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Multiple sclerosis–associated retrovirus particles cause T lymphocyte–dependent death with brain hemorrhage in humanized SCID mice model

R Firouzi,[1] A Rolland,[2] M Michel,[3] E Jouvin-Marche,[2] JJ Hauw,[4] C Malcus-Vocanson,[5] F Lazarini,[4] L Gebuhrer,[6] JM Seigneurin,[3] JL Touraine,[1] K Sanhadji,[1] PN Marche,[2] and H Perron[5]

[1]Laboratoire des déficits Immunitaires, Faculté de Médecine Laënnec, Lyon, France; [2]INSERM, Grenoble, France; [3]Laboratoire de Virologie, Faculté de Médecine de Grenoble, La Tronche, France; [4]Laboratoire de Neuropathologie Raymond Escourolle, Hôpital de la Salpêtrière, Paris, France; [5]BioMérieux, Research and Development Department, Chemin de l'Orme, Marcy l'Etoile, France; and [6]Laboratoire d'Histocompatibilité/EFS, Lyon, France

A retroviral element (multiple sclerosis–associated retrovirus, MSRV) defining a family of genetically inherited endogenous retroviruses (human endogenous retrovirus type W, HERV-W) has been characterized in cell cultures from patients with multiple sclerosis. Recently, MSRV retroviral particles or the envelope recombinant protein were shown to display superantigen activity *in vitro*, but no animal model has yet been set up for studying the pathogenicity of this retrovirus. In the present study, the pathogenicity of different sources of MSRV retroviral particles has been evaluated in a hybrid animal model: severe combined immunodeficiency (SCID) mice grafted with human lymphocytes and injected intraperitoneally with MSRV virion or mock controls. MSRV-injected mice presented with acute neurological symptoms and died within 5 to 10 days post injection. Necropsy revealed disseminated and major brain hemorrhages, whereas control animals did not show abnormalities ($P < .001$). In ill animals, reverse transcriptase–polymerase chain reaction (RT-PCR) analyses showed circulating MSRV RNA in serum, whereas overexpression of proinflammatory cytokines such as tumor necrosis factor (TNF)-γ and interferon (IFN)-γ was evidenced in spleen RNA. Neuropathological examination confirmed that hemorrhages occurred prior to death in multifocal areas of brain parenchyma and meninges. Further series addressed the question of immune-mediated pathogenicity, by inoculating virion to SCID mice grafted with total and T lymphocyte–depleted cells in parallel: dramatic and statistically significant reduction in the number of affected mice was observed in T-depleted series ($P < .001$). This *in vivo* study suggests that MSRV retroviral particles from MS cultures have potent immunopathogenic properties mediated by T cells compatible with the previously reported superantigen activity *in vitro*, which appear to be mediated by an overexpression of proinflammatory cytokines. *Journal of NeuroVirology* (2003) **9,** 79–93.

Keywords: animal model; blood-brain barrier; cytokine; endogenous retrovirus; superantigen

Introduction

Multiple sclerosis (MS) is a multifactorial inflammatory disease of the central nervous system characterized by plaques of demyelination associated blood-brain barrier breakdown followed by intraparenchymal lymphocytic infiltrates and prominent T-cell activation. Epidemiological and clinical data suggest that both genetic and environmental factors

Address correspondence to Herve Perron, BioMérieux, Research and Development Department, Chemin de l'Orme, 69280 Marcy l'Etoile, France. E-mail: herve.perron@eu.biomerieux.com

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could be involved in the etiology of this disease. MS pathogenesis is thought to consist in an autoimmune process directed against myelin components, probably triggered by environmental factors, among which viruses are favored candidates. Viruses such as herpesviruses (Ascherio and Munch, 2000; Ferrante *et al*, 2000; Soldan *et al*, 1997; Wandinger *et al*, 2000) and retroviruses (Haahr *et al*, 1991; Koprowski *et al*, 1985; Perron *et al*, 1991b) have been suggested to be associated with MS, and different groups have detected retroviral particles in cultured cells from MS patients (Haahr *et al*, 1991; Lan *et al*, 1994; Perron *et al*, 1991b).

Our previous studies on RNA associated with viral particles produced in choroid plexus/leptomeningeal cell or B-lymphocyte cultures from patients with MS had provided sequences corresponding to overlapping regions of a retroviral genome (Komurian-Pradel *et al*, 1999; Perron *et al*, 1997b), which was provisionally named MSRV (for multiple sclerosis–associated retrovirus element). MSRV revealed to have genetically homologous elements in human DNA, defining a novel family of human endogenous retroviruses, HERV-W, for human endogenous retrovirus type W (Blond *et al*, 1999; Perron *et al*, 1997b).

This HERV-W family comprises multiple copies of a prototypic retroviral genome that probably entered the germ line cell lineage by an infectious route more than 25 million years ago, before the species radiation of the ancestors of the old world monkeys (Kim *et al*, 1999; Voisset *et al*, 1999). Consequently, members of this endogenous family can be found in normal DNA of old world monkeys, apes, and humans, but are absent in all other animal species, including new world monkeys. In normal human DNA, most HERV-W copies are truncated and/or lack open reading frames (orf), but several chromosomal copies have retained potential orfs for retroviral proteins (Voisset *et al*, 2000). A complete HERV-W provirus on chromosome 7q, in a region associated with genetic susceptibility to MS (Perron *et al*, 2000), encodes an envelope protein strongly expressed in placenta (Blond *et al*, 1999). This protein is involved in the physiological process of syncytiotrophoblast fusion (Blond *et al*, 2000). The HERV-W7q provirus is not fully functional and cannot account for virion production as observed in MS (Perron *et al*, 2000). In addition, the HERV-W copy number in human DNA appears to vary between individuals and with ethnical origin (Mirsattari *et al*, 2001). Therefore, MS retroviral particles are not likely to be encoded by the normal inherited HERV-W copies, but may originate from a "modified" (e.g., retrotransposed and/or recombined) or exogenous member of the same family (Komurian-Pradel *et al*, 1999; Perron *et al*, 2000; Voisset *et al*, 2000). Particle-associated retroviral RNA has now been detected in MS sera by different groups (Dolei *et al*, 2002; Garson *et al*, 1998; Olsson *et al*, 1999; Serra *et al*, 2001); these data also appear to significantly correlate particular features of the clinical history.

The pathogenicity of retroviral elements belonging to genetic families with endogenous copies usually results from interactions between pathogenic members and the "normal" endogenous gene homologs (Contag and Plagemann, 1989; Gardner, 1990). This has also been reported for the superantigen encoded by members of the mouse mammary tumor virus (MMTV) family in mice (Kubo *et al*, 1996; Xu *et al*, 1996). Our recent findings of superantigen activity associated with virions and encoded by MSRV envelope protein (Lafon *et al*, 2002; Perron *et al*, 2001) therefore suggest that, apart from a major contribution in immune dysfunctions of MS disease, immunopathogenic properties of human MSRV/HERV-W members could also result from such retroviral interactions. Given recent evidence of active HERV-W proviruses in human cells (Yi *et al*, 2002), together with variability of DNA copy number among humans (Mirsattari *et al*, 2001), retrotransposition—and possibly recombination—events should occur within the HERV-W family under certain circumstances. Consequently, most animal models that could be set up for studying the pathogenicity of these MSRV particles might appear inappropriate because, as mentioned above, most animals lack these endogenous ERV-W elements in their genome, or, when present, e.g., in old world monkeys, these ERV-W copies are not identical to their human counterpart, HERV-W (Kim and Crow, 1999; Kim *et al*, 1999; Voisset *et al*, 1999).

In order to address the potential pathogenicity of MSRV virion *in vivo*, we have chosen a hybrid human/animal model: the severe combined immunodeficiency (SCID) mouse humanized with human lymphocytes, called hu-SCID mouse. This T cell– and B cell–deficient mouse supports grafting of functional human lymphoid cells from human peripheral blood mononuclear cells (PBMCs) that will survive and circulate in place of the mouse lymphoid cells. Such a model, called hu-PBL-SCID (considering peripheral blood lymphocytes as major elements of the graft), allows studies of human immunopathological mechanisms *in vivo* (Chargui *et al*, 1995; Okamoto *et al*, 1998; Persidsky, 1999; Scaramuzzino *et al*, 2000; Vanzieleghem *et al*, 2000). In our present protocol, the survival of the engrafted human cells was optimized by previous irradiation and anti–natural killer (NK) cell treatment, and therefore allowed studies over a rather long period (generally, up to 3 months with good confidence).

As previously discussed, mice do not harbor any chromosomal copy of the ERV-W family. The Hu-PBL-SCID mouse therefore allows the study of pathogenic mechanisms that may involve interactions between MSRV virus particles and endogenous HERV-W expression, within the human cells only. In addition, physiopathological effects within mice tissues should mostly result from retroviral expression

and/or immune response linked to human lymphoid cells.

Results

Different series of humanized mice have been prepared according to the protocol presented in Figure 1. In human PBMCs prior to engraftment, the CD3/CD45 ratio ranged from 50% to 80%, the CD4/CD45 ratio from 30% to 45%, and the CD19/CD45 ratio from 10% to 25%. Five days after engraftment with human PBMCs, peritoneal fluid from all mice was punctured and analyzed by cytofluorometry: CD3 ranged from 35% to 75%, CD4 from 20% to 30%, and CD19 from 10% to 25%. Mice series once found with less than 25% of CD3 and less than 10% of CD4 were not included in the study.

Occasionally, a mouse died prior to virus injection (three cases in all series), as normally observed with low frequency within the first days after x-ray irradiation, anti-NK treatment, and human PBMC grafting. In order to control the rate of such "accidental" death during the "viral" study period in our series, phosphate-buffered saline (PBS)-inoculated mice were always kept in parallel with mice from the same pre-engraftment group, grafted with the same PBMC sample, but inoculated with MSRV virion or mock preparation. During this period (after the first week post engraftment), we did not observe death of PBS-injected animals in our series.

As presented in Table 1, summarizing successive series of experiments, mice were grafted with PBMCs from five different blood donors with various human leukocyte antigen (HLA) class II phenotypes.

MS virions, as visualized by electron microscopy shown in Figure 2, were prepared from different sources of cell culture (Perron *et al*, 1989, 1991a, 1997a). They were pelleted from supernatants presenting a peak of reverse transcriptase (RT) activity above the background signal (mean + 3 SD determined from cell type–matched, non-MS control cultures), as previously described (Perron *et al*, 1991b, 1993, 1997a). In addition, most cellular debris were eliminated by precentrifugation (prior to freezing) and by using a glycerol cushion at the bottom of ultracentrifugation tubes, collection of the material pelleted under this glycerol barrier avoided eventual soluble factors from culture medium. Also, virion particles were preserved by adding 10% of glycerol in culture supernatants and resuspended pellets prior to any freezing step.

The RT activity count in the samples injected to mice was about 10,000 dpm and approximately corresponded to 10^4 particles, as determined elsewhere with negative-stained preparations on electron microscope (EM) grids. Our recent data (unpublished) indicate that MSRV particles can be detected in sera from MS patients at concentrations 3 logs below that of the suspensions injected to hu-SCID mice.

As indicated in Material and methods, cell cultures and virion batches were screened for possible contaminants and all samples were obtained with the same batch of culture medium. As expected, no Epstein-Barr virus genome was detected in choroids plexus cultures from MS or non-MS control. In addition, preliminary experiments on few nonhumanized SCID mice had shown no clinically detectable effect during 3 weeks after intraperitoneal (IP) injection of all preparations used for the humanized (hu-PBL-SCID) series (not shown). Consequently pathogenic contaminants that could have directly affected SCID mice were absent from our preparations.

Circulating particle-associated MSRV RNA was detected in cell-free serum collected from cardiac blood at necropsy, according to J. Garson's protocol (Garson *et al*, 1998), as indicated in Table 1. These data confirmed virion access to the bloodstream after IP inoculation of virion preparations, but its absence in mock-infected mice.

Hu-PBL-SCID mice with MSRV virion die with brain hemorrhage

In mice series grafted with total PBMCs (not T-depleted), we could observe a striking effect that rapidly appeared in all mice inoculated with MS-virion preparation from both sources (choroid plexus or B cells): mice presented with neurological symptoms such as partial or generalized paralysis followed by death of the animals. Such features were not observed in animals injected with mock preparations from non-MS cultures, nor in animals injected with PBS, during the few months they were kept alive. This was similarly reproduced in separate experiments: one with donors 1 and 2, another with donor 3, and one with T-depleted versus nondepleted PBMCs from donors 4 and 5 (presented in Table 1). A first series with lower numbers of animals (not shown in Table 1) had also included mock virion from non-MS choroid plexus cultures, in parallel with the other preparations mentioned in Table 1. These hu-PBL-SCID mice injected with mock choroid plexus virion survived as well as the PBS controls (over 2 months).

The difference between the numbers of ill/dead animals in virus-inoculated animals and mock- or PBS-injected controls, appears significant in all cases: comparing single donors (e.g., 1, 2, or 3) with one source of virion versus mock controls (5/5 versus 0/5) yields a chi-square value with Yate's correction of 6.4 ($P < .02$); comparing both sources of virion versus mock controls for single donors in the same series (10/10 versus 0/5) yields a chi-square value with Yate's correction of 10.84 ($P < .01$); comparing all virus-injected hu-PBL-SCID mice versus all mock-controls in Table 1 (46/46 versus 0/23, excluding T-depleted series) yields a chi-square value of 69 ($P < .001$).

Most virion-inoculated mice were examined postmortem, but a few ill mice, in the last series, were sacrificed when neurological symptoms appeared.

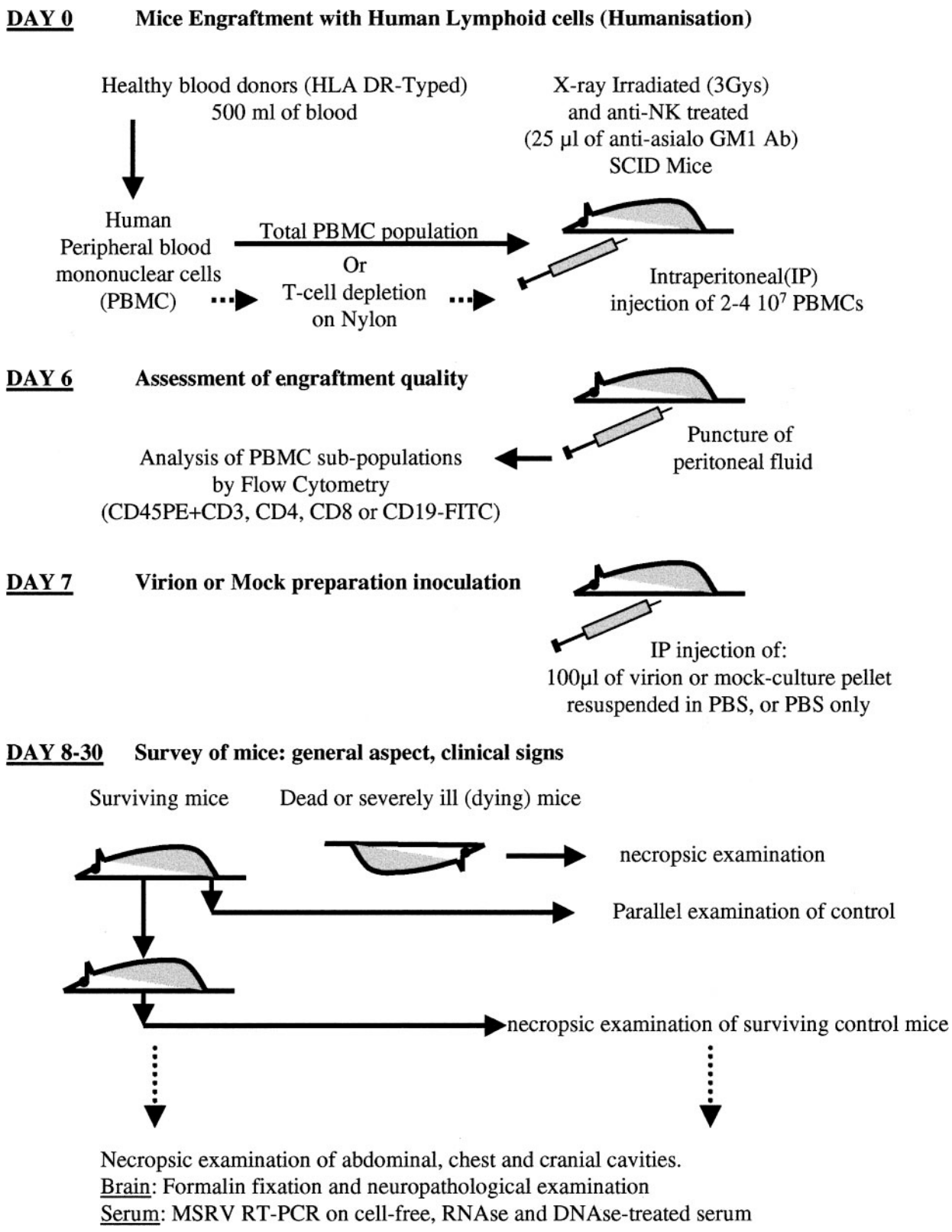

Figure 1 Hu-PBL-SCID grafting with human PBMCs followed by intraperitoneal injection of virus or control preparations: schematic representation of the experimental protocol.

Table 1 Hu-PBL-SCID series grafted with PBMCs from human blood donors with various HLA-II phenotypes

	Donor + HLA-DR	*1: DR2/DR4 DRB1*02/04*	*2: DR2/DR4 DRB1*02/04*	*3: DR2/DR2 DRB1*02*	*4: DR2/DR13 DRB1*1501/1303*	*4: T-Depleted DRB1*1501/1303*	*5: DR7/DR11 DRB1*1101/07*	*5: T-Depleted DRB1*1101/07*
Inoculum								
MSRV virion from MS	Death with brain haemorrhage	5/5 mice 5–10 days p.i.	5/5 mice 5–10 days p.i.	5/5 mice 5–10 days p.i.	4/4 mice 5–10 days p.i.	1/4 mice 5–10 days p.i.	4/4 mice 5–10 days p.i.	0/4 mice alive > 2 mo.
Choroid Plexus cultures	MSRV RT-PCR	+	+	+	*ND*	*ND*	*ND*	*ND*
MSRV virion from MS	Death with brain haemorrhage	5/5 mice 5–10 days p.i.	5/5 mice 5–10 days p.i.	5/5 mice 5–10 days p.i.	4/4 mice 5–10 days p.i.	0/4 mice alive > 2 mo.	4/4 mice 5–10 days p.i.	1/4 mice 5–10 days p.i.
B-Cell cultures	MSRV RT-PCR	+	+	+	*ND*	*ND*	*ND*	*ND*
Mock virion from Non-MS	Death with brain haemorrhage	0/5 mice alive > 3 mo.	0/5 mice alive > 3 mo.	0/5 mice alive > 3 mo.	0/4 mice alive > 2 mo.	0/4 mice alive > 2 mo.	0/4 mice alive > 2 mo.	0/4 mice alive > 2 mo.
B-Cell cultures	MSRV RT-PCR	≈	≈	≈	*ND*	*ND*	*ND*	*ND*
PBS alone	Death with brain haemorrhage	0/4 mice alive > 3 mo.	0/4 mice alive > 3 mo.	0/4 mice alive > 3 mo.	0/3 mice alive > 2 mo.	0/3 mice alive > 2 mo.	0/3 mice alive > 2 mo.	0/3 mice alive > 2 mo.
	MSRV RT-PCR	≈	≈	≈	*ND*	*ND*	*ND*	*ND*

Note. Hu-PBL-SCID mice were inoculated intraperitoneally in parallel with (i) MSRV virion preparations from two different cell cultures (choroid plexus and B cells); (ii) mock virion from non-MS B cells (series including non-MS choroid plexus mock preparation are not presented in this table because complete (series only) are considered here); and (iii) PBS used to resuspend virion or mock virion ultracentrifugation pellets. Three successive series of experiments are grouped: first series correspond to donors 1 and 2 with identical DRB1 phenotypes; second series correspond to donor 3; third series correspond to donors 4 and 5 with parallel T-cell depletion on nylon membranes of PBMCs before grafting. When mentioned, the period of death corresponds to that of animals that were not sacrificed before the fatal outcome. Similarly, the period during that mice were kept and survived in a healthy state excludes animals that were sacrificed in parallel with ill ones, for comparative histopathological examination. In addition, all surviving animals from control series were sacrificed after the period mentioned in the table and necropsied. None of such mice presented pathological signs at necropsic examination.
ND: not done.

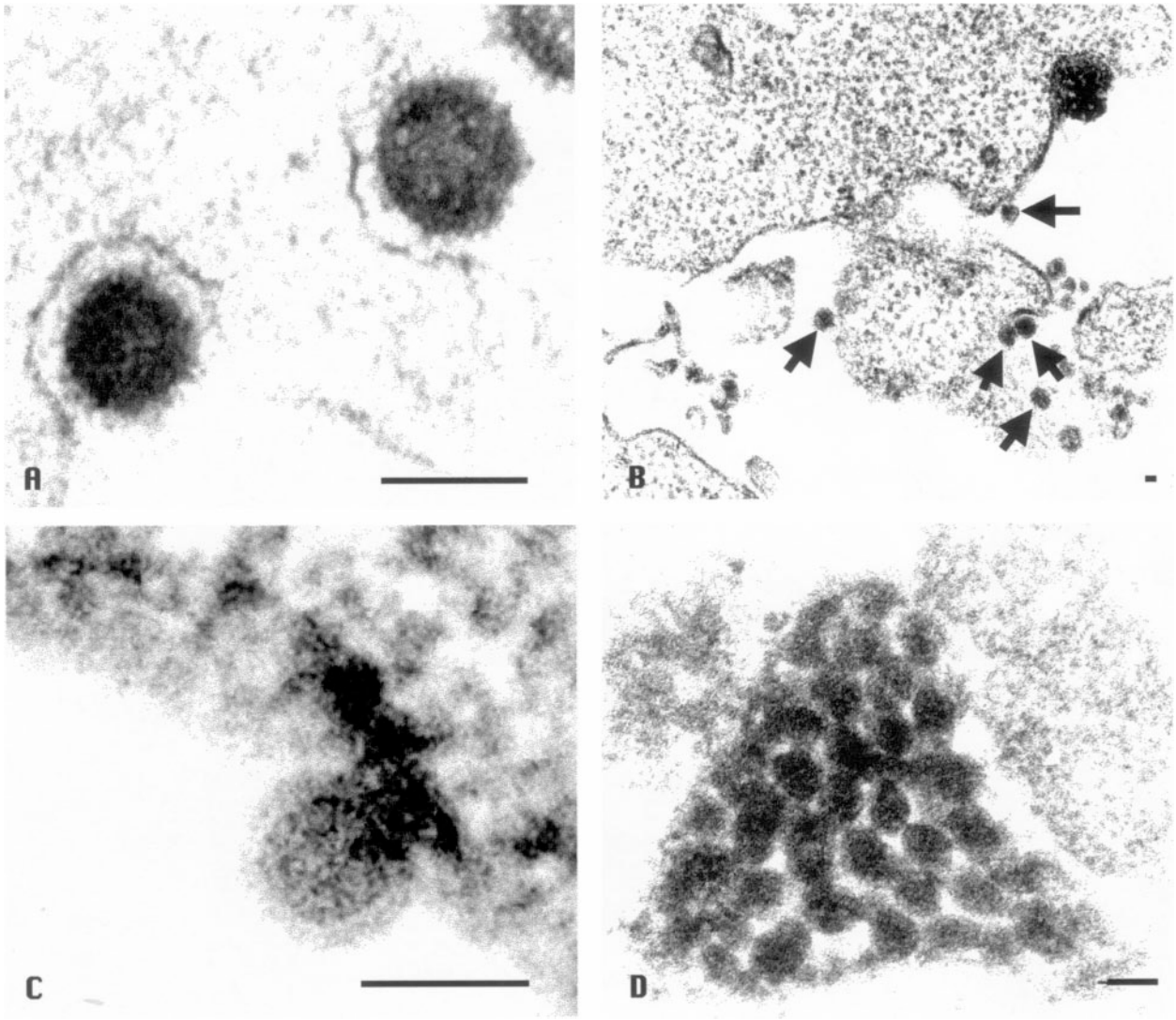

Figure 2 Retroviral particles (MSRV) in multiple sclerosis cell cultures. Electron microscopy. No retroviral particle could be seen either in the cytoplasm or at the cell surface in control B-cell or choroid plexus (leptomeningeal cells) cultures. In MS cultures studied over a 3-week period, retrovirus particles in isolated cells were observed one or two times corresponding to peaks of RT activity. Long periods of observation were necessary for such findings. Extracellular particles were observed at the surface of the cytoplasmic membrane in isolated cells, whereas clusters of retrovirus-like particles in vacuoles were also occasionally seen. (**A**) Extracellular virion particles at the surface of the cell membrane; MS leptomeningeal cells. (**B**) Arrows indicate retrovirus-like particles at the surface of the cell membrane or in the extracellular space; MS B-cell culture. (**C**) Extracellular virion particle at the surface of the cell membrane; MS B-Cell culture. (**D**) Cluster of electron-dense retrovirus-like particles (capsids) within an MS B cell. The bar represents 100 nm.

Control mice were sacrificed in parallel at the same day, and others were kept in order to estimate their survival time.

As shown in Figure 3, dissection of mice regularly showed a marked splenomegaly and relative hepatomegaly in MSRV virion–inoculated mice, whereas spleen from controls only reflected successful humanization with human lymphoid cells. Surprisingly, no other significant finding was made either in the abdominal cavity or in the chest. When the cranial cavity was dissected, we were greatly surprised to observe macroscopic and frequently massive brain hemorrhages in all dead MSRV-injected animals. As shown in Figure 4, displacement of the brain sometimes revealed massive hemorrhage with blood collected at the bottom of the cranial cavity, thus strongly suggesting that these animals could have died from cerebral compression as well as from the cerebral hemorrhage itself. This was not seen in control animals (mock-infected or PBS-inoculated), either sacrificed on the day when another mice died in infected series, or sacrificed after 2 or 3 months of survival. In ill mice sacrificed before death, hemorrhage appeared less important at the brain surface but mostly as disseminated patches at the meningeal surface.

In order to confirm the circumstances in which these brain hemorrhages occurred, the animals from the last series were formalin-fixed immediately

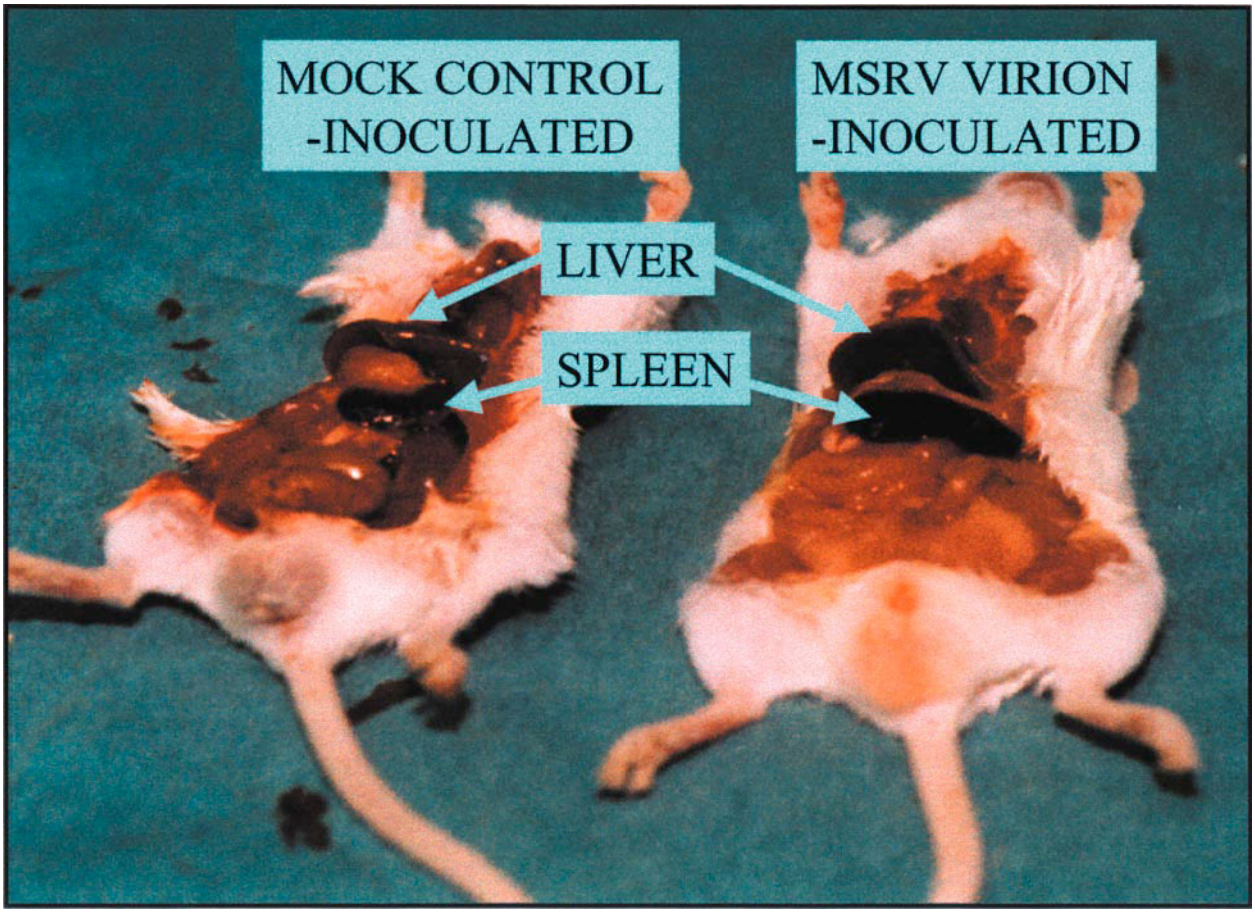

Figure 3 Autopsic examination: Abdomen and chest. Parallel dissection of dead animals from the virion-injected series and mice sacrificed the same day in mock-infected series revealed marked splenomegaly and relative hepatomegaly only in MSRV + hu-PBL-SCID mice.

after death without dissection and examined by a neuropathologist expert (Prof. J. J. Hauw, l'Hôpital Salpétrière, Paris, France).

As shown in Figure 5, autopsic examination performed in appropriate conditions confirmed the absence of visible abnormalities in control brains (Figure 5**A**), the presence of hemorrhagic patches at the brain surface (upper or lower side) of virion-injected animals sacrificed prior to death when neurological symptoms were detected (Figure 5**B, C**), and the presence of massive hemorrhage involving large portions of the brain with important blood leakage in the cranial cavity, after death (Figure 5**D–F**).

Neurohistological examination of brain sections from these brains (Figure 6) confirmed that the hemorrhagic phenomenon was not artefactual and occurred before death. Leptomeningeal (Figure 6**A**) as well as intraparenchymal (Figure 6**B, C**) hemorrhage was observed and anoxi-ischemic neurons were found in gray matter as well as multifocal necrotic areas (Figure 6**C, D**). These features indicate that corresponding hemorrhages must have occurred at least 6 h prior to death.

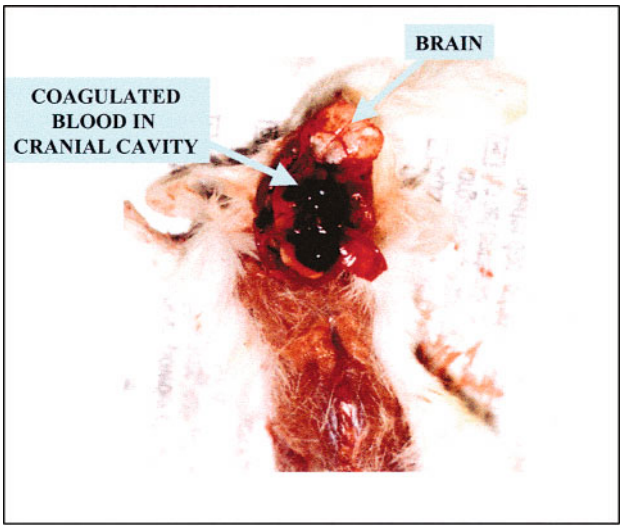

Figure 4 Autopsic examination: Head. Dissection of dead animals from the virion-injected series revealed major brain hemorrhages, with massive blood leakage into the cranial cavity in MSRV + hu-PBL-SCID mice. Parallel necropsy of control mock-infected hu-PBL-SCID mice was normal.

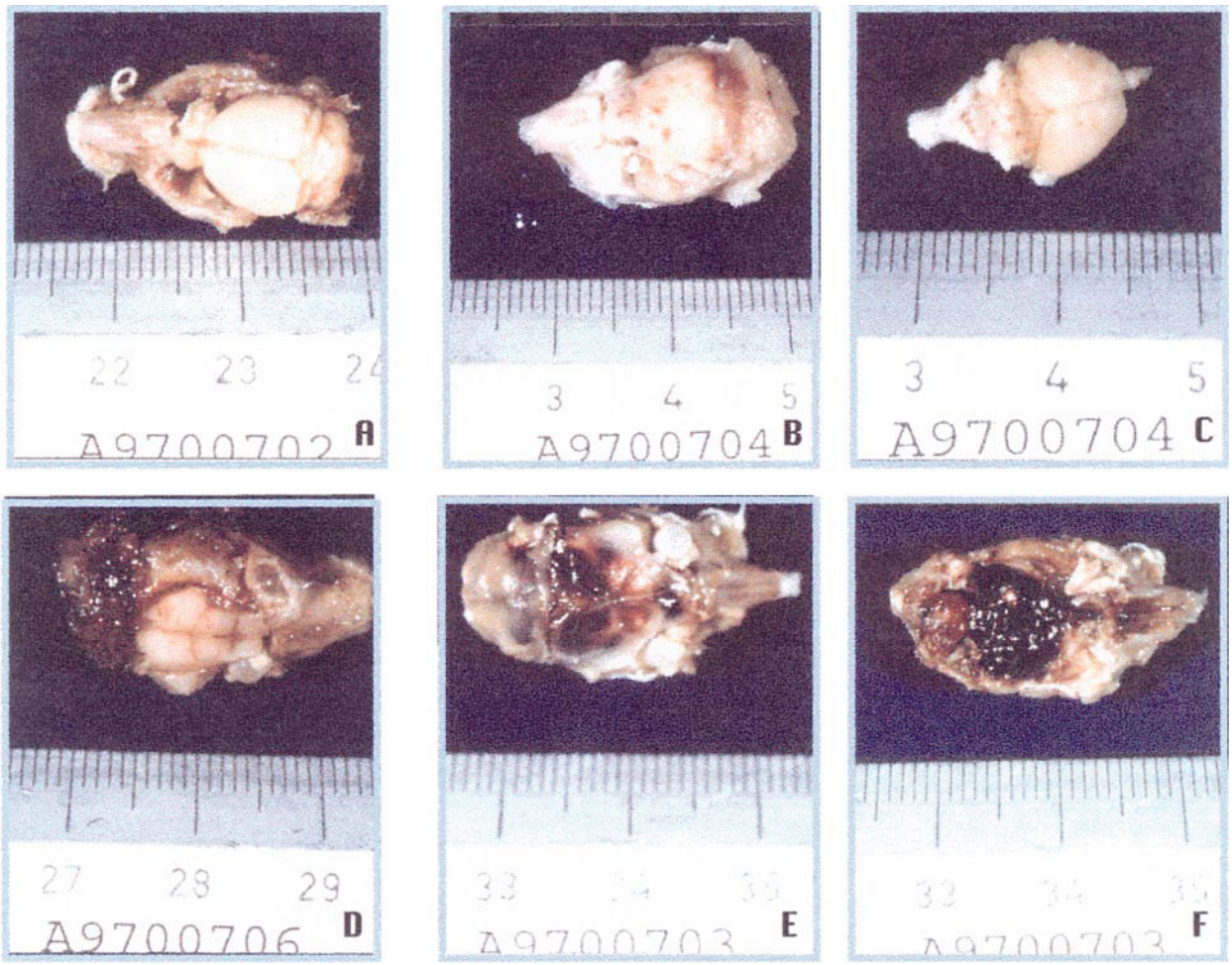

Figure 5 Neuropathological examination: Formalin-fixed heads. Dead animals from the virion-injected series and mice sacrificed the same day in mock-infected series were formalin-fixed prior to dissection. (**A**) Mock-infected control. No abnormality on the brain or in head structures can be noticed. (**B**, **C**) MSRV virion–injected hu-PBL-SCID mice presenting neurological symptoms (marked paralysis of hind legs and tremor) sacrificed before death. Multiple petechia can be seen all over the surface of the brain, at the level of the meninges; **B**, top side; **C**, bottom side. (**D**) MSRV virion–injected hu-PBL-SCID mice fixed after death. A major meningeal hemorrhage can be seen in the posterior part of the brain. (**E**, **F**) MSRV virion–injected hu-PBL-SCID mice fixed after death. An important hemorrhage is seen at the surface of the brain (**E**) and blood collected in the bottom of the cranial cavity is seen after displacement of the brain (**F**). The features observed in this case are clearly similar to the one (non-formalin fixed) shown in Figure 4.

Pathogenicity correlates with overexpression of proinflammatory cytokines in spleen

Because we had observed *in vitro* that MSRV virions induced proinflammatory cytokine production in association with polyclonal expansion or depletion of human T lymphocytes, mainly skewed towards Vμ16 subpopulation, we have analyzed these parameters by quantitative reverse transcriptase–polymerase chain reaction (RT-PCR) in RNA extracted from $\approx$80°C frozen spleen samples.

As shown in Table 2, the level of human T cells detected in control mice spleen was either below the detection limit or rather low, whereas it was significantly elevated in virion-injected mice. Human tumor necrosis factor (TNF)-μ and interferon (IFN)-μ RNA were not detected in spleen RNA from any control (including unstimulated normal human peripheral blood lymphocyte [PBL] cDNA) but were readily detected at significant levels in virion-injected ill mice.

In Table 3, human T-cell receptor (TCR) Vμ RNA was under the level of detection in control mice. In spleen from ill mice, all Vμ tested were detected as in normal human PBL cDNA but, interestingly, Vμ 16 remained selectively undetectable.

In Figure 7, the length polymorphism of human TCR Vμ transcripts was plotted and reflects the clonal diversity of each subpopulation tested, as previously described (Garban *et al*, 2000). As expected, the gaussian distribution found in phytohemaglutinin A (PHA)-stimulated normal human PBLs reflects a polyclonal T-cell expansion. The baseline signal in

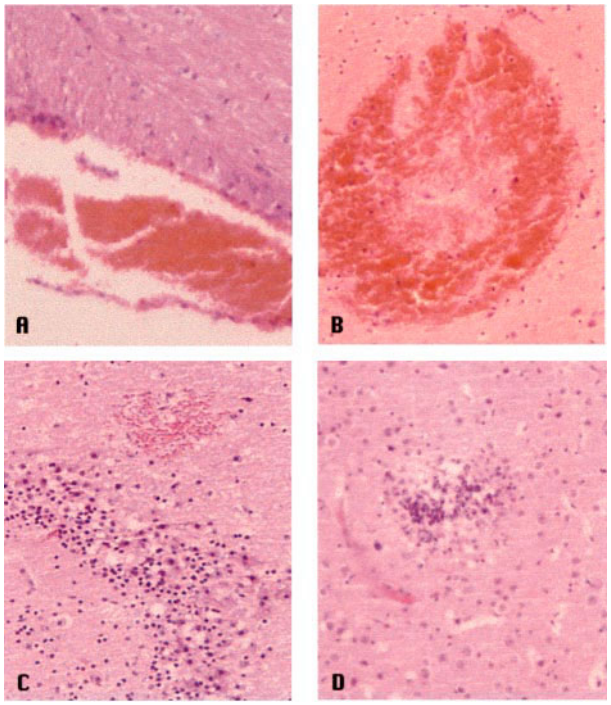

Figure 6 Neuropathological examination: Hematoxylin-eosin coloration of fixed brain sections. (**A**) MSRV virion–injected hu-PBL-SCID mouse no. A97003 (corresponds to Figure 5**D**). Leptomeningeal hemorrhage on the cortex surface. (**B–D**) MSRV virion-injected hu-PBL-SCID mouse no. A97004 (corresponds to Figure 5**B**, **C**). (**B**) Intraparenchymal hemorrhage in the cerebellum. (**C**, **D**) Intraparenchymal hemorrhage in the cortex with focal zone of necrosis. Anoxi-ischemic neurons (appeared >6 h before death) with few apoptotic-like cells can be seen.

control mice corroborates the undetectable level of human T-lymphocyte RNA within these spleens, as observed previously.

Interestingly, virion-injected mice showed a wide polyclonal Vμ 17 expansion, an oligoclonal Vμ 2 population, and as also shown in Table 3, a selective depletion of the whole Vμ 16 subpopulation.

Table 2 Quantitative real time RT-PCR of TCRμ, CD4, CD8μ, IFN-μ, and TNF-μ transcripts in spleen samples of different series of humanized mice

	Cytokines and T-cells RNA				
	CD4	*CD8*	*TCR A C*	*TNF-μ*	*INF-μ*
MSRV-Virion	4.01	1214	10000	91	120
Mock-Virion	0	0	0	0	0
human PBL cDNA	34550	24970	409600	0	0
mouse PBL cDNA	3.5	0	73	*ND*	*ND*

Note. cDNA from one representative spleen sample of each condition tested in donor 2 series was amplified using oligonucleotide specific for the different transcripts analysed. Amplification was followed in real time on a LightCycler. Quantification of transcripts was expressed as copy number of target molecule. Results were confirmed for two to three individuals and three independent quantitative assays.
CD4: human T-helper lymphocytes; CD8: human T-cytotoxic lymphocytes; TCR: T-cell receptor; IFN-μ interferon gamma; TNF-μ: tumour necrosis factor alpha.

Table 3 Summary of human TCRμ chains found in spleen of humanized SCID mice inoculated with MRSV virion or mock preparation

	Human PBL-SCID mice injected intraperitoneally with:			
TCR Vμ	*huPBL cDNA*	*MSRV-virion*	*Mock-virion*	*Control PBS*
2	+	+	≈	≈
7	+	+	≈	≈
14	+	+	≈	≈
16	+	≈	≈	≈
17	+	+	≈	≈
4	+	+	≈	≈
12	+	+	≈	≈

Note. The TCRμ transcripts analyzed are listed. Results were confirmed for two individual mice (in donor 2 series) and two independent amplification. + = detected; ≈ = nondetected.

Pathogenicity reveals mediated by T cells

In such retroviral systems, the immune response plays an important role in pathogenicity (Choi *et al*, 1992; Marrack *et al*, 1991; Myer *et al*, 1988; Ortin *et al*, 1998; Rudge, 1991). Therefore, in order to further evaluate the relative contributions of the virus infection versus the immune response, we have grafted total PBMCs from two donors in parallel with an equivalent number of cells from the same PBMC preparation, but previously depleted in T lymphocytes on nylon membranes. Control cytofluorometric analyses confirmed the efficient T-cell depletion in the PBMC graft (≈80% depletion, compared to non-depleted PBMCs), and even increased depletion in the resulting graft after 5 days in Hu-PBL-SCID mice (≈95%, when compared to mice grafted with non–T-depleted PBMCs).

As shown in Table 1, this last series with donors 4 and 5 reproduced the previous observations in mice grafted with total PBMCs. However, a high rate of survival was observed among mice grafted with T-depleted PBMCs from the same donors (and from the same samples). All mice grafted with total PBMCs from both donors and injected with MSRV virion from both sources died with brain hemorrhage within 2 weeks post injection. In parallel, only 10% of virion- injected mice with T-depleted grafts (one with each donor) died and showed less important hemorrhage than in previous cases. In parallel, control mice—with mock virion or PBS—showed survival similar to previous series.

Discussion

The purpose of this study was a preliminary evaluation of an *in vivo* experimental pathogenicity associated with retroviral particles (MSRV) produced from MS cell cultures, after having provided evidence for

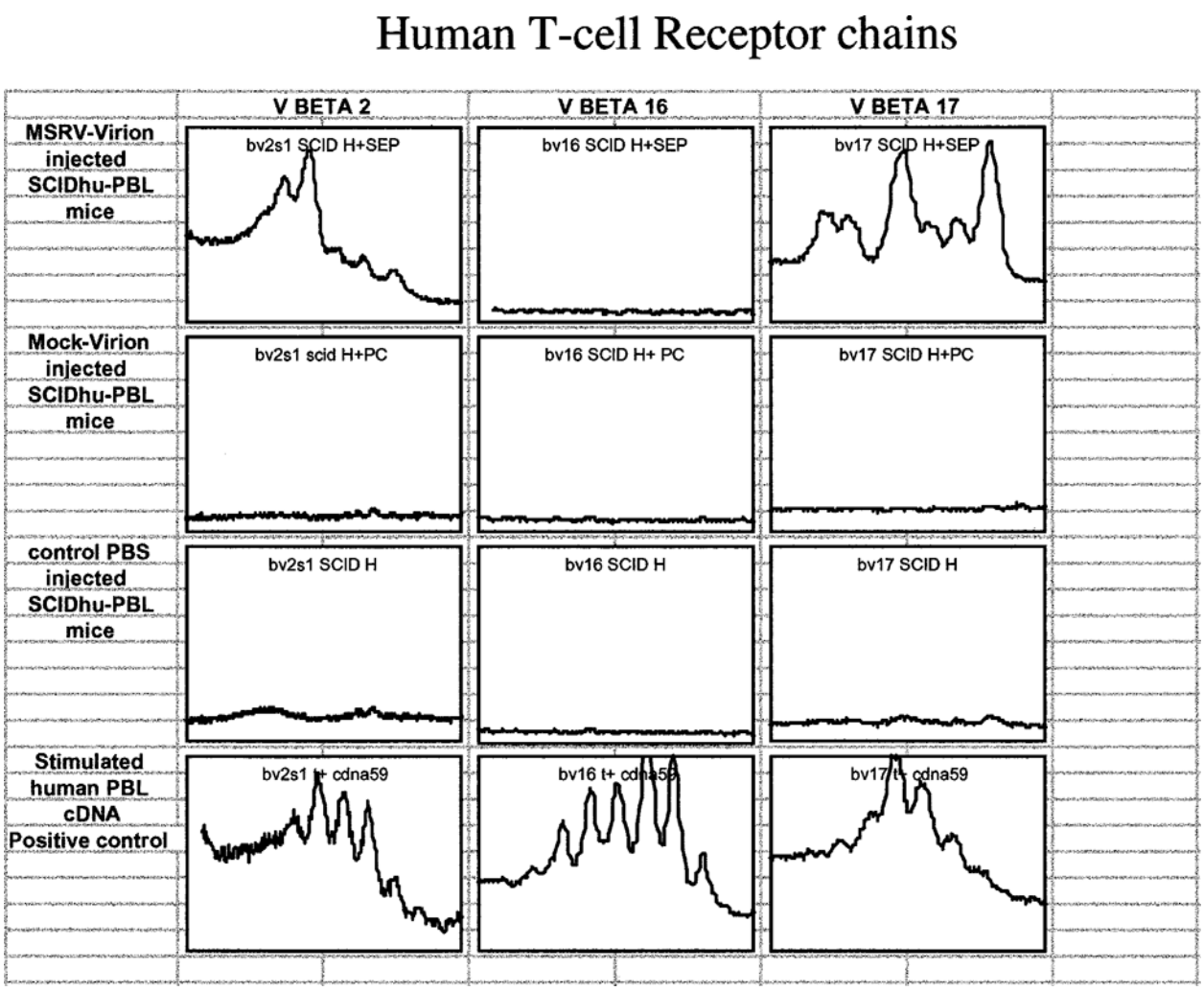

Figure 7 Representative profiles of the TCRμ chain diversity in spleen of humanized SCID mice injected with MRSV virion or mock preparation. TCRμ transcripts were amplified using the BV primers indicated and a common BC primer. RT-PCR products were labeled using an internal Texas Red primer. The graphs represent the intensity of fluorescence in arbitrary units (*y* axis) as a function of CDR3 length of BV-BC elongation products (*x* axis). Each peak corresponds to a CDR3 length.

an *in vitro* pathogenicity on human PBLs compatible with superantigen activity (Lafon *et al*, 2002; Perron *et al*, 2001).

The results from the present study show that an obvious pathogenicity is associated with MSRV particles inoculated IP to Hu-PBL-SCID mice. The observed pathogenicity is clinically characterized by an acute neurological impairment followed by death of animals, by massive brain hemorrhage, by overexpression of proinflammatory cytokines, and by human T-cell expansion and selective depletion in parallel. This pathogenicity appears not to be influenced by the HLA-DRB1 type of the PBMC donors represented in this series nor by the source of MS retroviral particles—choroid plexus or B-cell cultures. The short delay of onset, the particular features of the lesions, and, before all, the complete absence of symptoms in control animals grafted in parallel with the same PBMCs, rules out any deleterious "graft-versus-host" reaction (GvHR).

Brain pathology

Surprisingly, though the virion was injected intraperitoneally, no observation made in the abdominal and chest cavities could explain the systematic death of the animals in the infected series. The splenomegaly, noticeable relatively to control Hu-PBL-SCID mice, obviously reflects hyperstimulation and proliferation of the human lymphoid cells, which has definitely been confirmed by molecular analysis of human specific T-cell RNA in virion-injected hu-SCID spleen. Brain necropsy was convincing and could explain both neurological signs preceding death and the death itself, as a consequence of brain hemorrhage and/or cerebral compression by blood occuring prior to death. These features could indicate a particular neurotropism of this retrovirus. However, the rapid and systematic apparition of symptoms followed by the death of Hu-PBL-SCID mice and the apparent inocuity of these retroviral particles in nonhumanized SCID mice inoculated

for our preliminary evaluations were not a priori in favor of an explanation based on neurotropism and interspecies retrovirus transmission. More notably, the role of grafted human T cells appeared decisive.

T cell–mediated pathogenicity

Given the low RT activity of our virion samples and the estimated low number of particles injected (approximately 10^4), we were greatly surprised to obtain such a dramatic effect in this model. We consequently suspected an immunopathological amplification as encountered with superantigens or other immunopathogenic molecules, known to be produced by bacteria or by such retroviral elements (Choi *et al*, 1991; Kotzin *et al*, 1993; Kubo *et al*, 1996; Lafon *et al*, 1992; Marrack *et al*, 1991; Marrack *et al*, 1993; Pullen *et al*, 1992; White *et al*, 1989). We already had the *in vitro* confirmation that superantigen effects were associated with MSRV virions and revealed to be induced by MSRV-encoded envelope protein itself (Lafon *et al*, 2002; Perron *et al*, 2001). The limited number of blood donors who gave the PBMCs used for mice immunization provides few different HLA haplotypes, as indicated in Table 1, but no major difference in the results was noticed between the represented DR specificities. This is in agreement with the significant data obtained *in vitro* on a larger pannel of HLA DR phenotypes, which indicated a HLA DR-independent polyclonal activation of Vμ 16 T cells by MSRV virions, compatible with a superantigen activity (Perron *et al*, 2001).

Indeed, our results with T-depleted human lymphoid grafts confirmed the predominant (if not sole) contribution of human T cells to the pathogenic effects observed in our model. Because T cell depletion—though rather drastic—was never absolute, the existence of residual and attenuated pathogenicity (12%) in Hu-PBL-SCID with T-depleted grafts can be considered as logically occurring. The difference between T-depleted and non-depleted series is clearly significant (2/16 versus 16/16; chi-square test with Yate's correction for low numbers: 21.5; $P < .001$). We can therefore consider that grafted human T cells are mediating this dramatic effect of MS retroviral particles on SCID mice brains. Given results from molecular analysis of TNF-μ RNA transcripts in hu-SCID spleen, such hemorrhagic effects are likely to result from an immune amplification phenomenon causing huge proinflammatory cytokine production in the bloodstream, as observed in septic shocks caused by superantigens (Bernal *et al*, 1999; DeWinter *et al*, 1999; Krakauer, 1999; Muraille *et al*, 1999; Schlievert *et al*, 2000). Interestingly, TNF-μ has been directly involved in hemorrhages targeting the brain endothelium in cerebral malaria (Porta *et al*, 1993; Turner, 1997), though it revealed not sufficient by itself to induce similar pathogenicity in healthy animal models (Kido *et al*, 1991). A particular association between infected blood cells and high TNF-μ levels in the case of cerebral malaria is likely account for the brain endothelial cell tropism and the hemorrhagic phenomenon (Porta *et al*, 1993). Such pathogenic mechanisms are likely to be relevant in our observations.

Nonetheless, we cannot exclude a possible contribution of a gliotoxic factor, previously described from human MS macrophages expressing MSRV particles and RT activity (Menard *et al*, 1997), which was also shown to cause blood-brain barrier (BBB) breakdown in Lewis rats (Rieger *et al*, 1996). This parameter could not, however, be adequately dosed in hu-SCID mouse sera with our present bioassay technique (Malcus-Vocanson *et al*, 2001).

Consequently, from the results of both *in vitro* (Lafon *et al*, 2002; Perron *et al*, 2001) and *in vivo* (present report) studies, we can now assume that these retroviral particles from MS cultures have potent immunopathogenic properties, mediated by T cells, involving an MSRV envelope protein displaying superantigen-like effects on T cells and causing oversecretion of proinflammatory cytokines such as TNF-μ. This immune-mediated inflammatory pathogenicity may cause BBB disruption when certain cytokine levels are reached after systemic virion inoculation, as in this model. Alternatively, the gliotoxic factor coexpressed with MSRV virions in human macrophages from MS patients (Menard *et al*, 1997) may account—at least partially—for this MSRV-induced brain hemorrhage; but, in this case, the effect of T-cell depletion would mean that gliotoxin production from MSRV-expressing macrophages would require T-cell presence and/or that gliotoxin has synergistic effects on BBB with T-cell activation products such as IFN-μ. The ongoing molecular characterization of this gliotoxin (Malcus-Vocanson *et al*, 2001), and our efforts for setting up its immunodosage with appropriate monoclonal antibodies, may help addressing this particular point in further hu-SCID series.

Material and methods

Choroid plexus cell cultures for virion or mock preparations

Choroid plexus (CP) cells from two MS patients and one control without neuropathological abnormalities at necropsy were obtained from the brain-cell library (Prof. Hauw), Hôpital de la Salpêtrière, Paris, France. CP cells were cultured as previously described (Perron *et al*, 1991a, 1997a) with rabbit polyclonal antibody against leucocyte-produced interferon (Sigma), at a final neutralizing activity of 100 U/ml in the culture fluids, fresh medium added at each renewal of the corresponding culture medium. These cultures were controlled by PCR for Epstein-Barr virus (EBV) genome detection and were confirmed negative. CP cultures from MS patients, but not from the non-MS control, produced retroviral

particles as already described (Perron *et al*, 1991a, 1997a).

B-cell cultures for virion or mock preparations
Blood from patients with definite MS were obtained from Grenoble University Hospital, Department of Neurology (Prof. Pellat). Blood from healthy controls was obtained from blood transfusion center (EFS) in Lyon (Dr. Gebuhrer). B-cell cultures from MS patients, but not from the non-MS control, produced retroviral particles as already described (Perron *et al*, 1997a, 1997b).

The protocol used for the establishment of EBV-transformed B-cell lines, either from MS patients or healthy controls, was as follows.

Human lymphocytes from heparinized blood diluted 1:2 with RPMI 1640 separated by Ficoll density gradient centrifugation were collected from the buffy coat and from occasional cellular aggregates floating underneath. Devices with filters or separating membranes were avoided. Cells were washed twice in RPMI 1640 and resuspended to $2 \approx 10^6$ cells/ml in RPMI 1640 with 200 U/mL penicillin, 200 mg/L streptomycin, 6 mM L-glutamin, 1% sodium pyruvate, 1% nonessential aminoacids, and 20% heatin-activated fetal calf serum (FCS). The cell flasks were incubated at 37℃ and inoculated with 1 ml (10^5 viral particles for [4–5] $\approx 10^6$ total lymphocytes) of filtered supernatant from the EBV B95-8 productive culture in the presence of 200 μl (2 μg cyclosporin A for [4–5] $\approx 10^6$ total lymphocytes) of Cyclosporin A (Sandoz) for 3 to 5 days. Medium was then changed twice a week with same medium supplemented with rabbit polyclonal antibody against leucocyte-produced interferon (Sigma), at a final neutralizing activity of 100 U/ml. Cells were maintained in the original flask until a significant number of proliferating clones formed aggregates and were further passaged after mechanical dissociation with a split ratio of 1:2. All the cell lines were obtained by immortalization with the same EBV B95-8 strain, cultured in the Department of Virology (Prof. J. M. Seigneurin), CHU, Grenoble. In control analyses, SMRV-H (Squirrel Monkey RetroVirus, human isolate) sequences, which can be found integrated in the B-95 EBV genome as described Sun *et al* (1995), were searched for by PCR with two pairs of specific SMRV-H primers in the different B-cell cultures used in this study, as well as in B-95 B-cell culture used for the production of immortalizing EBV virion. No amplification was obtained in any case, thus excluding such retroviral contamination. Possible HHV-6 coinfection of B-cell cultures was also searched for by PCR with human herpesvirus 6 (HHV-6)-specific primers (Wilborn *et al*, 1994), but negative results were obtained in all cultures. The constant absence of mycoplasma was confirmed in all cultures with an enzyme-linked immunosorbent assay (ELISA) detection kit (Roche).

RT activity
For RT-activity measurement, 30 ml of culture supernatants were centrifuged, first at 3000 rpm for 30 min at 4℃ to eliminate the cell debris, and then for 1 h 30 min at 3,5000 rpm at 4℃. The pellets were resuspended in 100 μl 0.05 M pH 8.3 Tris-HCl and a 50-μl aliquot was used for RT-activity test as previously described (Perron *et al*, 1993). The cut-off value was calculated in order to discriminate specific activity from background signal and represents the mean value plus 3 standard deviations of all points obtained from control cell lines.

Virion preparation from either CP or B-cell culture supernatants
Virion (from previously characterized virion-producing MS cultures) as well as control preparations (from previously characterized negative-control cultures) were similarly prepared. All culture media (obtained from B-cell and CP cultures at passages 10 to 20) were changed and collected twice a week, centrifuged at 3000 rpm for 30 min, and frozen at ≈80℃ after addition of glycerol (10%). RT activity was measured in culture supernatants as previously mentioned. Supernatants were pooled in order to obtain a homogeneous preparation of about 500 ml from either MS B cells, MS CP, control B cells, or control CP. One large-volume fixed-angle rotor was used for each homogeneous batch that was distributed in polycarbonate tubes. Five milliliter, of a "cushion" consisting of PBS buffer with 30% glycerol was deposited at the bottom of each tube. The supernatants were ultracentrifuged at $100{,}000 \approx g$ for 2 h and a 30-min period of slow deceleration. Pellets were collected from each tube, resuspended in 100 μl of PBS buffer, pooled, and gently homogeneized with glycerol (10%, final concentration). Pooled pellets were aliquoted (100 μl), stored at ≈80℃, and further used for inoculation in normal PBLs ($100\ \mu l/2 \approx 10^6$ cells). Importantly, all freezing steps used glycerol in order to obtain a sufficient proportion of intact viral particles (sucrose gradients were avoided).

Transmission electron microscopy
From MS cultures 10^7 cells were collected and fixed for 30 min with 4% glutaraldehyde prepared in 0.2 M cacodylate buffer (pH 7.4). Cells were rinsed 3 times in a solution (*v*/*v*) of 0.2 M cacodylate (pH 7.4) and 0.4M saccharose, post-fixed in a solution (*v*/*v*) of 2% osmium and 0.3 M cacodylate (pH 7.4) for 30 min at 4℃ and rinsed in distilled water. For dehydrating, cells were treated with graded ethanol series. The substitution, impregnation, and inclusion were made in Epon for 3 days. Sections were stained with uranyl acetate and subsequently examined with an electron microscope Jeol 1200 EX (Tokyo, Japan).

Protocol for the preparation of Hu-PBL-SCID mice
This protocol, which corresponds to previously described Hu-PBL-SCID model preparation (Chargui

et al, 1995; Sanhadji *et al*, 2000), is presented in Figure 1, with conditions indicated for each step.

Human blood was obtained from healthy blood donors and was HLA-typed in the histocompatibility laboratory of the blood transfusion center (EFS, Lyon). PBMCs were separated by centrifugation on Ficoll gradients, as previously described for B-cell cultures, but were not cultured and were used directly for mice humanization within a 2 to 3-h delay. In these conditions, MSRV particle production has never been detected in primary PBMC preparations from healthy blood donors.

MSRV RT-PCR

RT-PCR was performed according to the protocol already described by Garson *et al* (1998) on serum obtained from cardiac blood puncture in dead or sacrificed animals. This nested RT-PCR protocol did not allow quantitative estimations of virion load in tested mice.

Cytokine and T-cell receptor Vμ chain RT-PCR on spleen cells

RNA extraction and cDNA synthesis: Total RNA was extracted from frozen spleen using a acid guanidium thiocyanate/phenol/chloroform method (RNA-NOW kit; Biogentex-Ozyme, Montigny-le Bx, France) according to the the manufacturer's instructions. cDNA synthesis was performed with 1 μg of total RNA using the SuperScript II Reverse transcriptase kit (Life Technologies–Gibco BRL, Cergy Pontoise, France).

Primers: Primers pairs used in CDR3 analysis as previously described in Even *et al* (1995). Primers pairs used in PCR quantitative assays were designed using Primers Select software (DNA STAR Inc, Lasergene, Madison, USA) and were synthesised by Genset (France). The primers were designated to amplify specifically human TCRμ, CD4, CD8μ INF-μ, and TNF-μ transcripts and were chosen in two independent exons in order to avoid amplification of putative DNA contamination in RNA preparations. For TCRμ oligonucleotide sequences were as previously described (Jouvin-Marche *et al*, 2001), the remaining sequences were as follows (5′ → 3′):

CD4
sense: ACCGAAGGCGCCAAGCAGAG
antisense: GAGCAGTGGGGAGAGGGTCAGAGA

CD8
sense: GGGGCGGGGTGGGAAAGATTA
antisense: AGACAGGGGCCTCGGAAAGAAAGAC

INF-μ
sense: AGCTCTGCATCGTTTTGGGTTCT
antisense: ACACTCTTTTGGATGCTCTGGTCA

TNF-μ
sense: AGGGCTCCAGGCGGTGCTTGTT
antisense: ACGGCGATGCGGCTGATGGT

Real time quantitative RT-PCR assay: Real time PCR was carried out with the LightCycler system (Roche Molecular Biochemicals, Mannheim, Germany) using the following reaction mixture: 1≈ of LightCycler DNA master SYBRGreen I, 22 ng of TaqStart Antibody (TaqStart Antibody, Clontech), 3 mM of $MgCl_2$, 0.5 μM of each primers, and 2 μl of cDNA preparation or DNA external standards appropriate dilutions, in a total volume of 20 μl. After an initial denaturation at 95℃ for 2 min, amplification were cycled 38 to 42 times with a 95℃ denaturation for 5 s, annealing as follows: 60℃ 10 s for CD4 amplification, 62℃ 10 s for CD8 amplification, 65℃ for INF-μ amplification, 69℃ 10 s for TNF-μ, and elongation at 72℃ ranging from 10 to 18 s according to the size of the target sequence. Product specificity was determined by melting curve analysis as described in the LightCycler handbook and visualization of PCR product on 1.5% agarose gels staining with ethidium bromide. The TCRμ, CD4, CD8μ, IFN-μ, and TNF-μ quantification of transcripts were made with a DNA external standard generated by PCR as described in Jouvin-Marche *et al* (2001).

T- lymphocyte repertoire analysis

TCR diversity was analyzed by monitoring the junctional variability of μ-chain transcripts as previously described (Pannetier *et al*, 1995; Perron *et al*, 2001). Briefly, cDNA was amplified using BV and BC specific primers over 35 cycles: 94℃ 30 s, 60℃ 30 s, 72℃ 1 min, with a GeneAmp PCR System 9600 (Perkin Elmer, Saint-Quentin, France). Amplified products were used as template for an elongation reaction with Texas-Red labelled internal BC primer. CDR3 size distribution determination of BV-BC products was performed using ImageQuaNT and FragmentNT software (Amersham Pharmacia Biotech, Orsay, France).

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