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Multiple Sclerosis Retrovirus Particles and Recombinant Envelope Trigger an Abnormal Immune Response *in Vitro*, by Inducing Polyclonal Vβ16 T-Lymphocyte Activation

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A retroviral element (MSRV) defining a family of genetically inherited endogenous retroviruses (HERV-W) has recently been characterized in cell cultures from patients with multiple sclerosis (MS). To address the possible relationship with MS, direct detection of circulating virion RNA was proposed but revealed technically difficult to perform in standardized conditions, in the face of multiple endogenous HERV-W copies. A parallel approach has evaluated MSRV potential pathogenicity in relation to characteristic features of multiple sclerosis, in particular, T-lymphocyte-mediated immunopathology. We report here that MSRV particles induce T-lymphocyte response with a bias in the Vβ16 chain usage in surface receptor, whatever the HLA DR of the donor. A recombinant MSRV envelope—but not core—protein reproduced similar nonconventional activation. Molecular analysis of Vβ CDR3 showed that Vβ16 expansions are polyclonal. Our results thus provide evidence that MSRV envelope protein can trigger an abnormal immune response with similar characteristics to that of superantigens.

Key Words: endogenous retrovirus; MSRV; HERV-W; envelope protein; immunopathology; T-lymphocyte; T-cell receptor; superantigen; cytokines; multiple sclerosis.

INTRODUCTION

Multiple sclerosis (MS) is a multifactorial inflammatory disease of the central nervous system characterized by plaques of demyelination and gliosis, with lymphocytic infiltrates and prominent T-cell activation. Epidemiologic and clinical data suggest that both genetic and environmental factors could be involved in the etiology of this disease. One major effector of MS pathogenesis consists of 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 multiple sclerosis. Different groups have detected retroviral particles in cultured cells from MS patients (Haahr *et al.,* 1991; Lan *et al.,* 1994; Perron *et al.,* 1991b). Linking such avenues of research, transactivation of a particular retroviral element by herpesviruses in MS has been epidemiologically (Haahr and Munch, 2000) or experimentally (Perron *et al.,* 1993) argued.

Analyzing RNA associated with viral particles produced in MS choroid plexus or B-lymphocyte cultures, we had identified sequences corresponding to overlapping regions of a retroviral genome (Komurian-Pradel *et al.,* 1999; Perron *et al.,* 1997b) that 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) (Blond *et al.,* 1999), suggesting that retroviral particles could have an endogenous origin or, more likely here, that they could 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). To address the possible relationship between this retroviral expression and MS, direct detection of circulating virion particles was proposed (Garson *et al.,* 1998), but revealed highly difficult to perform in standardized conditions due to the existence of a multicopy HERV-W family in human DNA (Olsson *et al.,* 1999). A parallel approach consisted of studying the potential pathogenicity of this retrovirus in relation to characteristic features of this disease, such as the abnormal lymphocyte behavior resulting in autoimmunity and multifocal inflammation in brain tissue.

In the context of a disease with severe immunolog-

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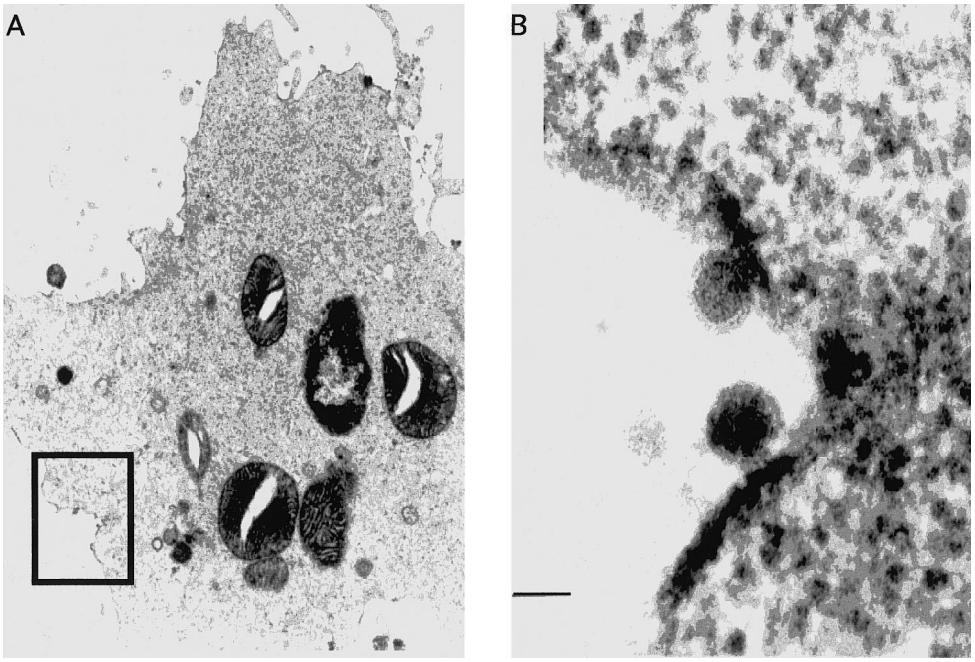

FIG. 1. Electron microscopy: retroviral particles in MS LCLs. Electron microscopy (EM) study was performed on samples collected and processed twice a week over 3 weeks from two MS, two non-MS patients, and one healthy control LCL cultures, in parallel with RT-activity detection. Sequentially fixed LCL cells were examined longitudinally (several hundreds of cells/sample). No retroviral particle could be seen either in the cytoplasm or at the cell surface in control LCLs. In MS LCLs, retrovirus particles in isolated cells (A) were observed at one or two different periods corresponding to peaks of RT activity. Long periods of observation were necessary for such findings. Extracellular particles (B, detail of A) were observed at the surface of the cytoplasmic membrane in isolated cells, whereas clusters of retrovirus-like particles in vacuoles were also occasionally seen. The bar indicates 100 nm.

ical impairment such as MS, the plausible role of a retroviral superantigen, possibly of endogenous origin, has been evoked from the origin of our observations (Rasmussen *et al.*, 1993; Rudge, 1991). The ability of superantigens to cause antigen-independent polyclonal activation of any T lymphocyte with a specific variable β chain (TCR Vβ) can result in abnormal autoreactive T-cell activation (Zhang *et al.,* 1995) and oversecretion of proinflammatory lymphokines (Arad *et al.,* 2000). Thus, a retroviral expression in early MS lesions would trigger an excessive proportion of infiltrating T lymphocytes resulting in (i) a disproportionate inflammatory response in focal areas mediated by locally oversecreted cytokines; (ii) a secondary "hyperactivation" of other lymphoid cells amplifying the inflammatory cascade; and (iii) an uncontrolled expansion of autoreactive T cells bearing (or not) the targeted TCR Vβ motif. Consequently, such a superantigen could be a key factor in MS immunopathogenesis (Rudge, 1991).

To address this issue, we have analyzed *in vitro* whether infection of human peripheral blood lymphocytes (PBL) from healthy donors with MSRV particles induced a selective Vβ expansion and whether a recombinant MSRV protein could mimic this eventual effect.

We report here that a significant and polyclonal activation of T cells bearing Vβ16 TCR chains has repeatedly been found after inoculation of MSRV virions to PBLs from healthy donors with various HLA class II haplotypes. We also report that the recombinant "Env" protein encoded by MSRV clones (Komurian-Pradel *et al.,* 1999) reproduces a similar superantigen activity to that observed with virion preparations.

RESULTS

Retroviral particles from MS cultures

Virion and control pellets were always prepared and aliquoted from the same culture material, obtained from MS and non-MS (i) early-passage B-lymphoblastoid cell lines -LCL-, and, (ii) choroid plexus -CP- cultures as already characterized (Perron *et al.,* 1997a, 1991a). Supernatants were collected over 2- to 4-week periods during which peaks of virion production were detected in MS cultures by RT activity as previously described (Perron *et al.,* 1993) and by electron microscopy as shown in Fig. 1. Therefore, collection of large volumes of supernatants from MS cultured cells, pooled over 3- to 4-week periods, allowed collection of virions produced by peak(s) over this period and further concentration from the total pool by ultracentrifugation in a single batch.

Proinflammatory cytokines in PBL cultures inoculated with MSRV virion

We evaluated the possibility that MSRV infection and/or expression is associated with pathogenic production of proinflammatory cytokine. Compared kinetics of IL-6, TNF-α, and IFN-γ production in 24 and 48 PBL cultures grown in the presence of MSRV+ or MSRV− virion pellets, TSST-1, or PHA, are presented in Fig. 2. Il-6 showed a significant increase in MSRV+ vs MSRV− PBL cultures. TNF-α appeared significantly increased at 24 h only when compared to mock controls, but only moderately increased when compared to the levels obtained with purified and concentrated bacterial TSST-1 superantigen or PHA. No statistical difference could be seen in IFN-γ production, given the low levels and the elevated standard deviation observed.

Production of IL-6, TNF-α, and IFN-γ was further tested in the 48-h supernatants of 12 new PBL cultures inoculated either with MSRV+ or with MSRV−. Unlike TNF-α which was equally undetected at 48 h (under the threshold of detection), concentration of IL-6 was significantly greater in MSRV+ PBL cultures supernatants and was detected with most PBL tested (10/12) production ranging from 1400 to 200 pg/ml in MSRV+ and 400 to 200 pg/ml in MSRV− cultures. In these new series analyzed at 48 h only, IFN-γ production was significantly higher in a few donors only (3/12; mean of 716 pg/ml in MSRV+, mean of 398 pg/ml in MSRV− cultures).

Selective bias in Vβ repertoire of PBL inoculated with MSRV viral particles

The induction of a selective bias in TCR Vβ repertoire is regarded as an intrinsic property of superantigens. To evaluate whether MSRV induces selective expansion or deletion of certain families of Vβ T cells, we performed a cytofluorimetric analysis of the Vβ repertoire among CD3-expressing T cells in PBL cultures from healthy donors. Cells were cultured for 24 or 48 h with either MS virion (MSRV+), mock-control (MSRV−), or with culture medium (control), and labeled with CD3 mAb in parallel with one of the antibodies directed against Vβ 2, 3, 5.1, 7, 8, 12, 13.1, 14, 16, 17, 21, or 22. Percentages of CD3+ cells expressing a specific Vβ were compared in the MSRV+, MSRV−, and control PBL cultures.

In preliminary evaluations, Vβ repertoire of cultured PBLs showed, when compared to either MSRV− or medium alone, that MSRV+ series displays a relative expansion of Vβ16 and a decrease of Vβ5.1 (not shown). The figures correctly reflected the general proportions of Vβ families in human PBLs and observed variations in the Vβ16 population were not found with other Vβ normally present in a similar proportion of T cells (e.g., Vβ7). As shown in Fig. 3 for one donor, virion (MSRV+) induced a specific Vβ16 expansion compared to mock-

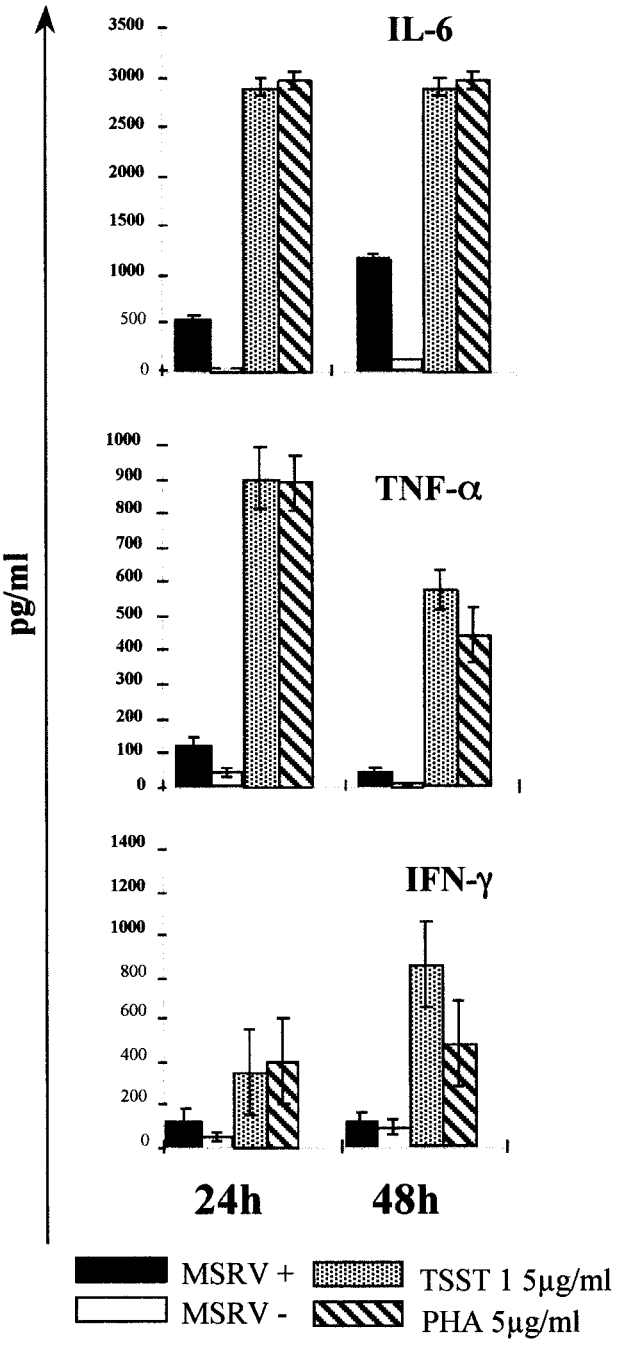

FIG. 2. Cytokine kinetics and pattern of production in PBL cultures inoculated with MSRV virion, MSRV− control, PHA, or TSST-1 bacterial superantigen. IL-6, TNF-a, or IFN-g were measured in 24- and 48-h supernatants of one donor PBL cultures stimulated with 5 μg/ml TSST-1, 5 μg/ml PHA, MSRV+, or MSRV−.

inoculated (MSRV−) cultures, whereas it did not modify other Vβ (illustrated with Vβ2).

The pattern of specific expansion defined by 12 Vβ families was further calculated for eight donors with various HLA-DR genotypes (Table 1). When compared to controls in conditions already determined in previous studies (Lafon *et al.*, 1992), a significant and predominant Vβ16 expansion was observed irrespective of the DR

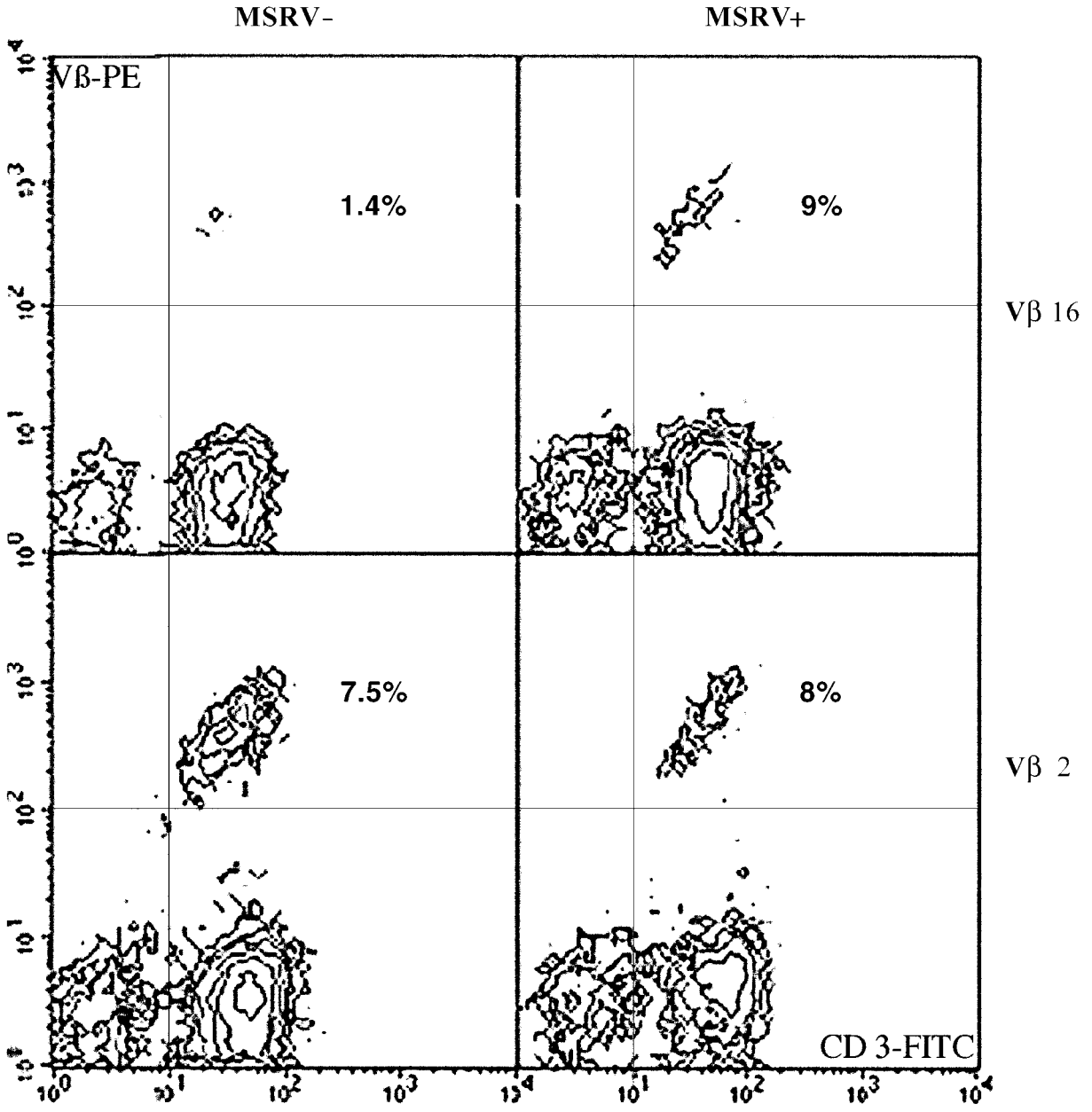

FIG. 3. Cytofluorimetric analysis: Vβ16 expansion in PBL culture with MSRV virion. MSRV induces expansion of Vβ16 (top) and not Vβ2 (bottom) in PBL cultures 48 h after virion inoculation (MSRV+, right) compared to mock preparation (MSRV−, left). Panels correspond to contour plots obtained by cytofluorimetry analysis. Cells were double stained with CD3 FITC (*x* axis) and biotinylated anti-Vβ antibody + PE-conjugated strepavidin (*y* axis).

genotype in 75% of donors (6/8). Increase in Vβ16 populations measured at a single time point (48 h) ranged from 31 to 315%. Moderate Vβ3 or Vβ12 expansions were also noticed in 37.5% of the donors (3/8) and Vβ2, 7, 8 14, 17, or 22 in 12.5% of donors (1/8), thus probably reflecting donor-dependent phenomenon.

Analysis of the relative size of Vβ families—with a restricted panel of 5 Vβ-specific antibodies focusing on Vβ16 variation within a representative selection (Vβ2, 7, 14, 16, and 17)—was repeated in 12 PBL cultures from new donors using a new MSRV virus preparation from CP cultures. As shown in Table 2 (no. 2 series), Vβ16 expansion was also observed irrespective of the DR genotype, in 50% of the cultures (6/12), while Vβ14 expansion was noticed once. In this last case, PBL were depleted in Vβ16 lymphocytes, down to a level representing 0.40% of the Vβ16 lymphocytes in control MRSV− culture (99.6% of depletion). In addition, these very few Vβ16 lymphocytes remaining in MSRV+ culture expressed a significantly lower level of TCR Vβ16 per cell: the mean peak channel of fluorescence decreased from 250 in MSRV− to 130 in MSRV+ (not shown). Few donors displayed a less pronounced "depletion" of specific TCR population ranging from 30 to 50%, for Vβ17, Vβ2,

TABLE 1

Cytofluorimetric Analysis of Vβ Variations among CD3 Cells in PBL Cultures of Healthy Donors with Various HLA DR Haplotypes (No. 1 series)

(No. 1) DR	Vβ:2	3	5	7	8	12	13	14	16	17	21	22
2/4				+								
13/8						+			+			
13/14		+							+			
3/12										+		
3/7		+							+			
3/13					+				+			
4/5		+				+		+	+			+
4/4	+					+			+			
%	12	37	0	13	13	37	0	12	75	12	0	12

Note. PBL cultures from eight different healthy women were analyzed at 48 h postinoculation of MSRV particles from MS B-lymphoblatoid cell line (LCL, batch 1), compared to mock-preparation from non-MS LCL. Increase of Vβ usage in MSRV+ compared to MSRV− cultures was determined as indicated under Materials and Methods. Only significant variations (ranging from +31 to +315%) are indicated with plus signs. Bottom percentage values correspond to the percentage of donors showing a significant Vβ expansion.

and Vβ14, but this was not accompanied by a decrease in the mean peak channel of fluorescence.

A third series with new virus preparation from MS and control LCLs (Table 2, no. 3 series) was analyzed with the same panel of Vβ-specific antibodies and showed more frequent Vβ16 depletions. All five donors reacted to MSRV+ compared to MSRV− either by a Vβ16 expansion (3/5) or by a Vβ16 depletion (2/5). Here again,

TABLE 2

Cytofluorimetric Analysis of Vβ Variations among CD3 Cells in PBL Cultures of Healthy Donors with Various HLA DR Haplotypes (No. 2 and 3 series)

(No. 2) DR	Vβ:2	14	16	17
11/13			+	
4/16	−			
4/4				
3/13			+	
1/15	−	−	+	
4/15			+	
15/14			+	
2/2	−			
4/16		+	−	

(No. 2) DR	Vβ:2	14	16	17
1/15				
4/11				
11/13			+	
(No. 3) DR				
13/14			−	−
3/12		+	+	
4/14			+	
3/13			−	
4/15			+	

Note. No. 2 series: PBL cultures from 12 different donors, 24 h after inoculation with MSRV particles from MS choroid plexus (CP, batch 2) cultures compared to mock-preparation from non-MS CP.

No. 3 series: PBL cultures from five different donors, 48 h after inoculation with MSRV particles from MS LCL (batch 3) compared to mock-preparation from non-MS LCL (new batch). Only significant expansions (as in Table 1) and significant depletions (ranging from −35 to −99.5%) are indicated with plus and minus signs, respectively.

TABLE 3

Cytofluorimetric Analysis: Vβ16 and Vβ17 Expansion or Depletion Induced by Env V14 Recombinant Protein

PBL Donor No.	Env V14 (ng/ml)	Vβ (%) 2	7	14	16	17
12	10	13	−7	7	159	151
	100	9	−9	8	212	156
13	10	10	4	32	−30	24
	100	7	2	1	−4	6
14	10	80	−60	ND	52	156

Note. Selective Vβ expansion of healthy human PBL taken from three different healthy women with various HLA DR, 48 h after inoculation of MSRV Env protein or diluant as control.

MSRV+ reactive cultures were not associated with a particular HLA DR.

In these series analyzed at a single time point (Tables 1 and 2), three different preparations of virus (two from MS LCL, one from primary CP cultures) yielded a significant modification in the size of the Vβ16 family in more than 70% of PBL cultures.

Selective expansion of Vβ16 and Vβ17 expressing T cells in PBL cultured with recombinant MSRV Env protein

Since retroviral proteins could be detected by FACS analysis in PBL cultured in the presence of MSRV virion preparation (not shown), we have evaluated the possibility that MSRV recombinant proteins could encode a superantigen-like activity. Recombinant MSRV Env and Gag proteins were produced in *Escherichia coli* and inoculated in PBL cultures. The Vβ repertoire was similarly analyzed with antibodies against an appropriate Vβ panel by cytofluorimetry, after incubation with 10–100 ng of protein per 2×10^6 cells in 1 ml of culture medium. Antibody against Vβ 2, 7, 14, 16, and 17 were tested with three donors for two concentrations of recombinant proteins. Similar to the data obtained with MSRV virion preparations, significant modulations were observed with Env protein in T lymphocytes expressing Vβ16, but also Vβ17, consisting of expansion for two of the three donors tested, and a moderate depletion restricted to Vβ16 of the remaining donor (Table 3). No significant variation was seen with the gag protein produced in the same bacterial system, purified with the same procedure, and tested at the same concentrations (not shown). A larger study with recombinant Env protein in PBLs from 13 donors indicated that significant Vβ16 modification occurred at 48 h postinoculation with seven donors (four cultures with expansion and three with depletion). Given variations observed at different times postinoculation (not shown), a kinetic study was performed to characterize the dynamics of expansion/depletion induced in nor-

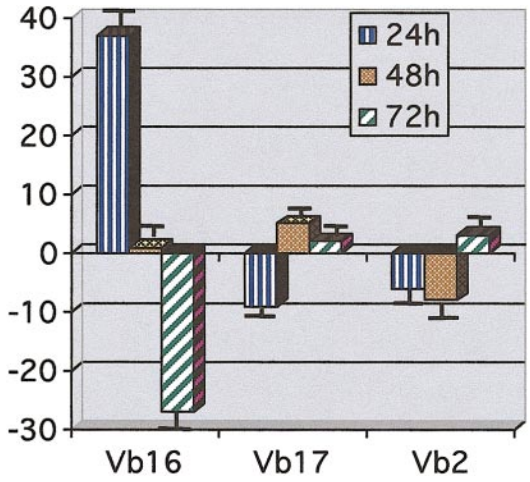

FIG. 4. Cytofluorimetric analysis: kinetics of Vβ16 expansion and depletion induced by recombinant Env protein. Selective Vβ expansion of PBL from one healthy donor 24, 48, and 72 h postinoculation of MSRV Env protein (100 ng/ml). The *y* axis shows relative percentage of variation.

mal PBL by this MSRV Env protein. Though the PBL culture presented in Fig. 4 yielded a moderate response, it revealed (i) an early expansion of Vβ16 detected at 24 h, followed by (ii) an apparent absence of effect at 48 h that obviously reflects equilibrium between expansion and depletion, and finally (iii) a marked depletion at 72 h. No significant variation was observed in the other Vβ subtypes analyzed in parallel. These features are compatible with a superantigen-like effect targeting Vβ16 TCR chains. They also indicate that since Vβ16 kinetics may differ among PBL cultures, several negative (non-significant) results observed in our series at a single time point may simply reflect a "point of equilibrium" between expansion and depletion, as observed here at 48 h in Fig. 4. Consequently, the frequency of significant Vβ16 variation in previous series might be underestimated.

Molecular analysis shows polyclonality of expanded TCR Vβ16-T cells

To determine whether the modifications of CD3+ T cell expressing Vβ16 correspond to oligoclonal or polyclonal expansions/depletions, we carried out a CDR3 spectratyping analysis (Garban *et al.,* 2000). This technique, derived from the original description based on the examination of CDR3 length (Pannetier *et al.,* 1993), gives a direct indication of the complexity of human T-cell repertoire. Thus, unstimulated healthy donor PBLs display a highly diverse and polyclonal TCR repertoire with a typically Gaussian distribution of CDR3 lengths. An immune response caused by, e.g., an infection, is associated with an oligoclonal distribution characterized by a reduction of CDR3 profile with a unique or predominant peak.

The comparison of Vβ 16 CDR3 lengths from cells collected 24 h after inoculation with either virion (MRSV+), mock-virion (MRSV−), envelope recombinant protein (*Env*), or medium preparation are illustrated in Fig. 5. The profiles of the patients displaying Vβ16 T-lymphocyte expansions appear as complex and diverse as those observed in the controls ("Gaussian-like" profiles with numerous peaks), thus excluding an oligoclonal response characterized by a sharply reduced number of peaks (corresponding to single clones acti-

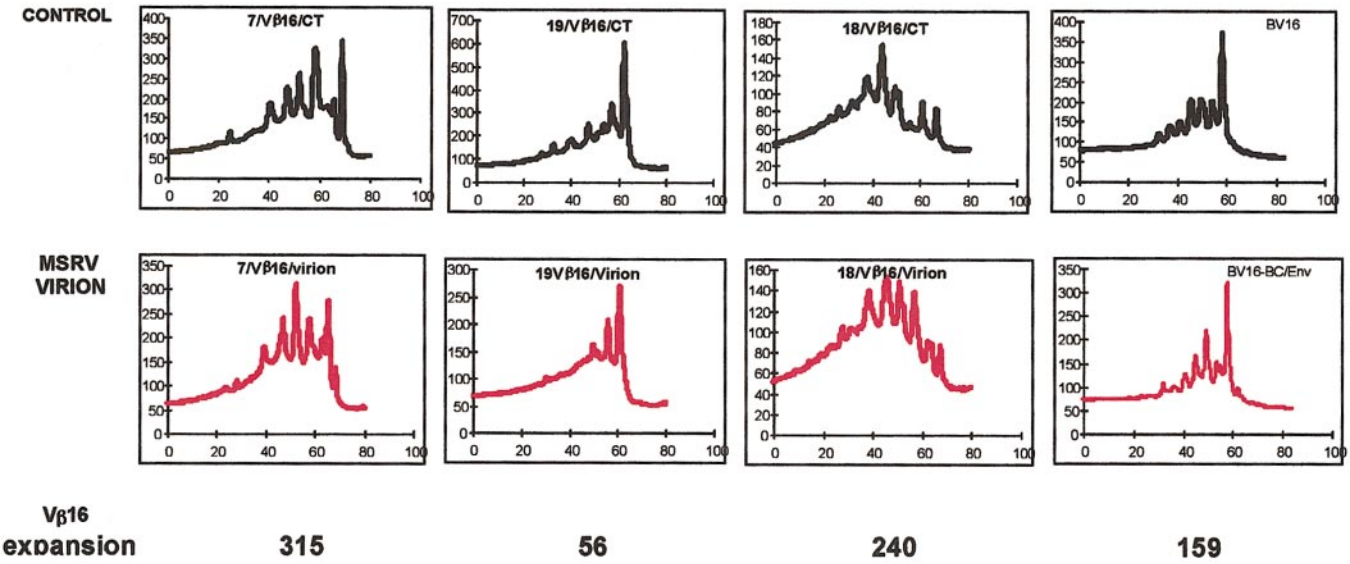

FIG. 5. Molecular analysis: diversity of Vβ CDR3 in normal PBLs after inoculation with MSRV virion or recombinant envelope protein. Each panel represents profiles of fragments obtained by PCR using BV16 and BC primers labeled by a runoff using a fluorescent internal BC primer. Graphs display the fluorescence intensity in arbitrary units (*y* axis) in the function of the CDR3 length (*x* axis). For each patient, the VB16 expansion is calculated as described under Materials and Methods.

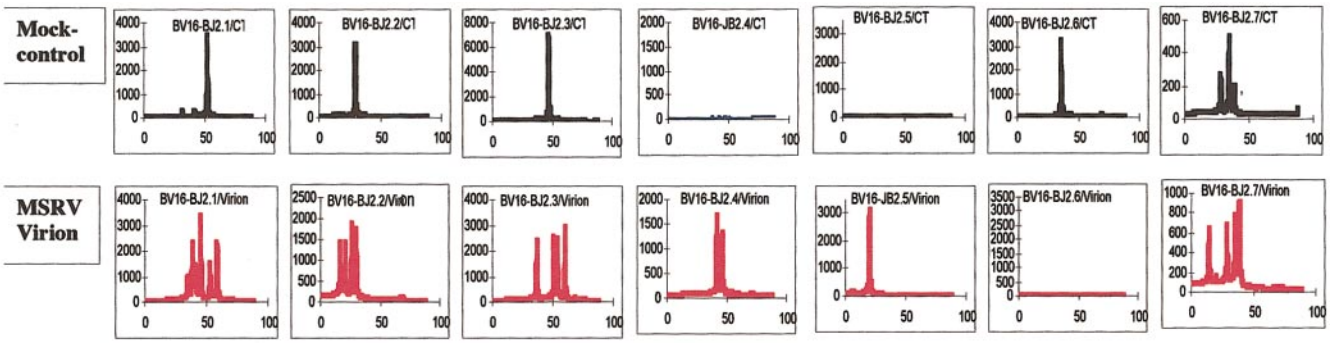

FIG. 6. Molecular analysis: CDR3 length distribution in individual BJ of Vβ16 lymphocytes. PBL were incubated for 24 h with MSRV virion. CDR3 length was analyzed as in Fig. 5 except that runoff was carried out with set of primers corresponding to BJ2 segment 1 to 7.

vated by an antigen). Similarly, in patients exhibiting a depletion of Vβ16 T lymphocytes, we observed a profile with a polyclonal distribution of spectratypes (not shown).

To further document that the Vβ16 population from patients inoculated with virion preparations or recombinant protein is polyclonally expanded, we carried out a refined analysis of CDR3 using a panel of seven oligonucleotides specific for each of the seven BJ2 gene segments. As shown in Fig. 6, the presence of virion or recombinant Env protein was associated with an increased number of Vβ16 peaks with variable CDR3 lengths. This was observed for most of the BV-BJ combinations, except for BJ2.6. The latter exception is not incompatible with an increase in all other BJ2 segments, since depletion can also occur and display variable kinetics in individual clones. These results confirm that these virus- or protein-induced Vβ16 variations appear not to result from conventional immune activation.

DISCUSSION

Polyclonal activation of naive T lymphocytes

In the present study, various MSRV virion preparations from different cultures and corresponding Env recombinant protein generated a reproducible pattern of T-lymphocyte activation in a series of PBL from non-MS donors. This pattern was independent from donors' HLA-DR genotype and from culture type (EBV-immortalized B cells or EBV-negative choroids plexus). Depending on culture kinetics, this activation yielded either an expansion or a depletion of Vβ16-bearing T lymphocytes. The molecular analysis of the TCR β chain in Vβ16 T-lymphocyte population revealed diversity of the CDR3 and thus confirmed the polyclonal nature of T-cell expansion. A parallel phenomenon was observed with Vβ17 bearing T lymphocytes but with less potent and/or less frequent activation. Other occasional activations observed within several Vβ populations were compatible with individual responses varying among donors, but cannot account for the superantigen-like effect on naive T lymphocytes. The identification of a recombinant MSRV envelope protein that reproduced the biased expansions of T lymphocytes bearing Vβ16—as well as of Vβ17—chain with polyclonal profiles strongly supports the hypothesis that this *Env* protein either displays or controls the superantigen-like activity of MSRV. Furthermore, the Vβ16 kinetics in PBL inoculated with this protein confirm the "superantigen-like" characteristics of this T-cell response. Further studies on linkage with DR and TCR molecules, as well as with animal models, are now envisaged to define whether, though less potent than bacterial superantigens, this retroviral envelope is a superantigen *stricto sensu*. Nonetheless, present data show an "abnormal" immune response induced by MSRV virion or *Env* protein (HLA-independent, Vβ-specific, and polyclonal activation of naive T cells), which should have immunopathogenic effects similar to those described for superantigens.

TCR Vβ studies in MS

Previous studies on Vβ expansions in MS have reported heterogeneous populations (Heard *et al.,* 1999) occasionally showing moderately biased Vβ usages either in circulating autoreactive T lymphocytes (Offner and Vandenbark, 1999) or in the general T-lymphocyte population (Birebent *et al.,* 1998). Different reasons can explain why biases in TCR Vβ16 or 17 chains have not yet been reported: (i) these studies used limited sets of TCR Vβ and did not mention Vβ16 nor 17 analysis; (ii) locally biased (and multifocal) expansions in brain may not be detected in circulating lymphocytes because they are diluted in the bloodstream or in CSF; (iii) Vβ usages seen in isolated autoreactive T-lymphocyte clones selected after *in vitro* MBP stimulation do not afford relevant information about superantigen activity; (iv) the superantigen-induced expansion may occur during the preclinical phase or in early stages of the disease only and may become progressively replaced by a depletion, or an anergic status, of the T lymphocytes bearing the Vβ target; and (v) even the *in situ* analysis of MS plaques may yield diverging results depending on pathologic

stage of lesions and on patient's status. Further "ex vivo" TCR studies might therefore be performed with classified patient subpopulations and, importantly, should take into account kinetics of reactivity in the time course of the disease, including expansion, depletion, and anergy analyses.

Biological significance

The superantigen effect reported here was detected using a straightforward strategy based on *in vitro* stimulation of naive T lymphocytes by virion preparations and viral proteins. Interestingly, other studies of superantigen activity required highly sensitive and complex techniques for detecting Vβ-biased stimulations, as reported for HIV nef (Tanabe *et al.,* 1997) or for another endogenous retrovirus family, HERV-K, putatively associated with autoimmune diabetes (Conrad *et al.,* 1997). Therefore, though intrinsically limited by the small percentage of the Vβ16+ T-cell population in normal PBLs, our results were significant and the standard techniques we have used should avoid difficulties encountered with HERV-K in autoimmune diabetes (Lapatschek *et al.,* 2000; Lower *et al.,* 1998). Indeed, our experimental conditions were previously used to state activity of well-known bacterial superantigens (White *et al.,* 1989) and viral superantigens (Lafon, 1992; Marrack, 1991). Moreover, results from a parallel *in vivo* study, in which we have explored the pathogenicity of this retroviral element in a humanized SCID-mouse model, support the T-lymphocyte-dependent pathogenicity of this retroviral element: intraperitoneal injection of MSRV retroviral particles induced brain hemorrhages that caused the death of animals within 5–10 days postinjection, but this phenomenon was abolished when T lymphocytes were depleted in the human lymphoid cell preparation used for SCID mice engraftment (submitted manuscript).

Thus, we can assume that the observed MSRV-induced effects are compatible with biological significance and could therefore play a role in MS disease by causing an inflammatory process with corollary uncontrolled autoimmunity, which is totally disproportionate to the initial antigenic challenge and leads to severe tissue lesions.

Which place for this immunopathogenic retroviral antigen in a multifactorial disease?

From a general point of view, the results presented in this study should provide new, but also "unifying" perspectives in the study of MS etiopathogenesis since their meaning extends to all aspects of this "immunovirogenetic" disease. As previously evoked (Rasmussen *et al.*, 1993; Rudge, 1991), "a retrovirally encoded superantigen" is an ideal culprit for disimmune reactions observed in MS, all the more if encoded by a retrovirus which does not belong to "purely exogenous" retroviruses, but to a family comprising endogenous retroviruses (ERVs).

Genetic factors

The known genetic contribution of ERVs to (retro)viral pathogenicity (Gardner, 1990) provides an intrinsic link with genetic susceptibility to associated diseases. The contribution of particular endogenous retroviral haplotypes in superantigen expression through interaction with exogenous strains of the same retroviral family has been described for mouse mammary tumor virus superantigen (Xu *et al.*, 1996, 1997). Therefore, the evidence of an immunopathogenic molecule encoded by a member of a retroviral family comprising endogenous copies in human genome (HERV-W) opens corollary perspectives in the study of MS genetic susceptibility. Interestingly, TCR Vβ chain gene is present on chromosome 7q35 region and, though rather distant from the HERV-W provirus present in 7q21–22 (Perron *et al.,* 2000), abnormal somatic rearrangements have rather frequently been reported in this chromosomal region (Kere, 1989; Kere *et al.,* 1989a,b; Stern *et al.,* 1989). In particular, HTLV-1 infected lymphocytes can present 7q chromosomal rearrangements between these regions and show impairment of immune function, including loss of antigen-specific responsiveness and the acquisition of alloreactivity (Jarrett *et al.,* 1986; Macera *et al.,* 1996). Endogenous retrovirus involvement has also been reported in rearrangement with 7q21 locus (Wahbi *et al.,* 1997). Consequently, known HERV-W copies within MS "genetic susceptibility loci" such as HERW7q (Perron *et al.,* 2000) should anyway provide preliminary elements for genetic evaluations in MS, all the more because associations between TCRVβ locus and MS have been claimed (Briant *et al.,* 1993; Buhler *et al.,* 2000), but also yielded conflicting results (Wood *et al.,* 1995).

Nonetheless, we cannot claim that this superantigen-like activity is linked to the "normal" endogenous HERV-W elements present in human DNA for, unless modified by e.g., retrotransposition and/or recombination, or by interaction with an exogenous strain, these inherited HERV-W elements are *a priori* unable to produce retroviral particles (Blond *et al.,* 1999; Perron *et al.,* 2000; Voisset *et al.,* 2000).

Infectious factors

The important contribution of infectious cofactors in the pathogenic expression of such retroviral elements is well known in relevant models (Contag *et al.*, 1989; Duc Dodon *et al.,* 2000; Nardiello *et al.,* 1994). A similar contribution, as proposed in MS (Haahr *et al.,* 1994; Perron *et al.,* 1993), also opens perspectives in the understanding of respective and relative roles of rather diverse infectious agents showing various degrees of association with MS (Perron *et al.,* 2000). Approaches attempting to prove that one of them is, alone, the etiological agent of MS may therefore be inappropriate. Interestingly, infectious agents evoked in MS studies,

including herpesviruses (HHV6, EBV, HSV1, and VZV), coronaviruses, and *Chlamydia pneumoniae*, have been shown to transactivate latent retroviruses or viruses (Garzino-Demo *et al.,* 1996; Perron, 2001; Wanishsawad *et al.*, 2000), including MS-associated retroviral element (Perron *et al.,* 1993).

For these reasons we now consider that the expression of such a superantigen-like molecule in MS would constitute a major pathogenic product of a biological "chain reaction" comprising variable infectious triggers, targeting a common retroviral core element, and consequently causing expression of pathogenic molecules such as retroviral superantigens (Perron, 2001; Perron *et al.,* 2000).

Finally, and beyond future characterization of yet unexplored features of this superantigen-like activity, the possibility of designing therapeutic molecules such as already described for other superantigens (Arad *et al.,* 2000) certainly indicates potential therapeutic perspectives which could be evaluated in MS in light of our present data, including the understanding that therapeutic approaches could be combined to control each step of such chain reaction.

MATERIALS AND METHODS

Choroid plexus cell cultures

Choroid plexus cells from two MS patients and one control patient without neuropathological abnormalities at autopsy were obtained from the brain-cell library (Professor Hauw), Hôpital de la Salpêtrière, Paris, France. CP cells were cultured as previously described (Perron *et al.,* 1991a, 1997a) with rabbit polyclonal antibody against leukocyte-produced interferon (Sigma), at a final neutralizing activity of 100 U/ml in the culture fluids, added in fresh medium at each renewal of the corresponding culture media. These cultures were controlled by PCR for EBV genome detection and were confirmed negative.

B-cell cultures: Lymphoblastoid cell lines

Blood from patients with definite MS—at distance from corticoid or immunosuppressive treatments—were obtained from Grenoble University Hospital, Neurological Dept. (Professor Pellat). Blood from healthy controls was obtained from the blood transfusion center (EFS) in Lyon (Dr. Gebuhrer).

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 2×10^6 cells/ml in RPMI 1640 with 200 U/ml penicillin, 200 mg/l streptomycin, 6 mM L-glutamin, 1% sodium pyruvate, 1% nonessential amino acid, and 20% heat-inactivated fetal calf serum (FCS). The cell flasks were incubated at 37°C and inoculated with 1 ml (10^5 viral particles for 4 to 5×10^6 total lymphocytes) of filtered supernatant from the EBV B95-8 productive culture in the presence of 200 μl (2 μg cyclosporine A for 4 to 5×10^6 total lymphocytes) of CSA (Sandoz) for 3–5 days; medium was then changed twice a week with same medium supplemented with rabbit polyclonal antibody against leukocyte-produced interferon (Sigma), at a final neutralizing activity of 100 U/ml. Cells were kept in the same 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 Dept. of Virology (Professor 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 by Sun *et al.* (1995) were searched for by PCR with two pairs of specific SMRV-H primers in the different LCLs used in this study, as well as in B-95 LCL 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 LCLs was also searched for by PCR with 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 ELISA detection kit (Roche).

RT activity

For RT activity kinetics study, 30 ml of culture supernatants was centrifuged, first at 3000 rpm during 30 min at 4°C to eliminate the cell debris and then at 1 h 30 min at 35,000 rpm at 4°C. The pellets were resuspended in 100 μl Tris–HCl 0.05 M pH 8.3 and a 50-μl aliquot was used for the RT activity test as previously described (Perron *et al.,* 1993). The cutoff value was calculated to discriminate specific activity from background signal and represents the mean value plus three standard deviations of all points obtained from control cell lines with identical phenotype.

Virion preparation

Virion, as well as control preparations, were similarly prepared. All culture media (obtained from LCL and CP cultures at passages 10–20) were changed and collected twice a week, centrifuged at 3000 rpm for 30 min, and frozen at −80°C after addition of 10% glycerol. RT activity was measured in culture supernatants as previously described (Perron *et al.,* 1993). Supernatants were pooled to obtain a homogeneous preparation of about 500 ml from either an MS LCL, an MS CP, a control LCL, or a control CP. One large volume fixed-angle rotor was used for each homogeneous batch that was distributed

in polycarbonate tubes. Five milliliters 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 *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 homogenized with glycerol (10% final concentration). Pooled pellets were aliquoted (100 μl), stored at −80°C, and further used for inoculation in normal PBLs (100 μl/2 $\times$ 10^6 cells). Importantly, all freezing steps used glycerol to obtain a sufficient proportion of intact viral particles (sucrose gradients were avoided).

Transmission electron microscopy

An amount of 10^7 cells was collected every week from the LCL cultures and fixed for 30 min with 4% glutaraldehyde prepared in 0.2 M cacodylate buffer (pH 7.4). Cells were rinsed three times in a solution (v/v) of cacodylate 0.2 M (pH 7.4) and saccharose 0.4 M, postfixed in a solution (v/v) of 2% osmium and 0.3 M cacodylate (pH 7.4) for 30 min at 4°C, 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).

Recombinants proteins

Recombinant MSRV proteins tested were produced in *E. coli* from clone CL2 (gag/AF123881) and from clone pV14 (env/AF331500), corresponding to cDNA clones amplified as previously described (Komurian-Pradel *et al.,* 1999). His-tagged proteins were produced and purified by Amplicon Express (WA) and controlled by SDS–PAGE and Western blot analyses.

PBL cultures

PBLs, used as "naive recipient cells," were collected from HLA-typed healthy blood donors (EFS, Lyon). PBLs were separated from fresh blood by standard Ficoll separation procedure, counted, and frozen with adequate stepwise protocol in decomplemented human AB serum + 10% DMSO under two-million-cell aliquots. They were stored in liquid nitrogen until thawed and washed in fresh medium for the study. Cells (6×10^6) were preincubated with 100 μl of virion or mock preparations for 30 min at 37°C. Cells were resuspended in RPMI with 2 mM L-glutamine and 100 U/ml gentamicin supplemented with 10% heat-inactivated human AB serum and cultured (2×10^6 per ml) for 5, 24, or 48 h before RNA extraction or cytofluorimetry analysis. Preincubation was omitted with TSST-1 or purified Env protein.

Determination of cytokines by immunocapture ELISA

Supernatants of cell culture (50 μl) and serial dilutions of cytokine standards were incubated 2 h at room temperature on ELISA plates previously blocked with 10% FCS in PBS and coated with an mAb for cytokine capture. Plates were washed five times with PBS–Tween. Fifty microliters of a mixture containing biotinylated detection Ab and streptavidin-conjugated horseradish peroxidase was added to each well. Plates were incubated for 1 h at room temperature. After washes with PBS–Tween, ABTS (2,2′-azino-di-[3-ethylbenthiazoline sulfonate(6)]-di-ammonium salt crystals) substrate was added and plates incubated at room temperature until color intensity was maximal. OD was read at 405 nm. OD of the control cell supernatant was subtracted and results expressed in pg/ml.

Cytofluorimetry: Analysis of Vβ repertoire

Cells were washed once with staining buffer (PBS, 0.1% (w/v) sodium azide, 1% FCS, pH 7.4) and incubated with one of the Vβ-specific biotinylated mAbs. PE-conjugated streptavidin was used as a second step together with FITC-conjugated anti-CD3 mAb. Cells were washed in staining buffer and resuspended into "Cell fix" (Becton-Dickinson, France) and 10,000 cells of FSC and SSC calibrated cells were analyzed by FACSCALibur cytofluorimeter (Becton-Dickinson). Usage of a given Vβ was expressed as a percentage of CD3+ T cells. Increase or decrease in MSRV+ cultures was determined by the formula: ((%CD3 Vβ+ cells in MSRV+ PBL culture) − (%CD3Vβ+ cells in MSRV-PBL culture))/(%CD3Vβ+ cell in MSRV-PBL culture). Variations over 30% were significant with this technical procedure, as previously determined (Lafon *et al.,* 1992).

Molecular analysis of TCR polyclonality

Cells (2×10^6) were collected from a culture well, washed with PBS, and resuspended in 200 μl of PBS for total RNA extraction using the "RNA Now kit" (Biogentec). Total RNA (0.5–1 μg) was reverse transcribed to cDNA using the Super Script RNASE H-Reverse Transcriptase Kit (Life Technology). PCR reactions were carried out in a Gene Amp 9600 (Perkin–Elmer) and performed using one of the BV subfamily primers and a BC primer as previously described for size spectratyping of TCR β CDR3 (Garban *et al.,* 2000). The PCR products were copied in run-off reactions primed with Texas red, labeled internal BC primer, over five cycles consisting of 2 min 94°C, 2 min 60°C, and 15 min 72°C. The run-off products were run on 6% polyacrylamide denaturing sequencing gels using a Vistra 725 DNA sequencer (Molecular Dynamics). Fragment sizes were determined automatically using ImageQuant software (Vistra Systems) and then graphically represented as peaks.

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