µl 0.3M $MgCl_2$, 23µl water, 10µl 2% NP40, 2µl poly-C/oligo-dG_{12-18} (10U O.D./ml; Pharmacia), 5µl ^{3}H-guanosine-tri-phosphate (NEN ref. NET 543) and incubated at 37°C for 75 minutes. To measure DNA-directed DNA polymerase activity poly-dC/oligo-dG_{12-18} was used instead of poly-C/oligo-dG_{12-18}. Reactions were stopped by adding 75µl of cold 20% trichloroacetic acid (TCA), 12.5% sodium phosphate and 12.5% sodium pyrophosphate. After 3 hours at -20°C the tubes were emptied and washed 4 times with 5% TCA onto a cellulose acetate membrane (0.45µm pore size) under suction. The membranes were dipped in a scintillation liquid (Ready-Safe, Beckman) and the enzyme activity evaluated in Counts Per Minute (CPM) and Decays Per Minute (DPM) in a β-counter. Each sample was tested in triplicate and the mean and standard deviation (SD) calculated. The intrinsic SD of this method had previously been determined and if the SD of a sample exceeded twice the intrinsic SD the sample was re-tested.

2.2 DNA Extraction

2.2.1 Preparation of buffer equilibrated phenol

Water saturated phenol (Rathburns Ltd.), was buffer equilibrated as follows. The upper aqueous phase was removed and replaced with an equal volume of 1M Tris pH 8.3 and thoroughly mixed by vigorous shaking. The phases were allowed to separate and the aqueous phase removed and replaced with an equal volume of 0.1M Tris pH 8.3. This was repeated until the pH of the aqueous phase remained above pH 7.6. The antioxidant, partial inhibitor of RNase, and weak chelator of metal ions, 8-Hydroxyquinilone was added at a concentration of 0.1% w/v.

2.2.2 Phenol chloroform extraction

DNA was extracted either from ~10^8 cultured cells or from 5ml blood. The PBMCs from 5ml of whole blood suspended in 1ml freezing medium were rapidly thawed by placing the cryo-tube in a 37°C water bath. PBS (9ml) was added and mixed by inversion. The cells were pelleted at 400xg for 10 minutes, the supernatant removed, the cells resuspended in 0.5ml of DNA extraction buffer (Appendix 1) and incubated at 60°C for 2 hours. The proteinase K was heat inactivated at 95°C for 10 minutes. The DNA was extracted by performing sequentially a phenol extraction, two phenol chloroform extractions followed by a final chloroform extraction. Each extraction

was performed using an equal volume of organic and aqueous phase. The DNA was precipitated overnight by adding two volumes of absolute ethanol and 0.1 volumes of 3M sodium acetate pH 7. The DNA was pelleted at 15000xg for 15 minutes, washed twice with 75% ethanol and resuspended in 10µl of water. The concentration and purity of the DNA was determined from its optical density at 260 and 280 nm (Sambrook *et al.* 1989).

2.2.3 DNA extraction from cryopreserved blood

Cryopreserved blood in Glycigel was melted at 37°C and 1ml centrifuged at 14000xg for 1minute. The DNA was extracted using a simple rapid nuclear preparation and lysis method (Kaye *et al.* 1991). Briefly, the lower 0.5ml of the centrifuged Glycigel sample was added to 0.5ml of Glycigel lysis buffer (Appendix 1), mixed well and centrifuged at 14000xg for 20 secs. The supernatant was discarded and the resulting nuclear pellet washed a further 2 times with 1.0 ml of lysis buffer. The pellet was resuspended in 100µl of PCR compatible Glycigel extraction buffer (Appendix 1) and incubated at 60°C for 2 hours. The proteinase K was inactivated by heating the extract to 95°C for 10 minutes.

2.2.4 Optical density measurement of concentration of nucleic acids.

1 to 10 µl of the DNA sample was made up to 1ml with TE (Appendix 1), placed in a glass cuvette and the optical density (OD) of the sample measured at 260 and 280 nm. The concentration of the sample was calculated using the following formula.

OD 260nm = 1 for 50µg/ml double stranded DNA

OD 260nm = 1 for 33µg/ml synthetic oligonucleotide DNA

Purity was determined from the OD 260/280 ratio, 1.8 to 2.0 being acceptable.

2.3 RNA Extraction

2.3.1 Modification of the method of Chomczynski and Sacchi

The denaturing solution used was 4M guanidinium thiocyanate, 25mM sodium citrate pH 7, 1% w/v N-laurylsarcosine and 0.72% v/v 2-mercaptoethanol.

RNAzol A was prepared by mixing 1volume of the denaturing solution with 0.1 volumes of 2M sodium acetate pH 4 and 1volume of water saturated phenol. A 200µl volume of sample was added to 800µl of RNAzol A. 100µl of chloroform was added, the solution vortexed for 15 seconds, cooled on ice for 15 minutes and the phases separated by centrifugation at 15000xg for 15 minutes at 4°C. The upper

aqueous phase was removed, an equal volume of propan-2-ol added and the nucleic acid precipitated overnight at -20°C, 3µg of glycogen was used as a carrier. The RNA was pelleted at 4°C and 15000xg for 15 minutes. The pellet was washed twice with 1ml of ice cold 75% ethanol by vortexing and subsequently re-spinning at 4°C and 15000xg for 8 minutes. The pellet was then left in an open tube at room temperature for 15 minutes for any remaining ethanol to evaporate. The dry pellet was resuspended in 20µl of RNase free water (Chomczynski and Sacchi, 1987).

2.3.1.1 RNA extraction from purified virions, serum, plasma and tissue culture fluid

Sucrose density gradient fractions of purified virions were mixed with an equal volume of 4M guanidinium thiocyanate, 20mM sodium acetate pH 5.2 and frozen at -70°C. The fractions were received frozen on dry ice, rapidly thawed at 37°C and 400µl of this (equivalent to 200µl of sucrose fraction) extracted. The buffer was adjusted and the RNA extracted according to the modification of the method of Chomczynski and Sacchi detailed above.

For serum, plasma and tissue culture fluid a 200µl volume was extracted according to the method detailed above.

2.3.2 Modified SNAP™ RNA extraction

Alternatively, RNA was extracted using the Simple Nucleic Acid Preparation (SNAP™) RNA extraction kit (Invitrogen™). This extracts RNA by guanidine-HCl / detergent lysis and adsorption to a silica matrix. The composition of the solutions used are described in Appendix 1. All centrifugations were performed at room temperature unless otherwise stated.

2.3.2 1 SNAP™ extraction of RNA from PBMCs

450µl of binding buffer was added directly to the cell pellet derived from 5ml of blood. If it was a stored frozen pellet the binding buffer was added immediately it was removed from the -70°C and the cells quickly dissolved by flicking the tube. The solution was thoroughly mixed by inversion. 150µl of TE pH 8 and 300µl of propan-2-ol was added and mixed by inversion 10 times.

Binding, washing and elution

500µl of the above mixture was transferred to the SNAP™ column, centrifuged at 15000xg for 1 minute and the flow through discarded. The remainder was added to the SNAP column and centrifuged at 15000xg for 1 minute and the flow through discarded. The column was washed with 600µl of Super wash and the flow through discarded. The column was washed with 600µl of 1x RNA wash and the flow

through discarded. This wash was repeated with a 2 minutes 15000xg spin and the flow through discarded. The bound nucleic acid was eluted by incubating with 135µl of RNase-free water for 5 minutes and centrifuged at 15000xg for 1minute.

DNasing

15µl of 10x DNase buffer and 3 µl (30 units) of DNase I, (RNase free, Boehringer Mannheim Cat. No. 776 785) was added and incubated for 30 minutes at 37°C. 450µl of Binding Buffer was added and mixed by inversion 6 times. 300µl of propan-2-ol was added and mixed by inversion 10 times.

The binding, washing and elution steps were again performed, but omitting the Super wash, and the DNasing repeated but using 20 units of enzyme. Finally the binding, washing and elution steps were repeated but the nucleic acid was eluted in 105µl of RNase-free water.

2.3.2.2 SNAP™ extraction of RNA from brain tissue

The protocol was slightly modified for extracting RNA from brain tissue by inclusion of a chloroform step to aid the removal of lipids. Once the tissue had been dissolved in the binding buffer and 150µl of TE pH 8 had been added, an equal volume of chloroform (500µl) was added and the solution vortexed for 15 seconds. The phases were separated by centrifugation at 15000xg for 15 minutes and the

upper aqueous phase removed. 300µl of propan-2-ol was then added prior to binding the RNA to the SNAP column as detailed above.

2.3.2.3 SNAP™ extraction of virion associated RNA from serum

200-500µl of serum was filtered using 0.45µm spin filters (Nanosep MF™, Flowgen Catalogue No. U3-0126 Ref. ODM45) to remove cells and cellular debris. The serum was centrifuged for 5 minutes at 13000xg (or for further 10 minutes if not all filtered). 150µl of filtered serum was incubated with 10 units RNase ONE™ (Promega Catalogue No. M4261) for 30 minutes at 37°C. The filtered, RNased serum was then extracted using the SNAP™ RNA extraction kit.

10µg of poly-A RNA (Boehringer Mannheim Cat. No. 108626) was added to the 450µl of binding buffer to act as a carrier. This was added to the serum and mixed by inversion 6 times. 300µl of propan-2-ol was added and mixed by inversion 10 times. The binding, washing and elution steps were performed as above with a single round of DNasing (30 units). The RNA was eluted with 105µl of RNase-free water.

2.4 cDNA synthesis

2.4.1 "In house" cDNA synthesis method

5 µl of the RNA extracted from 200 µl serum or tissue culture supernatant by the method of Chomczynski and Sacchi (Chomczynski and Sacchi, 1987) was incubated for 30 minutes at 37°C with 0.3 units RNase-free DNase-1 (Boehringer) in a 20 µl reaction containing 5 mM Hepes-HCl pH 6.9, 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 50 mM Tris-HCl pH 7.5, 7.5 µM random hexamers, 0.5 mM each dNTP, and 20 units recombinant RNase inhibitor (Promega). The DNase was heat inactivated at 80°C for 10 minutes and a further 20 units of RNase inhibitor and 20 units MMLV RT (Pharmacia) were added to each tube in a Genesphere™ enclosure (Safetech, Ireland). cDNA was synthesised for 90 min at 37°C. Following reverse transcription, the cDNA was boiled for 5 min then cooled rapidly on ice.

2.5 PCR

2.5.1 Theory of PCR

The polymerase chain reaction is the enzymatic process whereby DNA is exponentially amplified from two adjacent sections of known sequence using two oligonucleotide primer sequences, in a series of thermal cycles. It was invented by Kary Mullis in 1985 (Saiki *et al.* 1985). The DNA is first thermally denatured, then the two primer sequences, one sense the other antisense, are annealed to the target DNA by reducing the temperature below the anneal temperature of the primers. The DNA is copied by a DNA polymerase extending from the annealed primers and the process repeated. With each thermal cycle the amount of DNA is theoretically doubled as each strand is copied.

The process of PCR was greatly simplified with the incorporation of a thermostable DNA polymerase in the protocol (Saiki *et al.* 1988). This meant that the procedure could be performed for as many cycles of amplification as desired without having to add more enzyme at each cycle.

Nested PCR is the sequential amplification of the product of a PCR reaction using another PCR reaction and a new set of primers internal to the original set. The reason for performing a nested PCR is to increase the sensitivity and specificity of

the PCR. Typically the sensitivity of a nested PCR is at the single copy level. The specificity is also typically such that no PCR product bands other than the intended one are visible upon gel electrophoresis.

2.5.2 Principles of primer design

PCR primers were designed according to the following criteria:

The physical characteristics of each primer in a pair were matched as closely as possible, theoretical anneal temperatures were ideally designed to be between 50°C and 65°C, and primers to be 20 to 30 bases in length with an approximately 50% GC content. The human genome contains ~$3x10^9$ bp and 4^{16} is $4.3x10^9$, so if the sequence of the bases in the human genome was randomly composed then, in theory, a 16 base primer would be totally specific. However, this is not the case, as functional constraints on genes impose sequence constraints and hence gene families of similar function have similar sequences. In practice, carefully designed primers of 20-30 bases can give the required degree of specificity.

Primers were selected to have a 3' terminal T where possible (Kwok *et al.* 1990), and to avoid ending on the third base of a codon, which is often variable, since mis-match at the 3' end of the primer is likely to prevent amplification. Primer sequences were searched against the EMBL and Genbank databases to ensure that only the target of interest would be amplified. If a sequence was sufficiently similar to other related, but different, non target sequences so that they aligned with more

than 50% homology then primers were selected such that their 3' section was in an area of mis-match. The primers in a given pair were checked to avoid complementarity of their 3' ends to prevent the possibility of concatamerisation. In addition, each primer was checked for self complementarity to avoid the formation of hairpin loop structures.

2.5.3 Principles of PCR optimisation

The aim of optimising each nested PCR was to be able to detect a single target molecule. Sensitivity was determined by the end point of the PCR on ten fold serial dilutions of the target DNA. Each PCR was initially performed using Cetus *Taq* with the appropriate anneal temperature and standard amount of primer. If optimisation was required the initial parameters which were varied were the anneal temperature and enzyme/buffer, then the Mg^{2+} concentration and primer concentration. In practice it was found that the majority of PCRs only required optimisation of the anneal temperature.

2.5.4 Standard PCR protocol

The contamination prevention measures of Kwok and Higuchi (1989) were strictly applied throughout. PCRs were performed in a 500µl micro-centrifuge reaction tube (Treff labs.) with a 25 or 50µl reaction volume. The target material was either 0.5µg of DNA, 2.5-25µl of Glycigel nuclear extract, 2.5-5µl of cDNA, or a small loop

inoculum of bacterial culture for the PCRs; or 5-25µl of RNA for the combined RT-PCRs. The number of cycles in the first round of PCR was between 35 and 40, and between 25 and 35 in the second round. If a second round PCR was required, 0.5µl of first round product was transferred to the second round mix. The optimised conditions for the various PCRs are listed in Table 2. Normally each first round PCR was preceded by a 4 minute 95°C denaturation step, and all PCRs were followed by a final 7 minute extension to allow for full completion of the final DNA synthesis step.

2.5.4.1 "Pan Retrovirus" PCR

The first round, 25 µl PCR mix was incubated with 0.3 units DNase-1 at 37°C for 30 minutes and then heat inactivated at 80°C for 10 minutes. 2.5 µl of cDNA was added in the Genesphere™ enclosure and the tubes heated to 80°C before adding 0.5 units of *Taq* polymerase (Perkin Elmer) individually to each tube ('hot start'). The tubes were maintained at 80°C until cycling commenced. The PCR conditions were as detailed in Table 2. 0.5µl of first round PCR product was transferred to the second round DNase-treated PCR mix using the 'hot start' procedure. The second round PCR was performed as described in Table 2.

2.5.4.2 Intra-*Alu* PCR.

Human *Alu*-repeats were amplified by intra-*Alu* PCR (236 bp product) using the primer set ALI described by Cole *et al.* (1991). The PCR mix was DNased, cDNA was added in the Genesphere™ enclosure, and the 'hot start' procedure employed. PCR conditions were as described in Table 2.

2.5.4.3 HTLV *tax/rex* PCR

0.5μg of sample DNA or 25μl of nuclear extract (equivalent to approximately 1μg DNA, (Kaye *et al.* 1991)) was amplified by nested PCR. A 10μl aliquot of the second round reaction products was analysed by electrophoresis on a 2% agarose gel. Samples which produced a second roundband of the predicted size (128bp) were typed by restriction enzyme analysis using the endonucleases *Sau 3A* and *Taq* 1 (Tuke *et al.* 1992) .

2.6 Combined RT-PCRs

2.6.1 Titan™ RT-PCR

Combined Reverse Transcriptase and Polymerase Chain Reaction (RT-PCR) for the detection of MSRV-*pol* RNA (Section 3.5.2) was performed using the Titan one tube RT-PCR system. This contains a mixture of AMV reverse transcriptase and *Taq* plus *Pwo* DNA polymerases (Boehringer Mannheim Cat. No. 1 855 476). 25µl of SNAP extracted RNA was used in the combined RT-PCR reaction. The total reaction volume was 50µl. Promega rRNAsin (Cat. No. N2511, 10 units) was the RNase inhibitor used. 1 M dithiothreitol (DTT) (Pharmacia) was used instead of the supplied 100mM DTT in order to reduce the reagent volume. 170 ng of the first round primers were used (Table 2; Garson *et al.* 1998). A single master mix was prepared and the sample RNA added last. This was performed at room temperature, not on ice.

The RT step consisted of two sequential 30 minutes incubations at 50°C and then 60°C. This was immediately followed by the first round PCR as detailed in Table 2. The second round PCR was performed using the Expand™ long template PCR system (Boehringer Mannheim Cat. No. 1681 842). 0.5 µl of the RT-PCR mix was transferred to 25µl of second round PCR mix, cycling completed (details given in

Table 2; Garson *et al.* 1998) and 10µl of the final PCR products run on a 2% agarose gel.

The no-RT controls were performed in parallel using Expand™ for both rounds. The first round used 0.75µl of the Expand™ enzyme mix (3.5 U/µl *Taq* plus Pwo DNA polymerases) in the same buffer as for the Titan™ RT-PCR, and was incubated in the same PCR machine (Robocycler™, Stratagene), thus including a mock RT step consisting of two sequential 30 minutes incubations at 50°C and then 60°C. This was immediately followed by the PCR.

As a positive control a human DNA dilution series was used in both the RT-PCR and the no-RT PCR. For an assay to be acceptable the RT-PCR and no-RT PCRs had to have detected human DNA equivalent to between 1 or 0.1 cells.

2.6.2 Pyruvate dehydrogenase (PDH) *Tth* RT-PCR

Pyruvate dehydrogenase is a single copy cellular housekeeping gene. The PDH RT-PCR primers had been designed to flank an intron, so that a size difference in the PCR product would be observed between amplified RNA and DNA (Howard 1998). The PDH RT-PCR was used to check for cellular RNA or DNA contamination, both of which may produce false positives in MSRV PCR assays (Garson *et al.* 1998).

RT-PCR was performed using the *Tth* one tube RT-PCR system (Perkin Elmer Cat. No. N8080179). *Tth* is a thermostable DNA polymerase with both RNA and DNA dependent activity. 25µl of RNA was used in the combined RT-PCR reaction with a total reaction volume of 50µl. A single master mix was prepared and the sample RNA added last.

The RT step consisted of two sequential 30 minutes incubations at 56°C and then 60°C. This was immediately followed by the PCR as detailed in Table 2. The PCR products were run on a 4% Nu-sieve agarose gel to distinguish RNA derived (61 bp) signals from DNA (143 bp) (Garson *et al.* 1998).

The current protocol for the specific detection of virion associated MSRV-RNA in serum is detailed in Appendix 2.

2.7 Cloning and sequencing

2.7.1 Cloning

2.7.1.1 Restriction endonuclease digests

Restriction endonuclease digests were performed according to the enzyme manufacturer's instructions (Pharmacia). Digests of second round PCR products were performed on 5µl in a 20µl reaction mix containing 10 units of the restriction enzyme and 2µl of the appropriate 10x reaction buffer. Digestion was performed at the recommended temperature for 90 minutes. A 10µl aliquot of the digest was analysed by electrophoresis on a 2-4% NuSieve agarose gel as appropriate.

2.7.1.2 Gel electrophoresis

Sequencing gels were made using Sequagel™ reagents with 8% acrylamide and run for 2 hours at 70 W. Sodium acetate was then added to the buffer in the cathode chamber to a final concentration of 1.0M and the gel run for a further hour.

Agarose gel electrophoresis was performed on a 2-4% agarose gel as appropriate, and gels run at 7.5V/cm for between 30 minutes and an hour and a half. DNA bands were visualised by ethidium bromide staining and photographed at 302nm.

2.7.1.3 Ligation, transformation and selection of transformants

PCR products were analysed on 2-4% agarose gels as appropriate. Bands of the correct size were excised and the DNA purified using 'Mermaid' reagents (Bio 101, La Jolla, Ca). DNA was cloned using either the TA-cloning™ system (British Biotechnology) or the PCR cloning vector pGEM-T (Promega) according to the manufacturer's instructions. Briefly; purified PCR products were ligated into 12.5ng of the linearised PCR cloning vector at a 1:4 molar ratio (2.4ng of 142bp product). *E.coli* JM109 bacterial cells (50µl) were transformed by the products of the ligation reaction. JM109 Competent Cells High Efficiency (>10^8 colony forming units (cfu)/µg) were obtained from Promega. 450µl of Luria-Bertani (LB) medium (Appendix 1) was added to each transformation reaction, the tubes inverted to mix, incubated at 37°C for 1 hour and 200µl plated onto LB/amp/IPTG/X-Gal plates (Appendix 1) and incubated at 37°C overnight.

Blue/white selection in combination with PCR screening of colonies was used to identify positive clones. White colonies, together with any light blue ones, were picked and plated onto a gridded LB/amp plate and the loop inoculated into a PCR mix. The plate was incubated at 37°C whilst the PCR was performed and the gel run. Colonies which were PCR positive were picked from the gridded plate and inoculated into bijoux containing 3ml LB/amp medium (Appendix 1). These broths were incubated overnight on an orbital shaker at 37°C.

2.7.2 Culture of R408 phage and purification of single stranded DNA for sequencing using "magic minipreps"

The following morning, 50µl of overnight culture was inoculated into 3ml of LB broth containing ampicillin and incubated at 37°C for 30 to 40 minutes to achieve logarithmic phase growth. The culture was then infected with R408 helper phage (Promega) at a multiplicity of infection (MOI) of 10 (10µl of R408 helper phage at a concentration of $5x10^{10}$ plaque forming units/µl was added) and incubated with vigorous agitation and good aeration for between 4.5 and 6 hours. This allowed the phage to replicate efficiently, without the *E.Coli* beginning to lyse. The broths were split into 3 x 1.5ml micro centrifuge tubes and centrifuged at 3000xg for 15 minutes at 4°C to pellet the cells, the supernatant was then removed and transferred to a new micro-centrifuge tube. This centrifugation was repeated and the phage harvested by precipitating overnight at 4°C using 0.25 volumes of 3.75M ammonium acetate pH 7.5, 20% polyethylene glycol (RMM 8000).

The precipitated phage were harvested by spinning at 13000xg for 15 minutes at 4°C and the supernatant discarded. The pellet was resuspended in 50µl of TE and the 3 x 50µl from each culture pooled, to which equal volumes of lysis mix and neutralisation mix from Promega's "magic miniprep" kit were added. This was mixed with 1ml of DNA purification resin and added to a mini-column using a 2ml syringe. The column was washed twice with 1ml of wash buffer and centrifuged at 13000xg for 20 seconds. 25µl of water was added to the column and incubated at room temperature for 5 minutes. This dissolved the single stranded phage DNA, but

left contaminating genomic DNA still bound to the column. The column was placed in a new micro centrifuge tube and centrifuged at 13000xg for 25 seconds to elute the phage DNA, which was stored at -20°C until required.

2.7.3 Sequencing

Manual dideoxy sequencing was performed as described below. Alternatively, automated cycle sequencing (Prism cycle sequencing kit and 373A sequencer, Applied Biosystems) was performed by Perron and colleagues in Lyon.

2.7.3.1 Manual dideoxy sequencing

The sequencing reaction was performed as a modification of the Sequenase™ Version 2.0 T7 DNA Polymerase Sequencing Protocol (United States Biochemical (USB)), the sequencing reactions being performed in V bottomed microtitre plate wells. Briefly, this was as follows:

In a 500µl micro-centrifuge tube 35ng of M13 sequencing primer (-40), 500ng of single stranded DNA, 1 µl DMSO, and 2µl 5x Sequenase™ reaction buffer were mixed, and the volume made up to 10 µl with water. The primer was annealed to the template DNA by denaturing the samples at 70°C for 5 minutes and cooling to 25°C over 30 minutes in a Techne™ PHC-3 PCR machine. Simultaneously a V bottomed

microtitre plate was prepared, containing the termination reactions with 4 separate wells, labelled G, A, T, C, for each sample. Each well contained the appropriate dideoxy termination mixture, (2μl of the ddNTPs and 0.2 μl dimethyl sulfoxide (DMSO) per well (Appendix 1)). The microtitre plate containing the termination reactions was covered with a plate sealer and placed on top of wet tissues (to ensure good thermal contact) on a hot block at 50°C.

The annealed sequencing primer and template were pulse spun to bring down any condensation. 3.5μl of the labelling reaction mix (Appendix 1) was added to the 10μl of the annealed sequencing primer and template and incubated at room temperature for 2 minutes. 3.1μl of the completed labelling reaction was added to each pre-heated termination reaction (wells labelled G A T C). After 2 minutes, or when the last sample had been terminated, which ever was the longer, 4 μl of stop solution (Appendix 1) was added to each well. This was also performed whilst the samples were heated at 50°C on the hot block. The samples were heated to 80°C to denature the DNA prior to loading 4μl on a sequencing gel which had been pre-run for 30 minutes. Alternatively, the plate was covered with a plate sealer and stored at -20°C until the samples were run on a sequencing gel.

Modified nucleotide mixes were used, these mixes have a lower GC content than those provided with the USB Sequenase™ kit. For details of labelling reaction mix, dNTP mix and termination mix see Appendix 1.

2.8 Hybridisation analysis of PCR products: MSRV-*pol* detection by Enzyme Linked Oligo-Sorbent Assay (ELOSA)

The protocol was essentially as previously described (Mallet *et al.* 1993) but was modified for the detection of MSRV and ERV-9. Nunc MaxiSorp™ microtitre plates were coated with 100ng per well of capture probe CpV1b, either by passive adsorption (Mallet *et al.* 1993) or preferably by using streptavidin coated plates and biotinylated CpV1b (Table 2; Figure 2).

Nunc MaxiSorp™ plates were coated with streptavidin as follows: 100µl per well of 5 µg/ml streptavidin (Promega) in carbonate-bicarbonate buffer (Sigma) was incubated overnight at room temperature in a humidified box. The wells were washed twice with PBS and blocked by filling with 1% Bovine Serum Albumin (BSA) in PBS containing 0.1% sodium azide. The plates were stored at +4°C in a humidified box until required. The plates were washed twice with PBS/Tween (Appendix 1) prior to binding the biotinylated capture oligonucleotide (bioCpV1b).

The biotinylated capture oligonucleotide was bound to the streptavidin coated wells by incubating 100µl of bioCpV1b at a concentration of 1ng/µl in 3x PBS for 2 hours at 37°C. The plates were then washed twice with PBS/Tween.

The PCR products were denatured in one of the following two ways, preferably using the latter method.

(i) 10µl of PCR product was added to 15µl of water and 65µl of hybridisation buffer (Appendix 1). 10µl of 2M NaOH was added, the sample vortexed and incubated at room temperature for 8 minutes. This was neutralised by adding 10µl 2M acetic acid. 15µl of hybridisation buffer was added, and the sample vortexed.

(ii) 10µl of PCR product was added to 15µl of water, 80µl of hybridisation buffer and 20µl of 2M sodium acetate. The sample was vortexed and incubated at 95°C for 10 minutes, then plunged into wet ice to prevent re-annealing.

50µl of denatured PCR product was added in duplicate to coated wells. Detection solutions were prepared by diluting the peroxidase-labelled detector probe DpV1 (Figure 2; Table 2) to a concentration of 0.1ng/µl in hybridisation buffer and 50µl was added to each well. The plates were incubated at 37°C for 1 hour then washed 3 times with PBS/Tween. Fresh o-phenylenediamine (OPD) substrate was prepared (Appendix 1), 100µl added to each well and the plate incubated in the dark for 30 minutes at room temperature. The reaction was stopped with 100µl of 0.5M H_2SO_4 and the plate read at 492nm with a second cut off filter of 630nm.

The assay cut-off was defined as the mean of 4 negative controls plus 0.2 OD_{492} units. Each sample was tested in duplicate and was only scored as positive if it generated signals above the cut-off in two independent experiments.

2.9 Serology

All commercial serological tests were performed in accordance with the manufacturers' instructions. HTLV seroreactives were defined as those samples which tested positive in the Fujirebio HTLV-I particle agglutination test. These samples underwent confirmatory serological testing, by the Abbott HTLV-I ELISA, UBI HTLV-I and HTLV-II ELISA, an "in-house" HTLV-I specific competitive ELISA (adapted from (Tedder *et al.* 1984)), and Du Pont Western blot.

3 RESULTS

3.1 HTLV in MS

3.1.1 HTLV in MS. Introduction

As discussed in Section 1.2.5.2.1 the aetiology of MS is thought to involve an environmental factor, which has been postulated to be viral, and possibly retroviral. HTLV-I has come under scrutiny in this context as discussed earlier (Section 1.1.1.6.1.8).

In order to address the question of the potential involvement of HTLV in MS an HTLV PCR study was undertaken. The rationale for this approach was that PCR is the most sensitive method for obtaining evidence of retroviral infection and the two initial reports of HTLV involvement in MS were both PCR based (Reddy *et al.* 1989; Greenberg *et al.* 1989). An HTLV nested PCR which had been developed and successfully employed in the clinical diagnostic setting (Tuke *et al.* 1992) was used to test PBMC derived DNA from MS patients. We had previously established that this was the most sensitive and specific test available for detecting HTLV infections (Tuke *et al.* 1992).

3.1.2 HTLV in MS. PCR with HTLV specific primers

3.1.2.1 HTLV in MS. PCR with *tax/rex* primers

3.1.2.1.1 Introduction

Diagnosis of multiple sclerosis was made according to the criteria of Poser (Poser *et al.* 1983) and supported by magnetic resonance imaging in most cases. MS patient DNA samples were analysed for the presence of HTLV genomes as described (Section 2.5.4.3; Table 2) and more fully in (Tuke *et al.* 1992). This nested PCR assay is capable of detecting single copies of HTLV-I and HTLV-II in genomic DNA extracted from PBMCs. We have demonstrated that the set of nested oligonucleotide primers, based on the highly conserved *tax/rex* region of the viral genome, was able to detect both HTLV-I and HTLV-II proviral sequences in clinical samples of diverse geographic origins, from the United States, Great Britain, Japan, the Caribbean, Italy, Greece, Iraq and West Africa (Figure 3). Rapid discrimination between HTLV-I and HTLV-II infections was achieved by restriction enzyme analysis of unpurified second round PCR products, even in those cases in which serological assays had failed to provide a definitive result (Figure 4). 53 HTLV infections (37 HTLV-I and 16 HTLV-II) were identified by this technique and complete concordance with serological typing, where possible (41 cases), was observed (Tuke *et al.* 1992).

3.1.2.1.2 Results

Twelve patients with MS, whose blood samples were analysed, were all negative for HTLV proviral DNA when tested by the optimised *tax/rex* PCR method. Positive control DNA was obtained from C1218M (HTLV-II) and C81-66-45 (HTLV-I) cell lines. The detection limit for HTLV-I and HTLV-II was a single copy. The possibility remained however, that there was a different but related retrovirus present in the samples which was associated with MS. To investigate this possibility the stringency of the PCR was relaxed by reducing the anneal temperature, and the experiment repeated. It was found that if the conditions were sufficiently relaxed in the second round (40°C) a faint band of the expected size could be amplified by PCR from the MS patient samples. However, under these very low stringency conditions control DNA samples also produced a band of the expected size (Figure 5), suggesting that the product of the amplification was not an exogenous viral sequence but an HTLV related endogenous one. In an attempt to determine whether the amplified sequences from the DNA of patients with MS were endogenous or exogenous in origin, amplification of other regions of the genome was attempted.

3.1.2.2 HTLV in MS. PCR with *env* primers

DNA samples from twelve patients with MS were analysed for the presence of HTLV *env*-related sequences as described (Section 2.5.4; Table 2). A semi-nested set of HTLV *env* PCR primers were used and the PCR performed under conditions of low stringency (56°C anneal for the first round and 40°C for the second round). As with the *tax/rex* low stringency PCR, a band of the predicted size could be amplified from MS patient genomic DNA, but control DNA also amplified.

3.1.2.3 HTLV in MS. PCR with *protease* primers

12 MS patient DNA samples and 4 control DNA samples were analysed for the presence of HTLV I *gag/protease* genome sequences. A nested set of HTLV-I *gag/protease* primers was used as described (Section 2.5.4; Table 2). Using a 60°C 30 cycle second round PCR, strong bands were produced in the HTLV-I positive control samples, but a smear including weak bands of the expected 235 bp size, were also present in most (11/12) of the MS samples and the negative control DNA. One of the 11 MS patient DNA samples tested gave a fairly strong band, but this was not reproducible (Figure 6). A 5 degree increase in the second round anneal temperature of the PCR resulted in complete loss of product, including that of the HTLV-I positive control. The stringency of the PCR was relaxed further by reducing the anneal temperature 5 degrees to 55°C and the experiment repeated. Again it was found with this reduced stringency that a weak band of the expected size was

amplified from the MS patient samples, along with other spurious bands. However under these low stringency conditions control DNA samples also produced a band of the expected size.

3.1.3 HTLV in MS. Discussion

From the results of these PCR studies it can be concluded that neither HTLV-I nor HTLV-II is present in the PBMC DNA of the 12 MS samples tested. This is further data in support of the hypothesis that HTLV is not associated with MS. The results of the low stringency PCRs, in which the majority of DNA samples from MS and control subjects were positive, merely provides further evidence that there are HTLV related endogenous sequences in the human genome, as previously established (Shih *et al.* 1989) e.g. HRES1 (Banki *et al.* 1992).

Despite the interest caused by the initial publications on the presence of the retrovirus HTLV, detected by PCR in patients with multiple sclerosis (Reddy *et al.* 1989; Greenberg *et al.* 1989), subsequent publications and presumably far more unpublished findings, such as those presented above (Section 3.1.2), have failed to confirm such an association (Dekaban and Rice, 1990; Fugger *et al.* 1990; Jocher *et al.* 1990; Nishimura *et al.* 1990; Oksenberg *et al.* 1990; Ehrlich *et al.* 1991; Menzo *et al.* 1992; Rasmussen and Clausen, 1992; Merelli *et al.* 1993). It is now widely accepted that these initial and subsequently unsubstantiated claims were due to PCR contamination (Ehrlich *et al.* 1991). Another group has published relatively recently

on the detection of only the HTLV-I *tax/rex* gene, which they intermittently detected in patients with MS sampled sequentially during relapse (Ferrante *et al.* 1997). However, the majority of evidence, including that presented above (Section 3.1.2), does not support the involvement of HTLV in MS.

The lack of detection of HTLV in patients with MS does not preclude the involvement of another retrovirus in the disease. Indeed, despite these negative findings and the general scepticism surrounding the initial HTLV claims, Perron and colleagues (Perron *et al.* 1991; Perron *et al.* 1991b; Perron *et al.* 1997a) and others (Haahr *et al.* 1991; Haahr *et al.* 1994) have presented electron microscopic, immunological and biochemical observations suggesting that a new member of the retrovirus family may be involved in MS. In the following studies (Sections 3.2-3.4) we investigated this possibility by means of a 'Pan-Retrovirus' detection system based on the PCR amplification of retroviral *pol* sequences with degenerate oligonucleotide primers. This strategy was dependent upon the high degree of sequence conservation that occurs in the RT region of the *pol* gene shared by all known retroviruses (Mack and Sninsky, 1988; Donehower *et al.* 1990).

3.2 Development of a "Pan-Retrovirus" PCR system

3.2.1 Development of a "Pan-Retrovirus" PCR system. Introduction

All retroviruses have a common overall genomic structure, which at its simplest level can be expressed as the possession of 2 LTR sequences, *gag*, *pol*, and *env* genes. Relatively well conserved regions exist within the various genes of retroviruses, this is true of *gag*, *pol* and *env*, but the most conserved is the RT region of *pol*. On the basis of the sequence homology of these genes retroviruses can be grouped into families and their evolutionary relationships assessed (Section 1.1.1.2). The degree of sequence conservation also enables the detection of various families e.g. oncoviridae (Dube *et al.* 1997) lentiviridae (Gelman *et al.* 1992) and spumaviridae (Rozenberg *et al.* 1991) and/or members to be based on these conserved motifs. The most useful for the purpose of detecting potentially all retroviruses is the active site motif of RT (Mack and Sninsky, 1988; Donehower *et al.* 1990).

The defining feature of a retrovirus is its ability to flout the central dogma of molecular biology and copy DNA from an RNA template. This it achieves via the product of its RT gene. The unique characteristics of RT can potentially be used to detect all known and as yet undiscovered retroviruses. Currently two main methodological approaches have been developed, one detects the functional enzyme activity, the other the gene which encodes it. There are several conserved motifs in

RT which have been used to detect retroviral sequences (Medstrand *et al.* 1992; Medstrand and Blomberg, 1993), but the two most conserved are the amino acid sequences VLPQG and YXDD which forms the active site (Toh *et al.* 1983; Larder *et al.* 1989). These can be used as target regions for degenerate oligonucleotide PCR primers which should then be capable of detecting all retroviruses (Mack and Sninsky, 1988; Donehower *et al.* 1990). This was the approach we determined to employ in our search for a novel retrovirus in MS. This strategy had already proved successful in the identification of previously uncharacterised proviral sequences (Shih *et al.* 1989; Donehower *et al.* 1990). The initial stage in this quest was the development of a PCR-based method capable of detecting, with great sensitivity, diverse known retroviruses. The evolution of this 'Pan-Retrovirus' detection system is described.

Environmental contamination is potentially a major problem with any PCR method that employs degenerate *pol* primers. This is because the human genome and therefore environmental dust, contains thousands of copies of endogenous retroviral sequences and retrotransposons which are potential targets for amplification. These endogenous sequences account for between 0.6 and 6% (Temin, 1985; Baltimore, 1985) of the total genome.

In order to detect a putative novel retrovirus present in MS patients, the following broad experimental protocol was designed. Viral RNA was extracted from samples rather than proviral DNA. Cell free samples (e.g. serum/ CSF/ tissue culture supernatant) were analysed, in the first instance, to avoid the associated problems of

transcribed endogenous retroviral sequences and of contaminating genomic DNA. The viral RNA was reverse transcribed and the resulting cDNA subjected to degenerate primer PCR amplification. PCR products of the expected size (~140bp) were cloned and sequenced. These sequences were searched (on EMBL/Genbank) for any homology with known retroviral sequences.

3.2.2 Development of a "Pan-Retrovirus" PCR system. Results

Successful amplification of cDNA derived from the target retroviral RNA, required removal of contaminating DNA from the sample and all reagents. Otherwise PCR products from endogenous retroviral DNA sequences would out-compete products derived from the putative exogenous MS-associated retrovirus. Initial experiments demonstrated that contamination of the extracted RNA with genomic DNA, derived either from the patient or from the environment, remained a significant problem, unless specific measures were taken to avoid it. The standard contamination avoidance measures of Kwok and Higuchi (1989) which we had previously employed successfully in many other PCR-based projects (Brennan *et al.* 1993; Makris *et al.* 1993; Tuke *et al.* 1992; Brillanti *et al.* 1991; Garson *et al.* 1991; Parry *et al.* 1991; Tedder *et al.* 1991; Denic *et al.* 1990; Garson *et al.* 1990d; Garson *et al.* 1990b; Garson *et al.* 1990a; Garson *et al.* 1990c), proved entirely inadequate when used in this context with degenerate *pol* primers.

3.2.2.1 Contamination prevention

The MOP primers (Table 2) of Shih *et al.* (1989) were tested in the first instance. RNA was extracted by the method of Chomzynski and Saachi, from sera obtained from patients known to be infected with HIV, or HTLV, or Hepatitis C Virus (HCV) and serum from a negative control. The MOP PCRs were negative on random primed cDNAs derived from these after a single round of 35 cycles. A second round, for a further 25 cycles using the same primers, resulted in all these samples producing a band of the expected size. It also gave a secondary upper (~220bp) ribosomal RNA band (Mark Boyd personal communication). From this result it was evident that the RNA extraction method of Chomzynski and Saachi, despite the authors' claims, was not sufficiently RNA specific and that DNA was co-purifying and being amplified. The RNA preparation was confirmed as the source of the contaminating DNA by performing a no-RT "mock" cDNA synthesis as a control; the MOP PCR was still positive on the majority of samples.

Similarly, contamination of cDNA synthesis and PCR reagents, tubes and pipette tips with human genomic DNA from the atmosphere (human squames are a significant component of dust particles) was a major obstacle, giving rise to an unacceptably high rate of false-positive PCR signals. It was also found that certain manufacturers', or batches, of Moloney Murine Leukaemia Virus (MMLV)-RT enzyme preparations always gave positive "Pan-Retrovirus" signals even on water controls. This was later shown by sequencing of these products to be due to MMLV nucleic acid sequences contaminating the commercial RT preparations.

The following additional contamination prevention measures were therefore investigated: i) ultra-violet irradiation of PCR reagents prior to addition of the template, as described by Sarkar and Sommer (1993), ii) reaction set-up in an enclosed 'Genesphere' device supplied with HEPA (High Efficiency Particulate Air filter) filtered air, iii) DNase digestion of extracted RNA, and of all reaction components, including primers.

The ultra-violet irradiation of PCR reagents, prior to addition of the template, prevented successful amplification of the target. Reaction set-up in an enclosed 'Genesphere' device supplied with HEPA filtered air produced a marginal reduction in contamination.

However, DNase digestion of all reaction components (except enzymes) adequately controlled genomic DNA contamination. The concentration of DNase (1.75 Units optimum) employed and the manufacturer (Boehringer) were found to be critical variables. Similarly, the temperature and duration (80°C, 10 minutes optimum) of the DNase inactivation steps were also found to be critical. These conditions enabled complete digestion of any contaminating genomic DNA, whilst avoiding destruction of the viral RNA.

3.2.2.2 Primer design

Nested degenerate oligonucleotide *pol* primers ("Pan-Retrovirus", an outer set and a partially overlapping inner set) were designed according to the principles outlined in Section 2.5.2 and by Donehower *et al.* (1990) and Shih *et al.* (1989). Briefly, the longest primer sequences with the minimum degree of overlap possible were designed to recognise the conserved VLPQG and YXDD motifs of the active site of RT. Positions where degenerate bases were incorporated, and the number of alternative bases were kept to a minimum. All "Pan-Retrovirus" primers were constructed with a 9 base tail at the 5' end, incorporating a restriction site. This raises the melt temperature (Tm) of the primer/template hybrid after the first cycle and facilitates cloning of PCR products for sequence analysis.

Two alternative versions of this set of "Pan-Retrovirus" primers were synthesised (Table 2). The first set were primer mixes containing alternative bases ('standard degenerate') at non-conserved positions and were synthesised on an Applied Biosystems 381A DNA synthesiser. The second set had inosine-substituted bases at these non-conserved positions and were obtained from Pharmacia Biotech Ltd. Inosine had been shown to be capable of base pairing with all four standard bases (Patil and Dekker, 1990). The MOP primers designed by Shih *et al.* (1989) were evaluated in parallel as a comparison.

3.2.2.3 Optimisation of PCR parameters

These experiments were performed using the HIV plasmid pBH10 as the model template. This was chosen because from the available retroviral sequences HIV was predicted to be a rather poor target (i.e. multiple mismatches) for the degenerate *pol* primers described above. The efficiency of non-nested, nested (Tuke *et al.* 1992) and semi-nested PCRs were compared using various combinations of primers. This included trying 3 rounds of amplification, with the MOP primers in the first round and the "Pan-Retrovirus" primers in the two subsequent rounds. In addition, the performance of primers with inosine-substituted bases at non-conserved positions was compared with that of primer mixes containing alternative bases ('standard degenerate') at non-conserved positions.

The inosine primers did not perform as well as the standard degenerate "Pan-Retrovirus" primers when first evaluated, so a checkerboard experiment was performed to determine which of the inosine primers were sub optimal. It was found to be (PAN-UO, Table 2), which had the inosine nearest the 3' end of the primer. The remaining three inosine containing primers were tested in combination with the standard degenerate primers. It was found that a semi nested format with 2 rounds of PCR using 3 of the 4 "Pan-Retrovirus" PCR primers was the best combination. This was presumably because it avoided using the PAN-DO primer which contained the majority of the mismatch positions with respect to the HIV target.

Initially maximum sensitivity and specificity was obtained by employing a semi-nested format in which one inosine-substituted and one 'standard degenerate' primer were used in a 35 cycle first round and in which two inosine-substituted primers were used in a 30 cycle second round. However, after optimising the Mg^{2+} concentration and first round anneal time it was found that using the standard degenerate primers was preferable.

A wide range of annealing temperatures was tested and optimum results were obtained with 34°C for 30 seconds in the first round and 45°C for 1 minute in the second. A stepped increase in the anneal temperature by 10°C after the first 10 cycles was performed in an attempt to improve the sensitivity and specificity of the PCR, but had no demonstrable beneficial effect. Denaturation and extension temperatures were 95°C and 72°C respectively. A dramatic reduction in non-specific amplification products and consequential increase in sensitivity was achieved by means of the 'hot start' technique (Chou *et al.* 1992) (Figures 7 and 8).

Titration established that the optimum amount of degenerate primer was 200ng (100ng for the inosine primers) per 25µl reaction for both first and second rounds. Less inosine primer was necessary in the PCR reaction presumably because the effective concentration of primer matching the target sequence was greater than with the 'standard degenerate' primers, where only a small proportion exactly complement the target.

The optimum Mg^{2+} concentration was found to be 2.5mM. The PCR buffer used was also found to influence the performance of the PCR, with an alternative to the standard Cetus *Taq* buffer giving the best results (Degenerate buffer Appendix 1). The type of thermostable DNA polymerase used was found to be another critical variable; poor results were obtained with *Pyrococcus furiosus* (*Pfu*) polymerase (Stratagene), and Stoffel fragment (Cetus), whereas recombinant *Thermus aquaticus* (*Taq*) polymerase (Cetus) consistently performed well. No improvement was obtained by addition of the 'specificity enhancer' protein gp32 (Pharmacia) to the PCR reaction mix.

3.2.2.4 Ability of the 'Pan-Retrovirus' PCR system to detect diverse retroviruses

In order to demonstrate the ability of the PCR system to detect retroviruses other than lentiviruses (HIV-1) we selected a human C-type oncovirus (HTLV-1) and a simian D-type virus (Mason-Pfizer monkey virus, MPMV) as alternative targets (Figure 2). The sensitivity limit was approximately ten copies and one copy for the HIV-1 plasmid pBH10 (Ratner, 1985) and for the HTLV-I plasmid pMT2 (Hatanaka and Nam, 1989), respectively (Figures 9 and 10). MPMV RNA, (equivalent to approximately 200 plaque forming units), was also reliably detected following random hexamer primed reverse transcription (Figure 11). In addition, Moloney murine leukaemia virus (MMLV) sequences were unexpectedly identified in PCR products generated from some water controls. The origin of these sequences was found to be trace amounts of contaminating viral nucleic acid in certain batches of

commercial MMLV RT. The specificity of the ~140bp PCR products generated by each of these retroviral targets was confirmed by restriction enzyme digestion using *Sphaerotilus natans*1 (*Ssp*1) for HIV-1, *Deinococcus radiophilius*1 (*Dra*1) for HTLV-1 and *Haemophilus aegyptius*III (*Hae*III) for MPMV (Figure 12) and in some instances also by sequencing. Restriction enzyme fragments of the predicted size were produced in every case. The identity of the MMLV product was discovered by dideoxy sequencing.

3.2.3 Development of a "Pan-Retrovirus" PCR system. Discussion

Several regions of amino acid sequence similarity between the reverse transcriptases of diverse retroviruses have been described (Mack and Sninsky, 1988; Doolittle *et al.* 1989; Medstrand *et al.* 1992; Medstrand and Blomberg, 1993). We have exploited two of the most highly conserved of these regions, VLPQG and YXDD, to design a set of semi-nested 'universal' primers for the detection of novel retroviruses. In order to compensate for the degeneracy of the genetic code we have used low annealing temperatures and primer mixtures composed of 8 or 16 distinct oligonucleotides. A similar 'degenerate primer' approach has been employed successfully by others to search for retrotransposons in the human genome (Shih *et al.* 1989) and for the identification of an unknown, lymphoma-associated retrovirus (Donehower *et al.* 1990). However, the general utility of such systems has been limited by inadequate sensitivity, requiring relatively large amounts ($> 10^7$ virions) of highly purified virus to reliably generate specific PCR products (Donehower *et al.*

1990). We overcame the limitation of low sensitivity by adopting a semi-nested assay format in combination with a 'hot start' technique designed to minimise pre-PCR mispriming and subsequent competitive amplification of non-specific product (Chou *et al.* 1992). Together, these improvements of the "Pan-Retrovirus" PCR have increased the sensitivity to approach the single copy level attainable with standard non-degenerate nested PCR. Unfortunately, this increase in sensitivity was accompanied with an increased susceptibility to contamination by both sample-derived and 'environmental' human genomic DNA, but this initially intractable problem was eventually overcome by extensive DNase-1 digestion of samples and reagents. A system was now available to search for novel retroviruses.

3.3 "Pan-Retrovirus" PCR studies in multiple sclerosis

3.3.1 Detection of a novel *pol* sequence in serum from a patient with MS

3.3.1.1 Detection of a novel *pol* sequence in serum from a patient with MS. Introduction

Having established the ability of the "Pan-Retrovirus" PCR system to detect a wide range of known retroviruses with great sensitivity we proceeded to search for evidence of any novel retroviral sequences in RNA extracted from the sera of MS patients.

3.3.1.2 Detection of a novel *pol* sequence in serum from a patient with MS. Methods

RNA was extracted from the serum of 3 patients with MS using the modified version of the method of Chomczynski and Sacchi (Section 2.3.1). The RNA was DNased and the cDNA synthesised as detailed in Section 2.4.1. The "Pan-Retrovirus" PCR was performed as described in Section 2.5.4.1. Any bands of the expected size (~140bp) were excised from the gel, purified, cloned and sequenced as described (Section 2.7).

3.3.1.3 Detection of a novel *pol* sequence in serum from a patient with MS. Results

"Pan-Retrovirus" PCR amplification of random-primed cDNAs, derived from the serum of 3 patients with MS, resulted in bands of the expected size from 2 cases (only one shown (Figure 13)). These ~140bp bands from the MS patients serum samples were excised from the gel, purified, cloned and sequenced. One of the bands generated a sequence which contained a potential open reading frame and did not appear in either GenBank or EMBL databases. The sequence was characteristic of a retroviral *pol*, and was approximately 75% homologous to the equivalent region of the human retroviral endogenous element ERV-9 (La Mantia *et al.* 1991). The 136bp sequence, obtained from a 30 year old woman (patient 'C') who had developed MS 12 years previously, was designated Multiple Sclerosis associated RetroVirus (MSRV)-c*pol* (Tuke *et al.* 1997; Perron *et al.* 1997c; Figure 2)

3.3.2 "Pan-Retrovirus" PCR studies in MS. Studies on purified virions

3.3.2.1 "Pan-Retrovirus" PCR studies in MS. Studies on purified virions. Introduction

In 1989, Perron and colleagues described the production of extracellular virions which possessed RT activity, by a culture of leptomeningeal cells (LM7) obtained from the CSF of a patient with MS (Perron *et al.* 1989). This was followed by similar findings in monocyte cultures from a series of patients with MS (Perron *et al.* 1991). Neither viral particles nor viral RT-activity were found in control individuals. Furthermore, the LM7 virus could be transmitted to non-infected leptomeningeal cells *in vitro* (Perron *et al.* 1992). To enable full evaluation of its possible role in MS, molecular characterisation of the "LM7" retrovirus was required. Considerable difficulties arose in using "conventional" molecular virological techniques, because of the very limited amount of viral particles available, due to the low levels of expression in the few transiently productive cultures (Perron *et al.* 1997b). Therefore, in order to characterise the retrovirus produced, we applied the highly sensitive "Pan-Retrovirus" PCR to these cultures. Extracellular virions from tissue culture supernatants were used exclusively, to avoid non-specific detection of non virion-packaged RNA and of endogenous elements from contaminating human DNA.

3.3.2.2 "Pan-Retrovirus" PCR studies in MS. Studies on purified virions. Methods

All cells were cultured, virions purified on sucrose density gradients and RT assays performed as described (Section 2.1) by Perron and colleagues in Lyon. RNA was extracted from the sucrose density gradients fractions using the modified version of

the method of Chomczynski and Sacchi (Section 2.3.1). The RNA was DNased and the cDNA synthesised as detailed in Section 2.4.1. The "Pan-Retrovirus" PCR was performed as described in Section 2.5.4.1.

3.3.2.3 "Pan-Retrovirus" PCR studies in MS. Studies on purified virions. Results

Choroid plexus cells (Perron H *et al.* 1991b), obtained post-mortem, and EBV-immortalised peripheral blood B-lymphocytes (Perron *et al.* 1991) from patients with MS frequently gave rise to cultures expressing 100-120nm viral particles associated with RT-activity similar to that of the first LM7 isolate (Perron *et al.* 1989). Similar cell-types from non-MS donors produced neither this RT-activity nor detectable virions. All the 'infected' cultures were poorly and/or transiently productive and/or had a limited lifespan (Perron *et al.* 1997b). Therefore, in order to analyse the genomic RNA present in the very limited quantity of extracellular virions, we used the "Pan-Retrovirus" RT-PCR described in Sections 2.4.1 and 2.5.4.1. "Pan-Retrovirus" RT-PCR experiments were performed on sucrose-gradient purified virions from supernatants of different types of cell culture and the equivalent density gradient fractions of non-infected controls. The cultures included (i) choroid plexus cells from non-MS brain autopsy, infected by co-culture with irradiated LM7 cells (LM7P), (ii) choroid plexus cells sampled post-mortem from the brain of a patient with MS (PLI-1), and B-cell lines obtained by spontaneous *in vitro* transformation of two EBV-seropositive individuals, (iii) one MS patient and (iv) one non-MS control.

Figure 14 illustrates the RT-activity in sucrose-gradient fractions obtained from the B-cell cultures. The RT-PCRs were performed 'blind' on coded aliquots of sucrose gradients. Systematic cloning and sequencing of the PCR products generated in the Department of Virology UCLMS was undertaken in an independent laboratory in Lyon. After complete sequencing of 20 to 30 clones per sucrose gradient fraction, the codes were broken and the results analysed in parallel with the RT-activity data. Table 3 presents the distribution of sequences obtained from the sucrose gradient fractions containing the peak of viral RT activity in MS-derived cultures (i, ii, iii) and also the sequences amplified from the corresponding RT activity-negative fractions of uninfected cultures (iv).

The predominant sequence present in bands of the expected size (~140 bp) amplified from the RT-activity positive fractions was MSRV-c*pol*, the sequence which had previously been independently isolated from patient C (Section 3.3.1.3) This was not the case in the RT-activity negative fractions. As previously stated MSRV-c*pol* sequences exhibit partial homology (70-75%) with ERV-9 (La Mantia *et al.* 1991). A few ERV-9 sequences (>90% homology) were also present but clearly represented a minority of clones (Table 3).

In order to clarify the relationship between MSRV-c*pol* and ERV-9, 46 clones, containing ERV-9-related sequences, obtained in separate "Pan-Retrovirus" RT-PCR experiments, from 3 different cultures from 3 patients with MS were analysed and a frequency distribution histogram of similarity to the prototype ERV-9 sequence (La Mantia 1991) was plotted (Figure 15). A bimodal distribution clearly defined two

groups of sequences. The first group (>90% homology with ERV-9) constitutes only about 10% of the clones and represents true ERV-9 sequences. The second group, distinct from ERV-9 (approx. 70-75% homology), constitutes the major sequence group of the clones and represents MSRV-c*pol*. Only 2 of the 46 clones contained stop codons between VLPQG and YXDD. A number of isolate-specific amino acid substitutions were observed.

In addition to typical *pol* sequences, numerous PCR artefacts (primer multimers, concatamers or single-primer amplifications) related to the use of degenerate primers and low-temperature annealing, were found in all samples (Table 3).. There was a reciprocal relationship between the presence of MSRV-c*pol* and MMLV sequences in the PCR products. This suggested that the MSRV-c*pol* viral sequences and the MMLV sequences were competitively amplified and due to their low level the contaminating MMLV sequences were only detected in the absence of MSRV-c*pol*. Figure 2 is an alignment of a consensus sequence of MSRV-c*pol* with the corresponding VLPQG / YXDD region of diverse retroviruses. Figure 16 shows a phylogenetic tree based on the evolutionarily conserved amino acid sequences of both exogenous and endogenous retroviruses in this region.

3.3.3 "Pan-Retrovirus" PCR studies in multiple sclerosis. Discussion

Having demonstrated the ability of the optimised 'Pan-Retrovirus' PCR to detect minute quantities of a wide range of known retroviruses, we were able to detect the

presence of an unknown retroviral *pol* sequence in RNA extracted from the serum of a patient with MS. This finding was independently corroborated by the discovery of the same *pol* sequence in density gradient purified viral isolates derived from a further three MS patients. From these results it can be concluded that the sequence MSRV-c*pol*, originally isolated from the serum of an MS patient, is associated with the novel retrovirus previously referred to as LM7 (Perron *et al.* 1989; Perron *et al.* 1991; Perron *et al* 1991b; Perron *et al.* 1992; Perron *et al.* 1993).

The degree of sequence heterogeneity observed may possibly be due to the accumulation of point mutations during prolonged (40 - 50 passages) culture *in vitro* and may reflect the presence of defective particles. From the phylogenetic tree (Figure 16), it can be seen that the *pol* gene of MSRV is related to the C-type group of oncovirinae. The presence of ERV-9 sequences (Table 3) suggests that ERV-9 may be co-expressed in infected cells and be heterologously co-packaged (Seifarth *et al.* 1995; Patience *et al.* 1998) in MSRV virions.

It is of interest to note (Figure 2) that the protein encoded by the open reading frame of MSRV-*pol*, like that potentially encoded by the homologous region of ERV-9, is two amino acids shorter than that of most other retroviruses. Such 'deletions' appear to be compatible with function as indicated by the RT-activity associated with purified viral particles (Figure 14) and by the presence of similar deletions in human foamy virus *pol* (HSRV) and in the *pol*-like genes of duck hepatitis B (Mack and Sninsky, 1988) and cauliflower mosaic viruses (Shih *et al.* 1989).

Although clearly related, the phylogenetic distance between ERV-9 and MSRV appears to be greater than that between HIV-2 and SIV-1 (Figure 16). It thus seems reasonable to consider MSRV as a separate entity. Apart from ERV-9, the closest retroviral element is RTVL-H, a human endogenous sequence known to have a subtype with a functional *pol* gene (Wilkinson *et al.* 1993). The probable phylogenetic affiliation of MSRV with the C-type oncoviruses contradicts the preliminary assumption of Perron and colleagues, based on electron microscopy, which favoured a B- or D-type classification (Perron *et al.* 1989; Perron *et al.* 1992). However, it is well recognised that even a single amino acid substitution can convert retrovirus morphology to that of a different type (Rhee and Hunter, 1990).

3.4 "Pan Retrovirus" PCR ELOSA studies in multiple sclerosis

3.4.1 "Pan Retrovirus" PCR ELOSA studies in multiple sclerosis. Introduction

On the basis of these findings (Section 3.3 above) it was decided to investigate the prevalence of MSRV-c*pol* in patients with MS by means of the "Pan-Retrovirus" PCR. However, identification of MSRV-c*pol* in PCR products by cloning and sequencing is both laborious and time consuming. Accordingly, an enzyme-linked oligosorbent assay (ELOSA) was developed (Perron and colleagues, Lyon), using a capture probe (CpV1B) and a peroxidase-labelled detector probe (DpV1), for the

rapid identification of MSRV-c*pol* sequences in "Pan-Retrovirus" PCR products (Figure 2; Perron *et al.* 1997b).

3.4.2 "Pan Retrovirus" PCR ELOSA studies in multiple sclerosis. Methods

RNA was extracted as described in Section 2.3.1, cDNA synthesised as described in Section 2.4 and PCRs performed as specified in Sections 2.5.4.1 and 2.5.4.2. The enzyme-linked oligosorbent assay (ELOSA) was performed as described in Section 2.8.

3.4.3 "Pan Retrovirus" PCR ELOSA studies in multiple sclerosis. Results

3.4.3.1 Prevalence of MSRV-c*pol* in serum of patients with MS and controls. Results

The specificity of this sandwich hybridisation-based assay for MSRV-c*pol* was tested with both distantly related (HIV and MMLV) and closely related (ERV-9) *pol* sequences. No significant cross reactivity with such targets was observed despite the ability of the ELOSA to detect as little as 0.01 ng of MSRV-c*pol* DNA.

A molecular epidemiological study to determine the prevalence of MSRV-c*pol* sequences in serum samples was undertaken. RNA was extracted from the serum of 40 patients with MS (none were on treatment at time of venesection) and concurrently from 30 healthy blood donors. To avoid any possibility of the outcome being influenced by the position of the specimens within a run, MS and control samples were always alternated within each experiment. All positives were confirmed by repeat testing and each ELOSA test was performed in duplicate. The results of the ELOSA analysis of these 70 samples is illustrated in Figures 17 and 18. MSRV-c*pol* sequences were detected in 24 of the 40 sera from patients with MS (60%) but not in any of the 30 blood donor controls ($P<0.0001$).

Since it has yet to be established whether MSRV represents an exogenous retrovirus or a replication-competent endogenous one, it was necessary to exclude the

possibility that these results were simply due to different amounts of human genomic DNA, despite the DNasing, contaminating the two sets of samples. We therefore looked for evidence of such residual genomic contamination in the MS and control cDNAs by means of an intra-*Alu* PCR capable of detecting sub-femtogram quantities of human DNA (Cole *et al.* 1991). This was in addition to performing a mock cDNA synthesis on the extracted RNA, which had to produce a negative result in the PCR-ELOSA. Extremely high sensitivity for human DNA is achieved because *Alu* repeats are present at a level of several hundred thousand copies per haploid genome. The intra-*Alu* PCR demonstrated that there was no significant difference in the amount of human genomic DNA contaminating the two sets of samples. Neither set of cDNA samples (MS or controls) contained appreciable residual human genomic DNA. This excluded the possibility that genomic DNA was responsible for these results and demonstrated the effectiveness of the DNase digestion step which preceded the reverse transcription (Figure 19).

3.4.3.2. Prevalence of MSRV-c*pol* in CSF of patients with MS and controls. Results

A small scale molecular epidemiology study to determine the prevalence of MSRV-c*pol* sequences in the CSF of patients with MS was undertaken. Cerebrospinal fluid (CSF) samples were available from 10 patients with MS and from 10 patients with other neurological disorders (Table 1). Total RNA was extracted from CSF cell pellets, treated with 10x the concentration of DNase used for serum samples, reverse

transcribed and amplified (as above, Section 3.4.2). ELOSA analysis of the PCR products revealed MSRV-c*pol* sequences in 5 of the 10 MS patient samples but in none of the 10 samples from patients with other neurological diseases ($P<0.05$) (Table 1). The presence of MSRV-c*pol* did not appear to correlate with age, sex, or type of MS but did have a negative correlation with treatment. None of the 5 patients with MS in whom MSRV-c*pol* sequences were detected were on corticosteroids, cyclophosphamide or any other form of immunomodulatory treatment. 4 of the 5 patients with MS in whom MSRV-c*pol* sequences were not detected were on high-dose corticosteroids, on cyclophosphamide treatment or had received many immunosuppressive treatments. No correlation between MSRV-c*pol* detection and CSF cell count was observed.

3.4.4 "Pan Retrovirus" PCR ELOSA studies. Prevalence of MSRV-c*pol* in serum and CSF of patients with multiple sclerosis and controls. Discussion

The novel retroviral sequence MSRV-c*pol* was detected in the serum of 60% of patients with MS but not in any of 30 healthy blood donors (Figures17 and 18), and in the CSF of 5 (50%) patients with MS but not in any of 10 patients with other neurological disease. These epidemiological results provide molecular evidence that the presence of MSRVc-*pol* RNA in serum and CSF is associated with MS. MSRV may represent the exogenous member of an ERV-9-like endogenous family. Alternatively, the extracellular virions may be the product of a replication-competent endogenous provirus. Since *pol* is the most conserved retroviral gene, it may well be

an unsuitable region to permit PCR discrimination between exogenous and endogenous sequences. Human genomic DNA was positive by this "Pan Retrovirus" PCR ELOSA analysis, which would suggest that this region of *pol* has an endogenous counterpart.

Further experiments are required to explore other possible disease associations and the prevalence of the virus in the general population. However, the "Pan-Retrovirus" PCR ELOSA technique is not suitable for routine molecular epidemiological screening due to the inherent susceptibility to and difficulty in overcoming environmental DNA contamination. This results in it being a long, complex and technically demanding experimental technique.

3.5 MSRV specific PCR studies in multiple sclerosis

3.5.1 MSRV specific DNA PCR studies in multiple sclerosis.

3.5.1.1 MSRV specific DNA PCR studies in multiple sclerosis. Introduction

Further molecular characterisation of MSRV from cultured virions by Perron and colleagues (Perron *et al.* 1997b and personal communications) has enabled us to design specific primers to various regions of the MSRV genome. All these primers

were designed according to the principles outlined in Section 2.5.2. In addition, the primers were designed to specifically amplify the MSRV sequence and to discriminate from the known related endogenous retrovirus ERV-9 and from the endogenous counterparts of MSRV for which sequence information was available (H Perron, personal communication).

3.5.1.2 MSRV specific DNA PCR studies in multiple sclerosis. Method

In an attempt to detect MSRV proviral sequences, genomic DNA was extracted as detailed in Section 2.2 and 0.5 μg amplified by nested PCR using the appropriate primers and conditions as specified in Section 2.5.4 and Table 2. The PCR products were analysed by agarose gel electrophoresis as described in Section 2.7.1.2.

3.5.1.3 MSRV specific DNA PCR studies in multiple sclerosis. Results

3.5.1.3.1 MSRV LTR specific DNA PCR studies in multiple sclerosis. Results

The MSRV LTR primers were sensitive down to the equivalent of 1/10th of a cell and produced a band of the predicted size in all the DNA samples tested. The samples tested were: 3 PBMC DNAs from patients with MS, 2 MS cell line DNAs (PLI-2 and LM7PC), 7 PBMC DNAs from patients infected with HTLV-I and 3 normal human control PBMC DNAs.

3.5.1.3.2 MSRV *gag* specific DNA PCR studies in multiple sclerosis. Results

The MSRV *gag* primers were sensitive down to the equivalent of 1/10th of a cell and produced a band of the predicted size in the majority of the human DNA samples tested (Figure 20). The PCR results were: 2/4 PBMC DNAs from patients with MS were positive, 15/24 (62.5%) laboratory staff normal control PBMC DNAs were positive. In addition, DNA extracted from B cell lines established from patients with MS and controls were tested. The results of the PCRs on the DNA extracted from B cell lines were: 4/4 MS B cell lines, 4/4 neurological control B cell lines, 3/3 rheumatological control B cell lines, 3/3 healthy control B cell lines, 0/2 cottontop-tamarin (*Saguinus oedipus*) B cell lines were all positive. Although the DNA from the cotton-top tamarin was negative with these MSRV *gag* primers, this genomic DNA had been demonstrated to be of amplifiable quality by PCR of the evolutionarily conserved PDH gene.

3.5.1.3.3 MSRV *pol* specific DNA PCR studies in multiple sclerosis. Results

The MSRV PT*pol* AB/EF primers were sensitive down to the equivalent of 1/10th of a cell and produced a band of the predicted size in all the DNA samples tested (Figure 21). The samples tested were PBMC DNAs from 11 patients with MS, 4 normal controls and 10 neonates. Two DNAs from cell lines from patients with MS (PLI-2 and LM7PC) were also positive.

3.5.1.3.4 MSRV *env* specific DNA PCR studies in multiple sclerosis. Results

The MSRV *env* specific PCR produced a band of the predicted size in all the DNA samples tested (Figure 22). The samples tested were PBMC DNAs from 7 patients with MS and 10 normal controls. The limit of sensitivity of this PCR on a DNA dilution series was apparently greater than that for the other PCRs, detecting down to the equivalent of 1/100 th of a cell.

3.5.1.4 MSRV specific DNA PCR studies in multiple sclerosis. Discussion

The results obtained on DNA samples, with the MSRV specific PCRs for the various regions of the genome, all led to essentially the same conclusion. Discrimination between patients with MS and controls was not possible, almost all samples produced a positive result, i.e. a PCR band of the expected size (the identity of some of these PCR products was confirmed by DNA sequencing in Lyon). The limit of sensitivity of these PCRs was also similar, being capable of detecting genomic DNA down to 1/10 th or a 1/100 th of a cell. This suggests that if MSRV is an endogenous sequence in the human genome it is at the level of between 10 and 100 per cell. Alternatively, if MSRV is exogenous, it has a closely related endogenous counterpart which is present at a level of approximately 10-100 copies per cell.

The only PCRs which failed to amplify all samples were using the *gag* primer set. This is unlikely to have been due to a lack of sensitivity of this set of primers as the

same end point on a DNA dilution series was obtained. It may however suggest that there is a genetic polymorphism within the population in respect to this particular sequence, as has been observed for other HERVs (Mager and Goodchild, 1989; Kambhu *et al.* 1990; Zhu *et al.* 1994). Interestingly this region of the genome was not expressed in cellular RNA (Section 3.5.2.4).

The reason for testing the neonates' DNAs was that the epidemiological evidence suggests that any infectious agent involved in the aetiology of MS is acquired around puberty (Kurtzke, 1993). Hence, neonates should be uninfected by any exogenous environmental infectious agent. The fact that the neonates' DNAs were positive demonstrates that at least in the PT*pol* AB/EF primer sequence regions there are highly conserved endogenous counterparts. It would have been interesting to examine these DNAs with the primers from the other regions of the genome and sequence the products. These could then have been examined for any consistent differences with respect to that of virion associated MSRV genomic RNA.

If MSRV is not endogenous, but is an exogenous human retrovirus, then there is a highly related endogenous retroviral sequence or sequences. The possibility exists that the endogenous retroviral sequences detected with the various MSRV specific PCRs are not contiguous within the human genome (i.e. not a complete endogenous MSRV proviral genome) but represent signals derived from separate members of a family of MSRV related, incomplete endogenous sequences. This raises the further possibility that MSRV may be a recombinant virus, derived by recombination from various defective proviruses within the human genome. Another explanation of the

observed results is that the situation with MSRV is similar to that of MLV, MMTV (Liegler and Blair, 1986) and JSRV (Palmarini *et al.* 1996; Bai *et al.* 1996), where the exogenous form of the virus is highly related to a multicopy family of endogenous retroviruses but the pathogenic exogenous form differs subtly in sequence.

Although, as stated earlier, primers were designed in an attempt to avoid known endogenous sequences, no set has as yet been identified which is capable of discriminating between the virion associated sequence and the endogenous counterpart(s). These results were somewhat surprising, since the primers had been designed to avoid both ERV-9 sequences and all known MSRV related endogenous sequences. These findings are however entirely consistent with those of Perron and colleagues (personal communication), who also found endogenous counterparts for all MSRV genomic regions so far examined.

3.5.2 MSRV *pol* specific RT-PCR studies on cellular RNA from patients with multiple sclerosis and controls.

3.5.2.1 MSRV *pol* specific RT-PCR studies on cellular RNA from patients with multiple sclerosis and controls. Introduction.

In order to investigate the expression of MSRV RNA in the cells of patients with MS and controls a MSRV *pol* specific RT-PCR was developed. This was used to

analyse RNA expression in both cryopreserved brain sections and PBMCs. The reasons for examining these tissues were that each would represent a likely target for MSRV infection and replication, both on the basis of other known retroviral models (Section 1.1.1.3) and on the *in-vitro* replication of MSRV itself (Perron *et al.* 1991; Perron *et al.* 1997). Similarly, if MSRV was an endogenous retrovirus which was specifically expressed in MS, these would be likely target tissues for such expression.

3.5.2.2 MSRV *pol* specific RT-PCR studies on cellular RNA from patients with multiple sclerosis and controls. Methods

PBMCs from normal blood donor controls and patients with MS were obtained as detailed in Section 2.1. The PBMCs were washed in PBS and pelleted at 400xg for 10 minutes. The supernatant was removed and the cell pellet "snap" frozen by immersing the cryotube (Nunc) in liquid nitrogen. The frozen pellets were then stored at -70°C until required. Post mortem cryosections of white matter from normal brains and from plaques of patients with MS were obtained frozen on dry ice and stored at -70°C until required. When extracting the RNA, the chaotropic agent was added directly to the frozen pellet or cryosection before thawing. RT-PCR was performed as described in Section 2.6. A 10µl aliquot of the cellular RNA extract was run on a 1% agarose gel to determine its quality and quantity. Both ribosomal RNA bands (28S and 18S) should be distinct and clearly visible in undegraded RNA (Figure 23).

Cellular RNA was extracted by guanidine-HCl / detergent lysis and adsorption to a silica matrix as detailed in Section 2.3.2.1 for PBMCs or 2.3.2.2 for brain tissue. The cellular RNA was subjected to two sequential DNase digestion steps, then a combined reverse transcription-PCR (RT-PCR) reaction was performed using MSRV-specific primers *pol*-4 and *pol*-5 (Section 2.5.1). A second round of amplification with semi nested primers *pol*-6 and *pol*-5 (Table 2) generated a 626 bp PCR product which was identified by gel electrophoresis (Section 2.7.1.2). Control reactions without reverse transcriptase were performed to ensure that the products were RNA derived. In addition, aliquots were tested by nested RT-PCR for the presence of pyruvate dehydrogenase (PDH) DNA and RNA (Table 2) (Section 2.6.2) (Howard 1998). The PDH RNA RT-PCR signals provided a further check on the quality of the cellular RNA. However, the greater abundance of PDH RNA, its smaller RT-PCR product size and hence reduced requirement for RNA integrity, made it of only limited use in this context. Samples which generated a signal for PDH RNA but not for the MSRV RNA could therefore still have been false negatives due to RNA degradation or low yield. Samples which generated a signal either for PDH DNA or the 'no-RT' MSRV PCR assay were excluded from the analysis.

3.5.2.3. MSRV *pol* specific RT-PCR studies on cellular RNA from patients with multiple sclerosis and controls. Results

When tested using the MSRV *pol* 4/5/6 primers, all 8 PBMCs from blood donors that were tested were positive for MSRV cellular RNA (Figure 24). The ribosomal bands were not visible on the RNA extracts from the PBMCs from the 5 patients with MS which were tested (Figure 23). However, despite this 2 of the 5 PBMCs from patients with MS were still positive for MSRV cellular RNA. Similarly, when using the MSRV PT*pol* AB/EF primers all 10 PBMCs from blood donors that were tested were positive for MSRV cellular RNA. If the cellular RNA was diluted by a factor of one in ten, then it was generally found that the RT-PCR signal was lost. When brain tissue was tested using the MSRV *pol* 4/5/6 primers, it was found that post mortem cryosections of the white matter from three normal brains and from plaques of a patient with MS were all positive for MSRV cellular RNA (Figure 24).

3.5.2.4 MSRV *pol* specific RT-PCR studies on cellular RNA from patients with multiple sclerosis and controls. Discussion

MSRV *pol* RNA was detected in the PBMCs of all the healthy blood donors that were analysed. Similarly MSRV *pol* RNA was detected in the cryosections of all three normal control brains and the MS patient brain that were analysed (Figure 24). If the cellular RNA was diluted by a factor of one in ten, then it was generally found that the RT-PCR signal was lost. This suggests that the level of expression of

cellular MSRV RNA was very low, or restricted to a very limited subset of cells. The apparent lack of MSRV *pol* RNA in the PBMCs from the patients with MS is hence not surprising, since the ribosomal bands were not visible on the RNA extracts from these samples suggesting degradation of the cellular RNA (Figure 23). The probable explanation for this is that these samples had undergone a freeze-thaw cycle due to a freezer failure. As mentioned in Section 3.5.1.4 the *gag* primer set failed to amplify cellular RNA from all samples, PBMCs and brain tissue (Figure 24). This may reflect a genuine lack or lower level expression of this region, or a possible genetic polymorphism (sample numbers were low; 3 normal PBMCs, 3 normal and 1 MS brain tissue) within the population in respect to this particular sequence, as has been observed for other HERVs (Mager and Goodchild, 1989; Kambhu *et al.* 1990; Zhu *et al.* 1994).

These results suggest that the expression of cellular MSRV RNA, in contrast to the expression of virion associated MSRV RNA (Section 3.5.3), at least in the *pol* region of the genome, is ubiquitous and is not specifically associated with MS. The finding of expression of MSRV *pol* sequences in PBMCs and the white matter of the brain is consistent with the findings of other groups on the expression of endogenous retroviral sequences (Shih *et al.* 1989; Medstrand *et al.* 1992; Medstrand and Blomberg, 1993). However, quantitative differences between patients with MS and controls cannot be excluded by this RT-PCR analysis.

3.5.3 MSRV *pol* specific RT-PCR studies on serum from patients with multiple sclerosis and controls

3.5.3.1 MSRV *pol* specific RT-PCR studies on serum from patients with multiple sclerosis and controls. Introduction

The MSRV specific PCR studies described above demonstrated that the human genome contains a number of endogenous DNA sequences closely related to the *pol* gene of MSRV. At least a proportion of these were also found to be expressed at the RNA level in the leukocytes of normal individuals. A protocol was therefore designed to detect only cell free virion-encapsidated MSRV RNA. Any closely related endogenous sequences that might be co-packaged within MSRV virions may also be detected, but these would not affect the interpretation of a positive result, since they would also indicate the presence of circulating MSRV virions.

3.5.3.2 MSRV *pol* specific RT-PCR studies on serum from patients with multiple sclerosis and contols. Method

A cohort of 17 patients with clinically active MS and 44 controls were tested. The control group consisted of 8 patients with non-neurological disorders and 36 healthy UK adults.

The detailed, optimised, current protocol as available from the Lancet (Garson et al. 1998), where this study was published, is reproduced in Appendix 2 and summarised below:

Following 0.45μm filtration to remove cellular debris and RNase digestion to remove any residual non-encapsidated RNA (e.g. MSRV-related RNA released from lysed leukocytes during sample preparation), serum was processed to extract viral RNA by guanidine-HCl / detergent lysis and adsorption to a silica matrix, as detailed in Section 2.3.2.3. Viral RNA was subjected to DNase digestion, then a combined reverse transcription-PCR (RT-PCR) reaction was performed using MSRV-specific primers PT*pol*-A and PT*pol*-F (Table 2). A second round of amplification with nested primers PT*pol*-B and PT*pol*-E generated a 435bp PCR product which was identified by gel electrophoresis. The specificity of the products were confirmed by dideoxy sequencing (performed by Perron and colleagues) and cross-contamination between samples was excluded by the observed degree of inter-patient sequence variation. Control reactions without reverse transcriptase were performed to ensure that the products were derived from RNA. In addition, to exclude the possibility that the extracted viral RNA might be contaminated with host cell derived nucleic acids containing homologous but irrelevant endogenous sequences, aliquots were tested by nested PCR for the presence of pyruvate dehydrogenase (PDH) DNA and RNA. Samples which generated a signal in either the PDH or the 'no-RT' PCR assays were excluded from the analysis. The cross-contamination prevention measures of Kwok and Higuchi, (1989) were strictly employed throughout.

Coded sera from French patients with clinically active MS and controls were sent frozen on dry ice from Lyon to London where RT-PCR was performed 'blind'. The PCR products, again coded, were returned to Lyon for 'blind' sequencing.

3.5.3.3 MSRV *pol* specific RT-PCR studies on serum from patients with multiple sclerosis and controls. Results

MSRV-RNA was detected in the serum of 9 of 17 (53%) patients with clinically active MS but in only 3 of 44 controls without MS (Table 4). Figure 25 shows MSRV PT*pol* AB/EF RT-PCR second round products of RNA from sera of patients with MS and controls. Figure 26 shows the MSRV PT*pol* AB/EF 'no RT' PCR second round products of the serum RNA from the same patients and controls. Figure 27 shows the PDH RT-PCR second round products on the RNA from the sera of those patients and controls positive by MSRV PT*pol* AB/EF RT-PCR.

All the patients with non-neurological disorders were MSRV-RNA negative (P=0.0002 by Fisher's Exact Test). In the patients with MS, detection of MSRV-RNA was not associated with age, sex, disease duration, or MS type, however a statistically significant negative correlation with treatment was observed. 21 serum samples were obtained from the 17 patients; 6 of 6 sera from untreated patients contained detectable MSRV-RNA whereas it was detectable in only 4 of 15 samples obtained during treatment with high dose corticosteroids and/or azathioprine (P= 0.004).

3.5.3.4 MSRV *pol* specific RT-PCR studies on serum from patients with multiple sclerosis and controls. Discussion

The MSRV specific RT-PCR results described above showed that overall 53% of patients with clinically active MS were viraemic, but this figure rose to a 100% if just those not undergoing immunosuppressive treatment were considered and fell to 27% if only those receiving immunosuppressive treatment were analysed. These results suggest that MSRV viraemia might be reduced by this treatment. The reason (or reasons) for this apparent loss of viraemia during treatment is unknown, but the finding is in agreement with our previous observations on the detection of MSRV in cerebrospinal fluid (Section 3.4.3.2). Possible explanations for these observations are discussed in Section 4.1.2.

Previously, using the "Pan-Retrovirus" ELOSA detection system MSRV RNA was detected in the serum of 60% of 40 patients with MS but not in any of 30 healthy blood donors (Figures 17 and 18; Section 3.4.3.1). All of these 40 patients with MS were in clinical remission at the time blood was taken and were not undergoing treatment. Also using the "Pan-Retrovirus" ELOSA technique MSRV was detected in the CSF of 5 (50%) patients with MS but not in any of 10 patients with other neurological disease (Section 3.4.3.2). MSRV viraemia might be more prevalent and/or at higher levels during episodes of relapse (i.e. active disease). This hypothesis would appear to be supported by the above MSRV specific RT-PCR findings of 100% viraemia in untreated patients with clinically active MS, albeit only six cases. Alternatively, it could be argued that this specific RT-PCR technique is

more sensitive than the previously described "Pan-Retrovirus" system. Further possible explanations for these observations are discussed in Section 4.1.2.

Although all these results on the prevalence of MSRV viraemia in patients with MS are significant when compared to the controls, they do not prove an aetiological link, but simply demonstrate an association. If MSRV is involved in the aetiology of MS the lack of detection of viraemia in between 40% and 50% of cases is perhaps surprising. MS may represent a common clinical manifestation of more than one underlying biological/ virological process. Further possible explanations for these findings are discussed in Section 4.1.2.

4 GENERAL DISCUSSION

The hypothesis of a retroviral involvement in the aetiology of multiple sclerosis has been discussed in Section 1 but some of the major arguments in favour of this hypothesis are worth reiterating here. I) There are existing human and animal retroviral diseases which closely model the autoimmune and neurological character of multiple sclerosis. II) Cross reactive antibody responses to various retroviruses have been demonstrated in patients with multiple sclerosis. III) Retroviruses have been isolated in culture from patients with multiple sclerosis by two independent groups. IV) Most recently, molecular epidemiological data in support of these culture based findings have been presented by these two groups. V) Finally, the epidemiology of multiple sclerosis suggests, that in genetically susceptible individuals, an infectious agent acquired a decade prior to clinical onset triggers the disease. This is consistent with a 'slow' retroviral infectious disease model.

4.1 Discussion of results

4.1.1 Characterisation of a novel retrovirus from MS patients

Using the "Pan Retrovirus" RT-PCR a novel retroviral sequence c-*pol* was isolated from the serum of a patient with MS. This sequence was subsequently confirmed to be identical to the sequence of the LM7 virus cultured from multiple sclerosis patients. LM7 was later renamed MSRV (Multiple Sclerosis associated Retrovirus).

4.1.2 Molecular epidemiology of MSRV

Using the "Pan Retrovirus" ELOSA detection system MSRV *pol* RNA was detected in the serum of 60% of multiple sclerosis patients but not in that of any healthy blood donors (Figure 17 and 18). All the multiple sclerosis patients were in clinical remission at the time blood was taken and were not undergoing treatment. However, it must be borne in mind that the presence of cellular RNA was not formally excluded in this set of samples. Also using this technique MSRV-RNA was detected in the CSF of 50% multiple sclerosis patients but in none of the patients with other neurological disease. The MSRV specific RT-PCR results on clinically active multiple sclerosis patients, with 53% of patients overall being viraemic, but 100% of those not undergoing immmunosuppressive treatment and only 27% of sera from those undergoing treatment, suggests that MSRV viraemia might be more prevalent during episodes of relapse (i.e. active disease) but be decreased by immunosuppression.

If MSRV is involved in the aetiology of MS, the lack of detection of viraemia in 40% of untreated cases ("Pan-Retrovirus" ELOSA analysis, Section 3.4.3.1) is perhaps surprising. Several explanations are possible; viraemia levels may be below the limit of sensitivity of detection with the "Pan-Retrovirus" technique in some cases. Not all of the infected individuals may be viraemic, the virus may just act in a 'hit and run' fashion as a trigger to the underlying autoimmune process. Sequence heterogeneity among different 'strains' of MSRV may prevent detection due to mismatch between the RNA template and the primers. All these multiple sclerosis

patients were in clinical remission at the time blood was taken and MSRV viraemia might be more prevalent during episodes of relapse, as suggested by the 100% prevalence in untreated patients with active disease (Section 3.5.3.3). Finally, multiple sclerosis may represent a common clinical manifestation of more than one underlying biological/virological process.

The wide variation in the clinical spectrum of disease in multiple sclerosis might suggest that there is more than one underlying biological/virological process. The MRI appearance of patients with primary and secondary progressive multiple sclerosis are significantly different (Thompson *et al.* 1991) and thus the diseases and consequently their causations may be different. However, no association with a particular type of the various forms of multiple sclerosis has been observed in these studies, although the number of patients analysed was low. It is interesting to note that the proportion of multiple sclerosis patients in whom serum MSRV-*pol* sequences were detected (60% of patients in clinical remission and 53% of clinically active patients overall) was approximately the same as the proportion (60%) in whom antibodies against the LM7 (MSRV) virus were previously detected by radioimmunoprecipitation (Perron *et al.* 1992). Whether this concordance proves to be anything other than coincidental remains to be determined.

The reason for the apparent loss of circulating virion-associated MSRV-RNA from the serum during immunosuppressive treatment (Section 3.5.3.3) is unknown (27% versus 100%), but this finding is in agreement with our observations (Perron *et al.*

1997b) on the detection of MSRV in cerebrospinal fluid (Section 3.4.3.2). It is interesting to speculate on possible explanations for this observation.

It is possible that the immunosuppression results in a reduced viraemia by suppressing the number or activity of the cells (e.g. lymphocytes and/ or monocytes) that support viral replication. Alternatively, it could involve a specific downregulation of the virus by corticosteroids acting on hormone responsive elements of the LTR, as has been demonstrated to occur in HIV (Mitra *et al.* 1995). Finally, it is conceivable that there could be a competitive suppression effect due to general upregulation of endogenous retroviral expression. This could possibly result in competition for cellular transcription factors which then become limiting, in turn resulting in decreased levels of MSRV expression. Such competitive suppression effects have been observed for steroid hormone receptors in other experimental systems (Meyer *et al.* 1989).

Although these epidemiological results provide molecular evidence that the presence of MSRV RNA in serum and CSF is associated with multiple sclerosis they do not prove an aetiological link. The exact role of MSRV in the aetiology and pathogenesis of multiple sclerosis remains to be determined by further studies. It is also possible that the detection of MSRV in multiple sclerosis patients simply represents an interesting epiphenomenon. If this is the case it may nonetheless prove useful as a diagnostic and/or prognostic marker of the disease.

4.1.3 Characteristics of the MSRV virus

Sucrose density gradient purified virions from multiple sclerosis patient cultures are observed at a density of 1.17 g/ml. These MSRV retroviral particles, are 100-120 nm in diameter (Figure 1; Perron *et al.* 1991, Perron *et al.* 1997). They have been demonstrated to possess an intact ORF encompassing the entire PR-RT region (Perron *et al.* 1997b) and display functional RT activity. This observed ORF of MSRV is consistent with it encoding the enzymatic RT activity detected.. The expression of recombinant proteins (RT, protease, Gag and Env) from the cloned sequence of MSRV confirms the existence of functional open reading frames. Similarly the MSRV LTR has been shown to have functional activity in a chloramphenicol acetyltransferase (CAT) reporter assay (Perron, personal communication; Blond *et al.* 1999).

MSRV has been demonstrated to be transmissible *in-vitro* by tissue culture of leptomeningeal and choroid plexus cells. Some of the data from the *in-vitro* culture experiments suggests that MSRV has an immortalising effect on infected cells, as indicated by their tumourigenic potential in nude mice and ability to achieve high passage number (Perron *et al.* 1997b).

The optimum conditions for assaying MSRV reverse transcriptase include use of a poly-Cm/oligo-dG_{12-18} template and 20 mM Mg^{++} at pH 8.2. These conditions are distinct from those required by either HIV or HTLV. The virus has been

demonstrated to possess a typical but unique human retroviral antigen profile. A Western blot of purified MSRV proteins using antibodies from the sera of MS patients reveals bands of 90, 65, 60, 50, 45 and 15 kDa (Perron *et al.* 1992; Perron *et al.* 1997).

4.1.4 MSRV phylogeny

The phylogeny of MSRV, based on the conserved amino acid sequence of RT in retroviruses (VLPQG.....YXDD), suggests that it is a mammalian C-type retrovirus and is illustrated by the phylogenetic tree in Figure 16. The closest related retroviral sequences are human endogenous sequences. ERV-9 is the closest known named retroviral element, followed by RTLV-H, which is known to have a subtype with a functional *pol* gene (Wilkinson *et al.* 1993). The MSRV related RT sequences of ERV-9 and RTVL-H, like that of MSRV itself, have a two amino acid deletion with respect to most other retroviral *pol* genes (Figure 2), (apart from foamy viruses), which reflects their close phylogenetic relationship. This phylogenetic classification as a C-type retrovirus apparently contradicts the earlier assumptions of Perron and colleagues based on the general morphology of the particles observed by electron microscopy (EM), which were compatible with a B or D-type retrovirus (Perron *et al.* 1992; Perron *et al.* 1989). However, preliminary *env* sequence data does demonstrate a greater phylogenetic proximity with D-type retroviruses (Perron personal communication). Such differences in the phylogenies of the *pol* and *env* genes have been described in MPMV and suggest a recombinatorial origin in D-type

retroviruses (Sonigo *et al.* 1986). D to C type morphological conversion is also possible since it has been reported that a single amino acid substitution in the Gag protein can convert retrovirus morphology to that of a different type (Rhee and Hunter, 1990). The endogenous counterpart of MSRV probably entered the primate genome after the divergence of the primates, since MSRV PCR findings were negative on genomic DNA from the New World primate, the cotton-top tamarin (Saguinus oedipus) (Section 3.5.1.3.2). In support of this conclusion Blond *et al.* (1999) have estimated in their recent publication on the MSRV related endogenous retroviral family HERV-W, that a prototypic member of this family (RG083M05) entered the genome 6 million years ago.

4.1.5 Endogenous or exogenous nature of MSRV

PCR analysis of genomic DNA, from multiple sclerosis patients and normal controls, with a variety of primers from different regions of the retroviral genome (Section 3.5.1), has demonstrated the existence of a family of endogenous elements closely related to MSRV. The copy number of this endogenous family of retroviruses has been estimated at between 10 and 100 per haploid genome on the basis of PCR end point titrations on genomic DNA. This has been confirmed by Southern blot analysis with MSRV probes, which showed hybridisation with a multicopy endogenous family. This family of endogenous elements, closely related to MSRV, has recently been characterised and named HERV-W on the basis of its unique primer binding site motif (Blond *et al.* 1999). The existence of this family of

endogenous elements closely related to MSRV prevented the simple detection and isolation of a replication competent provirus from MSRV-producing cells.

In order to characterise this family of endogenous elements closely related to MSRV and to search for the existence of a replication competent member Blond *et al.* investigated their expression in normal tissues. They found using Northern blotting, that expression was apparently limited to the placenta. The HERV-W family consists of between 13 (*env*) and 260 (*protease*) copies per genome, which is in broad agreement with our PCR findings. The LTR of this family is apparently unique, in that along with MSRV it possesses a primer binding site specific for a tryptophan transfer RNA molecule. Hence, the HERV-W nomenclature for this family of human endogenous retroviruses. Although full length genomic clones were identified no replication competent member has as yet been identified. This contrasts with the predominantly apparently functional open reading frames obtained from MSRV virions. Placental expression of 8kb, 3.1kb, and 1.3kb transcripts was observed.

Additional results with RT-PCR in *pol* and other regions of the genome suggested very low-level expression of MSRV RNA in both blood cells and brain tissue from multiple sclerosis patients and normal healthy controls (Section 3.5.2.3). This is in agreement with the previous findings of Lefebvre and colleagues (1995) on the expression of ERV-9 related sequences in brain and other tissues (Brahic and Bureau 1997). However, lower sensitivity Northern blot and RNA dot blot analysis with

HERV-W probes, only demonstrated expression of this multicopy endogenous family in normal human placenta, other tissues being negative (Blond *et al.* 1999).

The apparent specific expression of MSRV RNA in CSF cells of patients with MS and not controls (Section 3.4.3.2) might initially seem surprising with respect to these other results. However, the number of cells analysed in the CSF study were very much lower and the types of cell probably different from either the blood or brain cells analysed with the specific MSRV RT-PCR. It remains possible that there is simply higher level expression of MSRV RNA in CSF cells of patients with MS than in controls. Consequently, whether MSRV is an exogenous retrovirus or an endogenous retrovirus producing extracellular virions still remains uncertain.

This uncertainty could possibly be resolved by using probes derived from other less well conserved regions of the MSRV genome such as *env*, although currently all regions analysed have proven to have endogenous counterparts. As previously stated (Section 3.5.1.4), it is however possible that these different endogenous sequences are not contiguous within the genome, and that MSRV is a recombinant virus. The possibility also exists that there are subtle sequence differences between MSRV and the highly related endogenous sequences now known to be present in the human genome (Section 3.5.1 and Blond *et al.* 1999). Sequence analysis of the endogenous homologues of MSRV would appear to support this assertion, as they frequently possess stop codons (Perron personal communication and Blond *et al.* 1999).
Similarly, the recently described sequences present in the EMBL database do not possess large intact reading frames, nor correspond exactly to the observed genetic

organisation of MSRV. The BAC clone RG083M05 from 7q21-7q22, the T-cell receptor alpha delta locus homologue BAC378 (chromosomal location 14q11-12) and the chromosome 21q22.3 cosmid Q11M15, all possesses a ~2kb insertion around the primer binding site. The clones BAC378, Q11M15 and the U134E6 cosmid containing DNA from chromosome Xq22 all have a large deletion in *env* (Blond *et al.* 1999).

MSRV would therefore appear to be conceptually similar in certain aspects to a particular category of retroviruses, exemplified by MLV, MMTV and JSRV, which are intermediate between being strictly exogenous and strictly endogenous non-virion producing retroviruses. Unlike the normally defective endogenous retroviral elements, these are known to produce infectious and pathogenic virions, to cause neurological disease (Portis, 1990) solid tumours / leukaemias (Gardner *et al.* 1979; Palmarini *et al.* 1996) and to express endogenous superantigens (Hugin *et al.* 1991; Marrack *et al.* 1991). Furthermore, in MLV infections, the endogenous retroviral background of the mouse strain can determine susceptibility or resistance to disease (Gardner, 1990; Marrack *et al.* 1991).

Such interactions between an infectious retrovirus and its endogenous counterpart may be relevant in the pathogenesis of MS, since endogenous retroviral genotypes are not always identical in all individuals (Mager and Goodchild, 1989; Kambhu *et al.* 1990; Zhu *et al.* 1994; Eberhart and Curran, 1998; Medstrand and Mager, 1998). A genetic control due to related endogenous retroviral genotypes could therefore contribute to the known genetic susceptibility to MS. Recently

Murine AIDS has been shown to be due to transmission of a defective retrovirus, which is closely related to the LP-BM5 endogenous retrovirus (Kubo *et al.* 1996). The immmunosuppressive effect is due to a change in reading frame and aberrant expression of the Gag protein. It is possible that a similar complementation mechanism could be occurring between exogenous and endogenous counterparts of MSRV in MS.

4.2 Expression of endogenous retroviruses in autoimmunity

It is possible that there is a general upregulation of expression of endogenous retroviruses in autoimmune conditions such as multiple sclerosis. Such generic upregulated expression could be either a causative factor or a consequence of the autoimmune disease. Evidence which would argue against such a general upregulation includes the lack of other retroviral sequences detected in both the "Pan-Retrovirus" PCR clones derived from the serum of the original multiple sclerosis patient 'C' and those derived from the virions generated by the multiple sclerosis patient cultures. Also, the finding of a different novel endogenous retrovirus, expressed in Type I diabetes (Conrad *et al.* 1997), supports the specificity of these associations.

4.2.1 Expression of endogenous related retroviruses in multiple sclerosis

A recent publication by Christensen and colleagues (Christensen *et al.* 1998) is particularly pertinent to this discussion. The first molecular data on the C-type retrovirus which this group has been working on since 1991 (Haahr *et al.* 1991), is presented. They describe detecting RGH-2 sequence variants, initially in purified virions from multiple sclerosis patient PBMC cultures and subsequently in 22 of 31 (71%) multiple sclerosis patient plasmas and 0 of 47 controls. RGH is a member of the RTVL-H or HERV-H (human endogenous retrovirus with histidine tRNA primer binding site) family of endogenous retroviruses which are present at particularly high copy number and are highly expressed (Wilkinson *et al.* 1993). RTVL-H is the second most closely related, characterised human endogenous retrovirus, to MSRV after ERV-9 (Figure 16). The similar overall rates of detection of RGH (71%) and MSRV (53-60%) in multiple sclerosis may be significant, or may simply be coincidental.

The fact that two groups have independently isolated C-type retroviruses from multiple sclerosis patients and that the detected genomes are either expressed endogenous retroviruses, or highly related to expressed endogenous retroviruses is intriguing. The close phylogenetic relationship of these endogenous retroviral sequences suggests that there may be a common factor or factors involved in their activation and expression (Section 4.2.1 below).

In this context it is worth considering that ERV-9 sequences were detected in a minor proportion of the clones derived from the from the "Pan-Retrovirus" PCRs performed on purified virions from multiple sclerosis patient cell cultures (Section 3.3.2.3). We did not detect RGH-2 related sequences, but from the published *pol* sequence (Hirose *et al.* 1993) it would appear that these might easily be missed by the "Pan-Retrovirus" PCRs performed. The conserved VLPQG YXDD motifs are actually VMLQG YIGD in RGH-2 and consequently a worse match for the PAN PCR primers used (PAN-UO, PAN-UI and PAN-DI) than even HIV-1 (Figure 2). The presence of RGH-2 related sequences could equally be a result of co-packaging, as hypothesised for the ERV-9 sequences detected with the MSRV virions from tissue cultures (Section 3.3.3; Perron *et al.* 1997b). RTVL-H co-packaging has also been observed with HERV-K (Patience *et al.* 1998) which is far more distantly related to it than is MSRV.

A recent publication from another group (Olsson *et al.* 1999), has provided independent confirmation of our initial finding, the isolation of the MSRV c*pol* sequence from virions present in the serum of a patient with MS. Using our published Pan-Retrovirus PCR detection method (Tuke *et al.* 1997) Olsson *et al.* examined stored sera from 9 Swedish patients with MS and 21 healthy blood donor sera. "One of the sera (LUMS9) gave a positive amplification" and all their no-RT controls were negative. LUMS9 was from a patient with MS, which produced a band of ~142bp after RT and PCR. When this band was cloned and sequenced "despite the potential of the primers to amplify a broad variety of retroviral sequences", "the only retroviral sequence found (144 bp insert) gave the highest

similarity score in a BLAST search with genomic ERV-9 like sequences and the earlier published ERV-9/MSRV sequence". The sequence of LUMS9 "was 87% identical at the nucleotide level to the MSRV variant of ERV-9".

Not only has this provided independent confirmation of our original findings and validation of the usefulness of the Pan-Retrovirus RT PCR detection method that we developed but it has also generated intriguing new data. Their "new sequence variant" LUMS9 of MSRV had a 'normal' full length RT sequence between the VLPQG YXDD motifs, containing an extra two amino acids with respect to MSRV, ERV-9 and the other HERV-W family members, as well as possessing an open reading frame. LUMS9 was obtained from a pregnant patient with MS sampled 2 weeks after onset of relapse i.e. during active disease, all the other 8 patients with MS did not have active disease and were sampled between 25 years and 3 months after their last bout of disease. This finding is in agreement with our own detection of MSRV in association with active disease.

In conclusion it is worth reiterating the statement by Olsson *et al.* "We therefore agree with Perron *et al.* (1997b) that retroviral elements related to ERV-9 are worthy of further investigation for a role in the pathogenesis of MS. The alternative possibility, that the presence of ERV-9/MSRV RNA in serum is related to pregnancy should also be investigated."

If MSRV is an endogenous retrovirus, or a common exogenous retroviral infection, then the role of hormonal influence on the LTR may be important in activating viral

expression, as perhaps suggested by the detection of LUMS9 in a pregnant woman with MS. This might also help to explain the observed sex bias in the prevalence of MS, female hormones acting on hormone responsive elements within the LTR. Female steroid hormones have been noted to result in elevated expression of certain endogenous retroviral sequences (Ono *et al.* 1987). It is interesting to note that a recently described endogenous retrovirus $IDDMK_{1,2}22$ has been suggested to have a role in the aetiology of type I diabetes (Conrad *et al.* 1997), although recent studies have failed to confirm this association (Murphy *et al.* 1998; Lower *et al.* 1998; Lan *et al.* 1998; Conrad, 1998). The highest rate of insulin-dependent diabetes mellitus (IDDM) occurs at 10-14 years of age in both sexes, which coincides with puberty (Bruno *et al.* 1993). It has been suggested that infection with ubiquitous herpes viruses may act synergistically with an increase in circulating levels of steroids to enhance expression of the superantigen (SAG) of $IDDMK_{1,2}22$ (Conrad *et al.* 1997).

Whether MSRV is an endogenous or exogenous retrovirus, it is worthy of note that the environmental factor or "infection" which triggers multiple sclerosis is thought to be acquired at a similar age (11-15 years (Kurtzke, 1993)) as the age of onset for IDDM. Similar mechanisms of activation of MSRV have been postulated (Perron *et al.* 1997b) as for $IDDMK_{1,2}22$ and the activation of these retroviruses during puberty may represent part of a common theme in retroviral induced autoimmunity.

The expression of MSRV in culture has been demonstrated to be enhanced, presumably by transactivation of the LTR, by coinfection of the cells with HSV-1 and also by transfection with the immediate early genes ICP-0 and ICP-4 in isolation

(Perron *et al.* 1993). Further data on the potential role of herpesvirus infection in multiple sclerosis is provided by recent studies on HHV-6 (Soldan *et al.* 1997), which it has been suggested, may act as a co-factor in activating MSRV (Perron *et al.* 1997b). Subsequent reports from some other researchers have failed to confirm the HHV-6 association (Coates and Bell, 1998; Fillet *et al.* 1998), but Soldan and colleagues have continued to present data in support of HHV-6 involvement in MS, finding 27% of multiple sclerosis patients sera positive by PCR (Jacobson *et al* 1998). Also the findings of Haahr and colleagues, on EBV and retroviral particles in multiple sclerosis patients' B-cell lines, are supportive of the hypothesis of a dual involvement of herpesviruses and a retrovirus in the aetiology of the disease (Haahr *et al.* 1992).

Interferon treatment has been demonstrated as having a beneficial effect on patients with relapsing remitting multiple sclerosis (Section 1.2.6.4; Anonymous, 1993; Sandberg Wollheim *et al.* 1995; Cardy, 1997; Abdul Ahad *et al.* 1997; Anonymous, 1998a). Recently IFN β1b has been demonstrated as having a similarly beneficial effect on patients with secondary progressive MS (Anonymous, 1998b). In the light of our findings on the association between MSRV and multiple sclerosis and additionally the possible involvement of a herpes virus or viruses, it is interesting to speculate that the relevant mode of action of interferon may be antiviral. This could be either by a direct antiviral effect, perhaps on herpes viruses or on MSRV itself, or via an immunomodulatory effect. Interferon has been demonstrated to have a limited but beneficial effect in HIV infections (Lane, 1994) and in herpes infections (Lebwohl *et al.* 1992; Cantell, 1995).

4.2.2 Cross reactive antibody responses in disease

It is perhaps tempting to overplay the role of retroviruses, be they endogenous or exogenous, in the aetiology of disease, if serological data is overinterpreted. Cross reactive antibody responses, predominantly directed towards Gag, have been observed in a diverse range of disease conditions, including SLE (Banki *et al.* 1992; Brookes *et al.* 1992), Sjogren's syndrome (Talal *et al.* 1990; Garry *et al.* 1990; Walchner *et al.* 1997; Sauter *et al.* 1996; Krieg and Steinberg, 1990; Krieg *et al.* 1992; Jimenez *et al.* 1995; Blomberg *et al.* 1994; Bengtsson *et al.* 1996), Grave's disease (Jaspan *et al.* 1995; Jaspan *et al.* 1996) and primary biliary cirrhosis (Mason *et al.* 1998).

The findings of cross reactive antibody response in multiple sclerosis patients was one of the initial spurs to searching for a novel retrovirus in this disease (Ohta *et al.* 1986; Dalgleish *et al.* 1987; Sandberg Wollheim *et al.* 1988; Perron *et al.* 1991; Rasmussen *et al.* 1992). Although investigation of the possible role of retroviruses in diseases in which cross reactive antibody responses were observed has led to the identification of novel retroviruses, namely HIV-2, HTLV-II, any agent responsible for the observed cross reactive antibody responses remains to be identified in the majority of cases. The retrovirus cultured by Haahr and colleagues from multiple sclerosis patients and a control was demonstrated to have antigenic determinants in common with the Env glycoproteins of HTLV (Christensen *et al.* 1997).

It is quite possible that such serological reactivities are directed against expressed endogenous sequences rather than against exogenous infections. When considering Gag antibody responses, predominantly observed in autoimmune diseases and germ cell tumours (Lower *et al* 1996), it should be borne in mind that on antibody screening of 100,000 blood donors for HTLV, only five genuine HTLV positives were detected (Brennan *et al.* 1993). However, 37 Western blot indeterminants were found which were negative by PCR for known retroviral infections, but showed multiple reactivity (4 or more bands), including Gag. This could be interpreted as representing an immune response to either an exogenous retroviral infection, which is present in this healthy subgroup of the general population, or to expressed endogenous sequences, or even simply cross reactive antibody responses to non-retroviral antigens. Whether the observed serological associations with disease represent a marker of infection or are a result of the disease pathology, is open to interpretation. Higher prevalences of HTLV Western blot seroreactivity have been detected in other populations than can be confirmed as true HTLV infections (Elm *et al.* 1991, Lal *et al.* 1992; Soriano *et al.* 1996).

4.3 Possible mechanisms of action of MSRV in the aetiology and pathogenesis of multiple sclerosis

Whether MSRV proves to be an exogenous retrovirus or a replication-competent endogenous one, there are numerous possible mechanisms (Mims, 1985) by which such an agent may be involved in the pathogenic process; antigenic mimicry (Perl

and Banki, 1993; Banki *et al.* 1994; Wucherpfennig and Strominger, 1995), retrovirally encoded superantigens (Rudge, 1991; Skov and Baadsgaard, 1995), direct cytotoxicity (Rasmussen *et al.* 1993), trans-activation of host genes (Perl and Banki, 1993; Rasmussen *et al.* 1993) and interactions with herpesviruses (Haahr *et al.* 1994; Haahr *et al.* 1992; Perron *et al.* 1993; Challoner *et al.* 1995) can all be plausibly invoked.

4.3.1 Possible Autoimmune Mechanisms

Multiple sclerosis is generally regarded as a disease which has an autoimmune pathology and there are several mechanisms by which MSRV could be implicated in this. It could act simply as a trigger for the development of the autoimmune condition, either by antigenically mimicking self proteins and giving rise to a cross reactive immune response, or by destruction of infected cells leading to an immune response to host cell proteins which are not normally targeted by the immune system. It has recently been demonstrated that bystander damage is responsible for causing the autoimmune diabetes experimentally induced in mice by Coxsackie B4 virus (Horwitz *et al.* 1998). A similar phenomenon is theoretically possible in human autoimmune diseases including multiple sclerosis.

Enveloped viruses may take up cellular membrane components such as MHC and HLA molecules during the budding process. These in turn may carry peptides which

could then be presented to the immune system in an aberrant fashion, which could result in an autoimmune response (Dalgleish, 1987).

The location of an endogenous related counterpart of MSRV in the T-cell receptor alpha delta locus could conceivably lead to aberrant expression of T-cell receptor genes. The established genetic susceptibility conferred by the T-cell receptor locus is intriguing in the context of the recent finding of an endogenous homologue of MSRV in this location (Alliel *et al.* 1998; Blond *et al.* 1999).

A recently published hypothesis is that multiple sclerosis is due to a failure of activation-induced apoptosis of autoreactive T-cells (Pender, 1998). It is conceivable that MSRV might be exerting such an effect by immortalising T-cells. Some of the data from the *in-vitro* MSRV culture experiments does suggest that MSRV has an immortalising effect on infected cells, as indicated by their tumourigenic potential in nude mice and ability to achieve high passage number (Perron *et al.* 1997b). Although this is a theoretical possibility, it has not yet been established whether MSRV can or does infect and replicate in T-cells.

4.3.2 Gliotoxin

MSRV could possibly exert a directly toxic effect on the glial cells of the CNS, although such an effect, or infection of these cells, remains to be demonstrated. MSRV has been shown to produce almost synchronous massive apoptosis in

leptomeningeal and choroid plexus cultures (Perron *et al.* 1997). Also, a gliotoxic factor has recently been identified in the CSF of multiple sclerosis patients (Menard *et al.* 1997b; Menard *et al.* 1998a; Menard *et al.* 1998b). This gliotoxin was shown to cause apoptosis *in-vitro* in astrocytes and oligodendrocytes, but not in fibroblasts, Schwann cells, endothelial cells or neurones (Menard *et al.* 1998a). Monocyte/macrophage cell culture supernatants from multiple sclerosis patients were shown to possess this gliotoxic activity, which also correlated with reverse transcriptase activity and the presence of the MSRV genome (Menard *et al.* 1997a).

The gliotoxic factor has been partially characterised and shown to be a stable 17 kDa glycoprotein. The sequence of this glycoprotein remains to be determined, but if it is encoded by the MSRV genome, or its production is induced by MSRV, this would provide a direct mechanism for the demyelination observed in MS. Matrix Assisted Laser Desorption/Ionisation Time Of Flight (MALDI-TOF) mass spectrometry analysis of trypsin digested fragments of the purified gliotoxin has demonstrated that the protein is compatible with a region of the Pol protein of MSRV (Alliel *et al.* 1998). However the resolution achieved in this analysis was far from optimal and the relationship of the gliotoxin with MSRV Pol currently remains speculative (J. Belliveau personal communication). If MSRV does in fact encode this gliotoxin or causes it to be expressed then a possible direct mechanism for the pathogenesis of MSRV in multiple sclerosis will have been established.

Injection of approximately 10ng of the partially purified 17kDa gliotoxin into the CSF of rats produced widespread apoptosis and also some necrosis within the CNS

(Perron, personal communication). Ten days after injection of the gliotoxin the majority of the cells to have undergone apoptosis were astrocytes, but also included oligodendrocytes. However, three months after injection of the gliotoxin demyelination and death of oligodendrocytes was a far more important feature of the damage caused to the CNS (Perron, personal communication). Astrocytes maintain the structure and function of the blood brain barrier (Stewart and Wiley, 1981), the loss of integrity of which is one of the primary features of an acute MS lesion, with oligodendrocyte depletion occurring secondarily (Hawkins *et al.* 1991; Gay and Esiri, 1991; Poser, 1993). These observations provide tantalising evidence for a potential animal model of multiple sclerosis in which the gliotoxin acts as a trigger for the development of disease.

Most recently this gliotoxic factor has been shown to be present in the urine from 32/35 (91%) of multiple sclerosis patients but 0/35 (0%) of healthy controls (Marcus Vocanson *et al.* 1998). The gliotoxic activity causes a form of apoptosis to occur in a human glial hybrid cell line (cell line CLTT 1-1 (Galiana *et al.* 1990)).

4.4 Possible models for MSRV pathogenesis

Graves' disease, another autoimmune condition with an environmental component to its aetiology, has been suggested to be the product of genetic susceptibility determined by HLA haplotypes and expression of the endogenous retroviral sequence, human intracisternal type A retroviral particle (HIAP) (Jaspan *et al.* 1996).

It is possible that if MSRV is an endogenous retrovirus then there could be a similar interaction between it and the observed genetic susceptibility loci (Section 1.2.5.1).

There are several animal models which are highly relevant when considering the possible pathogenic mechanisms of MSRV in MS. One is murine age dependent poliomyelitis, which is caused by the interaction of an exogenous viral infection and genetic susceptibility of the host (Contag and Plagemann, 1989; Contag *et al.* 1989). This genetic susceptibility is due to an endogenous murine leukaemia virus. Age dependent poliomyelitis only develops in mice which are infected with lactate dehydrogenase virus (LDV) after a certain age, and is enhanced in old mice by cyclophosphamide treatment or X-ray irradiation. This would suggest that enhanced expression of the endogenous murine leukaemia virus results in increased susceptibility to age dependent poliomyelitis (ADPM) perhaps by upregulating the levels of the viral receptor. If the endogenous retrovirus which encodes susceptibility is itself the receptor for LDV, this would make sense, since X-ray irradiation is known to activate the expression of endogenous retroviruses (Weiss *et al.* 1971). The normal target-cell for infection by LDV is the macrophage, but the motor neurones of the spinal cord are the target for the neuropathogenic replication in age dependent poliomyelitis. Although the endogenous MLV is expressed in a range of tissue types including the macrophages, it is only the motor neurones where it is responsible for providing the viral receptor. Here there is a novel neuronal LDV receptor which is thought to involve at least two separate epitopes. This receptor is thought to be either MLV encoded or an MLV induced neuronal surface protein (Contag *et al.* 1989).

The age dependent susceptibility in multiple sclerosis could conceivably have a similar basis, in which case MSRV might represent an expressed endogenous retrovirus, which provides a genetic component to the disease. A normally innocuous viral infection, such as a herpes virus, could be the viral trigger of neurological damage. Alternatively, MSRV could represent the exogenous, disease triggering, viral infection and an uncharacterised endogenous retrovirus represent the genetic susceptibility. Viral interference could have a role, perhaps MSRV related endogenous retroviruses protect the majority of individuals (or the majority of genetically susceptible individuals) against infection with the exogenous, neurovirulent, strain of MSRV.

It has recently been shown that mice which are transgenic for an autoreactive T-cell receptor develop destructive polyarthritis (Kouskoff *et al.* 1996). Rheumatoid arthritis is another autoimmune disease in which a retroviral aetiology has been proposed (Wilder, 1994). The sequencing of a human T-cell receptor gene (BAC378) has revealed that there is an MSRV related endogenous sequence present within it, albeit with multiple stop codons and a deleted *env* gene (Section 4.3.1; Alliel *et al.* 1998; Blond *et al.* 1999). It is theoretically possible that this MSRV related LTR could be acting as a promoter and/ or enhancer for this T-cell receptor gene, which could conceivably lead to the inappropriate expression of autoreactive T-cell receptors and hence to autoimmunity in some individuals.

It must however be stressed that all the potential mechanisms outlined here are still speculative and entirely theoretical at present.

4.5 Future work

4.5.1 Detection and analysis of virion-associated retroviral RNA

An important requirement is to establish whether the observed association between MSRV viraemia and multiple sclerosis is confirmed in larger molecular epidemiological studies. Further epidemiological studies could also be performed to determine if MSRV was involved in a range of other autoimmune conditions. In view of the observed tumourigenic properties of LM7PC cells (MSRV infected) in nude mice (Perron *et al.* 1997) it would be of interest to examine patients with a range of cancers for virion associated MSRV-RNA. The question of MSRV involvement in the aetiology of multiple sclerosis could also be addressed by studies of identical twins discordant for MS.

Another priority is to fully characterise the virion associated genome of MSRV. In addition, the endogenous counterpart(s) should be cloned and sequenced. This may help elucidate whether MSRV is itself endogenous or an exogenous member of an endogenous family. Recent work has characterised a family of endogenous retroviruses related to MSRV (HERV-W) but as yet this question is not fully answered (Blond *et al.* 1999).

It is also necessary to establish whether the observed expression of RGH-2, MSRV, LUMS9 and ERV-9 in multiple sclerosis patients is part of the same phenomenon. It

is possible that they are the result of co-packaging of related sequences within virions encoded by a single functional retrovirus (Golovkina *et al.* 1994). The recent demonstration of RTVL-H sequences in virions derived from embryonal carcinoma cells known to produce HERV-K virions has been suggested to be due to such retroviral co-packaging (Patience *et al.* 1998).

The RTVL-H family has particularly high transcriptional activity and copy numbers (Wilkinson *et al.* 1993). Therefore, it might not be surprising if these related sequences were copackaged along with MSRV. Alternatively, it is possible that each represents a sub-population of the total endogenous retroviral expression that occurs in multiple sclerosis. As stated by Christensen and colleagues, "Whether the retroviral proteins in the particles are encoded by RGH (or MSRV or LUMS9 or ERV-9) needs to be assessed". In either scenario the retroviral expression that is observed in multiple sclerosis could represent a causal or casual association with the disease. If all three virus families were searched for simultaneously, a better understanding of the role of the expression of these C-type retroviruses in multiple sclerosis might be achieved.

4.5.2 Immunoassay development and seroepidemiology

In order to address the issue of which sequence encodes the virus particles, PCR products from MSRV, LUMS9, ERV-9 and "RGH-2 related sequences" *gag, pol* and *env* genes should be cloned and expressed and the recombinant proteins purified.

Serological assays including Western blotting, RIPA and enzyme linked immunosorbent assay (ELISA), could then be developed and evaluated. Viral antigen specific, oligoclonal band immunoblotting techniques on CSF could also be undertaken. Additionally, synthetic peptides could be used as an alternative strategy for immunoassay development. Antibody-based assays such as these will be capable of a much higher throughput than the PCR-based assays so far employed and will therefore be more suitable for large scale epidemiological studies using both serum and CSF. However the polyclonal B-cell activation that occurs in MS may complicate the interpretation of such experiments.

4.5.3 Immunohistology on post-mortem central nervous system tissue

A number of polyclonal antibodies have been raised against recombinant MSRV Pol, Gag and Env proteins by Perron and colleagues. Preliminary and as yet unconfirmed studies have shown that these antibodies immunostain MSRV infected B-lymphocytes from multiple sclerosis patients and plaques from MS brain samples (Perron, personal communication). Confirmatory studies with these antibodies should be undertaken to investigate the expression of MSRV antigens in plaque and non-plaque CNS tissue from multiple sclerosis patients and from other neurological disease controls.. Standard neuropathological staining techniques should also be performed in parallel.

4.5.4 Pathogenesis and MSRV

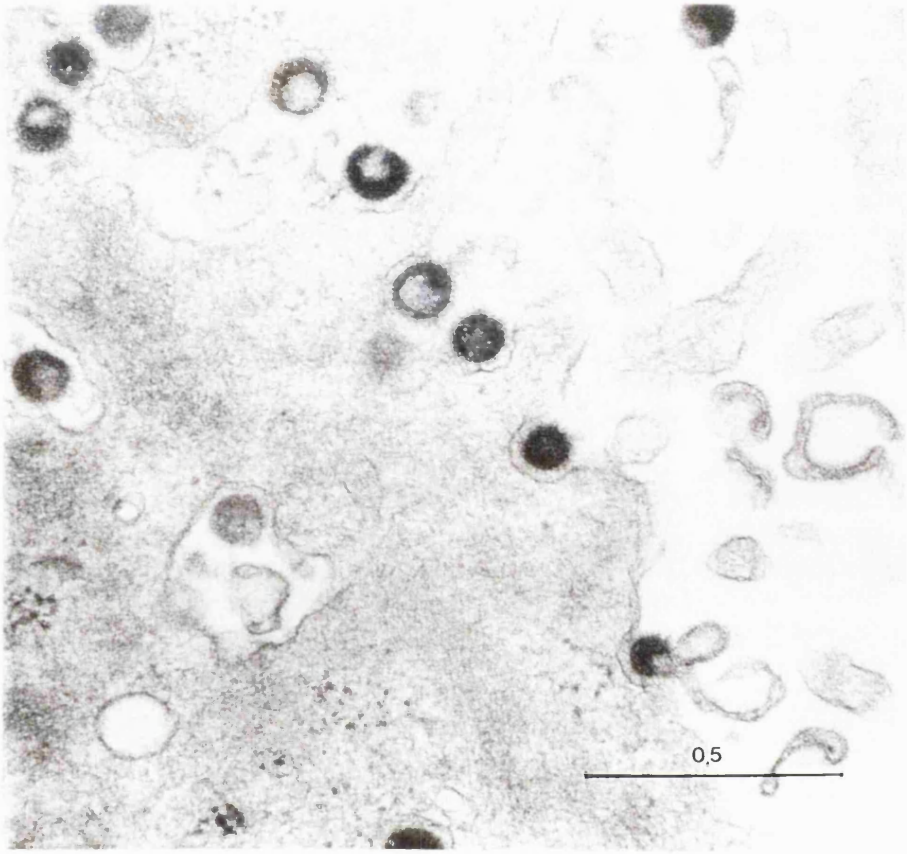
Whether MSRV is an exogenous or endogenous retrovirus, further experiments should be performed to determine the mechanisms by which its expression is activated, including identifying any viral co-factor involvement. If MSRV is an exogenous retrovirus then transmission studies should be performed.

It is possible that MSRV is having a direct cytolytic effect on the oligodendrocytes of the CNS. Hence it would be of interest to investigate whether MSRV will grow in oligodendrocytes *in-vitro* and to observe any cytopathic effect. Similarly astrocytes should be investigated to see if they are permissive for MSRV replication and to determine whether they exhibit apoptosis, as might be predicted.

The partially characterised gliotoxin associated with multiple sclerosis and its possible correlation with a fragment of MSRV Pol requires further investigation (Alliel *et al.* 1998). Microsequencing of the gliotoxin or higher resolution MALDI-TOF (Matrix Assisted Laser Desorption/Ionisation Time Of Flight) mass spectrometry will confirm whether this protein is encoded by MSRV. Expression of the putative gliotoxic fragment of MSRV Pol and toxicity studies should equally help to address this question.

Figure 1

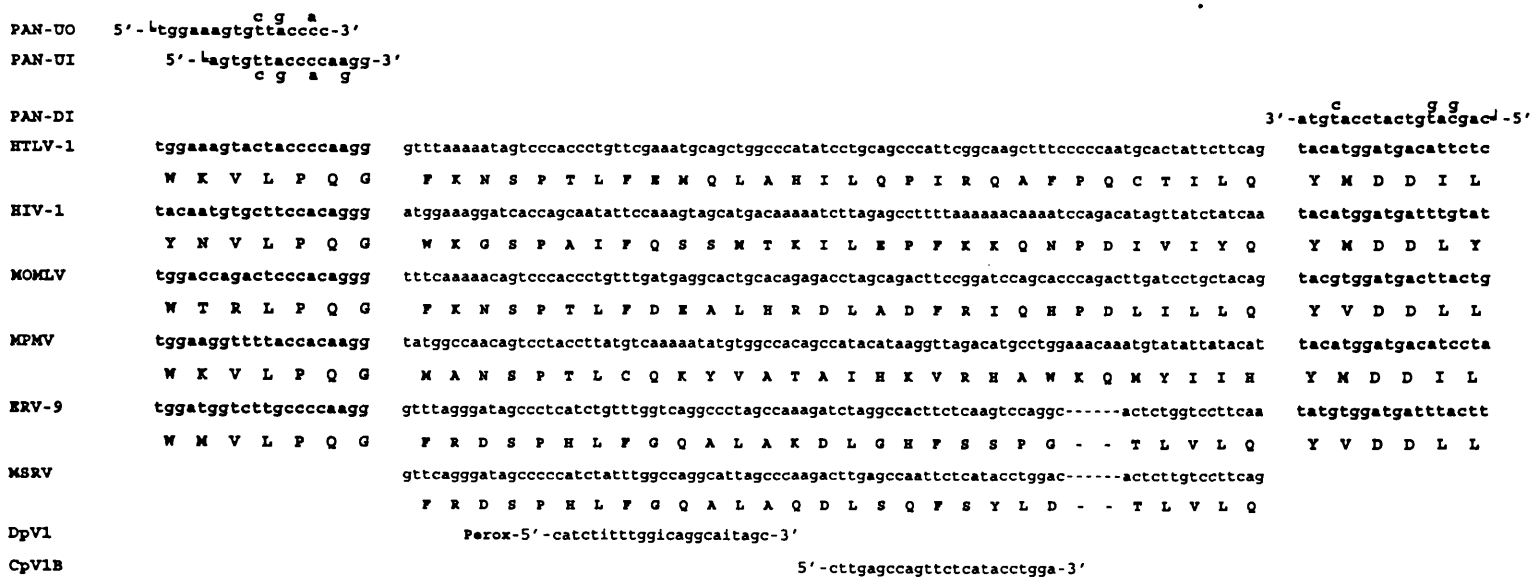
Multiple sclerosis-associated retrovirus



Electron micrograph of MSRV virions in a culture of leptomeningeal cells from a patient with multiple sclerosis. A number of viral particles are seen along the cell membrane. The scale bar is 0.5μm. Adapted from Perron *et al.* (1989).

Figure 2

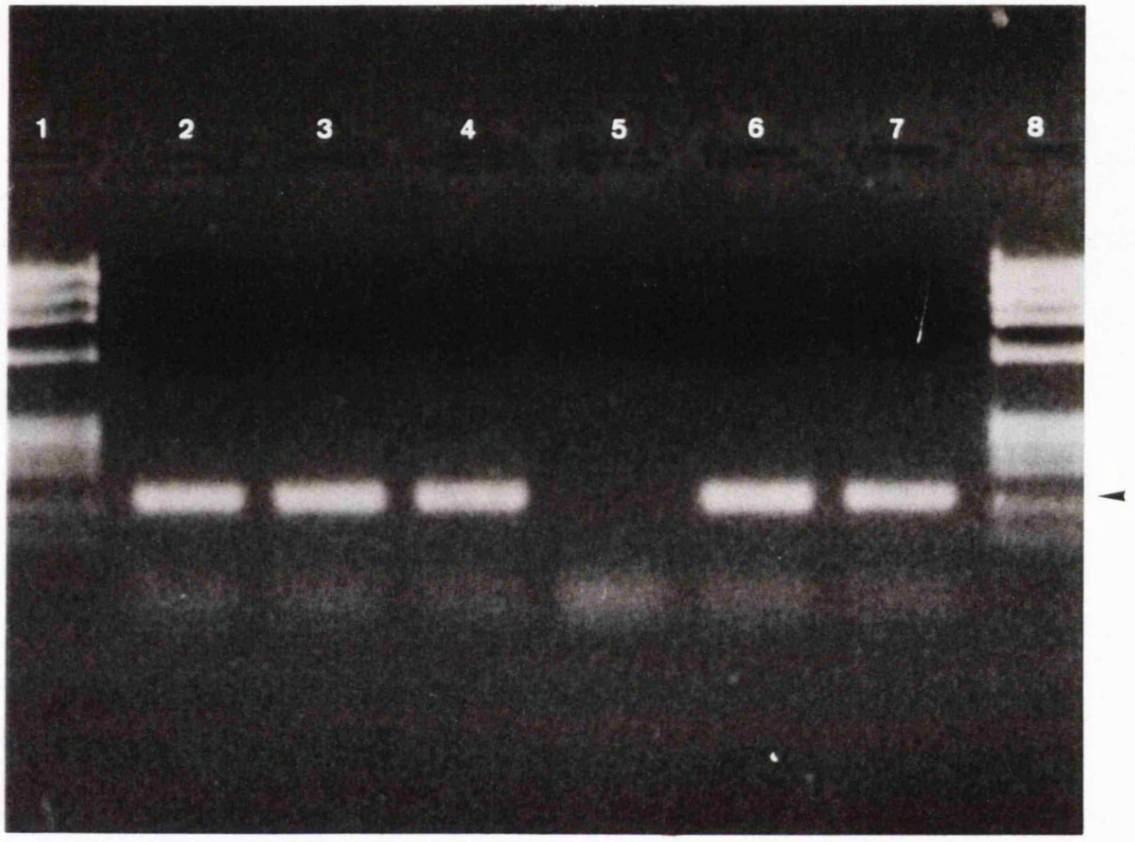
MSRV *cpol* sequence amplified between the conserved VLPQG YXDD motifs in *pol*: alignment with other retroviruses



Nucleotide and amino acid alignment of the conserved *pol* regions of viruses detected in the present study by the 'pan-retrovirus' PCR. 'Deletions' are represented by dashes and standard single-letter abbreviations are used to designate amino acids and nucleotides (i = inosine). The most highly conserved VLPQG and YXDD regions are shown as separate blocks in bold type at the end of each sequence. PCR primers PAN-UO and PAN-UI are orientated 5' to 3' (sense) whereas primer PAN-DI is 3' to 5' (antisense). Degeneracies are shown above (PAN-UO & PAN-DI) or below (PAN-UI) the PCR primer sequences. ʟdenotes the nine base 5' extension *cttggatcc*. ɹdenotes the nine base 5' extension *ctcaagctt*. The capture and detector probes DpV1 and CpV1b used in the ELOSA assay are shown below a representative MSRV-*cpol* sequence. DpV1 is peroxidase labelled and that CpV1b may be biotinylated at the 5' end if streptavidin coated plates are used. The name of each sequence is indicated on the left. HTLV1: Human Leukaemia Virus type 1; HIV1: Human Immunodeficiency Virus type 1; MoMLV: Moloney-Murine Leukaemia Virus; MPMV: Mason-Pfizer Monkey Virus. ERV9: Endogenous Retrovirus 9. MSRV-cpol: Multiple Sclerosis associated RetroVirus conserved pol region.

Figure 3

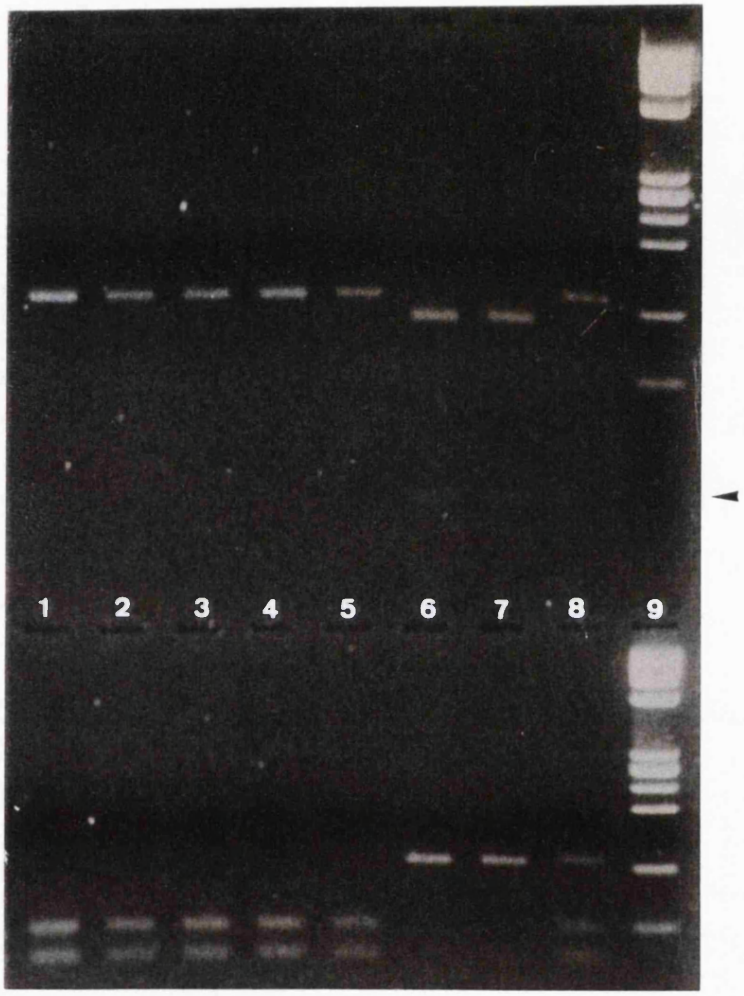
HTLV *tax/rex* PCR of DNAs from HTLV patients and controls



Ethidium bromide stained 2% agarose gel:
The MW marker is a *Hae* III digest of phage ϕX174 DNA
Lanes 1 and 8 contain MW marker
Lanes 2, 3 and 4, HTLV-II patients
Lane 5 water control
Lanes 6 and 7, HTLV-I patients
Arrow points to 128bp bands

Figure 4

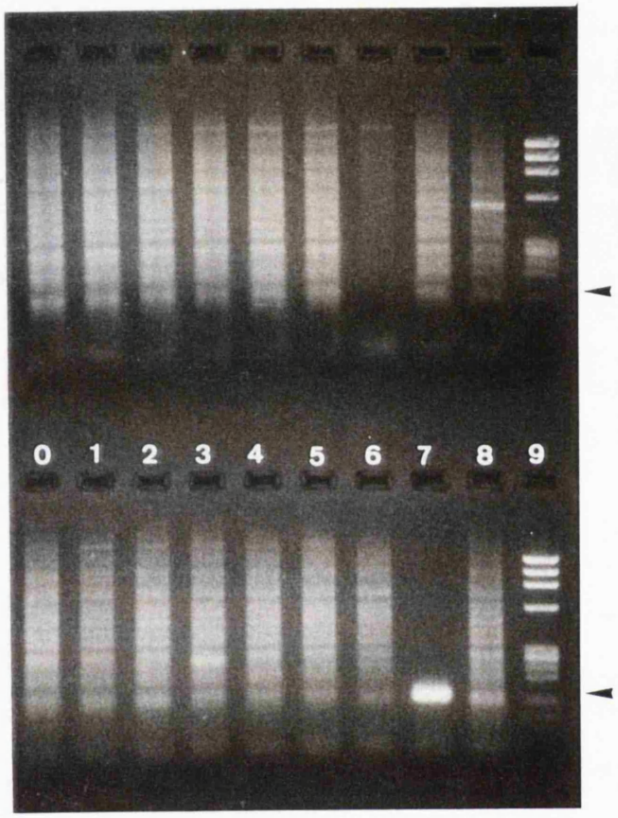
Restriction Enzyme Analysis of HTLV-I/II *tax/rex* PCR Products



Ethidium bromide stained 4% agarose gel:
Upper row *Sau* 3a digests, lower row *Taq* 1 digests of second round PCR products. Lanes 1 to 5 HTLV-II patients, lane 6 HTLV-I patient, lane 7 HTLV-I control DNA and lane 8 a mixture of HTLV-I and HTLV-II control DNAs. Lane 9 MW marker is a *Hae* III digest of ϕX174 DNA. *Sau* 3a does not cut HTLV-II products but cuts HTLV-I products to generate 104bp and 24bp fragments. *Taq* 1 cuts HTLV-II products to generate 69bp, 53bp and 6bp fragments, and cuts HTLV-I products to yield 122bp and 6bp fragments (all 6bp fragments not visible). Arrow points to 24 bp bands

Figure 5

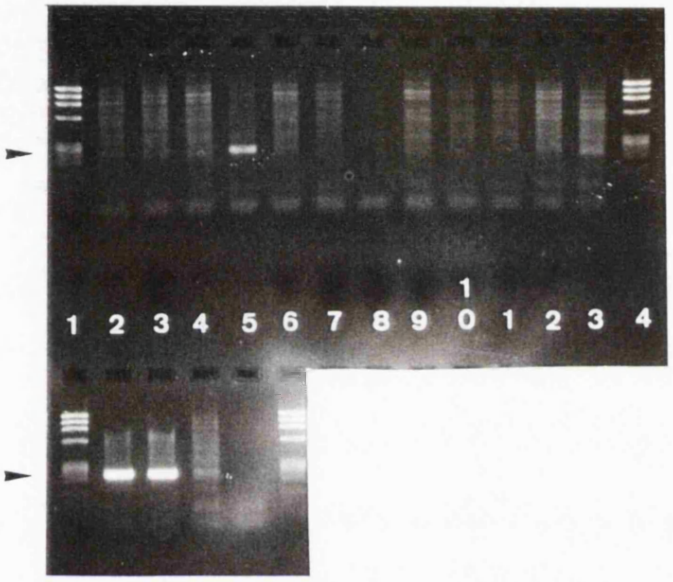
Very low stringency HTLV *tax/rex* PCR of DNAs from patients with MS and controls



Ethidium bromide stained 2% agarose gel:
The MW marker is a *Hae* III digest of phage ϕX174 DNA
Upper row and lower row: lane 9 contains MW marker
Upper row lanes 0-8 patients with MS
Lower row lanes 0-6 controls, lane 7 HTLV-I , lane 8 control
Arrow points to 128bp bands

Figure 6

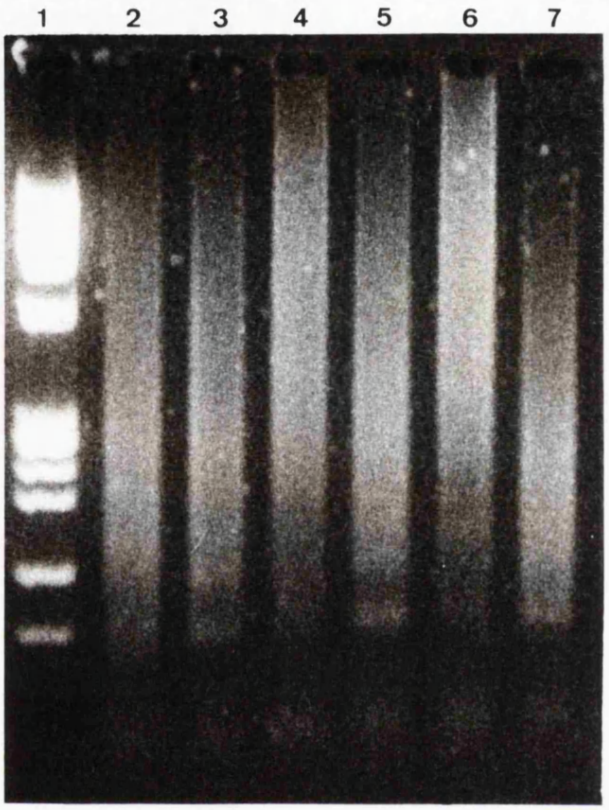
Very low stringency HTLV *gag/protease* PCR of DNAs from patients with MS and controls



Ethidium bromide stained 2% agarose gel:
The MW marker is a *Hae* III digest of phage ϕX174 DNA
Upper row: Lanes 1 and 14 MW marker
Lanes 2-13 patients with MS
Lower row: Lanes 1 and 6 contain MW marker
Lane 2, 10 copies of HTLV-I, lane 3, 1 copy of HTLV-I, lane 4 control DNA, lane 5 1/25 copy of HTLV-I
Arrow points to 235 bp bands

Figure 7

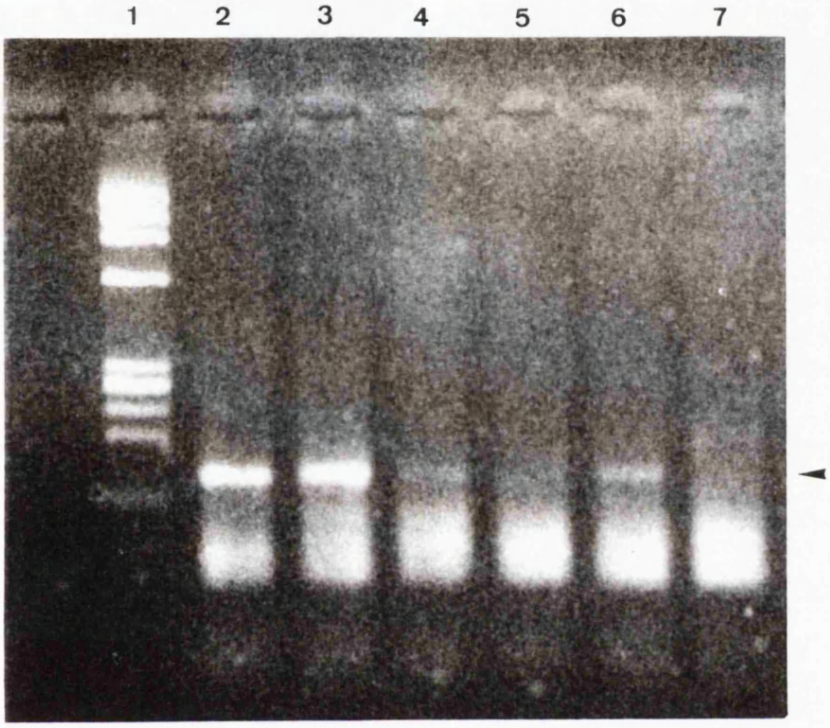
"Pan-Retrovirus" PCR products of HIV pBH10 DNA without 'hot-start'



Ethidium bromide 2% agarose gel:
The MW marker is a *Hae* III digest of phage ϕX174 DNA
Lane 1 MW marker
Lanes 2 and 3 10,000 copies, lanes 4 and 5 1,000 copies,
Lanes 6 and 7 100 copies

Figure 8

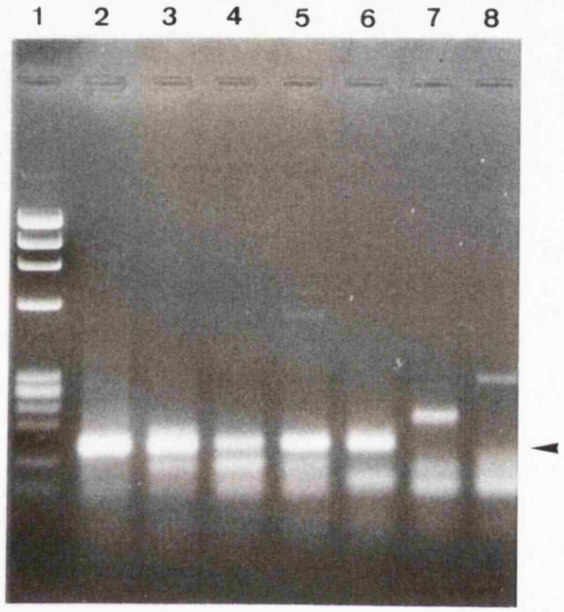
"Pan-Retrovirus" PCR on HIV pBH10 DNA showing effect of 'hot-start'



Ethidium bromide 2% agarose gel:
The MW marker is a *Hae* III digest of phage ϕ X174 DNA
Lane 1 MW marker
Lanes 2 and 3 10,000 copies, lanes 4 and 5 1,000 copies,
Lanes 6 and 7 100 copies
Arrow points to 142 bp band

Figure 9

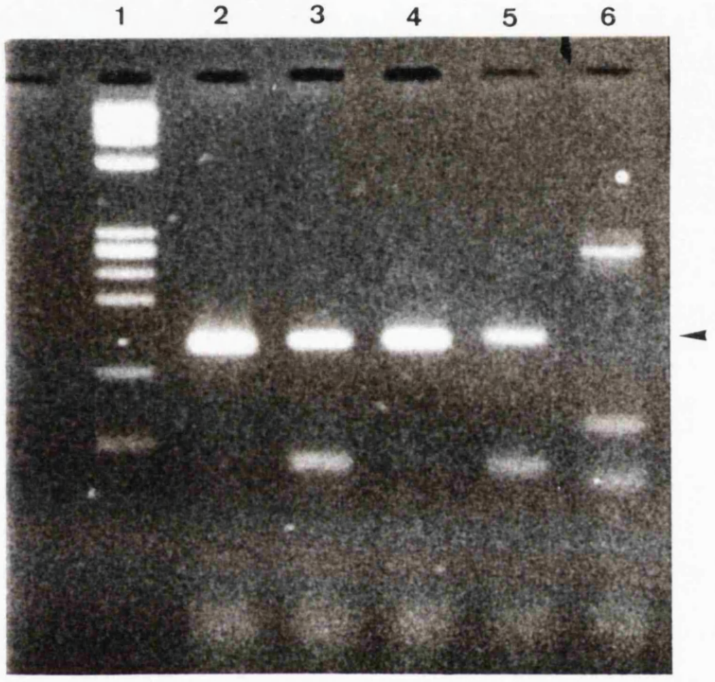
"Pan-Retrovirus" PCR showing sensitivity with HIV-1 plasmid pBH10 DNA



Ethidium bromide 2% agarose gel:
The MW marker is a *Hae* III digest of phage ϕX174 DNA
Lane 1 MW marker
Lane 2 10,000 copies, lane 3 1,000 copies, lane 4 100 copies, lane 5 10 copies, and lane 6 10 copies
Lanes 7 and 8 water controls
Arrow points to 142 bp band

Figure 10

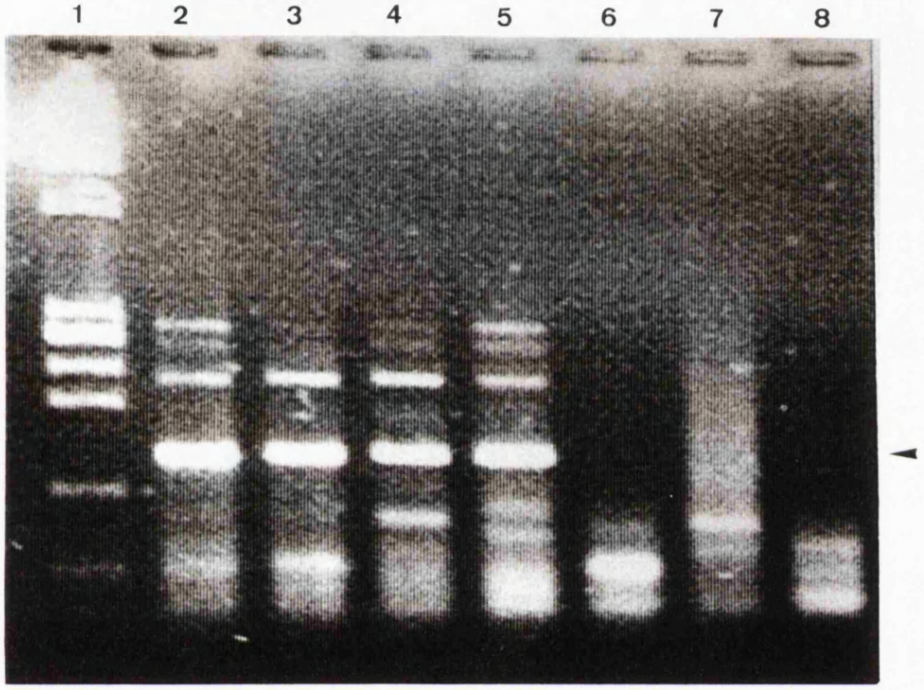
"Pan-Retrovirus" PCR showing sensitivity with the HTLV-I plasmid pMT2



Ethidium bromide 2% agarose gel:
The MW marker is a *Hae* III digest of phage ϕX174 DNA
Lane 1 MW marker
Lane 2 1,000 copies, lane 3 100 copies, lane 4 10 copies, lane 5 1copy,
and lane 6 0.1 copies
Arrow points to 142 bp band

Figure 11

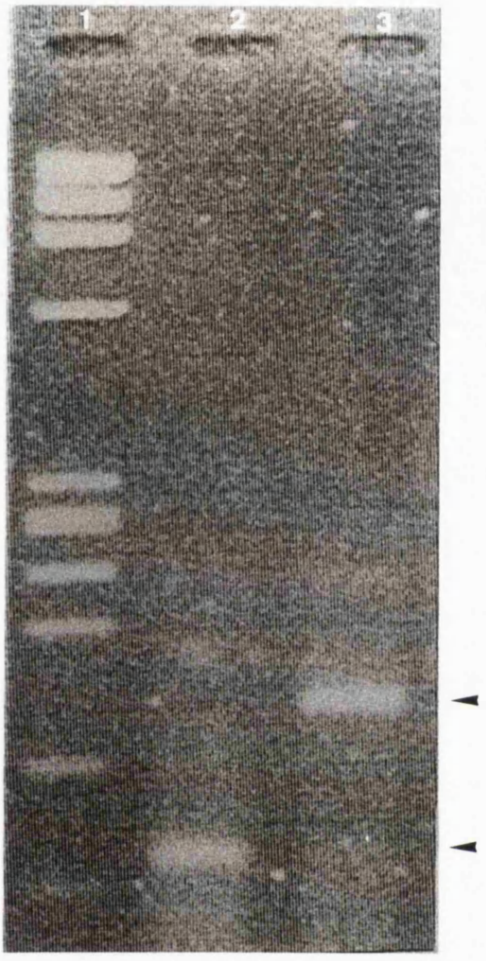
"Pan-Retrovirus" PCR of MPMV cDNA and controls



Ethidium bromide 2% agarose gel:
The MW marker is a *Hae* III digest of phage ϕX174 DNA
Lane 1 MW marker
Lanes 2, 3, 4, and 5 PCR products derived from approximately 100 pfu of MPMV
Lanes 6, 7 and, 8 water controls
Arrow points to 142 bp band

Figure 12

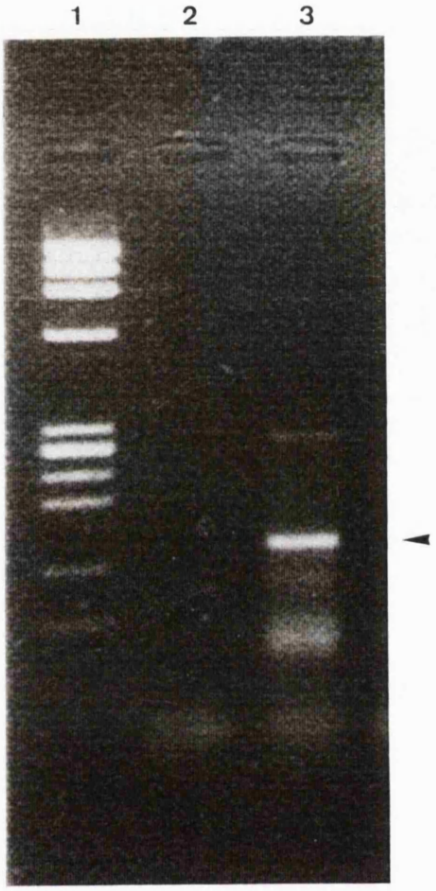
Hae III digest of "Pan-Retrovirus" PCR product of MPMV cDNA



Ethidium bromide 2% agarose gel:
The MW marker is a *Hae* III digest of phage ϕX174 DNA
Lane 1 MW marker
Lane 2 *Hae* III digest
Lane 3 uncut
Arrows point to 142 and 80 bp bands

Figure 13

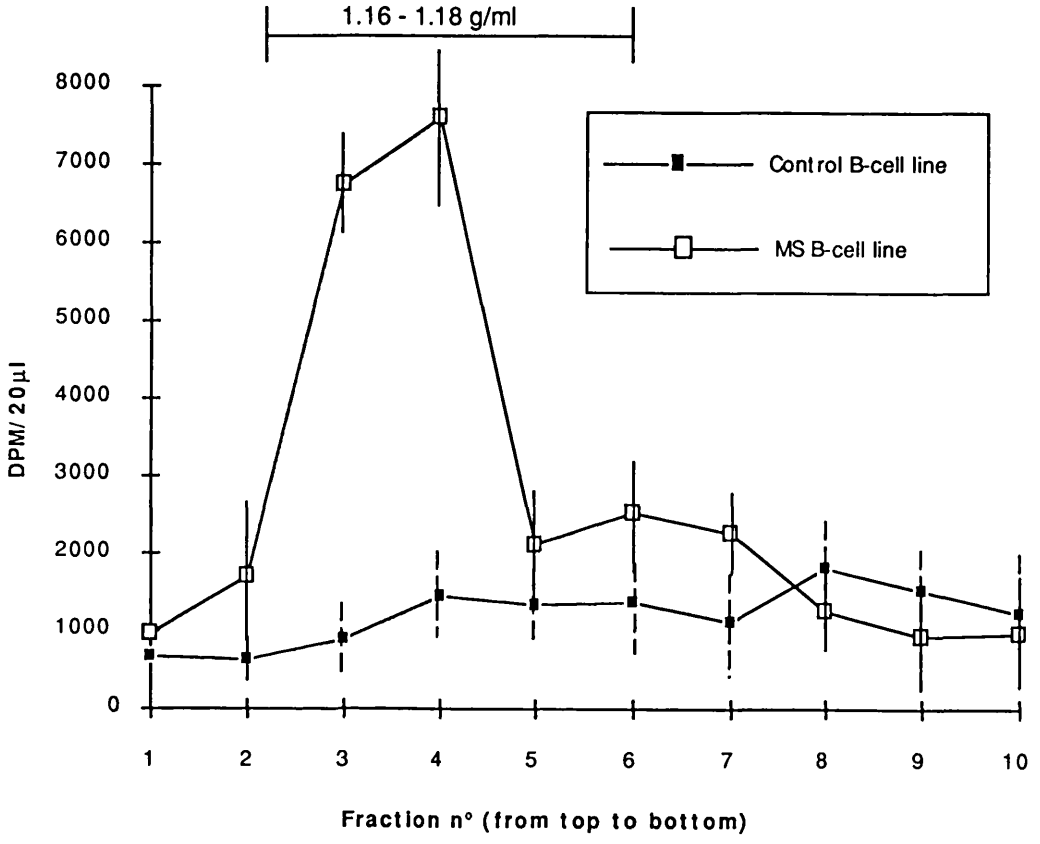
"Pan-Retrovirus" PCR on cDNA of RNA from sera of patients with MS



Ethidium bromide 2% agarose gel:
The MW marker is a *Hae* III digest of phage ϕX174 DNA
Lane 1 MW marker
Lane 2 MS patient B
Lane 3 MS patient C
Arrow points to 136 bp band

Figure 14

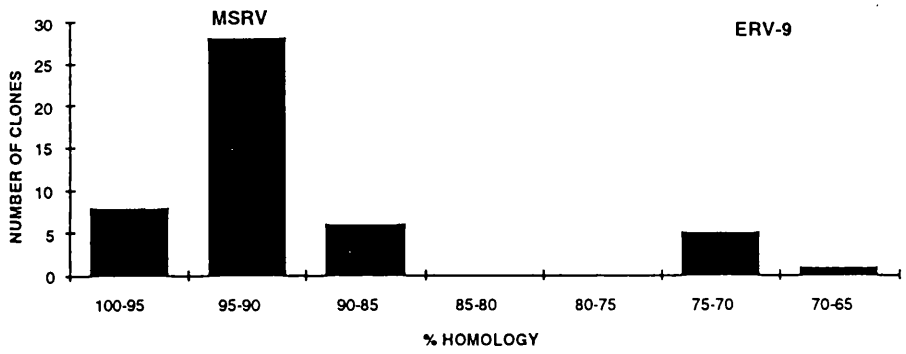
RT activity profile of sucrose density gradients



RT-activity profile in sucrose density gradient fractions of B-cell culture supernatants. Data presented as mean of triplicate readings ± two standard deviations. Note peak RT-activity at approximately 1.17 g/ml in the supernatant from the MS patient-derived B-cell culture. The control B-cell line was obtained from an unaffected adult relative of a patient with a mitochondriopathy. (Adapted from Perron *et al.* 1997b)

Figure 15

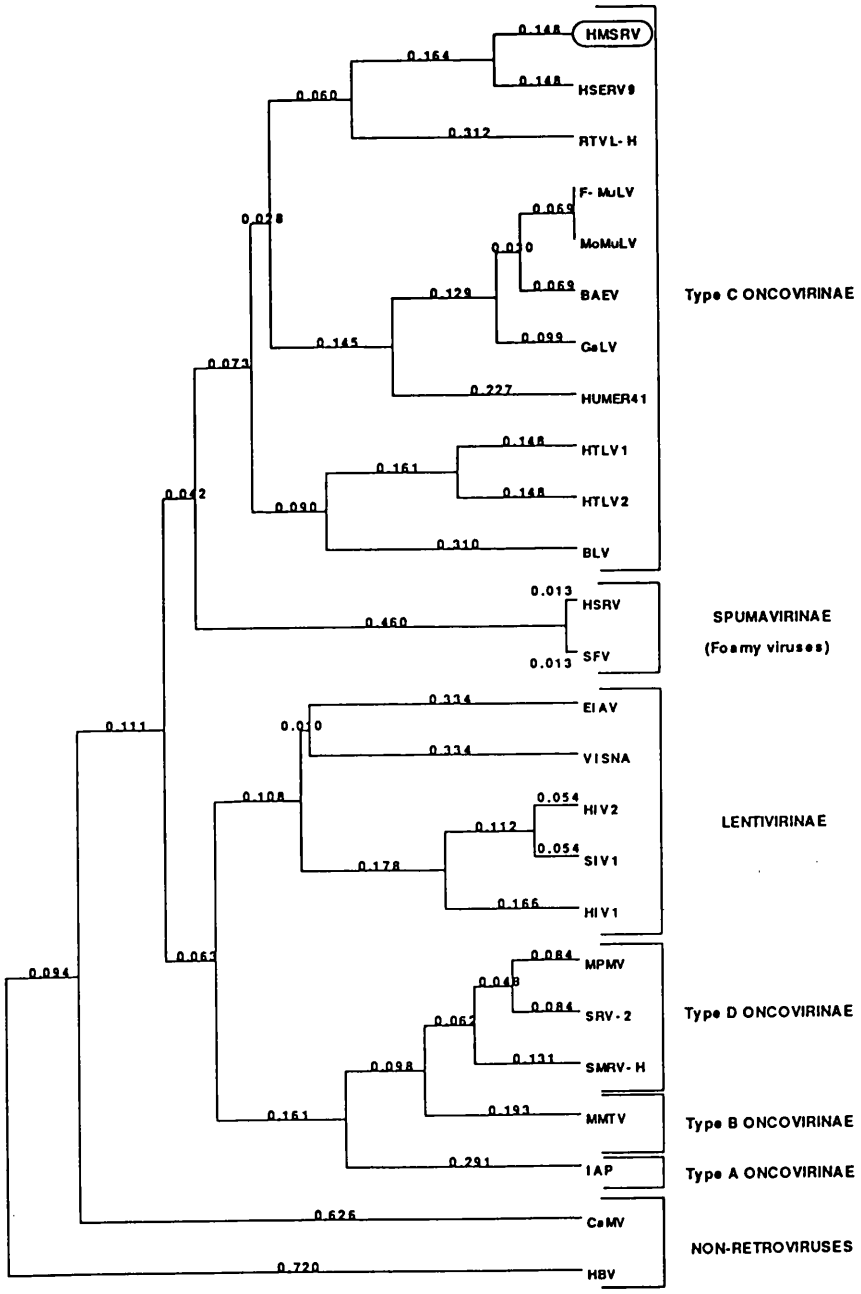
Nucleotide homology of clones with a reference MSRV-c*pol* clone



Frequency distribution of 30 ERV9-related sequences derived from the LM7PC and PLI2 sucrose fractions containing the peak of RT-activity (Table 3). Note the bimodal distribution of % nucleotide homology with a reference MSRV-*pol* sequence. The major cluster on the left represent MSRV-*pol* sequences, the small cluster of clones on the right (65-75% homology) represent ERV9 sequences.

Figure 16

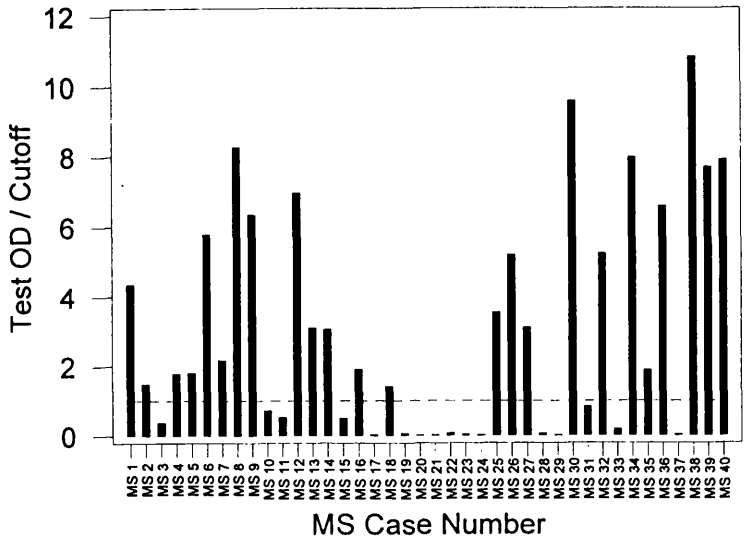
Phylogenetic analysis of the of the YMDD VLPQG region of *pol*



A phylogenetic tree, computer generated by Geneworks® (Intelligenetics Inc.), based on amino acids of the highly conserved VLPQG-YXDD region of *pol* (kindly provided by H. Pérron). A representative selection of exogenous and endogenous retroviruses is included plus two DNA viruses that possess reverse transcriptase. Figures on branches show degree of divergence. All sequences other than HMSRV were obtained from GenBank. Abbreviations as follows - HMSRV, Human Multiple Sclerosis associated Retrovirus; HSERV9 (ERV9), Human Sequence of Endogenous Retrovirus 9; RTVL-H, Retrovirus-Like Human endogenous sequence; F-MuLV, Friend-Murine Leukaemia Virus; MoMLV, Moloney-Murine Leukaemia Virus; BAEV, Baboon Endogenous Virus; GaLV, Gibbon Ape Leukaemia Virus; HUMER41, Human Endogenous Retroviral sequence, clone 41; HTLV1 and HTLV2, Human T-cell Leukaemia Viruses types 1 and 2; BLV, Bovine Leukaemia Virus; HSRV, Human Spumaretrovirus; SFV, Simian Foamy Virus; EIAV, Equine Infectious Anaemia Virus; VISNA, Visna virus; HIV1 and HIV2, Human Immunodeficiency Virus types 1 and 2; SIV1, Simian Immunodeficiency Virus type 1; MPMV, Mason Pfizer Monkey Virus; SRV-2, Simian Retrovirus type 2; SMRV-H, Simian Monkey Retrovirus H; MMTV, Mouse Mammary Tumour Virus; IAP, Intracisternal A-type Particle; CaMV, Cauliflower Mosaic Virus; HBV, Hepatitis B Virus.

Figure 17

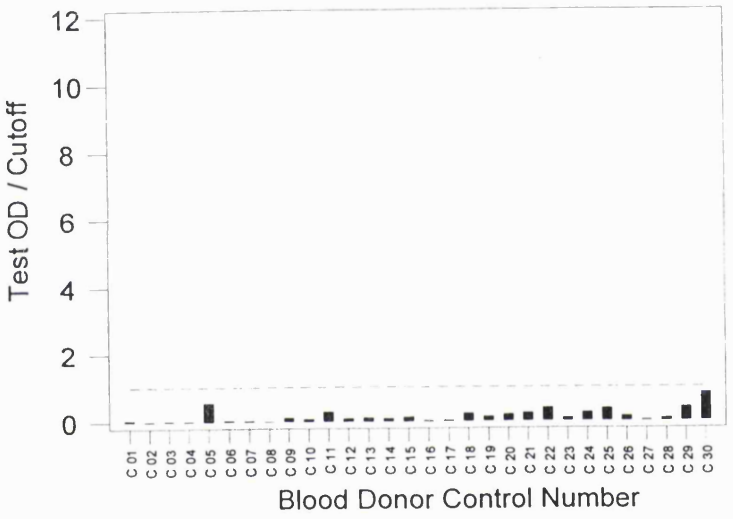
MSRV-*pol* ELOSA data from 40 multiple sclerosis patients



MSRV-*pol* ELOSA data generated from the analysis of sera from 40 patients with multiple sclerosis. Each bar represents the mean of two ELOSA readings. The cut-off is represented by the horizontal dashed line.

Figure 18

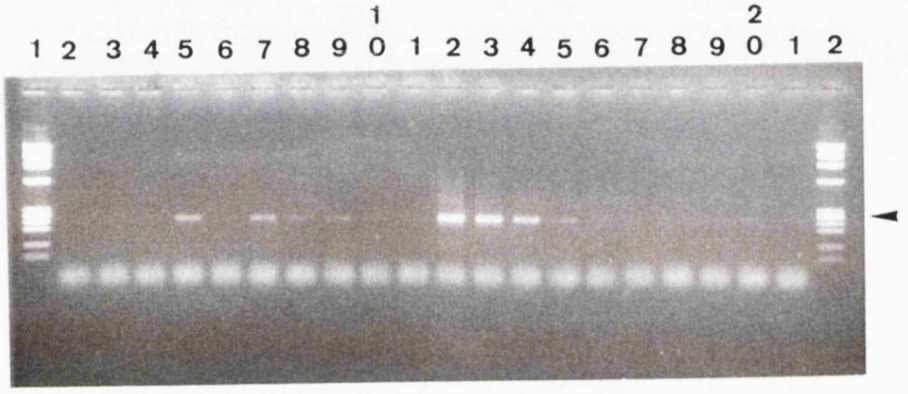
MSRV-*pol* ELOSA data from 30 blood donor controls



MSRV-*pol* ELOSA data generated from the analysis of sera from 30 healthy blood donors. Each bar represents the mean of two ELOSA readings. The cut-off is represented by the horizontal dashed line.

Figure 19

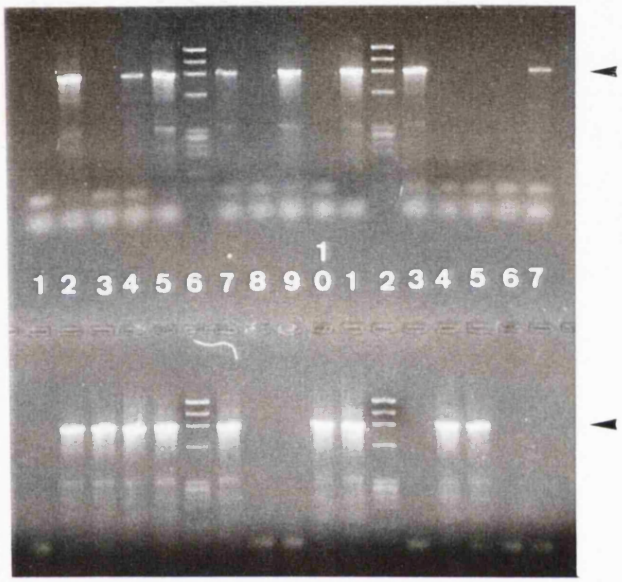
Alu PCR on cDNA samples from patients with MS and controls



Ethidium bromide stained 2% agarose gel:
The MW marker is a *Hae* III digest of phage ϕX174 DNA
Lanes 1 and 22 MW marker
Lanes 2-11 patients with MS and normal controls alternately
Lanes 12-15 human DNA dilution series 10, 1, 1/10th, 1/100th of a cell
Lanes 16-21 water controls
Arrow points to 236 bp bands

Figure 20

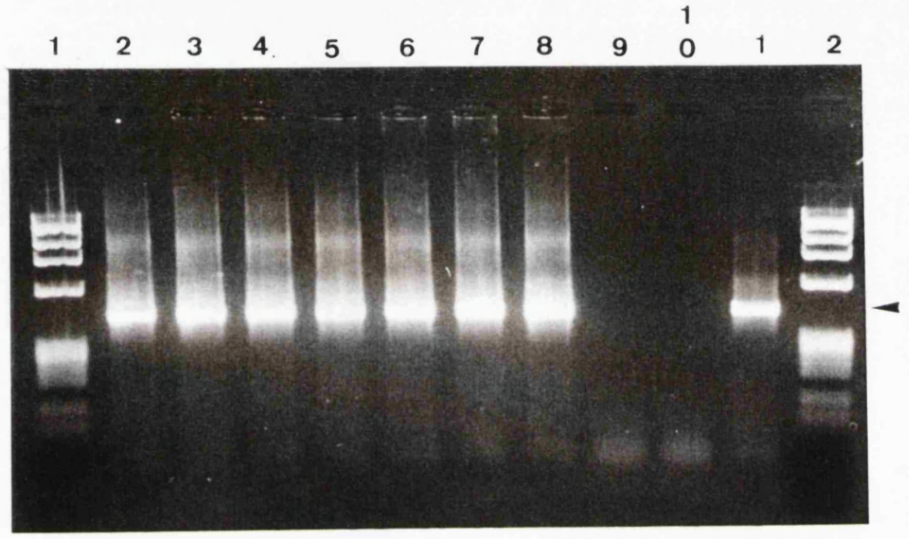
MSRV *gag* specific PCR on DNA samples



Ethidium bromide stained 2% agarose gel:
The MW marker is a *Hae* III digest of phage ϕX174 DNA
Upper and lower row lanes 6 and 12 MW marker
Upper row:
Lanes 1-4 patients with MS
Lanes 5, 7-11 and 13-17 normal controls
Lower row:
Lanes 1-5, 7-11 and 13-17 normal controls
Arrow points to 865 bp bands

Figure 21

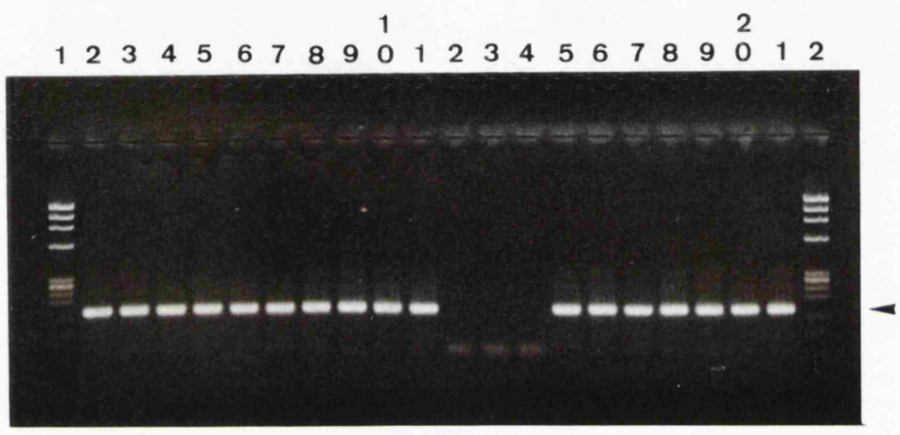
MSRV PT*pol* AB/EF specific PCR on DNA samples



Ethidium bromide stained 2% agarose gel:
The MW marker is a *Hae* III digest of phage ϕX174 DNA
Lanes 1 and 12 MW marker
Lanes 2-4 neonates
Lanes 5 and 6 normal controls
Lanes 7 and 8 patients with MS
Lanes 9 and 10 water controls
Lane 11 positive control (previously amplified first round PCR product)
Arrow points to 435 bp bands

Figure 22

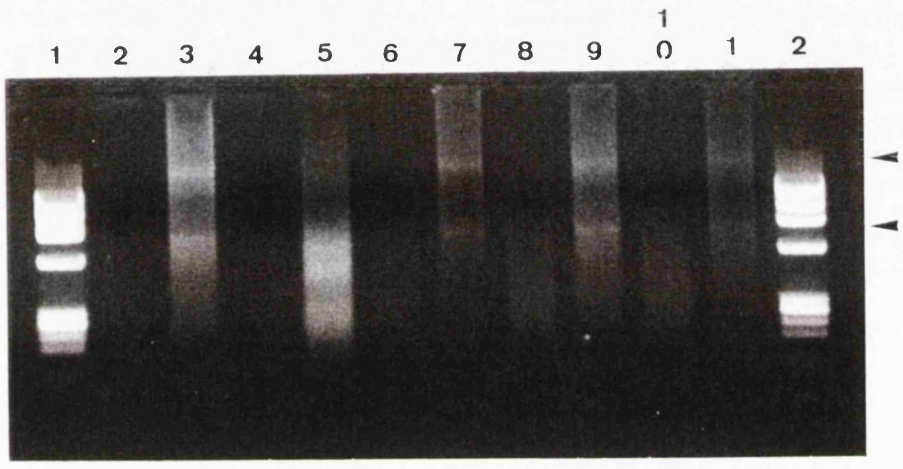
MSRV *env* specific PCR on DNA samples



Ethidium bromide stained 2% agarose gel:
The MW marker is a *Hae* III digest of phage ϕX174 DNA
Lanes 1 and 22 MW marker
Lanes 2-11 normal healthy controls
Lanes 12-14 water controls
Lanes 15-20 patients with MS
Lane 21 PLI-2 MS cell line
Arrow points to 137 bp bands

Figure 23

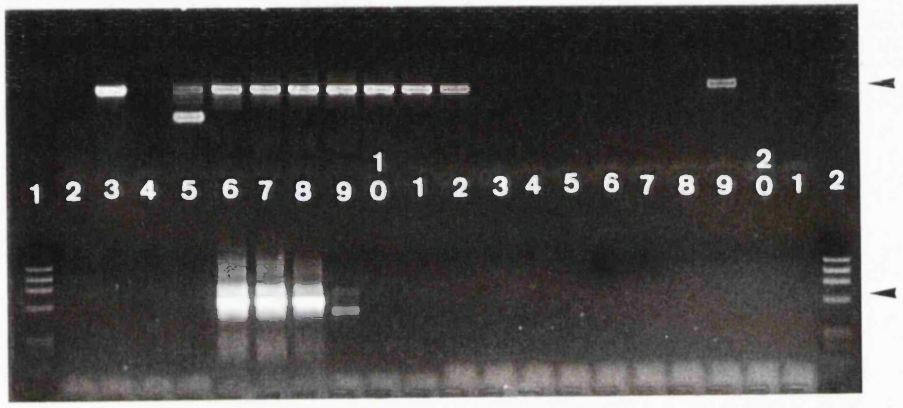
Total cellular RNA from PBMCs



Ethidium bromide stained 2% agarose gel:
The MW marker is a *Hae* III digest of phage ϕX174 DNA
Lanes 1 and 12 MW marker
Lanes 2-11 PBMC cellular RNA from alternately, patients with MS and controls
Note that the RNA from the patients with MS, lanes 2,4,6,8 and 10, is degraded
Arrows point to 28S and 18S ribosomal RNA bands

Figure 24

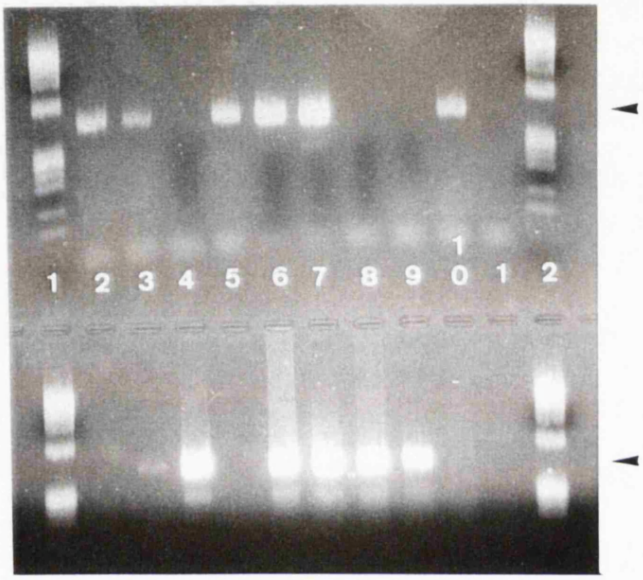
MSRV *pol* 4/5/6 and *gag* RT-PCR and *pol* 4/5/6 'no-RT' PCR on total cellular RNA from PBMCs and brain cryosections



Ethidium bromide stained 2% agarose gel:
The MW marker is a *Hae* III digest of phage ϕX174 DNA
Upper and lower row lanes 1 and 22 MW marker (faintly visible)
Upper row lanes 2-13 are *pol* RT-PCR products, and 14-21 *pol* 'no-RT' PCR products, lower row lanes 2-5 are *pol* 'no-RT' PCR products, lanes 6-11 are *pol* RT-PCR products and lanes 12-21 are *gag* RT-PCR products
Upper row: lane 2 water control, lane 3 normal PBMC, lane 4 control serum, lanes 5 and 6 PBMCs of patients with MS, lanes 7-11 control brain cryosections, lane 12 patient with MS brain cryosection, lane 13 water control. lane 14 water control, lane 15 normal PBMC, lane 16 control serum, lanes 17 and 18 PBMCs of patients with MS, lanes 19-21 control brain cryosections
Lower row: Lanes 2 and 3 control brain cryosections lane 4 patient with MS brain cryosection, lane 5 water control lanes 6-9 control human DNA dilution series 10, 1, 0.1, 0.01 cell equivalents, lanes 10-12 water controls, lane 13 normal PBMC, lane 14 control serum, lanes 15 and 16 PBMCs of patients with MS, lanes 17-21 control brain cryosections
Arrow points to 626 bp bands

Figure 25

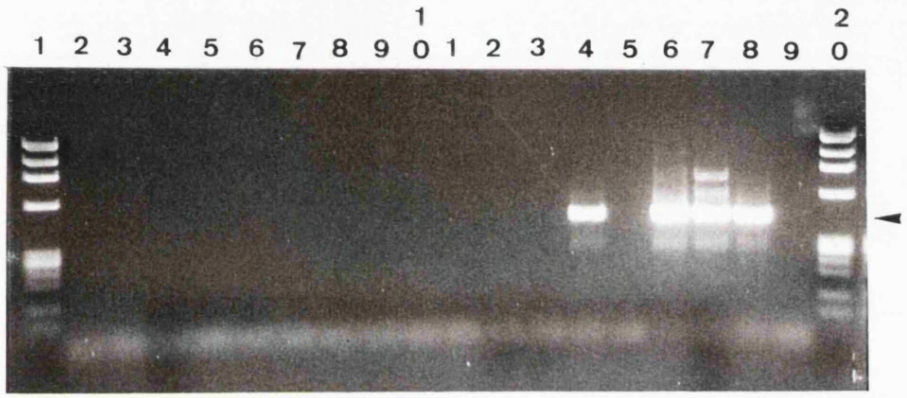
MSRV PT*pol* AB/EF RT-PCR on RNA from sera of patients with MS and controls



Ethidium bromide stained 2% agarose gel:
The MW marker is a *Hae* III digest of phage ϕX174 DNA
Upper and lower row lanes 1 and 12 MW marker
Upper row: Lanes 2-8 patients with MS, lanes 9-11 controls
Lower row: Lanes 2 and 3 controls, lane 4 control PBMCs, lane 5 HIV patient lanes 6-9 control human DNA dilution series 100, 10, 1, 0.1 cell equivalents
Lanes 10 and 11 water controls
Arrow points to 435 bp bands

Figure 26

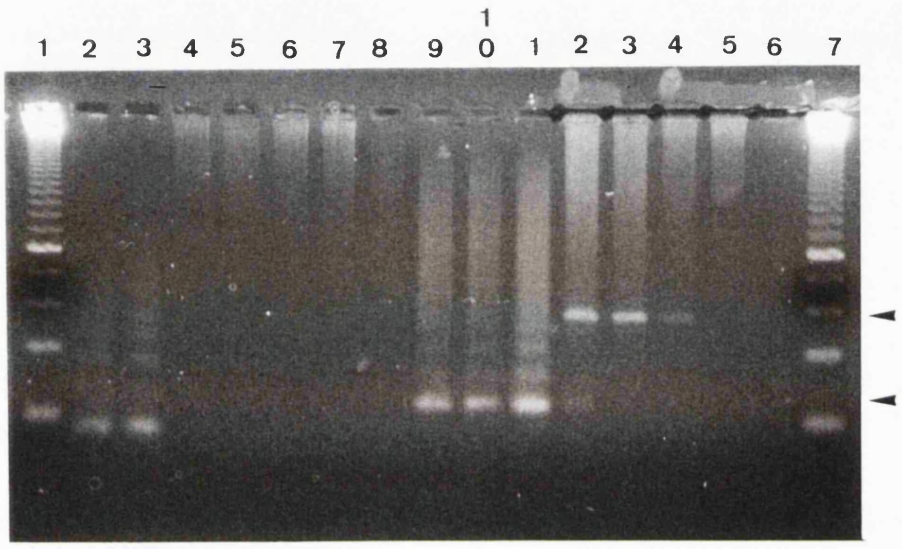
MSRV PT*pol* AB/EF 'no RT' PCR on RNA from sera of patients with MS and controls



Ethidium bromide stained 2% agarose gel:
The MW marker is a *Hae* III digest of phage ϕX174 DNA
Lanes 1 and 20 MW marker
The patients and controls are the same as in Figure 25
Lanes 2-8 patients with MS
Lanes 9-13 controls
Lane 14 control PBMCs lane 15 HIV patient
Lanes 16-19 control human DNA dilution series 100, 10, 1, 0.1 cell equivalents
Arrow points to 435 bp bands

Figure 27

PDH RT-PCR on RNA from sera of patients with MS and controls



Ethidium bromide stained 4% NuSieve agarose gel:
The MW marker is a 50 bp ladder
Lanes 1 and 17 MW marker
Lanes 2 and 3 water controls
The patients and controls are the same as in Figure 25
Lanes 4-8 MSRV PT*pol* AB/EF RT-PCR positive MS patients
Lanes 9 and 10 MSRV PT*pol* AB/EF RT-PCR positive control patients
Lane 11 control PBMCs
Lanes 12-15 control human DNA dilution series 100, 10, 1, 0.1 cell equivalents
Lane 16 water control
Arrows points to 143 bp (PDH DNA) and 61 bp (PDH RNA) bands
Note that lanes 9 and 10 reveal that the RNA extracted from the serum of these two healthy controls contained cellular RNA and therefore gave 'false positive' signals in Figure 25 (Upper row lane 10 and lower row lane 3)

Table 1

Detection of MSRV-*cpol* sequence in CSF of patients with MS and other neurological disease controls

Age, sex	Diagnosis	MS Type	MS Activity	MS duration (years)	MS treatment	MSRV ELOSA
27 M	multiple sclerosis	2° progressive	slow progression	5	corticosteroids	negative
55 M	multiple sclerosis	1° progressive	slow progression	9	none	POSITIVE
51 F	multiple sclerosis	1° progressive	slow progression	2	none	negative
22 F	multiple sclerosis	relapse/remitting	in remission	8	none	POSITIVE
27 F	multiple sclerosis	1° progressive	slow progression	8	cyclophosphamide	negative
33 M	multiple sclerosis	2° progressive	slow progression	16	none*	negative
33 F	multiple sclerosis	2° progressive	slow progression	9	none	POSITIVE
25 F	multiple sclerosis	relapse/remitting	stable	3	none	POSITIVE
36 F	multiple sclerosis	2° progressive	slow progression	3	none	POSITIVE
38 M	multiple sclerosis	2° progressive	slow progression	7	corticosteroids	negative
37 F	cerebellar atrophy					negative
26 F	viral myelitis					negative
38 F	viral encephalitis					negative
28 F	viral encephalitis					negative
54 M	viral encephalitis					negative
32 M	Guillain - Barré					negative
54 F	cerebrovascular					negative
52 F	hydrocephalus					negative
25 F	1° cerebral tumour					negative
21 M	epilepsy					negative

*Patient was not undergoing treatment at this time but had undergone frequent treatment previously

Table 1 continued (summary)

"Pan-Retrovirus" PCR ELOSA detection of MSRV-RNA in CSF cells of patients with MS and other neurological disease controls

Patient group	Positive	Negative	Total	% Positive
Patients with MS	5	5	10	50%
Other Neuro Disease	0	10	10	0%
MS patients untreated at time of sampling	5	2	7	71%
MS patients treated at time of sampling*	0	3	3	0%

*Patients treated with corticosteroids or cyclophosphamide

Table 2

PCR Conditions

Target sequence	Initial denaturation	Denaturation	Anneal	Extension	Buffer	Cycles	Primers	Size of PCR product bp
HTLV *tax/rex*	94°C 5min	95°C 1min 95°C 1min	50°C 1min 50°C 1min	72°C 1min 72°C 1min	Cetus Taq Cetus Taq	R1 35 R2 25	670/671 30ng 670N/671N 125ng	159 128
HTLV-*gag/protease*	94°C 4min	95°C 1min 95°C 1min	60°C 1min 60°C 1min	72°C 1min 72°C 1min	Cetus Taq Cetus Taq	R1 35 R2 30	G0/G5 100ng G1/G2 100ng	814 235
HTLV-*env*	94°C 4min	95°C 1min 95°C 1min	56°C 1min 40°C 1min	72°C 2min 72°C 1min	Cetus Taq Cetus Taq	R1 35 R2 30	ENV A/ENV B 100ng ENV A/ENV C 100ng	1486 938
VLPQG / YXDD	94°C 4min	95°C 1min 95°C 1min	34°C 30sec 45°C 1 min	72°C 1min 72°C 1min	Degenerate Degenerate	R1 35 R2 30	PAN UO/DI 200ng PAN UI/DI 200ng	146 141
VLPQG / YXDD	94°C 4min	95°C 1min	45°C 1 min	72°C 1min	Degenerate	R1 35	MOP 200ng	137
Alu	94°C 4min	95°C 1min	65°C 1min	72°C 1min	Degenerate	R1 30	ALI 3/5 200ng	236
PDH	94°C 4min	95°C 30sec 95°C 30sec	56°C 30sec 56°C 30sec	72°C 30sec 72°C 30sec	Tth EZ Cetus Taq	R1 35 R2 35	PDH 1/2 200ng PDH 2/3 100ng	186 DNA / 102 RNA 143 DNA / 61 RNA

Table 2 continued

PCR conditions

Target sequence	Initial denaturation	Denaturation	Anneal	Extension	Buffer	Cycles	Primers	Size of PCR product bp
MSRV *gag*	94°C 4min	95°C 30sec 95°C 30sec	60°C 30sec 60°C 30sec	72°C 30sec 72°C 30sec	Cetus Taq Cetus Taq	R1 R2	GAG 1/2 100ng GAG 3/4 100ng	1449 865
MSRV *env*	94°C 4min	95°C 30sec 95°C 30sec	60°C 30sec 60°C 30sec	72°C 30sec 72°C 30sec	Cetus Taq Cetus Taq	R1 R2	ENV 1/2 100ng ENV 3/4 100ng	187 137
MSRV LTR	94°C 4min	95°C 30sec 95°C 30sec	50°C 30sec 50°C 30sec	72°C 30sec 72°C 30sec	Cetus Taq Cetus Taq	R1 R2	LTR 1/2 100ng LTR 3/4 100ng	110 72
MSRV PT*pol*AB/EF	94°C 2min	95°C 30sec 95°C 30sec	60°C 30sec 60°C 30sec	68°C 45sec 68°C 30sec	Titan Expand B° 3	R1 40 R2 35	PT*pol*A/F 170ng PT*pol*B/E 50ng	575 435
MSRV *pol*45/56	94°C 2min	95°C 30sec 95°C 30sec	60°C 30sec 60°C 30sec	68°C 45sec 68°C 30sec	Titan Expand B° 3	R1 40 R2 35	*pol*4/5 170ng *pol*5/6 50ng	670 626

Table 2 continued

Primer and probe sequences (5' to 3')

Primer / probe	Sequence
670 sense	CGGATACCCAGTCTACGTGT
671 antisense	GAGCCGATAACGCGTCCATC
670N sense	GTGTTTGGCGATTGTGTACA
671N antisense	CCATCGATGGGGTCCCA
G0 sense	CTCCAAGACCTCCTGCAGTACCTTT
G5 antisense	GGGATCTAACGGTATAACTGGCAGAAT
G1 sense	GTCAGACCTGGACCCCCAAAGAC
G2 antisense	GAGGGCATCTTCCTCTGGCTCTG
ENV A sense	ACCACCGGATCCATGGGTAATGTTTTCTTC
ENV B antisense	GCTAGCGGATCCTATTATAGCATGGTTTCT
ENV C antisense	ATATATGGATCCTCATTAACGGCGGCGTCTTGTCGC
PDH 1 sense	GGGTATGGATGAGGAGCTGGA
PDH2 antisense	TCTTCCACAGCCCTCGACTAA
PDH 3 sense	CTTGGAGAAGAAGTTGCCCAGT
PAN-UO sense	CTTGGATCCTGGAAAGTGYTRCCMC
PAN-DO antisense	CTCAAGCTTGGMGGCCAGCAGSAKGTCATCC
PAN-UI sense	CTTGGATCCAGTGYTRCCMCARGG
PAN-DI antisense	CTCAAGCTTCAGSAKGTCATCCAYGTA
CpV1b probe	bio-CTTGAGCCAGTTCTCATACCTGGA
DpV1 probe	Perox-CATCTITTTGGICAGGCAITAGC
MOP sense	TGGAAAGTGYTRCCMCARGG
MOP antisense	GGMGGCCAGCAGSAKGTCATCCAYGTA
ALI3 antisense	TGGAGTGCARTGGYRYRATCWYRG
ALI5 sense	TCAYRCCTGTAATCCCAGCACTTT
M13 -40 sequencing primer	GTTTTCCCAGTCACGAC

Table 2 continued

MSRV specific primer sequences (5' to 3')

Primer	Sequence
LTR1 sense	TGTCCCGTTTAGAGTGGG
LTR2 antisense	GATTGKTGYATTTACAATCC
LTR3 sense	AGGTGAAGCCAGCTGGACT
LTR4 antisense	TTTACAATCCTTTAGCTAGAC
GAG1 sense	AAGGAAAACTAGGAAGATATGAA
GAG2 antisense	GGATAATTTTAACCCTAAGTATTTA
GAG3 sense	AGGCATTGAGGAAGCATACCAGG
GAG4 antisense	TGGGTTTCCTTACTCCTAAAATTGG
ENV1 sense	CCACTAACTGGACAGGCACCTGAACCT
ENV2 antisense	TAAACCGGCTATTCCAGTTCCTGTAGCA
ENV3 sense	TGAACCTTAGTCTTTCTAAGTCCCAACAT
ENV4 antisense	GTAGCCATTCCTAACCCTATAAATAGGGA
PT*pol*-A sense	GGCCAGGCATCAGCCCAAGACTTGA
PT*pol*-F antisense	TGCAAGCTCATCCCTSRGACCT
PT*pol*-B sense	GACTTGAGCCAGTCCTCATACCT
PT*pol*-E antisense	CTTTAGGGCCTGGAAAGCCACT
pol-4 sense	GGCTCTGCTCACAGGAGATTAGATAC
pol-5 antisense	GGTTTAAGAGTTGCACAAGTGCGCAGTC
pol-6 sense	AAAGGCACCAGGGCCCTCAGTGAGGA

S = G or C R = A or G Y = C or T M = A or C K = G or T W = A or T

Table 3

Sequences detected by 'Pan-Retrovirus' PCR in density gradient fractions with peak RT-activity*

Culture	MSRV-c*pol* sequences	MMLV-*pol* sequences	ERV9-*pol*[$] sequences	PCR[£] artefacts	Total clones
LM7P	16	0	4	6	26
PLI-2	9	0	1	13	23
MS-B cell line	9	0	2	8	19
Control B cell line	0	5	0	21	26

* Except Control B cell line (RT negative)

$ Clones with >90% homology with the GenBank sequence HSERV9

£ PCR artefacts include primer multimers and single primer amplifications

Table 4

Detection of circulating virion associated MSRV-RNA in patients with MS and controls

Samples	Positive	Negative	Total	Percent Positive
Patients with clinically active MS[a]	9	8	17	53%
Controls without MS[b]	3	41	44	7%
Sera from clinically active MS patients untreated at time of sampling	6	0	6	100%
Sera from clinically active MS patients treated at time of sampling[c]	4	11	15	27%

[a] In patients who were sampled on more than one occasion the result shown is that obtained on initial sampling.

[b] Controls were healthy hospital staff

[c] Patients treated with azathioprine and/or intravenous corticosteroid bolus (≥ 1 gm/day)

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Appendix 1 - Buffers and Solutions

PBMC isolation and DNA extraction

PBMC wash:

Roswell Park Memorial Institute tissue culture medium (RPMI) 1640 (Gibco) containing:

2.0 X 10^{-3} M L-glutamine

100 units / ml penicillin

100 µg / ml streptomycin

2.5 µg / ml Fungizone

10% (v/v) Foetal Calf Serum (FCS)

Tissue culture freezing medium:

RPMI 1640 (Gibco) containing:

2.0 X 10^{-3} M L-glutamine

100 units / ml penicillin

100 µg / ml streptomycin

2.5 µg / ml Fungizone

10% dimethyl sulfoxide (DMSO) and 30% Foetal calf serum

Glycigel:

10 mM NaCl, 0.5mM EDTA, 39% glycerol (v/v), 1.5% gelatine (w/v), 0.1% sodium azide (w/v)

Glycigel lysis buffer:

10 mM Tris-HCl pH 7.5, 1% Triton X100, 0.32M sucrose, 5mM $MgCl_2$

PCR compatible Glycigel extraction buffer:

10 mM Tris-HCl pH 8.3, 50mM KCl, 2.5mM $MgCl_2$ 0.001% w/v gelatine, 0.45% Tween 20, 0.45% Nonidet P40, proteinase K 1μg/μl

DNA extraction buffer:

10 mM Tris-HCl pH 8, 10mM EDTA, 10mM NaCl, 0.5% SDS,

100μg/ml proteinase K

SNAP™ RNA extraction kit buffers

Binding buffer:

7M Guanidine HCl, 2% Triton X-100

Super wash solution:

5.25M Guanidine HCl, 1% Triton X-100

RNA wash solution:

100mM NaCl

10X DNase buffer:

40mM Tris pH 8, 6mM $MgCl_2$, 2mM $CaCl_2$.

RT-PCR and PCR mixes

Perkin Elmer Cetus PCR mix:

10mM Tris-HCl pH 8.3

50mM KCl

1.5mM $MgCl_2$

200mM each dNTP

100ng of each of the primers

1 unit recombinant *Taq* polymerase (Perkin Elmer Cetus)

Degenerate primer mix:

20mM Tris-HCl pH 8.4

60mM KCl

2.5mM $MgCl_2$

200mM each dNTP

200ng of each of the primers

1 unit recombinant *Taq* polymerase (Perkin Elmer Cetus)

***Pfu* Polymerase mix:**

20mM Tris-HCl pH 8.75

10mM KCl

10mM $(NH_4)_2 MgSO_4$

2mM $MgSO_4$

0.1% Triton X-100

100µg/ml BSA

200mM each dNTP

100ng of each of the primers

1 unit recombinant *Pfu* polymerase

Titan™ polymerase mix:

10mM Tris-HCl pH 8.3

50mM KCl

1.5mM $MgCl_2$

5mM DTT

200µM each dNTP

100ng of each of the primers

10 units rRNAsin (Promega)

***Tth* polymerase mix:**

50mM Bicine

115mM Potassium acetate

8% glycerol pH8.2

Expand™ polymerase mix (system 3):

2mM Tris-HCl pH 7.5

10mM KCl

2.25mM $MgCl_2$

0.1mM DTT

10μM EDTA

0.05% Tween 20

0.05% NP40

500μM each dNTP

100ng of each of the primers

1 μl Expand™ polymerase (Boehringer Mannheim)

ELOSA Buffers:

PBS Phosphate buffered saline:

1.5×10^{-1} M NaCl

1.5×10^{-3} M KH_2PO_4

6.3×10^{-3} M $Na_2HPO_4.12H_2O$

2.6×10^{-3} M KCl

pH 7.2

PBS/Tween:

Phosphate buffered saline/0.05% Tween 20 w/v

Hybridisation buffer:

0.01M sodium phosphate (pH 7.0)

0.5M Na Cl

0.14 mg/ml salmon sperm DNA

2% PolyEthelyeneGlycol 4000

Tween 20 0.65% w/v

OPD (o-phenylenediamine) buffer:

0.05M citric acid

0.1M Na_2HPO_4 pH 4.9

0.1% hydrogen peroxide

o-phenylenediamine 2mg/ml Sigma Ref. no. P7288

Agarose gels and buffers

Agarose gel:

1 X TAE (from 50X stock)

2-4% (w/v) agarose

0.05% (w/v) ethidium bromide

Gel loading buffer:

30% (v/v) glycerol

0.25% (w/v) xylene cyanol

TAE (50x):

1.0 M Tris

6% acetic acid

5.0 X 10^{-2} M EDTA

pH 8

TE:

1.0 X 10^{-2} M Tris

1.0 X 10^{-3} M EDTA

Media

Luria-Bertani (LB) Medium:

bacto-tryptone 10g

bacto-yeast extract 5g

NaCl 10g

in 1litre deionised water final pH 7.0

Luria-Bertani (LB) Amp Medium:

bacto-tryptone 10g

bacto-yeast extract 5g

NaCl 10g

in 1litre deionised water final pH 7.0

Ampicillin 100μg/ml final concentration

LB/amp agarose plates:

bacto-tryptone 10g

bacto-yeast extract 5g

NaCl 10g

agarose 15g

in 1l deionised water final pH 7.0

Ampicillin 100μg/ml final concentration

LB/amp/IPTG/x-gal agarose plates:

bacto-tryptone 10g

bacto-yeast extract 5g

NaCl 10g

agarose 15g

in 1litre deionised water final pH 7.0

Ampicillin 100μg/ml final concentration

IPTG (Isopropylthio-β-D-galactoside) final concentration 1mM

X-gal (5-Bromo-4-chloro-3-indolyl-β-D-galactoside) final concentration 40μg/ml (stock 20mg/ml in dimethylformamide)

Sequencing mixes

Labelling reaction mix:

0.1M DTT	1µl/sample
1 in 30 dilution of labelling dNTP mix	2µl/sample
α^{35}S dATP (Amersham 1000 Ci/mM)	0.5µl/sample
Sequenase enzyme	0.16µl/sample
Water	0.84µl/sample

Labelling dNTP mix:

7.5 µM dCTP
7.5 µM 7-dGTP
15 µM dTTP

Termination mixes:

dNTP mixture:

160 µM dATP
160 µM dTTP
80 µM dCTP
40 µM dGTP

In addition to this dNTP mix the termination mixes contained the following concentrations of dideoxy nucleotides:

'A' termination mix 8 µM ddATP

'T' termination mix 8 µM ddTTP

'G' termination mix 4 µM ddGTP

'C' termination mix 4 µM ddCTP

Stop solution:

98% deionised water

10mM EDTA pH 8.0

0.025 % Xylene cyanol FF

0.025 % bromophenol blue

Appendix 2

Detailed protocol for the specific detection of virion associated MSRV-RNA in serum

This protocol is to be read in conjunction with the Lancet letter entitled, Detection of Virion Associated MSRV-RNA in the Serum of Patients with Multiple Sclerosis (Garson *et al.* 1998). The technique is designed to ensure that non-encapsidated MRSV-related RNA and DNA sequences are not detected. NB - All steps are at room temperature unless otherwise indicated and aerosol-resistant pipette tips (ART™, Promega Ltd) are used throughout.

Extraction of virion-associated MSRV-RNA from serum

1) Serum should be separated and frozen in aliquots at -70°C within 5 hours of venesection. It is important to avoid repeat freeze thaws.

2) 200µl of serum is filtered through a 0.45µm spin filter (Flowgen Ltd., Nanosep MF, Cat. No. U3-0126 Ref. ODM45) by centrifugation at 13000 x g for 5 to 10 min. Clarification of serum by brief centrifugation (1 min. at 13000 x g) is occasionally necessary prior to spin filtration.

3) 150 µl of the filtered serum is incubated at 37°C for 30 min with 10 units of RNase I (Promega Ltd, Cat. No. M4261).

4) 150 µl of the filtered and RNase I digested serum is processed to extract DNA-free viral RNA using a silica matrix spin column technique (Invitrogen Ltd., S.N.A.P reagents, Cat. No. K1950-01) as follows-

5) Add 10μg poly-A carrier RNA (Boehringer Mannheim Ltd., Cat. No. 108626) to 450μl of Binding Buffer (7M guanidine HCl, 2% Triton-X 100) and then add the 150 μl of serum.

6) Mix by inversion 6 times, add 300μl of propan-2-ol and mix by inversion a further 10 times.

7) Apply 500μl of this mixture to the S.N.A.P column, centrifuge at 13000 x g for 1 min and discard the flowthrough.

8) Apply the remainder of the mixture to the S.N.A.P column, centrifuge at 13000 x g for 1 min and discard the flowthrough.

9) Wash the column with 600μl of Super Wash, centrifuge at 13000 x g for 1 min and discard the flowthrough.

10) Wash the column with 600μl of 1x RNA Wash, centrifuge at 13000 x g for 1 min and discard the flowthrough.

11) Repeat step 10) but with centrifugation for 2 min.

12) Elute the bound nucleic acid by incubating for 5 min with 135μl RNase-free water and then centrifuge at 13000 x g for 1 min.

13) To the eluate add 15μl of 10x DNase buffer and 3μl (30 units) of RNase-free DNase I (Boehringer Mannheim Ltd., Cat. No. 776 785) and incubate at 37°C for 30 min.

14) Add 450μl of Binding Buffer, mix by inversion 6 times, add 300μl of propan-2-ol and mix by inversion a further 10 times.

15) Repeat steps 7), 8), 10) and 11)

16) Elute the bound nucleic acid by incubating for 5 min with 105µl RNase-free water and then centrifuge at 13000 x g for 1 min. The eluted RNA may be used directly in RT-PCR (see below) or frozen at -70°C for use within 1 week.

Reverse transcription and polymerase chain reaction (RT-PCR)

17) Combined RT-PCR is performed in a single tube system using 'Titan' reagents (Boehringer Mannheim Ltd., Cat. No. 1 855 476).

18) 25µl of the extracted RNA is used in a total reaction volume of 50µl in the presence of 10 units of recombinant RNAsin (Promega Ltd., Cat. No. N2515). The master mix, containing 170ng each of primers PTpol-A and PTpol-F, is prepared at room temperature and the RNA added last.

19) Reverse transcription occurs during two sequential 30 min. incubations, the first at 50°C and the second at 60°C.

20) First round PCR is performed immediately after the reverse transcription using the following cycling parameters (Robocycler™ thermal cycler, Stratagene Ltd. Cat. No. 400862 [N.B. This machine has extremely rapid temperature ramp rates]) -Initial denaturation of template at 94°C for 2 min; 40 cycles of 94°C for 30 sec., 60°C for 30 sec., 68°C for 45 sec; 1 cycle of 68°C for 7 min

21) Second round (nested) PCR is performed using 'Expand' reagents (Boehringer Mannheim Ltd., Cat. No. 1681 842). 0.5µl of first round PCR product is added to 25µl of the second round reaction mix (Buffer No. 3) which contains 50ng each of primers PTpol-B and PTpol-E. Cycling parameters - 35 cycles of 94°C for 30 sec., 60°C for 30 sec., 68°C for 30 sec; 1 cycle of 68°C for 7 min.

22) The 435 bp PCR product is demonstrated by ethidium bromide staining following electrophoresis on a 2% agarose gel.

Controls

23) Adequate sensitivity of the nested PCR is monitored by including (in place of the extracted RNA) a ten fold dilution series of human genomic DNA (100pg, 10pg and 1pg) in each experimental run. As several copies of MSRV-related endogenous sequences are present in the human genome, nested PCR generates a product of the expected size from as little as 1pg of genomic DNA (equivalent to approximately one tenth of a cell). For an experimental run to be valid the 435 bp band generated from 1pg of human DNA must be clearly visible.

24) To ensure that the PCR products generated from test sera are derived from RNA rather than from traces of related endogenous genomic DNA, a mock RT-PCR reaction must be performed without reverse transcriptase (the 'no-RT' control). The mock RT-PCR reaction is performed using a second 25µl aliquot of the extracted RNA, using the same buffer as the real RT-PCR but replacing Titan enzyme mix with 0.75µl of Expand enzyme mix, under the same thermal cycling conditions, in the same machine. The second round PCR is performed exactly as in step 21) above.

25) To exclude the possibility that the PCR products might be derived from cellular RNA rather than from encapsidated virion-associated RNA, the RNA extracts are also tested for the presence of cellular pyruvate dehydrogenase RNA (PDH-RNA is an abundant 'housekeeping' transcript) by RT-PCR. The PDH PCR primers flank an intron so that products derived from RNA can be distinguished by size from products derived from DNA.

26) PDH RT-PCR is performed using the bifunctional enzyme Tth in a single tube system (Perkin Elmer Ltd., Cat. No. N808 0178) with 25µl of the extracted RNA in a total reaction volume of 50µl. The master mix contains 200ng each of primers PDH1 and PDH2; the RNA is added last.

27) Reverse transcription occurs during two sequential 30 min. incubations, the first at 56°C and the second at 60°C.

28) First round PCR is performed immediately after reverse transcription using the following cycling parameters - Initial denaturation of template at 94°C for 4 min; 35 cycles of 94°C for 30 sec., 56°C for 30 sec., 72°C for 30 sec; 1 cycle of 72°C for 7 min.

29) Second round PCR is performed using *Taq* polymerase (Perkin Elmer Ltd., Cat. No. N808 0152) in buffer supplied by the manufacturer. 0.5µl of first round PCR product is added to 25µl of the second round reaction buffer which contains 100ng each of primers PDH2 and PDH3. Cycling parameters - 35 cycles of 94°C for 30 sec., 56°C for 30 sec., 72°C for 30 sec; 1 cycle of 72°C for 7 min.

30) PCR products are run on a 4% Nu-Seive (Flowgen Ltd., Cat. No. A3-0285) agarose gel to distinguish RNA derived signals (61 bp) from DNA derived (143 bp).

31) Sensitivity of the PDH PCR system is monitored by ensuring that one cell equivalent of human DNA (approximately 7pg) gives a band of the expected size.

32) Note that any serum sample which generates a signal in either the PDH or the 'no-RT' PCR assays is excluded from the analysis.

Oligonucleotide primers

The sequences of the oligonucleotide primers (HPLC purified) used in this protocol are as follows:

MSRV *pol* primers

PTpol-A 5'ggccaggcatcagcccaagacttga3'

PTpol-F 5'tgcaagctcatccctSRgacct3' [S = g or c R = a or g]

PTpol-B 5'gacttgagccagtcctcatacct3'

PTpol-E 5'ctttagggcctggaaagccact3'

Pyruvate dehydrogenase primers

PDH1 5'gggtatggatgaggagctgga3'

PDH2 5'tcttccacagccctcgactaa3'

PDH3 5'cttggagaagaagttgcccagt3'