Gliotoxicity, reverse transcriptase activity and retroviral RNA in monocyte/macrophage culture supernatants from patients with multiple sclerosis

Armelle Ménard[a], Rim Amouri[a], Marlène Michel[b], Frédérique Marcel[b,c], Arthur Brouillet[a], James Belliveau[d], Christian Geny[e], Lionel Deforges[f], Carine Malcus-Vocanson[c], Marilyn Armstrong[g], Olivier Lyon-Caen[h], Bernard Mandrand[c], Tomás Dobránsky[a], François Rieger[a], Hervé Perron[b,c,*]

[a] *INSERM, Laboratoire de Neuromodulations Interactives et Neuropathologies, Paris, France*
[b] *Laboratoire de Virologie moléculaire médicale, Université Joseph Fourier, La Tronche, France*
[c] *Unité mixte CNRS-BioMérieux UMR 103, Lyon, France*
[d] *Department of Chemistry, Providence College, Providence, RI 02918, USA*
[e] *Service de Neurologie, Hôpital Henri Mondor, Créteil, France*
[f] *Service de Bactériologie-Virologie, Hôpital Henri Mondor, Créteil, France*
[g] *Department of Microbiology and Immunology, The Queen's University of Belfast, Belfast, UK*
[h] *Fédération de Neurologie and INSERM U.360, Hôpital de la Salpêtrière, Paris, France*

Received 9 June 1997

Abstract In investigating a possible link between a novel retroviral agent (provisionally called MSRV), recently characterised in multiple sclerosis (MS), and the neuropathology of MS, it was found that there was a significant correlation between gliotoxicity and reverse transcriptase activity in monocyte/macrophage culture supernatants (MMCS) unique to MS patients. MMCS from healthy controls and patients with other neurological diseases did not display either gliotoxicity or reverse transcriptase activity. The observed gliotoxic effect was an initial, intermediate filament network disorganization and subsequent cell death which was specific to astrocytes and oligodendrocytes. The reverse transcriptase activity and MSRV-specific RNA were observed during the first 2 weeks of culture in MMCS from patients with active MS. The further elucidation of the molecular form(s) of this gliotoxic factor and its original source may be crucial in elucidating important etiopathogenic mechanisms in MS.

Key words: Multiple sclerosis; Monocyte; Astrocyte; Oligodendrocyte; Cytotoxicity; Retrovirus

1. Introduction

Multiple sclerosis (MS) is an autoimmune demyelinating disease of the human central nervous system, leading to a progressive motor and sensory disability, usually over years of remitting-relapsing and/or chronic progressive clinical evolution ([1–3] and references cited therein). It is characterised by multifocal plaques of demyelination with preservation of axons in the white matter of brain and the spinal cord, commonly associated with a fibrous astrogliosis in the old plaques [1]. Activated microglia-macrophages are associated with the earliest events of the lesional process in MS and contribute to phagocytosis of myelin debris. In acute inflammatory plaques, a disruption of the blood brain barrier is associated with oedema and massive lymphoid cell infiltration [4–8].

Despite extensive neuropathological studies and decades of multidisciplinary research, the primary cause of MS remains unknown. Epidemiological studies have suggested a viral origin of MS [9–11], but the etiopathological agent, if any, has remained elusive. A working hypothesis is that the cascade of pathological events as well as autoimmunity may be triggered by a viral agent. We have recently described retrovirus-like extracellular particles associated with reverse transcriptase (RT) activity in culture supernatants from patients with MS [12,13]. The first molecular data on this retrovirus (now designated MSRV, MS associated RetroVirus) have revealed phylogenic proximity with a human endogenous retroviral family represented by ERV-9 [14]. In contrast to the defective ERV-9 element [15], MSRV RNA is associated with extracellular particles co-sedimenting with RT activity on sucrose gradients [16]. The MSRV polymerase RNA sequence encoding the protease and the RT is compatible with that of an exogenous retrovirus sharing extensive homology with a family of human endogenous retroviruses or with that of a replication-competent endogenous retrovirus [16]. Cultured blood monocytes from MS patients with active disease were found to express low but significant RT activity in the cell culture supernatants [13,17]. Macrophage cells are quite active in MS lesions as previously mentioned, as well as in visna, a natural animal model of MS [18–20]. In sheep infected through natural routes with the visna retrovirus, blood monocytes harbouring a provirus are crucial in the initiation and spreading of the demyelinating lesions in the central nervous system. However, the link between the neuropathological phenomena

*Corresponding author at Unité mixte CNRS-BioMérieux UMR 103, Ecole Normale Supérieure de Lyon, 46 allée d'Italie, 69364 Lyon cedex 07, France. Fax: (33) 472 72 85 33.
E-mail: Herve.Perron@ens-bma.cnrs.fr
Correspondence can also be addressed to F. Rieger, INSERM U153, 17 Rue du Ferz Moulin, 75005 Paris, France.

Abbreviations: MS, multiple sclerosis; MMC, monocyte/macrophage culture(s); MMCS, monocyte/macrophage culture supernatant(s); MSRV, multiple sclerosis associated retrovirus; ERV, human endogenous retrovirus; PLL, poly L-lysine; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; RT, reverse transcriptase; GFAP, glial fibrillary acidic protein; PLP, myelin proteolipid protein; GC, galactocerebroside; MBP, myelin basic protein; ELOSA, enzyme-linked oligosorbent assay

PII S0014-5793(97)00889-2

and the retroviral expression which is genetically restricted and limited in space, is not obvious. Such interrogations have also arisen in HTLV-I and HIV-1 infections about the neuropathogenesis of these human retroviruses, and possible mechanisms of HIV-1 neurotoxicity have been explored. In AIDS encephalopathy, HIV-1 infected macrophages were found to produce a neurotoxin while HIV-1 infected and non-infected lymphocytes, and HIV-1 non-infected macrophages did not [21,22].

In considering the possible role of an exo/endogenous retrovirus in MS, it was postulated that a virus-related or virus-induced factor could be produced by monocyte/macrophage cell cultures from patients with active MS, and that this factor could affect cells from the brain parenchyma. We have therefore explored potential cytotoxic activities which could be co-expressed with RT activity and MSRV RNA in the monocyte/macrophage culture supernatant (MMCS) from active MS patients.

2. Materials and methods

All the cell lines were maintained at 37°C in a humidified atmosphere of 5% CO_2 in air.

2.1. Monocyte/macrophage blood cells

Blood cells from healthy donors, patients with MS and patients with other neurological diseases were obtained from Grenoble (CHU A. Michallon, Service de Neurologie, Pr. Pellat and Pr. Perret), Créteil (CHU H. Mondor, Service de Neurologie, Pr. J.D. Degos) and from Paris (Hôpital de la Salpêtrière, Pr. O. Lyon-Caen). All the MS patients were diagnosed according to the standard criteria of Poser et al. [23,24,8]. All blood samples and monocyte/macrophage cultures were processed as previously described [17].

2.2. Primary cortical cell cultures

Cells were prepared from 14 days gestation Wistar rat fetal cerebral cortex. The tissue was minced and dissociated in F12 medium (GIBCO BRL) by repeated gentle trituration. The dissociated cells were resuspended in F12 medium supplemented with 7.5% FCS, 7.5% heat-inactivated horse serum (GIBCO BRL), 50 U/ml penicillin (GIBCO BRL), 0.05 mg/ml streptomycin (Sigma) and plated onto poly L-lysine coated (5 μg PLL/ml in PBS, Sigma) eight-well polystyrene chamber slides (LAB TEK, NUNC Inc.) at a density 1×10^4 cells/well. Culture medium was changed on day 4 and then every 2 days. Cultures were used for experiments 14 days after preparation.

2.2.1. Primary rat cortical cells immunostainings. Cells were plated in eight-well polystyrene chamber slides and exposed to MS MMCS (dilution 1:4 in 200 μl) for 24 h. Cells on coverslips were fixed for 10 min at −20°C in acetone/methanol (vol/vol, 1/1) and then incubated overnight at −20°C with the appropriate rabbit Ab [against GFAP (dilution 1:50, Boehringer) for astrocytes, against MBP and GC (dilution 1:50, Boehringer) for oligodendrocytes and against neurofilaments (dilution 1:50, Boehringer) for neurons] followed by FITC-conjugated goat anti-rabbit Ig (dilution 1:100).

2.3. The mouse immortalised astroglial cell line CLTT 1-1

Astrocytes [25] were cultured in PLL coated (5 μg/ml in PBS) plates in DMEM-F12 (vol/vol, 1/1, GIBCO BRL) supplemented with 10% FCS, 0.05 mg/ml gentamicin (GIBCO BRL).

2.3.1. CLTT 1-1 astrocytes vimentin immunostaining. Cells were plated at a density 1×10^3 cell/well for 36 h before experimentation and exposed to MMCS for 72 h (dilution 1:20 in 200 μl). Cells on coverslips were washed with PBS and fixed in acetone for 10 min at −20°C. Cells were preincubated overnight at 4°C with mAb anti-vimentin IgM (clone VIM 13,2, Sigma) at a dilution of 1:200, followed by FITC-conjugated goat anti-mouse IGM (dilution 1:50). All washings were carried out with PBS supplemented with 5% FCS.

2.4. The mouse immortalised oligodendrocyte cultures 158N

Oligodendrocytes derived from secondary oligodendrocyte cultures of new-born mouse cerebral hemispheres [26] were transfected with a plasmid containing the SV40 T-antigen gene expressed under the control of the mouse metallothionein-I promoter [27]. The calcium phosphate method was used for cell transfection. Colonies were selected for their resistance to G418 (Geneticin sulfate, GIBCO-BRL). After cloning by limit dilution, selected clones were maintained in culture in PLL coated (5 μg/ml in PBS) plates in DMEM (CIBCO) supplemented with 10% FCS, 0.05 mg/ml gentamicin. The 158N oligodendrocyte culture used was immunoreactive in vitro for all three oligodendrocyte proteins: myelin proteolipid protein (PLP), myelin basic protein (MBP) and galactocerebroside (GC) (see below).

2.4.1. 158N oligodendrocyte cells immunostaining

MBP and PLP immunostainings were accomplished by using rabbit polyclonal Ab directed against MBP and against tridecapepide corresponding to the sequence 117–129 of PLP (dilution 1:50), and then revealed with FITC-conjugated goat anti-rabbit IgG (dilution 1:50) (Sigma). A mouse mAb directed against GC was used (dilution 1:1000) and then revealed with FITC-conjugated goat anti-mouse IgG (R&D systems; Immunotech, Luminy).

2.5. Qualitative live dead assay

The assay was performed according to the procedure of MacCoubrey et al. [28]. CLTT 1-1 astrocytes or 158N oligodendrocytes were cultured in PLL-coated eight-well polystyrene chamber slides (LAB TEK, NUNC Inc.) at a density of 2×10^3 cells/well 24 h before experimentation. Cells were then exposed to MMCS samples diluted in standard medium (dilution 1:20 in 200 μl) and incubation was continued for 72 h with no change of medium. The medium was then removed, the cells were washed with PBS and exposed to 2 μM calcein acetoxymethyl ester (Interchim) and 2 μM ethidium homodimere (Interchim) for 15 min at room temperature. Fluorescence microscopy was performed on a Carl Zeiss Axiophot microscope equipped with a mercury HBO 50 lamp, using BP 450-490 exciter filter (live cells) and BP 365 filter (both fluorophores).

2.6. Quantitative colorimetric MTT assay

CLTT 1-1 astrocytes were plated at a density of 2×10^3 cells/dish in 60 mm plastic dishes for 24 h before experimentation. Cells were then exposed to different dilutions of samples in standard medium (2 ml/dish) and incubation was continued for 3 days with no change in medium. The medium was then removed and 2 ml of MTT (0.5 mg/ml in DMEM, Sigma) were added. Living cells transform the tetrazolium salt into dark blue formazan crystals [29]. After a 2 h incubation, the reaction was stopped by addition of 2 ml of 40 mM HCl in isopropanol which solubilizes the formazan product. After a 5 min centrifugation at $5000\times g$, the OD was measured using a test wavelength of 570 nm and a reference wavelength of 630 nm. Cytotoxicity was expressed as a percentage: % cytotoxicity = % of dead cells = $100-[(OD_{sample}/OD_{control})\times100]$.

2.7. Purification of the protein fraction associated with gliotoxic activity

Samples (10 ml of MS MMCS – 42 mg of total proteins) were previously heated for 30 min at 56°C, pooled, centrifuged and dialysed overnight at 4°C against 2 liters of PBS. The proteins were then applied onto a protein A sepharose column CL-4B (Pharmacia) and the Ig-free fraction was collected. Ig were eluted from the protein G column with 0.1 M glycin-HCl, pH 3.0 buffer. Toxic fraction (Ig-free fraction) was loaded onto a Concanavalin A Sepharose column CL-4B (Pharmacia). Fractions containing glycosylated proteins were eluted first with 0.2 M D-glucopyranosid and then with 0.1 M glycin-HCl, pH 3.0 and desalted through a NAP-25 column (Pharmacia) in order to test the toxicity. When stored after purification, the gliotoxic material was pooled and/or aliquoted, snap-frozen in liquid nitrogen and placed at −80°C.

2.8. RT-PCR with ST2 primer sets

RNA was extracted as previously described by Chomczynski [32] from 2 ml of MMCS pooled from 400 μl aliquots of MMCS collected from the same flask on days 3, 6, 9, 12 and 15. The RNA extracted was resuspended in 20 μl of RNase-free water. In order to eliminate eventual traces of human DNA which contains irrelevant but genetically homologous endogenous retroviral sequences, 7.5 μl of RNA solution, the cDNA mix as well as round-I and round-II PCR mix, were incubated with DNase as described [14] and further used for RT-PCR as described [16] with the following modifications: first-round

primers (annealing temperature 54°C) were ST2.1 (upstream primer: 5′-GAAGATCCTTTGAACCCAACGTC-3′) and ST2.1 (downstream primer: 5′-TGGCACAAGGTTTCTGAACGG-3′), and second-round primers (annealing temperature 60°C) were ST2.2 (upstream primer: 5′-CAACGTCTCAACTCACCTGGACTG-3′) and ST2.2 (downstream primer: 5′-CGGGCGACTAAAAGTAAATCATC-3′); PCR parameters were: 5 min at 94°C, 1 min at annealing temperature and 2 min at 72°C, 39 cycles at 94°C for 1 min, annealing temperature for 1 min, 72°C for 2 min, with a final extension of 2 min at 72°C. One μl from the first round was transferred to the second-round PCR mix previously treated with DNase 1. For each sample, a second tube was always prepared in parallel with the same composition at the exception of the MoMLV reverse transcriptase, in order to perform a 'no-RT' control in subsequent PCR amplifications and validate the absence of contaminating DNA in the RNA sample and in the successive solutions used. An Aldolase RT-PCR was also made to confirm the abscence of cellular RNA in the samples.

2.8.1. Hybridization analysis of PCR products: MSRV-pol detection by ELOSA. The protocol was performed essentially as previously described [16,33].

3. Results

3.1. Cytotoxic effect of MMCS from MS patients on primary rat cortex embryonic cells

Phase contrast microscopy shows that supernatants collected from day 3 to day 9 in active MS cases induced cell death in primary cortical cell cultures from embryonic Wistar rat cerebral cortex (Fig. 1B) or spinal cord. Equivalent MMCS samples from 4 healthy controls, cultured and tested in parallel, did not induce detectable cytotoxicity (Fig. 1A). Also, there was no toxicity with MMCS from 6 patients with other neurological diseases (one each with normal pressure hydrocephalus, epilepsy, encephalopathy, migraine, peripheral neuropathy and Guillain Barré syndrome) (data not shown). An immunostaining with Ab against cell-specific antigens was performed at different stages of these cultures, i.e., against neurofilaments for neurons (Fig. 1C), against GFAP for astrocytes, and against MBP and GC for oligodendrocytes. The data for astrocytes and oligodendrocytes in treated cells are not shown since no staining was observed. In these primary brain cultures, the astroglial layer first exhibited a cytolytic process while the neurons appeared to be well preserved (Fig. 1B, C). During longer periods of incubation (48 to 72 h), nearly complete destruction of the astrocytes and oligodendrocytes populations was observed (data not shown). Significant astrocyte and oligodendrocyte cytotoxicity was detectable after 24 h of incubation. This immunoanalysis therefore suggests that astrocytes and oligodendrocytes are the primary target for this toxic activity. The neurons only started to degenerate after massive depletion of glial cells in vitro. This late neuronal degeneration was presumably due to the deprivation of the astroglial feeder-layer.

3.2. Cytotoxic effect of MMCS from MS patients on immortalised astrocytes and oligodendrocytes

The supernatants from the three day periods, day 3 to day 6 and day 6 to day 9, were pooled for both the control and MS samples. These pooled supernatants were added to immortalised astrocytes CLTT 1-1 cultures [25] and to immortalised oligodendrocyte-enriched cultures 158N [26]. A qualitative study of cell death was first performed using the fluorescence method of MacCoubrey et al. [28] in order to distinguish live and dead cells. A high incidence of death of CLTT 1-1 immortalised astrocytes was demonstrated by the nucleic staining of dying cells by ethidium homodimer (Fig. 2B) compared to the controls (Fig. 2A). This same effect was also observed on immortalised oligodendrocyte cultures (Fig. 2C, D). It confirmed that immortalised astrocytes and oligodendrocytes are sensitive to this toxic activity.

3.3. Partial purification and dose-response effect of partially purified MMCS on immortalised CLTT 1-1 astrocytes

In order to obtain quantitative data on the MMCS-induced glial cell death, we used the MTT assay. Since culture media could contain many active components, we performed a partial purification, as described in Section 2.7, which resulted

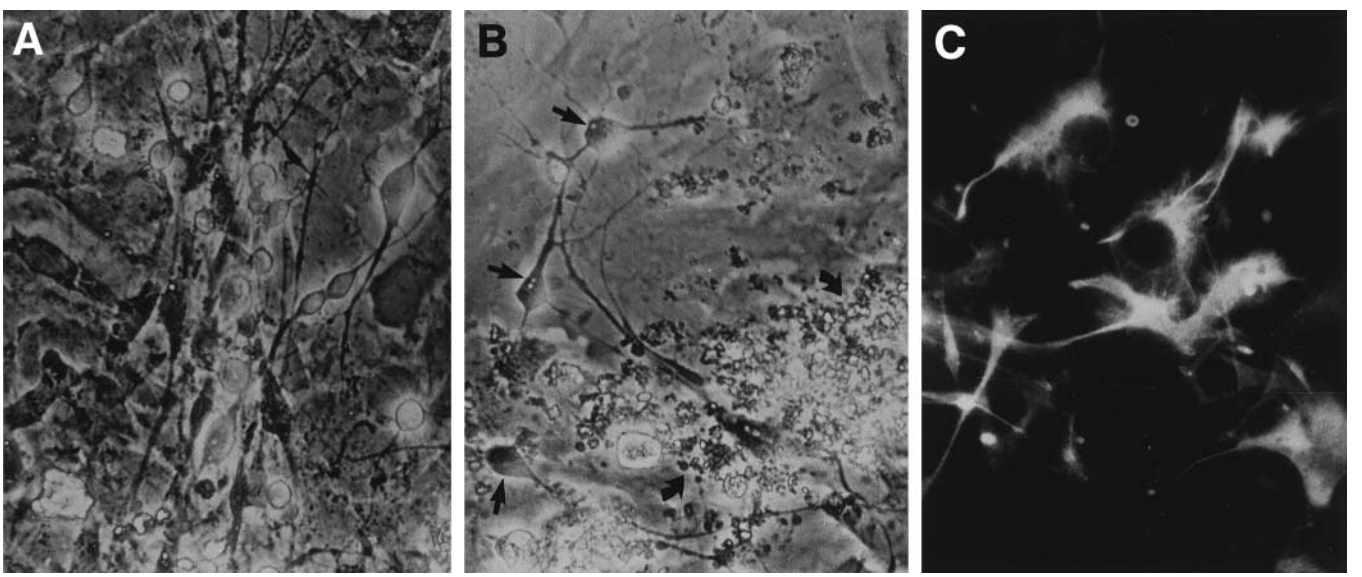

Fig. 1. Phase-contrast microscopy of primary rat cortical cells exposed to crude MMCS from healthy donor and MS patient. Primary mouse cortical cell culture generated from embryonic (E14) cortical tissue was exposed at 14 days post-seeding for 24 h to healthy donor MMCS (A) and MS MMCS (B) at a dilution of 1:4 in 2 ml of culture medium. Phase-contrast ×400. (C) represents the positive neurofilaments immunostaining of neurons exposed to MS MMCS (×400). Astrocytes and oligodendrocytes were not stained by their characteristic Ab (against GFAP for astrocytes and against MBP and GC for oligodendrocytes, data not shown).

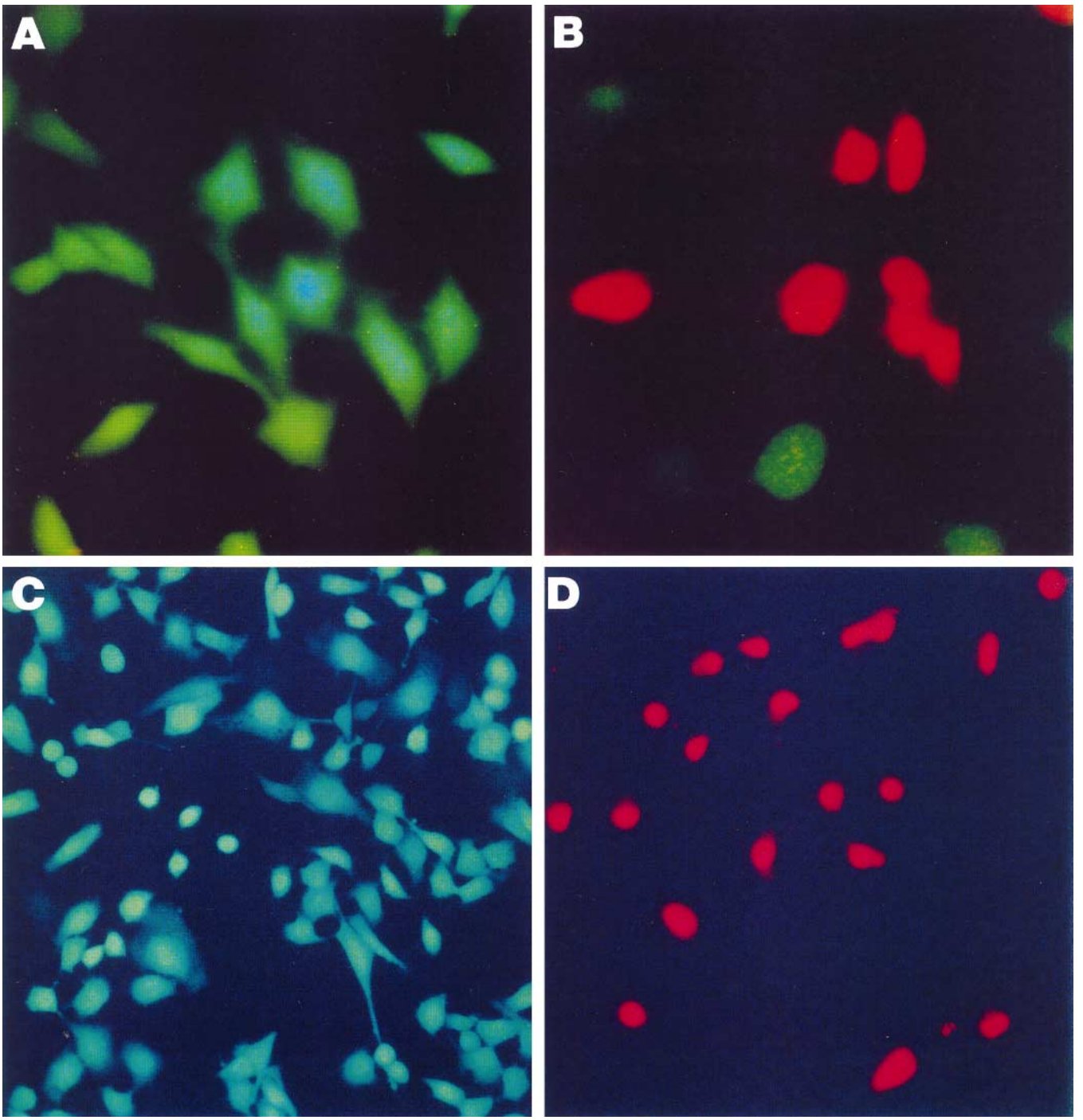

Fig. 2. Cytotoxic effect of MMCS from MS patients on immortalised mouse astrocytes and 158N oligodendrocytes cell lines. Non confluent astrocytes (A, B) and oligodendrocytes (C, D) cells were exposed to MMCS from a healthy donor (A, C) and from a MS patient (B, D) for 72 h (dilution 1:20). Live and dead cells were distinguished by green and red fluorescence, respectively (×40).

in a 76% recovery of toxic activity (Table 1). Purified toxic fractions were analysed for protein composition on SDS-PAGE (Fig. 3A). Elution of the protein from the gel and SDS removal demonstrated that gliotoxicity was essentially recovered in the 17-kDa gel region (data not shown). The same purification was also performed with 10 ml of MMCS from healthy donors. No toxicity was recovered at each step. Increasing dilutions of partially purified MMCS were tested starting from an initial 1:20 dilution with resulting cytotoxicity of about 53%. Fig. 3B illustrates the dose-response effect observed with the MMCS from active MS cases. Cell survival was decreased with increasing amounts of the toxic, partially purified MS MMCS sample, in a dose dependent manner. MMCS from controls corresponding to the highest concentration on this curve (1:20) did not induce cell death.

3.4. Disorganization of the vimentin network in immortalised CLTT 1-1 astrocytes exposed to MS MMCS

Some insight into the cellular modifications accompanying cytotoxic effects in glial cells was obtained by studying the cytoskeletal organization of the cells exposed to MS MMCS. Attention was paid to intermediate filaments, since we had observed important modifications in the glial cell shape as an early cytotoxic event, and in particular, to vimentin, since GFAP is synthesised at low levels in proliferating CLTT 1-1 cells. The GFAP synthesis increases at confluence [34], but our observations require low density cultures with proliferating astrocytes. In previous studies, the gliotoxic effect was only seen with rapidly dividing cells and not with cells at confluence (data not shown). Aliquots of MS MMCS with known gliotoxicity were added to eight-well chamber slides coated with dividing CLTT 1-1 astrocytes. After 24 to 48 h of incubation, many CLTT 1-1 cells displayed an abnormal distribution of vimentin, with disorganised cytoplasmic networks (Fig. 3D). This was not observed in wells incubated with control MMCS (Fig. 3C). It was also confirmed by Western blot that vimentin monomers were not hydrolysed in MS MMCS treated astrocytes (data not shown). This diffuse labeling of vimentin correlated with an initial cytotoxic effect and was therefore suggestive of intracytoplasmic depolymerization.

3.5. Correlation of cytotoxic activity and reverse transcriptase activity

Since a significant gliotoxicity associated with cultures from MS patients was observed, it was decided to analyze for any correlation between this cytotoxicity and the RT activity previously reported in MMCS from 'active' MS patients [13,17,30]. The results are presented for both the gliotoxic activity measured by the MTT assay on CLTT 1-1 cells (Fig. 4A) and the RT activity measured on ultracentrifugation pellets in MMCS (Fig. 4B) from 3 patients with differing MS stages and one healthy control. At sampling, patient MS1 had a chronic-progressive form in a quickly progressive phase, MS2 had a relapsing-remitting form and was in relapse, and MS3 had a relapsing-remitting form and was in remission for more than 6 months. The MMCS, harvested and completely renewed every three days, were pooled for the periods between days 3 to 6 and 9 to 15. The supernatants from day 1, which preceded macrophage differentiation, was tested separately [17]. Significant gliotoxicity (Fig. 4A) and RT activity (Fig. 4B) were detected in samples from MS1 and MS2, with highest levels from day 3 to 6. Lower activities were detected in the MS3 culture and only background activities were present in the control supernatant. Since the culture medium was completely replaced every three days and the cells washed before medium replacement, the presence of toxicity in subsequent media is consistent with an in vitro production of a gliotoxic factor in monocytes/macrophages cells. This in vitro production was also observed for RT activity. In Fig. 4C, mean RT activity data, abscissa, was plotted against mean gliotoxicity data, ordinate, for the MS1, MS2, MS3 and control data given in Fig. 4A, B. A significant correlation was observed between the RT activity data and gliotoxicity data with a correlation coefficient (r) of 0.89 and a probability (P) for the test of significance of the slope [35] of 0.0001, thus suggesting a pathophysiological link between retroviral and gliotoxic factors. The RT activity and gliotoxicity were observed in the MS1 and MS2 groups and are the basis for the observed correlation. MS3 and control data were at background levels (0–2000 dpm for RT-activity and 0–10% for gliotoxicity). Additionally, cultures from 10 different control patients and 6 patients with other neurological diseases were analysed. They did not present any toxicity and RT activity (data not shown).

3.6. Absence of correlation with cytokines potentially involved in the MS inflammatory response

In order to evaluate the kinetics of cytokine production in our MMCS from MS patients, patients with other neurological diseases, and healthy controls, we measured the levels of GM-CSF, TNF-α, IL-1β and IL-6 using the specific ELISA assays (R&D System Cytokine ELISA kits) between day 3 and day 15 in a series of cultures. GM-CSF was detected in practically all the MMCS tested. However its levels appeared to be lower in healthy controls and it was not detected in one MS MMCS. TNF-α was not detected in all the MMCS tested except in the MMCS from a patient with Wegener's disease. IL-1β and IL-6 were found at day 3 and 6 in several MS and controls MMCS, with higher levels in healthy controls. No correlation has been found between RT activity and the production of cytokines in MS MMCS (data not shown). None of the known cytokines tested in parallel on CLTT 1-1 astrocytes cultures displayed any significant cytotoxic activity under the conditions of our gliotoxicity assays (manuscript in preparation).

3.7. Detection of specific MSRV RNA in cell-free MS MMCS

Since the nucleotide sequence of a retroviral pol gene in RNA from MS virions has recently been characterised [16] and attributed to a novel retrovirus (MSRV), MS and controls MMCS were analysed by RT-PCR with specific MSRV primers and probes. Supernatants from each culture were pooled from day 3 to day 15 and RNA was extracted from 500 μl of each pool. A nested RT-PCR was performed with MSRV primers, followed by hybridization of the PCR prod-

Table 1
Summary of the purification steps

Purification steps	Total proteins (mg)	Total toxicity (dead cells)	Specific toxicity (dead cells/mg)	Toxicity yield (%)
Initial supernatant	42	700 000	16 600	100
Protein A sepharose Cl-4B	35.3	654 000	18 530	93
Con A sepharose+NAP-25	0.32	533 335	166 667	76

The protein concentrations were measured by the method of Bradford [40] using BSA as reference and toxicity was determined on immortalised mouse CLTT 1-1 astrocytes using the MTT assay. Each result is the average of three experiments, each run in triplicate. Cytotoxicity represents the cell death at 72 h. The Ig fraction, the proteins directly eluted from the Concanavalin A column and the protein eluted with 0.2 M D-glucopyranosid did not present any toxicity (data not shown). The yield represents the percentage of toxic activity recovered after the purification steps as compared to the initial supernatant toxicity.

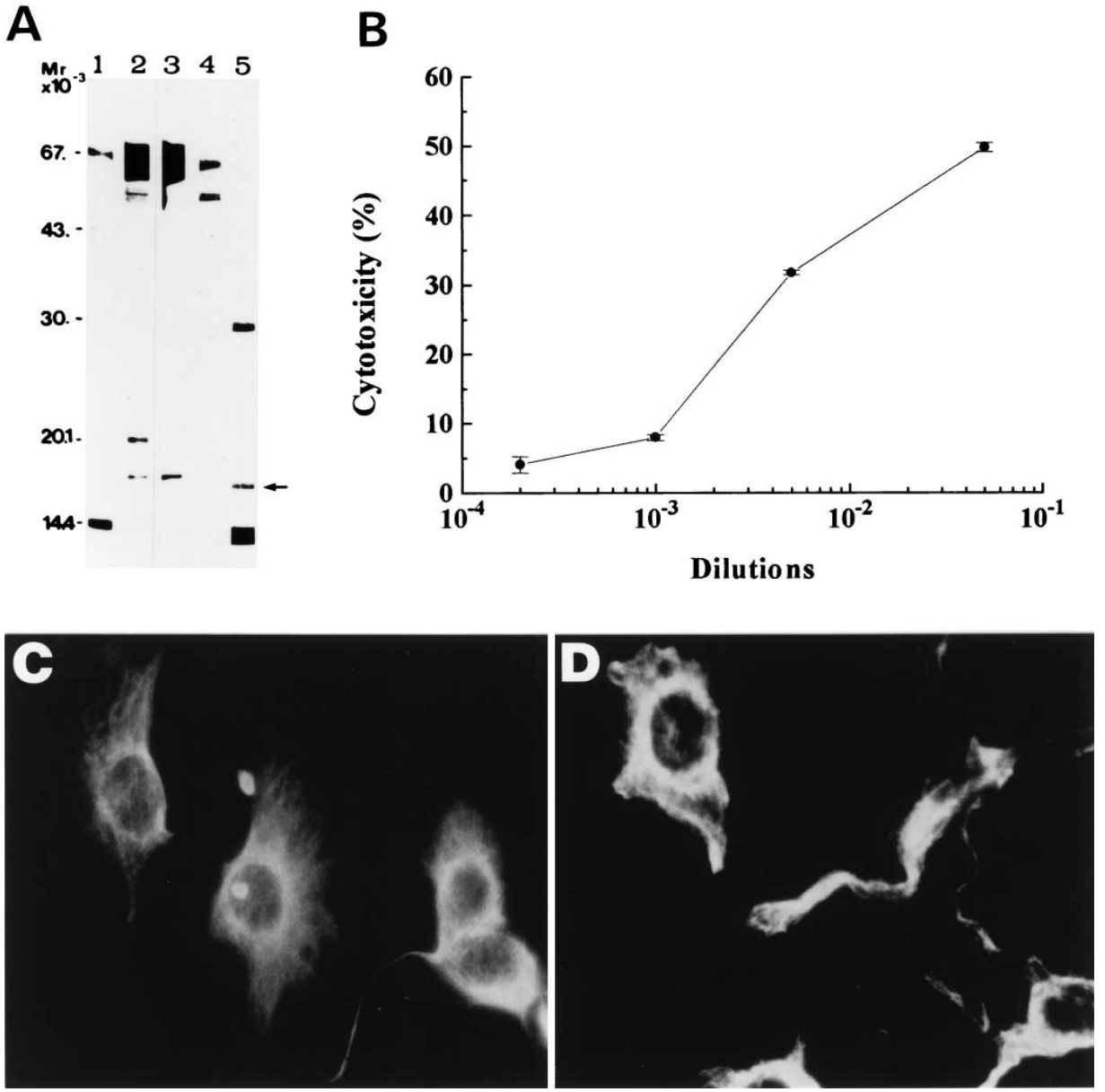

Fig. 3. (A) Protein composition from a 50 μl sample of partially-purified MS MMCS analysed on a 10% SDS-Page gel. Proteins were visualised by silver nitrate staining. Lane 1 represents the MW markers (albumin, ovalbumin, carbonic anhydrase, trypsin inhibitor and α-lactalbumin, sequentially). Lanes 2 and 3 correspond to the MMCS before purification and to the non retained proteins on concanavalin A, respectively. Lanes 3 and 4 represent the glycosylated proteins eluted with 0.2 M D-glucopyranosid and 0.1 M glycin HCl pH 3, respectively. The arrow indicates the toxic protein recovered after elution from the gel and SDS removal. (B) Dose-response curve of cell death in astrocyte cultures after exposure to toxic MMCS. CLTT 1-1 astrocytes were exposed to MS MMCS partially purified proteins at the indicated dilutions for 72 h. Cytotoxicity was evaluated by determining the surviving cells using the MTT assay and expressed as a percentage of cell death, compared to untreated control cultures. Each result is the mean of data obtained in three separate experiments, each performed in three replicate wells. Bars represent ±S.E.M. (C) and (D) Immunofluorescence analysis of vimentin network on mouse immortalised astrocytes exposed to MMCS. CLTT 1-1 astrocytes were exposed to purified healthy donor MMCS (C) or purified gliotoxic fraction from MS MMCS (D) for 48 h at a dilution of 1:20. Cells on coverslips were fixed in acetone for 10 min at −20°C. The cells were incubated with mAb anti-vimentin IgM, followed by FITC-conjugated goat anti-mouse IgM (see Section 2.3).

ucts with MSRV-specific capture and detection probes (ELOSA technique). In parallel, 'pan-retrovirus' pol primers were used in RT-PCR and the presence of a MSRV corresponding region was detected by specific hybridization with MSRV probes in the ELOSA technique [14,16]. In Table 2, the ELOSA results on RT-PCR products from MS and control MMCS, indicate the presence of MSRV RNA in cell-free MMCS from two MS patients only (K and LE). LE was found positive with two techniques and K with one only. This may be explained by sequence variations which cannot be amplified during RT-PCR using the chosen ST2 MSRV pol primers at the stringent annealing temperature, but are amplified by the corresponding 'pan-retro' primers and further detected by specific MSRV ELOSA. Alternatively, thermodynamics generated by the target, the primers and the RT-PCR conditions might render one technique more sensitive than the other. Two kinds of controls were measured in parallel with these RT-PCR, one consisted of a 'no-RT' control

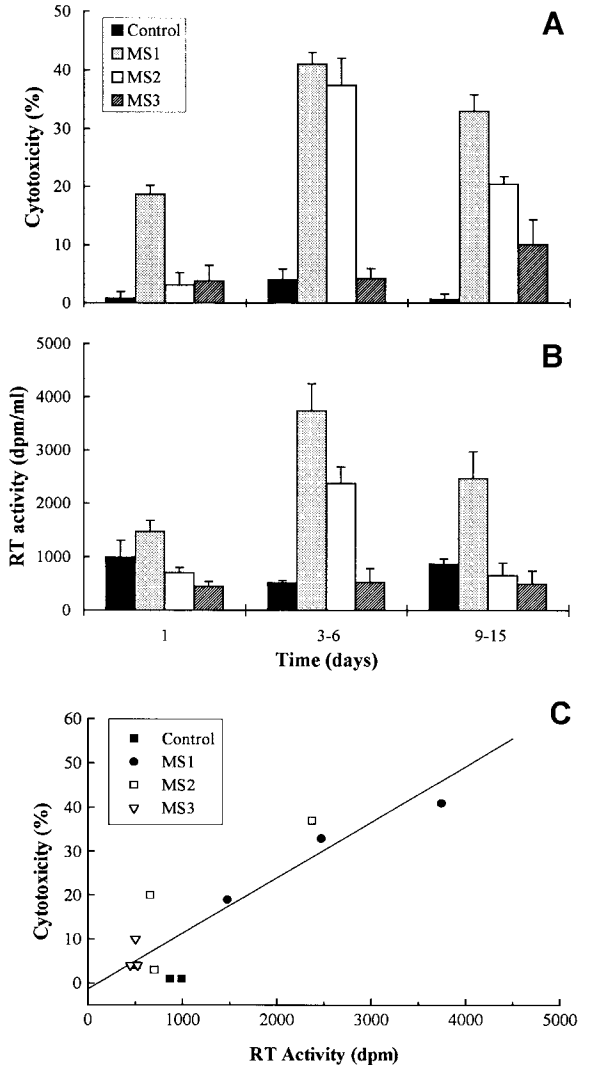

Fig. 4. Correlation between the presence of cytotoxic activity and reverse transcriptase activity in MS MMCS. MMCS from a healthy donor (control) and MS patients at different stages of the disease (MS1, chronic progressive; MS2, relapsing-remitting at relapse; and MS2, relapsing-remitting at remission) were removed, pooled (day 1, from days 3 to 6 and from days 9 to 15) and analysed for cytotoxicity (A) and RT activity (B). Cytotoxicity and RT values are the mean of three experiments run in triplicate and bars represent ±mean deviation. (C) illustrates the correlation between cytotoxicity and RT activities data. Correlation coefficient, $r = 0.89$.

which was designed to ascertain the absence of DNA, which contains endogenous homologs, in our RNA preparations (see Table 2), and another consisted of a RT-PCR with human aldolase primers in order to ascertain the absence of contaminating cellular RNA from occasional cells shedded or lysed, in the supernatants. None of these RNAs extracted from MMCS contained cellular DNA or RNA. The gliotoxicity assay and the RT-PCR were made in two independent laboratories by two different persons unaware of the origin (MS or control) of the samples and unaware of any result independently obtained with them. A strict coincidence is observed between the presence of a significant gliotoxic activity peak in MMCS during the time-course of the culture and the detection of a specific 'extracellular' MSRV RNA by RT-PCR+ELOSA in the MMCS pooled over the corresponding period.

4. Discussion

The etiopathogenesis of MS stands at the cross-roads of immunology, microbiology and neurobiology and we are convinced that its elucidation requires an integrated multidisciplinary approach. A few years ago, we had obtained a leptomeningeal cell-culture (LM7) from cerebrospinal fluid of a patient with an acute relapse of MS in which we detected retrovirus-like RT-activity and extracellular particles [12]. A similar observation was obtained in larger series of patients when we adapted blood monocyte culture to the detection of this retroviral activity [13]. We later confirmed the association of a specific retroviral RNA with these extracellular virions, and the apparent association between MS activity and their release in MMCS [17,14]. We also observed retroviral-like particles produced by MS MMC in phase with a burst of RT activity [13].

The retroviral genome associated with these virions has been characterised as that of an oncovirus and is closely related to a family of human endogenous elements (ERVs). The current nucleotide sequence information on this novel retrovirus, now designated MSRV, is compatible with that of an exogenous retrovirus sharing extensive homology with particular ERVs or with a replication-competent ERV harboured by the genome of susceptible individuals and activated by cofactors such as herpes viruses [36,16]. These notions are well-known in animal retroviral families such as murine leukaemia and mouse mammary tumour viruses in which pathogenic strains can be exogenous or endogenous and share more

Table 2
MSRV and pan-retro RT-PCR on MMCS: MSRV-specific ELOSA results

	MSRV RT-PCR				'Pan-retro' RT-PCR				Gliotoxicity MTT test (%)
	No RT-PCR		RT-PCR		No RT-PCR		RT-PCR		
	V1	V2	V1	V2	V1	V2	V1	V2	
Control cDNA	0.021	0.010	0.007	0.007	0.022	0.017	0.01	0.014	
MS K	0.007	0.006	0.005	0.005	0.026	0.011	**2.409**	**2.602**	8
MS L	0.010	0.028	0.013	0.015	0.020	0.017	0.018	0.013	0
MS LE	0.034	0.006	**0.118**	**0.102**	0.028	0.031	**1.684**	**1.469**	12
MS R	0.006	0.007	0.044	0.011	NI	NI	NI	NI	0
Healthy control	0.010	0.006	0.008	0.008	0.045	0.035	0.010	0.008	0

ELOSA results are expressed as OD units. The cut-off value of the technique is 0.050. V1 and V2 are the duplicate ELOSA values [16,31,33]. The control cDNA represents a cDNA made in parallel with the others, with water in place of the RNA extracted from each sample. NI, not interpretable. Gliotoxicity represents the maximum value during the considered period of culture; background activity with reference samples was inferior to 5% in these series.

than 90% of homology in their nucleotide sequences. However this field remains quite unexplored in humans, despite many studies arguing in favor of a pathogenic role of such retroviruses in autoimmune diseases (for review, see [37]). If we consider the considerable technical and ethical difficulties to definitely establish a direct or indirect link between this category of agents and a human disease, the present study, resulting from our multidisciplinary approaches, is certainly a way to evaluate such a link.

The results presented in this report address the following points:

1. Monocytes isolated from peripheral blood, and cultured in our conditions, produce significant levels of gliotoxic activity, when obtained from patients with active MS.
2. The corresponding culture supernatants induce both astrocytes and oligodendrocytes cell death in primary mouse cortical cultures. Cell death was also observed in immortalised astrocytes and oligodendrocytes in a characteristic dose-dependent way. The phenomenon of filament disorganization precedes cell death, but its relationship with the subsequent cell death must be further evaluated. However, such a relationship has been discussed in a study of viral infections [38,39] where there was an induced intermediate network disorganization triggered by adenoviral products.
3. This gliotoxic activity does not result from the progressive dilution in cell culture of a toxic factor present in MS plasma, since long-term kinetics indicate successive 'waves' of gliotoxic activity released in MS MMCS (data not shown). The variations observed are thus consistent with an active process associated with the cultured cells.
4. The release of a significant gliotoxic activity during the first two weeks of culture is apparently correlated with significant RT activity, and MSRV-specific RNA can be detected in cell-free 'active' MS MCCS.
5. The present results and those of complementary study (manuscript submitted) show that the observed gliotoxicity cannot be attributed to already characterised human cytokines, at present.

We cannot say at present whether the gliotoxic activity is due to an MSRV-encoded structural or regulatory protein, or to a host's cellular factor subsequently induced by this retrovirus. However, the apparent coincidence between gliotoxic and RT-activities in MMCS from patients with active MS suggests interesting directions for further studies addressing the potential pathogenic role of MSRV replication in MS.

The fact that monocytes/macrophages from MS patients are able to produce a gliotoxic activity may ultimately be related to several unique neuropathological features of MS, like blood brain barrier disruption and demyelination, and could also explain the presence of the gliotoxic activity in MS cerebrospinal fluid [41], if monocytes/macrophages are the privileged source of the gliotoxic factor(s). We are currently pursuing the molecular characterization of the protein associated with this gliotoxic activity which can also be purified from cerebrospinal fluid of MS patients.

Knowing the difficulties to address the relationship between endo/exogenous retroviruses and human diseases, the pathogenic link provided by this gliotoxic activity may be crucial in elucidating important etiopathogenic mechanisms in MS.

Acknowledgements: We are indebted to Dr. P. Rouget (Collège de France, Paris) for supplying the CLTT 1-1 astroglial cell line and to Dr. S. Gandhour for immortalised mouse oligodendrocytes. We thank Dr. D. Hantaz-Ambroise for help in the preliminary experiments on primary rat cortical and spinal cord cell cultures, to D. Reoutov for photographic artwork and to K. Belliveau for editing the manuscript. This work has been partially supported by the French National Multiple Sclerosis Associations, ARSEP, NAFSEP and local association SEP Rhône-Alpes. This research project has been performed under a formal agreement with the Comité Consultatif de Protection des Personnes dans la Recherche Biomédicale (La Pitié Salpêtrière Hôpital, Paris).

References

[1] Prineas, J.W. (1985) in: Koetsier, J.C. (Ed.), Handbook of Clinical Neurology: demyelinating diseases, vol. 3, no. 47, pp. 213–257, Elsevier, Amsterdam.
[2] Waksman, B.H. (1985) Nature 318, 104–105.
[3] Martin, R., MacFarland, H.F. and McFarlin, D.E. (1992) Annu. Rev. Immunol. 10, 153–187.
[4] Prineas, J.W. and Wright, R.G. (1978) Lab. Invest. 38, 409–421.
[5] Gay, D. and Esiri, M. (1991) Brain 114, 557–572.
[6] Hawkins, C.P., Mackenzie, F., Tofts, P. and McDonald, W.I. (1991) Brain 114, 801–810.
[7] Thompson, A.J., Miller, D., Youl, B., MacManus, D., Moore, S., Kingsley, D., Kendal, B., Feinstein, A. and MacDonald, W.I. (1992) Neurology 42, 60–63.
[8] Poser, C.M. (1993) J. Neurol. Sci. 115, (suppl.) 3–15.
[9] Carp, R.I., Warner, H.B. and Merz, G.S. (1978) Prog. Med. Virol. 24, 158–177.
[10] Waksman, B.H. and Reingold, S.C. (1986) Trends Neurol. Sci. 9, 338–391.
[11] Waksman, B.H. (1989) Nature 337, 599.
[12] Perron, H., Geny, C., Laurent, A., Mouriquand, C., Pellat, J., Perret, J. and Seigneurin, J.M. (1989) Res. Virol. 140, 551–561.
[13] Perron, H., Lalande, B., Gratacap, B., Laurent, A., Genoulaz, O., Mallaret, M., Shuller, E., Stoebner, P. and Seigneurin, J.M. (1991) Lancet 337, 862–863.
[14] Tuke, P.W., Perron, H., Bedin, F., Beseme, F. and Garson, J.A. (1997) Acta Neurol. Scand. 169S, 16–21.
[15] La Mantia, G., Maglione, D., Pengue, G., Di Cristofano, A., Simeone, A., Lanfrancone, L. and Lania, L. (1991) Nucleic Acid Res. 19, 1513–1520.
[16] Perron, H., Garson, J.A., Bedin, F., Beseme, F., Paranhos-Baccala, G., Komurian-Pradel, F., Mallet, F., Tuke, P.W., Voisset, C., Blond, J.L., Lalande, B., Seigneurin, J.M., Mandrand, B. and Collaborative Research Group on MS (1997) Proc. Natl. Acad. Sci. 94, 7583–7588.
[17] Perron, H., Firouzi, R., Tuke, P., Garson, J.A., Michel, M., Beseme, F., Bedin, F., Mallet, F., Marcel, F., Seigneurin, J.M., Mandrand, B. and Collaborative Research Group on MS (1997) Acta Neurol. Scand. 169S, 22–31.
[18] Nathanson, N., Georgsson, G., Palsson, A., Najjar, J.A., Lutley, R. and Petursson, G. (1985) Rev. Infect. Dis. 7, 75–82.
[19] Gendelman, H.E., Narayan, O., Kennedy-Stoskopf, S., Kennedy, P.G.E., Ghotbi, Z., Clements, J.E., Stanley, J. and Pezeshkpour, G. (1986) J. Virol. 58, 67–74.
[20] Haase, A.T. (1986) Nature 322, 130–136.
[21] Giulian, D., Vaca, K. and Noonan, C.A. (1990) Science 250, 1593–1596.
[22] Giulian, D., Wendt, E., Vaca, K. and Noonan, C.A. (1993) Proc. Natl. Acad. Sci. USA 90, 2769–2773.
[23] Poser, C.M., Paty, D.W., Scheinberg, L., McDonald, W.I., Davis, F.A., Ebers, G.C., Johnson, K.P., Sibley, W.A., Silberberg, D.H. and Tourtellotte, W. (1983) Ann. Neurol. 13, 227–231.
[24] Poser, C.M. (1986) Acta Neuropathol. 71, 1–10.
[25] Galiana, E., Borde, I., Marin, P., Rassoulzadegan, M., Cuzin, F., Gros, F., Rouget, P. and Evrard, C. (1990) J. Neurosci. Res. 26, 269–277.

[26] Feutz, A.C., Bellomi, I., Allinquant, B., Schladenhaufen, Y. and Gandhour, M.S. (1995) J. Neurocytol. 24, 865–877.
[27] Tennekoon, G.I., Yoshino, J., Peden, W.C., Bigbee, J., Rutkowski, J.L., Kishimoto, Y., DeVries, G.H. and McKhann, G.M. (1987) J. Cell Biol. 105, 2315–2325.
[28] MacCoubrey, I.C., Moore, P.L. and Hougland, R.P. (1990) J. Cell Biol. 111, 58a. abstract
[29] Mosmann, T. (1983) J. Immunol. Methods 65, 55–63.
[30] Perron, H., Gratacap, B., Lalande, B., Genoulaz, O., Laurent, A., Geny, C., Mallaret, M., Innocenti, P., Shuller, E., Stoebner, P. and Seigneurin, J.M. (1992) Res. Virol. 143, 337–350.
[31] Mallet, F., Oriol, G., Mary, C., Verrier, B. and Mandrand, B. (1995) Biotechniques 18, 678–687.
[32] Chomczynski, P. and Sacchi, N. (1987) Anal. Biochem. 162, 156–159.
[33] Mallet, F., Hebrard, C., Brand, D., Chapuis, E., Cros, P., Allibert, P., Besnier, J.M., Barin, F. and Mandrand, B. (1993) J. Clin. Microbiol. 31, 1444–1449.
[34] Evrard, C., Galiana, E. and Rouget, P. (1988) J. Neurosci. Res. 21, 80–87.
[35] Snedecor, G.W. and Cochran, W.G. (1974) in: Statistical Methods, 6th ed., Regression Chapter 6, p. 138, Iowa State University Press, Ames, Iowa.
[36] Perron, H., Suh, H., Gratacap, B., Laurent, A., Stoebner, P. and Seigneurin, J.M. (1993) J. Gen. Virol. 74, 65–72.
[37] Nakagawa, K. and Harrisson, L.C. (1996) Immunological Reviews 152 (Möller, G., Ed.), Munksgaard Copenhagen.
[38] Belin, M.T. and Boulanger, P. (1987) J. Virol. 61, 2559–2566.
[39] White, E. and Cipriani, R. (1989) Proc. Natl. Acad. Sci. USA 86, 9886–9890.
[40] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
[41] Ménard, A. et al. (1997) J. Neurol. Sci., in press.