Long-term human coronavirus-myelin cross-reactive T-cell clones derived from multiple sclerosis patients

Annie Boucher [a], Marc Desforges [a], Pierre Duquette [b], Pierre J. Talbot [a],*

[a] *Laboratory of Neuroimmunovirology, INRS-Institut Armand-Frappier, 531, boulevard des Prairies, Laval (Québec), Canada H7V 1B7*
[b] *MS Clinic, Hôpital Notre-Dame, Montréal, Québec, Canada H2L 4K8*

Abstract   Autoimmune reactions associated with MS involve genetic and environmental factors. Because murine coronaviruses induce an MS-like disease, the human coronaviruses (HCoV) are attractive candidates as environmental factors involved in a demyelinating pathology. We previously reported the isolation of HCoV-229E/myelin basic protein (MBP) cross-reactive T-cell lines (TCL) in MS patients. To investigate antigenic cross-reactivity at the molecular level, 155 long-term T-cell clones (TCC) were derived from 32 MS patients by in vitro selection with MBP, proteolipid protein (PLP) or HCoV (strains 229E and OC43). Overall, 114 TCC were virus-specific, 31 were specific for myelin Ag and 10 other were HCoV/myelin cross-reactive. Twenty-eight virus-specific TCC and 7 myelin-specific TCC were obtained from six healthy donors. RACE RT-PCR amplification of the Vβ chains of five of ten the cross-reactive TCC confirmed clonality and sequencing identified the CDR3 region associated with cross-reactivity. Our findings have promising implications in the investigation of the role of molecular mimicry between coronaviruses and myelin in MS as a mechanism related to disease initiation or relapses.
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Introduction

Multiple sclerosis (MS) is a chronic central nervous system (CNS) autoimmune disease, characterized by inflammation and T-cell-associated myelin destruction. Precise MS etiology remains unknown, but appears to be polygenic and multi-factorial, potentially involving HLA genes [1] and female hormones [2]. Environmental factors are also thought to act as key players in disease triggering, as there is low MS concordance in identical twins and specific patterns of worldwide disease distribution [3,4]. Even though no microbial agent has consistently been isolated from the tissue of patients, viral infection of the CNS can trigger autoimmune response [5]. Furthermore, exposure to infectious pathogens prior to puberty appears to increase the risk of developing MS, and respiratory tract infections often precede disease exacerbations [6,7]. Several infectious agents have been associated with MS but the direct implication of a viral agent has never been confirmed. By leading to T-cell activation, viruses could act in MS initiation or progression, although immunopathology could occur much later after disease onset [8] as strongly suggested in a model using SJL/J mice, where

* Corresponding author. Fax: +1 450 686 5566.
  E-mail address: Pierre.Talbot@iaf.inrs.ca (P.J. Talbot).

it is believed that virus infections can prime animals for the development of CNS autoimmunity that may appear long after the infection has been cleared [9]. Therefore, clinical associations between viral infections and the triggering or exacerbation of MS remain difficult to prove and poorly understood. A very good model for the study of viral etiological agents potentially associated with autoimmune neurological diseases is the Mouse Hepatitis Virus (MHV) infection of the CNS, which triggers a neurological disease similar to MS in genetically susceptible rodents [10]. Furthermore, Human Coronaviruses (HCoV), which are responsible for up to one third of common colds [11], are also neuroinvasive [12,13] and neurotropic [14−16], enhancing the potential relevance of their association with neuropathologies such as MS [15]. We therefore investigated HCoV as one possible etiological agent of MS.

Pro-inflammatory CD4 (+) Th1-helper cells reactive to myelin Ag are considered one of the key players in the induction and perpetuation of MS [17]. The primary activation or reactivation of autoreactive T cells has been explained in various ways: (1) Ag-independent activation of mature CD4 (+) T cells induced by dendritic cells that could shape the cytokine milieu, favoring autoimmune states such as MS [18], (2) superantigen binding to given TCRVβ families and activating non-specific family restricted T-cell proliferations [19], (3) epitope spreading associated with collateral inflammation and bystander activation resulting in the release of autoantigens [20] and (4) shared determinants between microbes and self-peptides, also known as molecular mimicry [21]. For neurotropic and neuroinvasive viruses such as HCoV, molecular mimicry provides an elegant framework to explain how cross-reactivity between foreign-Ag and self-proteins may trigger or perpetuate autoimmune diseases on the basis of an appropriate genetic background, Ag presentation and TCR repertoire, as well as earlier microbial encounters [22]. Moreover, sequence homologies between foreign (viral) and self-proteins are not essential for molecular mimicry to be relevant, as it was shown with combinatorial peptides libraries [23].

Even though proof of molecular mimicry as a phenomenon promoting an autoimmune pathology in humans is still controversial, its involvement in the triggering of Lyme arthritis by *Borrelia burgdorferi* [24] and in (HTLV-1)-associated myelopathy/Tropical spastic paraparesis (HAM/TSP) where HTLV-1 infected neurons were targeted by autoantibodies [25] has been suggested. Furthermore, animal models do exist to support pathological relevance of molecular mimicry. For instance, models of virus-infected mice represent examples where autoimmunity seems to be involved with either HSV-1 [26] or TMEV [27] infection.

Potential autoreactive lymphocytes are normally present in an individual, but in a non-activated state [28]. Moreover, T-cell-associated myelin destruction can be observed in association with T-cell activation and inflammation in MS [29] and the myelin-related Ag MBP and PLP were shown to activate T-cell lines in MS patients [30]. Furthermore, our previous work showed that 29% of the peripheral T-cell lines (TCL) derived from 10 out of 16 MS patients and only 1.3%, from 2 out of 14 controls donors, were activated by both HCoV and myelin Ag ($p<0.001$) [30]. Therefore, it appeared essential to characterize cross-reactivity at the clonal and molecular level. Therefore, our long-term goal aims at confirming the clonal origin of the antigenic cross-reactivity between the myelin antigens MBP and PLP and the common cold agents HCoV-OC43 and HCoV-229E, commonly found in the population [11]. Herein, we show that TCC derived from the blood of MS patients can be activated by either viral or myelin Ag and sometimes by both. Cross-reactive TCR were cloned and sequenced to identify the hypervariable CDR3 regions characterizing TCR recognition and establishing clonality. Our results strengthen the hypothesis of a possible coronavirus-induced activation of T-cell autoimmunity by molecular mimicry in MS patients and also provide a framework to explain MS relapses.

Materials and methods

Donors

Patients diagnosed with MS were selected with prior informed consent and approval of the experimental protocol from institutional ethics and scientific committees. Thirty-two patients were recruited at random for this study. Confidentiality was preserved at all times. Patients were not on immune modulating drugs at least 2 months prior the beginning of the study. HLA-typing of MS patients was done at the Histocompatibility laboratory of INRS-Institut Armand-Frappier, Laval, Québec, Canada.

Antigens

Preparation of Ag was performed as described elsewhere [30]. Briefly, human myelin basic protein (MBP) was prepared from a normal human adult brain (Montreal Brain Bank, Douglas Hospital, Verdun, Québec, Canada) [31]. Purity was monitored by SDS-PAGE. Dr. Mario Moscarello (Hospital for Sick Children, Toronto, Canada) graciously provided chromatographically purified proteolipid protein (PLP). HCoV-229E (ATCC VR740) and HCoV-OC43 (VR759) were respectively propagated in the human embryonic lung cell line L132 and in the human colorectal adenocarcinoma cell line HRT-18. Viral Ag was prepared from either 229E-infected L132 cells or OC43-infected HRT-18 cells. Infected cells were lysed in culture medium by three cycles of freezing at −80 °C and thawing at room temperature, followed by centrifugation to harvest the viral Ag-containing supernatant (both viral structural and non-structural proteins), while getting rid of cell debris. The presence of infectious virus was confirmed by titration on susceptible cells. Control Ag was prepared from parallel cultures of mock-infected cells. Viral Ag was prepared by UV-irradiating the whole virus-infected cell lysates (to inactivate infectious virus) and control Ag (mock-infected cultures) was also UV-irradiated.

Generation and maintenance of T-cell clones (TCC)

Competent nurse staff obtained a single blood sample of approximately 400 ml from control subjects and MS patients. PBL were then separated on Ficoll/Hypaque (Pharmacia) gradients, resuspended at a concentration of 1×10^6 cells/ml and seeded in 96-well flat-bottom plates (ICN) in a total volume of 200 μl of complete medium: RPMI 1640 (Invitrogen)

with 50 U/ml of penicillin and streptomycin (Gibco), 10% (v/v) autologous inactivated serum, 5×10^{-5} M β-mercaptoethanol (Bio-Rad). Initial T-cell cloning directly from the blood sample was attempted, but yielded a low production of clones for both viral and myelin Ag (data not shown), and the use of PHA led to unspecific proliferation and was considered inadequate as Ag-specific TCC were required in this study. TCL were therefore generated with selecting Ag (one plate/Ag): MBP and PLP at a final concentration of 6 μg/well and viral Ag at a final dilution of 1/20. Dilutions were always made in complete RPMI medium. Leftover PBL were quickly frozen on dry ice to serve as APC for later antigenic stimulations. Briefly, PBL were frozen at −80 °C with cellular concentration ranging between 2 and 10×10^6 cells/ml in RPMI 1640 with 50 U/ml of penicillin and streptomycin (Gibco) with 20% (v/v) autologous inactivated serum, 15% (v/v) DMSO (Bio-Rad) and 5×10^{-5} M β-mercaptoethanol (Bio-Rad).

After 7 days of incubation, IL-2 (Hoffman-La Roche) was added to TCL cultures, at a final concentration of 20 U/well. On day 12, primary TCL were tested for Ag specificity with a standard proliferation assay. Ag-specific TCL were cloned by day 16. Cloning following limiting dilutions of 3, 1 and 0.3 cell/ml was achieved in complete medium into 60 microwell-Terasaki plates (ICN) [32]. Autologous APC, gamma-irradiated at 2500 rads to avoid cellular proliferation (PBL prepared as described above), were quickly thawed and washed in culture medium, were then added to those Terasaki wells at a concentration of 5×10^5 cells/well, with either MBP or PLP at a final concentration of 300 μg/ml (6 μg/well) or UV-inactivated viral Ag at a final dilution of 1/20. To increase cloning efficiency, IL-2 was supplemented at a concentration of 5 U/well. Each well was checked for cell growth 10–13 days after cloning. The Poisson distribution suggests that a clone is generated when 33% or less of seeded wells show proliferation [32]. Cell growth was monitored and was characterized by round and clear blasts forming a tight colony. Wells exhibiting no cellular expansion revealed a layer of adherent APC with visible cytoplasmic structures covering most of the well surface. Subsequently, positive cell growth (higher cell population, acidic culture medium) yielding less than 33% positive wells per seeded plate, in the presence of irradiated autologous APC and the selecting Ag (MBP, PLP or HCoV-229E or OC43) reached a number of cells that allowed for testing of antigenic specificity in a standard proliferation assay. Selected TCC were then tested again for antigenic specificity, expanded and stimulated with the selecting Ag at bimonthly intervals (always with irradiated autologous APC). After a maximum of four antigenic restimulations, TCC were frozen at −80 °C in RPMI medium supplemented with 20% (v/v) fetal calf serum (Invitrogen) and 15% (v/v) DMSO (Sigma).

Antigen-specific proliferation assays

To ascertain Ag specificity, proliferation assays were performed by adding 1×10^4 T cells per well into 96-well round bottom microtiter plates (ICN) to which irradiated APC and various Ag were added separately in complete culture medium, 0.1 ml/well. These selecting Ag were MBP and PLP (6 μg/well), viral Ag HCoV-229E and HCoV-OC43 as UV-inactivated infected cell lysates (final dilution 1/20). Control Ag used were tetanus toxoid (1 μg/well), appropriate dilutions of UV-treated uninfected cell lysates and serum-free complete culture medium. Myelin and coronavirus Ag, each with appropriate controls being respectively medium alone or uninfected cell lysates, were tested on each TCC according to the selecting Ag used. After 72 h, tritiated-thymidine (1 μCi/well; Amersham) was added for 18 h. Cells were harvested onto glass microfiber filters (Skatron) on a 96-well Skatron model 11050 Micro Cell Harvester and counted in Ultimagold scintillation fluid (Packard), using a Canberra Packard Tri-Carb 2200a scintillation counter. A stimulation index (S.I.) was calculated, as the ratio of the radioactivity (counts per minutes (CPM)) incorporated in the presence of specific Ag over the CPM for its control Ag. S.I. value above 2.0, with counts higher than 800 incorporated CPM, was considered significant. Those CPM values are somehow lower then the usual 1000 CPM cutoff used when working with TCL [30], since there is sometimes a lower number of cells used for our assay with TCC.

Total RNA extraction

Primary TCC were frozen for future experimentations after their specificities were ascertained. TCC were not put back in culture prior to RNA extraction to avoid clonal exhaustion. Thus, frozen TCC were thawed quickly and the cell pellet (between 10,000 to 50,000 cells) was lysed and resuspended for RNA extraction using Trizol™ (Invitrogen). Because of the small number of cells used for RNA extraction, glycogen was added at the precipitation step as recommended by the manufacturer (Invitrogen). Concentration and ratio of total RNA were determined by spectroscopy at 260 nm and 260/280 nm.

RT-PCR and determination of the CD4/CD8 TCC profile

To ascertain the CD4+/CD8+ phenotype of TCC, clones resulting from different antigenic selections were chosen for total RNA extraction. For each sample, RT-PCR amplifying the CD4 or CD8 gene was performed. Samples (0.5 μg of total RNA) were reverse transcribed to cDNA with Moloney murine leukemia virus reverse transcriptase (M-MLV RT) using 1 μg of oligo d(T)$_{15}$ primer according to the manufacturer's protocol (Invitrogen). Briefly, the samples were put at 65 °C for 5 min then the reverse transcription was carried out for 90 min at 37 °C in a total volume of 50 μl with 20 mM dNTP mix (Roche), 5 Units of RNAse inhibitor (Roche) and 5 Units of M-MLV RT (Invitrogen). One fifth of the reaction was then used for PCR; hot start 5 min at 94 °C, annealing 5 min at 55 °C, followed by 35 cycles of 94 °C for 1 min, 55 °C for 2 min and 72 °C for 2 min in a Perkin-Elmer Thermocycler using rTaq™ (Pharmacia). The PCR mixture contained 200 μM dNTP mix and CD4 or CD8 gene-specific primers. Gene-specific primers for CD4 amplification were (GI 10835166): CD4-1: 5′ TTC CCA CTC GCC TTT ACA GTT G 3′ (nt 742–765) and for CD42: 5′ CAC CTG ACA CAG AAG AAG ATG CC 3′ (nt 1340–1317). For CD8 RT-PCR the following set of primers were used (GI 4826666): CD8-3: 5′ CAC CAA GAA GTC CAC CCT CAA G 3′ and CD8-4: 5′ GAA CTC CTA TCC TCA AAC CCG C 3′.

TCR region Vβ RACE RT-PCR amplification, cloning and sequencing

For each cross-reactive TCC (P7a, P8a, P12a, P13a, P13b), the total RNA sample (0.1 to 1 μg) was reverse transcribed by the standard GeneRacer™ (Invitrogen) kit protocol. Briefly, after dephosphorylation and uncapping of the total RNA sample, an RNA GeneRacer oligonucleotide was ligated at the 5′ RNA ends prior to reverse transcription with oligo(dT). This strategy allows for the amplification of an unknown 5′ RNA sequence with the use of gene-specific primer located in the constant 3′ region of the TCRβ chain (Cβ primer: 5′ CCT CCT TCC CAT TCA CCC ACC 3′ GI 1552511) and a 5′ primer corresponding to the GeneRacer™ RNA oligo previously ligated. The RACE RT-PCR amplification included a hot start of 5 min at 94 °C, then an annealing of 5 min at 55 °C, followed by 35 cycles of 94 °C for 1 min, 55 °C for 2 min and 72 °C for 2 min in a Perkin-Elmer apparatus. The DNA polymerase was the Expand high fidelity™ (Roche). Amplicons were run on 1% (w/v) agarose gels and the amplification product of about 600bp was cut from the gel and purified on S.N.A.P. columns (Invitrogen) for cloning with the Zero Blunt™ TOPO PCR Cloning Kit for Sequencing (Invitrogen) into the pCR4blunt™ TOPO vector using the standard protocol provided by the manufacturer. Plasmid DNA was isolated from colonies of transformed DH5-α bacteria and was sequenced at BioS&T (Montreal, Canada). For each clone, 10 bacterial colonies were sequenced in both orientations.

Plasmid DNA extraction and diversity-PCR

To validate molecular clonality for TCC P7a, P8a, P12a, P13a, and P13b, a diversity-PCR was performed on bacterial colonies obtained after transformation of the cloned amplicons in the Zero Blunt™ TOPO vector. Using primers specific to the CDR3 hypervariable sequences of each clone in combination with the Cβ primer described earlier, a hundred colonies were analyzed for each transformation. For each TCC the 5′ region primer corresponded to the following sequence: P7a-1 5′ AGT CTA AAT CCA GCG AGT TAT GG 3′, P8a-1 5′ AGC TTA AGC CTG TAT AGC GGT 3′, P12a-1 5′ AGC AGC TTA AAT GGG AGC CCA 3′, P13a-1 5′ AGC CTC AGC CTA TTA AGC CCA 3′. Briefly, bacterial colonies were boiled in 50 μl of sterile water and spun at maximal speed in a table centrifuge to clarify the supernatant. An aliquot of 5 μl was used for PCR performed as in the previous section except for the annealing, which was carried out at 50 °C for 30 cycles. Amplicons were run on 1% (w/v) agarose gels.

Results

MS donors and overall number of coronavirus and myelin monospecific TCC

Thirty-two MS patients, selected at random and designated P1 to P32 for confidentiality purposes, participated in the study but as MS affects female with predilection [2] the majority were female. Donors were between 28 and 69 years old with a median age of 43.9 for women and 58 for men. Referring to their medical files, none of them was treated with immunomodulating drugs at least 2 months prior to their enrollment in the study. T-cell lines (TCL) were generated with each selecting Ag and then cloned by limiting dilution. Table 1 indicates the total number of clones (TCC) obtained from the TCL and shows the global distribution of 145 monospecific TCC generated from MS patients and 35 TCC produced from healthy donors. No TCC responded to the control Ag tetanus toxoid. From 145 clones generated from the MS patients, 114 responded to coronaviral Ag and 31 recognized either MBP or PLP, the two myelin Ag tested. The protocol was designed to generate long-term CD4+ TCC, since the presentation was achieved with a purified protein or a pool of proteins presented by irradiated APC. A random sample of 28 TCC were tested by RT-PCR and were all CD4$^+$ (data not shown). Only the monospecific Ag-specific TCC coming from 22 patients from whom we could generate long-term TCC (4 antigenic stimulations), are listed in Table 2. Samples from the 22 out of 32 patients appearing in this list did allow for the production of long-term TCC of determined antigenic specificity. Fig. 1 illustrates typical proliferation profiles in cpm and stimulation index (S.I.) obtained with highly proliferating TCC established from MS patients by selection with either HCoV or myelin Ag. CPM values observed during the course of the study are stated with each selecting Ag following proliferation assays. Values seem to indicate that no specific patterns of proliferation could be established for any given Ag, as some TCC exhibited high proliferation and others yielded lower tritiated-thymidine intakes. The autoantigens (MBP and PLP) generally led to lower proliferation counts when compared to viral Ag. Those results were obtained after the fourth antigenic stimulation, with the corresponding selecting Ag. The coronavirus Ag led to a higher absolute measure of thymidine intake as measures went beyond 7000 cpm. However, the S.I. of all those TCC was high and constant throughout the four antigenic stimulations, for a period of about two months. Only those TCC that reached the fourth antigenic stimulation are included in the overall total of TCC produced during this experiment. The first antigenic stimulation established TCL and the second corresponded to cloning by limiting dilution. The third and fourth antigenic restimulations with selecting Ag were mostly expansion phases of Ag-specific TCC cultures. From an overall 229 TCC growing after the third stimulation, only 145 clones survived until the fourth stimulation, as there was a great variability in between patients regarding TCC production. Table 3 shows the distribution of the 145 long-term monospecific and 10 cross-reactive TCC obtained. Out of the 145 TCC, 114 clones responded to coronaviral Ag, 80 responding to 229E Ag and 34 to OC43 Ag, while 31 TCC recognized myelin Ag, as 19 TCC were selected with MBP, and 12 with PLP. In addition to these 145 monospecific TCC, 10

Table 1 Summary of TCC derived from 38 donors

	Donors *n*	Viral TCC	Myelin TCC
MS	32	114	31
Control	6	28	7

A total of 145 TCC were produced from the blood of 32 MS patients and 35 TCC were produced from 6 healthy donors. More TCC were activated with viral Ag than with myelin Ag.

MS donor	Sex	Age (year)	MS type	HLA-DR	T-cell clones			
					229E	OC43	MBP	PLP
P4	F	35	RR	8, 15	1	0	0	0
P5	F	65	CP	7, 17	2	0	2	0
P6	F	45	RR	ND	3	0	0	0
P7	F	34	CP	11, 17	1	1	0	3
P8	H	58	CP	15, 17	3	2	0	0
P9	F	34	CP	15, –	1	0	1	0
P11	F	42	RR	ND	1	0	0	0
P12	F	38	RR	13, 13	8	0	0	0
P13	F	54	RR	15, –	8	6	5	1
P14	F	45	CP	ND	1	0	0	0
P16	F	28	RR	11, 15	3	3	0	0
P17	F	32	RR	13, 15	3	0	0	0
P18	H	47	RR	ND	1	0	0	0
P22	H	58	CP	15, 13	16	2	0	2
P23	F	45	RR	7, 16	6	0	0	0
P24	F	46	CP	7, 17	7	7	2	1
P25	F	38	RR	15, 11	2	3	2	2
P26	F	53	CP	15, –	4	3	0	0
P27	F	58	RR	ND	8	2	4	0
P29	H	69	RR	ND	1	0	0	2
P30	F	56	CP	ND	0	2	1	0
P31	F	43	RR	7, 13	0	6	2	1
Total TCC					80	34	19	12

Participants were also HLA-typed. The total number of long-term specific TCC derived from each patient is indicated. Patients from whom coronavirus-myelin cross-reactive TCC were obtained are identified in bold. An overall number of 32 patients were tested during this study, but appearing in this table are only the 22 individuals from whom we could generate long-term primary TCC. About 80% of the TCC produced are monospecific and activated with viral Ag.

cross-reactive clones were obtained from 6 MS patients out of 32. Indeed, while some patients did not even lead to the production of monospecific TCC, some others generated several cross-reactive TCC.

Coronavirus-myelin cross-reactive TCC

Table 4 describes Ag specificities for 10 coronavirus-myelin cross-reactive TCC. CNS myelin Ag was tested in addition to coronaviral Ag for each HCoV-specific clones, and vice versa. All cross-reactive TCC remained specific to the selecting Ag while also responding to the heterologous Ag of interest. Clones that no longer recognized the selecting Ag while responding to a heterologous Ag were observed but eliminated from further investigation because they could represent a non-clonal population. The most frequent occurrence of this phenomenon was with TCC that lost their reactivity to myelin Ag after being selected by PLP or MBP. Six out of ten TCC were selected with viral Ag (four by HCoV-229E and two by HCoV-OC43), but also proliferated when stimulated with myelin Ag. Interestingly, two TCC selected with either HCoV-OC43 or HCoV-229E recognized

both MBP and PLP Ag. On the other hand, two TCC selected with PLP and two selected with MBP recognized 229E Ag (Table 4). Therefore, it appeared that HCoV-229E and MBP were the two Ag predominantly involved in the cross-reactivity. Among the 6 patients from whom we were able to generate cross-reactive TCC, four suffered from the chronic-progressive (CP) form of MS and two from the relapsing-remitting (RR) form and no relationship could be observed between MS severity for a given patient and coronavirus-myelin cross-reactive TCC. The patients from whom cross-reactive clones were derived were all HLA-typed and half of them (P8, P13, P22) were HLA-DR15 (Table 5), which represents a genetic susceptibility factor associated with MS [1].

Identification of the V(D)J regions of the TCRVβ chain for five cross-reactive TCC

To further characterize the TCR involved in cross-reactivities, we identified the TCR β chain of some TCC. Variable TCR regions of 5 of the 10 coronavirus-myelin cross-reactive TCC are identified in Table 5. Clones P7a, P8a, P12a, P13a and P13b were analyzed for their Vβ chains following RACE RT-PCR amplification with a Cβ gene-specific primer. Then for each of these 5 TCC, 10 sequencing reactions, in both orientations, were performed and results of sequencing are presented in Table 5. In order to get a higher statistical significance on the clonality of the 5 TCC that were sequenced, a diversity-PCR was performed on a hundred bacterial colonies per TCC. Except for some bacterial clones that had ingested only the vector during the transformation, each and every bacterial clone that was analyzed by PCR produced an amplicon of the expected size, demonstrating without any reasonable doubt the clonality of the cross-reactive T cells. The HLA-typing of the patient and the Vβ
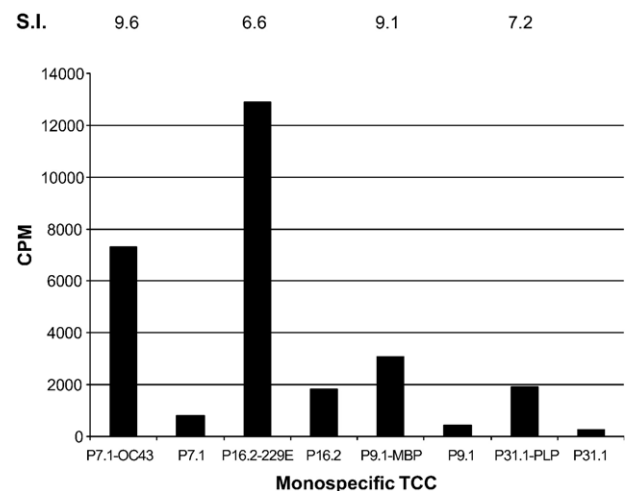

Figure 1 Typical Ag-specific proliferation profiles in CPM of monospecific myelin or coronavirus TCC established from MS patients or healthy controls. Selection was achieved with HCoV-229E or HCoV-OC43, MBP or PLP Ag. Activated TCC are identified by their name and selecting Ag. The background values of tritiated thymidine intake (CPM) appear for all TCC. Stimulation index (S.I.) are indicated at the top of the figure.

Table 3 Overall number of TCC generated throughout this study

Donors	n1	Monospecific				n2	Cross-reactive			
		Viral Ag		Myelin Ag			Viral Ag		Myelin Ag	
		229E	OC43	MBP	PLP		229E	OC43	MBP	PLP
		80	34	19	12		4	2	2	2
MS	32	114		31	6		6		4	

From the blood of 32 donors (n1), we generated 145 long-term primary TCC selected with either viral or myelin Ag and 10 HCoV-myelin cross-reactive TCC were produced from the blood of 6 out of these 32 MS patients (n2).

chain are indicated. Since region Vβ frequencies repertoire varies with ethnical origins, it is interesting to mention that all MS patients in this study were Caucasians. For all Vβ chains, the junction was clearly identifiable by the characteristic cysteines involved in disulfide bonds. The Vβ6 was observed with prevalence among the TCC we tested, being identified in 4 out of 5 TCC, with two being 6S7*2 and two being 6S5 (Table 5). Analogous amino acids were present in the CDR3 portion of all the identified TCR. For instance, four out of the five studied cross-reactive TCC begin with a leucine. The CDR3 portions were also similar in lengths of 6 to 7 amino acids. The Jβ portion of P12a, P13a and P13b was identical and contained the following threonine−arginine−leucine (TRL) segment, also found in the P8a TCC.

Discussion

HCoV-myelin Ag cross-reactivity was previously reported in a highly significant fraction of TCL from MS patients compared

Table 4 Antigenic specificities of coronavirus-myelin cross-reactive TCC

Clones	HCoV-229E	HCoV-OC43	MBP	PLP
P7a	NS	**5.9 (4772)**	2.4 (1584)	NS
P8a	**2.7 (5000)**	NS	3.5 (1231)	NS
P12a	3.1 (2300)	NS	NS	**3.2 (1198)**
P12b	4.5 (1542)	NS	NS	**5.2 (985)**
P12c	2.9 (1120)	NS	**3.4 (898)**	NS
P13a	11.3 (4293)	NS	**4.2 (4233)**	NS
P13b	NS	**9.0 (10800)**	8.4 (7365)	3.4 (2400)
P22a	**18.3 (6284)**	NS	3.8 (2628)	NS
P22b	**9.0 (2322)**	NS	4.9 (1014)	5.1 (1164)
P24a	**18.0 (9020)**	NS	3.4 (1205)	NS

The first number indicates the stimulation index (S.I.) and CPM counts are indicated in parentheses. The S.I. for a given antigen is the ratio of the radioactive CPM obtained after tritiated-thymidine incorporation when the T cells are stimulated with the selecting antigen over the CPM generated by stimulation with the corresponding control antigen. A T-cell clone was considered positive for an Ag if the CPM count was higher than 800 and if the S.I. was of 2 or more. The values in bold indicate the selecting Ag for a specific TCC. During antigenic specificity assays, viral Ag was tested against myelin Ag and vice versa. Viral and myelin Ag were not tested against one another. The name of the cross-reactive TCC in bold indicates the TCC that were further studied and sequenced. Proliferation that did not reach our criteria is considered non-significant (NS).

to healthy controls [30]. As cross-reactivity could have represented a mixture of antigenic reactivities due to a non-clonal population of T cells, clonality had to be proven by the production of TCC. Our results confirmed coronaviral-myelin cross-reactivity at the single cell level, involving two major myelin Ag (MBP, PLP) and two HCoV serotypes. Overall, from 6 healthy donors and 32 MS patients, 145 monospecific TCC were activated by viral Ag while 35 TCC responded to myelin Ag. As autoreactive T cells are present in healthy individuals as well as in patients with autoimmune disorders [5], detection of autoreactive cells (monospecific TCC) against myelin Ag in normal donor was not surprising. Moreover, 13 participants out of the 22 from whom we were able to get TCC, had the relapsing-remitting (RR) form of the pathology and 9 suffered from chronic-progressive (CP) MS, but no association with specific cell proliferation profile was noted.

A key feature of this protocol consisted in the use of the same stock of autologous APC throughout the study and the requirement for a single blood sample for each individual. Greater flexibility for antigenic restimulations was therefore achieved and experiments were independent of external elements like intake of medications and by possible fluctuating health of the patients, avoiding possible immune variations occurring between Ag restimulations. Furthermore, autologous APC adequately address the antigenic restriction necessary for presentation [33], avoiding the introduction of further foreign elements in the culture, a key feature to consider when working with study participants exhibiting various haplotypes. Because it significantly increased the production of TCC, Ag-specific TCL were generated but the evaluation of in vivo frequencies of activated TCC was thus forbidden since lymphocytes were previously activated/selected in vitro. The nature of the Ag is determinant to generate TCC. Even though, it has been known for a long time that peptides provide the advantage of being potent antigenic stimulators, which can allow the identification of specific epitopes [34], the use of complete proteins was chosen for our study as it increases the range of available epitopes and might favor the presentation of some conformations. Furthermore, it was shown that the use of peptides might not faithfully reproduce those generated by intracellular loading of peptides coming from the processing of native antigens [35]. Even though the S.I. was similar after stimulation with either coronavirus or myelin Ag (Fig. 1), the coronaviral Ag was better activators of T cells as nearly 80% of the TCC were selected against viral Ag (Table 1). Common seropositivity for coronavirus in the population [13], may be linked to this result and one has to keep in mind that myelin-reactive TCC have low in vivo frequency [36].

Table 5   Characterization of the TCRVβ chains

TCC	HLA-DR typing	Antigenic specificity		Vβ chain	V	N-D-N	J
P7a	11, 17	**OC43**	MBP	6S7*2	CASS	LNPASYG	EQYVGPGTM
P8a	15, 17	**229E**	MBP	6S5	CASS	LSLYSG	GNETRLGT
P12a	13	229E	**PLP**	6S7*2	CASS	SLNGSPL	GPGTRLTVT
P13a	15	229E	**MBP**	6S5	CASS	LGPASYE	GPGTRLTVT
P13b	15	**OC43**	MBP/PLP	8S2	CASS	LSLLSPH	GPGTRLTVT

Following RACE RT-PCR amplification, the Vβ chains of five cross-reactive TCC were sequenced in order to identify the hypervariable CDR3 region. Here the HLA-DR is indicated and the variable region (V), the hypervariable portion N-D-N and the joining (J) region are characterized.

The presence of activated CD4+ Th1 T cells reactive against PLP in the CNS of mice that were immunized with a PLP-specific peptide that induced EAE, adds to the relevance of autoreactive T cells in the induction of autoimmune disease [37]. Furthermore, the relevance of an autoimmunologic T cell memory as a critical factor in chronic autoimmune disease is suggested by the lifelong persistence of encephalitogenic T cell clones in the rat CNS [38] and in the long-lasting persistence of autoimmune T-cell clones against MBP or PLP in human blood [39,40]. Memory T cells have a lower threshold for activation: therefore, when they are activated during an immune response against high affinity foreign antigens, cross-reactive low-affinity self-ligands may potentially reactivate an autoreactive T cell response [41]. Another interesting observation was that cross-reactive TCC selected with viral Ag could in some instances recognize both myelin Ag (clones P13b and P22b described in Table 5). This might underline the fact that autoreactive CD4+ T-cell Ag recognition is associated to the degenerate/flexible nature of the TCR essential for a good recognition of foreign antigen [42]. Holler and Kranz [43] previously proposed the "conformer model" as a possible means to explain T cell cross-reactivity and it was recently shown that cross-reactive TCR possess a molecular flexibility to accommodate structurally very diverse ligands [44]. Both types of cross-reactivity, the initial selecting Ag being either a viral or a myelin Ag, could have a potential pathological significance. Indeed, autoreactive T cells are present in healthy individuals but the autoimmune potential of these cells appears to be in good part controlled by regulatory T cells. However, those regulatory T cells have an impaired capacity in MS patients and autoreactive cells are more easily activated [5]. Furthermore, it was previously shown that CD4+ myelin oligodendrocyte glycoprotein (MOG) autoreactive T cells isolated from MS patients had increased cytotoxic properties compared to those isolated from control subjects [45]. Therefore, even though our results do not demonstrate that the cross-reactive TCC obtained have a relevant implication in triggering or exacerbating disease, their presence in MS patients and not controls [30] makes it plausible that these cells play a direct or indirect role in inducing autoimmunity in vivo. With regard to potential pathological significance, a TCC selected with viral Ag could be activated in the periphery after a coronavirus infection, pass through the blood brain barrier and gain access to the CNS where it can damage myelin. Alternatively, a TCC selected with myelin Ag could be activated following myelin damage (resulting from a cytopathic infection or an injury) leading to the release of autoantigens. Subsequent corona-

virus infections can then occur and enhance or maintain the induced autoimmunity and since coronavirus reinfections are possible, it could explain or at least be linked to MS relapses [11]. Cytotoxic T lymphocytes attracted to the CNS during a murine coronavirus (MHV) infection need the help of the CD4 + population to be effective in clearing the virus from the CNS [46]. Moreover, we have recently demonstrated that MBP-reactive T cells were activated following MHV-A59 infection of the CNS [47]. Therefore, such CD4+ cells may also be essential to clear neuroinvasive HCoV [12] from the CNS and could react against myelin epitopes being exposed in significant concentrations after damage resulting from inflammation brought about by virus-mediated activation of glial cells, cytokines and nitric oxide [48]. Moreover, the presence of coronaviruses within the CNS could increase the number of MHC molecules on cell surface [49], which could also participate in the possible establishment of an autoimmune state. Cross-reactivity between self and other viral antigens was recently reported. Indeed, cross-reactivity between Human Herpesvirus-6 (HHV-6) and MBP [50] and a high level of cross-reactivity between myelin-derived peptide and Influenza HA for one specific CD4+ TCC [51] were recently reported and represent other examples of potential virus-induced molecular mimicry.

Further evidence is needed to confirm that such examples can really be explained by molecular mimicry and represent a relevant explanation to link infection and human neurological diseases like MS. Because of the degeneracy in the T-cell repertoire, cross-reactive activation of autoreactive T cells must be tightly regulated. Therefore, molecular mimicry-related activation of autoreactive T cells following an infection would require that the infectious agent encode a mimic epitope that can be processed from its native protein. Moreover, the pathogen should be able to induce the innate immune system in order to start the process of clonal expansion of the potentially pathologic cross-reactive T cells and it may be essential that an inflammation process occur in the target organ to initiate or maintain the putative autoimmune reaction [52]. A model of autoimmune disease resembling MS was induced using recombinant Theiler's murine encephalomyelitis virus (TMEV) expressing a mimic peptide related to PLP. A recent report describing the use of this virus and the initiation and exacerbation of the disease [53], showed that all the above requirements were necessary in order to produce the disease and demonstrated that virus-induced molecular mimicry was indeed a viable explanation for the onset of demyelinating diseases like MS. Furthermore, infections are often temporally associated with relapses in MS and these relapses lead to a more sustained

neurological deficit than non-infection-associated relapses [54]. Even though there is still no convincing evidence for a role of specific viral agent in the initiation or maintenance of a disease like MS, viruses have been major players in the search for the cause of multiple sclerosis and common ones causing upper respiratory tract infection are often associated with MS relapses [7,8].

A potential role for molecular mimicry in MS was reported in a paper describing a TCR derived from an MS patient that recognized both a DRB1*1501-restricted MBP and a DRB5*0101-restricted EBV peptide. Analysis of the crystal structure of each MHC-peptide complex revealed a strong degree of structural equivalence, providing structural evidence for molecular mimicry involving HLA molecules [55]. The HLA-DRB1*1501 haplotype, which appears to present an immunodominant MBP peptide [56], represents a genetic factor increasing the susceptibility to develop MS and it is known that several in vitro T-cell clones (TCC) generated from MS patients, were also activated with viral and bacterial peptides that contain the predicted motifs for binding to HLA-DRB1*1501 [22]. Moreover, a recent report using humanized transgenic mice models overexpressing HLA-DR15 coupled to a peptide coming from either MBP, myelin oligodendrocytes glycoprotein (MOG) or αB-crystallin [57] and MS-derived TCR specific for HLA-DR15, described spontaneous demyelinating disease strongly resembling MS. Therefore, our HLA-typing results (Table 5) are in concordance with the literature.

The presence of two TCR with distinct β chains on a single cell could be possible [58,59], so further molecular studies were performed to identify the Vβ region of the TCR. The high proportion of the TCR chain Vβ6 observed in our study is consistent with the literature, which indicates that Vβ6*5 is over expressed in MS patients [60]. It is believed that the CDR2 portion of that particular beta chain could favor interactions involving autoimmune peptides or autoreactive lymphocytes [61]. Interestingly, Vβ8*2, which was identified in one of our TCC, was also associated to autoreactive myelin TCC [62]. The associations between certain Vβ families and cross-reactivity in MS patients are interesting in the exploration of possible new genetic-related patterns that might lead to pathogenesis, as it is strongly suggested for HLA-DRB1*1501. Furthermore, as the hypervariable portion of cross-reactive TCR was identified, it may be interesting to see whether the hypervariable (CDR3) sequence of the cross-reactive clones could be used as a molecular probe in the CNS. Future investigations are required to examine TCR-peptide interaction and to pinpoint the epitopes involved in the cross-recognition we observe. However, even though our results from sequencing and diversity-PCR demonstrate the clonality of the cross-reactive T cells isolated from MS patients, the possibility that some of these cells bear two TCR remains. Indeed, even though it remains uncertain whether both TCR with a different α chain are always functional on dual receptor T cells, these TCR do exist and this type of T cell may represent up to 30% of the normal repertoire in human and mouse [63]. Furthermore, at least two important reports have shown that these dual receptor T cells could express two fully functional TCR with different α chain [64,65]. Therefore, dual recognition of antigens by the same TCC could involve either the same TCR recognizing both viral and self antigen on the same HLA haplotype, or two different TCR (with two different α chains on the same TCC) interacting with two different HLA-DR molecules on the APC. Nevertheless, the fact that three out of five cross-reactive TCC were derived from patients homologous in their DR haplotype does strongly suggest recognition of the same HLA-DR molecule by the TCC. Further studies could involve overlapping peptides and purified/fractionated viral proteins to assess the fine specificity of the TCC isolated in our study. TCR gene reconstitution in fast growing T-cell hybridomas [66] could allow further analysis and interpretations of the identified sequences through peptide affinity investigations and confirm whether all the tested TCC possessed only one TCR.

Although several viruses could be involved in virus-induced autoimmune pathologies like MS, human respiratory pathogens like HCoV represent attractive candidates. Furthermore, it will be interesting to look for this coronaviral-myelin cross-reactivity in other neurological pathologies where inflammation is also a key player. As coronaviral reinfections are possible, the neurotropic and neuroinvasive properties of the HCoV may explain the initiation or the exacerbation of symptoms in various other neuropathologies. In conclusion, our results concerning the cross-reactivity are consistent with molecular mimicry and suggest that such a mechanism could occur and be relevant in MS patients. The activation of a single T-cell recognizing both viral and myelin Ag could represent a mechanism involved in the triggering of the autoimmune attacks observed in MS or in amplifying/maintaining the autoimmunity in genetically predisposed individuals.

Acknowledgments

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