Thymic Export Function and T Cell Homeostasis in Patients with Relapsing Remitting Multiple Sclerosis

Andreas Hug, Mirjam Korporal, Isabella Schröder, Jürgen Haas, Katharina Glatz, Brigitte Storch-Hagenlocher and Brigitte Wildemann

J Immunol 2003; 171:432-437; doi: 10.4049/jimmunol.171.1.432
http://www.jimmunol.org/content/171/1/432

Why The JI?

- Rapid Reviews! 30 days* from submission to initial decision
- No Triage! Every submission reviewed by practicing scientists
- Speedy Publication! 4 weeks from acceptance to publication

*average

References This article cites 46 articles, 19 of which you can access for free at:
http://www.jimmunol.org/content/171/1/432.full#ref-list-1

Subscription Information about subscribing to The Journal of Immunology is online at:
http://jimmunol.org/subscription

Permissions Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Multiple sclerosis (MS) is an inflammatory and possibly autoimmune mediated demyelinating disease of the CNS. Autoimmunity within the CNS may be triggered by dysfunction of peripheral immune tolerance mechanisms via changes in the homeostatic composition of peripheral T cells. We have assessed the release of naive T lymphocytes from the thymus in patients with relapsing remitting MS (RRMS) to identify alterations in the equilibrium of the peripheral T cell compartment. Thymic T cell production was estimated by measuring TCR excision circles (TRECs) as a traceable molecular marker in recent thymic emigrants. A total of 46 treatment-naive patients with active RRMS and 49 gender- and age-matched healthy persons were included in the study. The levels of TREC-expressing CD4$^+$ and CD8$^+$ T lymphocytes were significantly decreased in MS patients, and TREC quantities overall matched those of 30 years older healthy individuals. The average concentrations of TRECs/10$^6$ CD4$^+$ and CD8$^+$ T lymphocytes derived from MS patients and healthy donors were 26 $\times$ 10$^3$/10$^6$ and 28 $\times$ 10$^3$/10$^6$ vs 217 $\times$ 10$^3$/10$^6$ and 169 $\times$ 10$^3$/10$^6$, respectively. To account for any influence of T cell proliferation on TREC levels, we assayed T lymphocytes from additional patients with MS and normal individuals for telomere length ($n = 20$) and telomerase activity (8 MS patients, 16 controls), respectively. There were no significant differences between CD4$^+$ and CD8$^+$ T cells from MS patients and controls. Altogether, our findings suggest that an impaired thymic export function and, as a consequence, altered ability to maintain T cell homeostasis and immune tolerance may play an important pathogenic role in RRMS. The Journal of Immunology, 2003, 170: 432–437.
Materials and Methods

Patients

MS patients and controls were recruited at the Department of Neurology, University of Heidelberg. For TREC analysis in peripheral lymphocytes, 46 individuals (mean age 37 years, range 18–56 years) with clinically or laboratory-supported definite MS according to the Poser criteria and 49 gender- and age-matched controls were included in the study (23). All patients had relapsing remitting MS (RRMS), presented with an acute relapse, and had not yet received treatment with corticosteroids or immunomodulatory agents. The median duration of disease was 2.0 years (average 3.9 years), and patients had previously experienced a median of 2.0 relapses (average 3.1). Seven patients were re-examined within 3 mo (n = 4), 4 mo (n = 1), 6 mo (n = 1), and 7 mo (n = 1) following resolution of relapse-associated symptoms. None of these patients had initiated treatment with immunomodulatory agents at second assessment. We additionally included four healthy donors aged between 60 and 80 years. Assessment of lymphocytic telomere length and telomerase activity was performed in 20 and 8 patients with active RRMS and comparable demographic profiles as well as in 20 and 16 age-matched healthy individuals, respectively. Patients and controls were informed about the scientific purpose of the study, and all individuals gave written informed consent. The study was approved by the University of Heidelberg ethics committee.

Cell separation

PBMCs were obtained from 10 ml EDTA blood by density-gradient centrifugation using Ficoll/Hypaque (Biochrom, Berlin, Germany). PBMCs were separated into CD4+ and CD8+ populations using magnetic anti-CD4 and anti-CD8 beads (Dynal, Hamburg, Germany), according to the manufacturer’s instructions. In selected experiments, magnetic beads were removed from CD4+ T cells using Detachabead CD4 solution (Dynal). This was followed by positive selection of CD4+ CD45RA+ and CD4+ CD45RO+ T lymphocytes by the use of CD45RA and CD45RO microbeads (Miltenyi Biotech, Bergisch-Gladbach, Germany).

Thymocytes were isolated from thymus tissue of a 6-mo-old infant undergoing cardiac surgery. Thymocyte suspensions were obtained by mechanical disruption of thymic tissue in a ground-glass tissue grinder and subsequent Ficoll density-gradient centrifugation. Cells were washed twice in serum-free RPMI medium and resuspended in 0.9% NaCl.

Quantification of TRECs by real-time PCR

Total DNA was isolated using DNazol (Invitrogen, Karlsruhe, Germany), according to the manufacturer’s instructions. Quantification of TRECs was performed by SYBR Green real-time quantitative PCR using a GeneAmp 5700 Sequence Detection System (Applied Biosystems, Weiterstadt, Germany). Each amplification was conducted in three independent reactions using SYBR Green reagents (Applied Biosystems) and reproduced at least once. Reaction mixtures contained 200 ng total DNA, 3 mM MgCl2, 1 mM dNTP, 100 nM of each TREC-specific primer (14), 0.75 U AmpliTaq Gold, and 1 U AmpErase in a total volume of 30 μl. Cycling proceeded under the following conditions: 50°C for 2 min, 94°C for 8 min, five cycles of 95°C for 1 min, 60°C for 45 s, 72°C for 1 min and 30 s, followed by 35 cycles of 94°C for 45 s, 65°C for 45 s, and 72°C for 1 min and 30 s. In each experiment, serial dilutions of a cloned TREC-PCR product were used (104, 105, 106 copies) as a standard for absolute quantification of TREC levels. Results were extrapolated to TREC content per 108 CD4+ and CD8+ cells or CD4+CD45RA+ and CD4+CD45RO+ cells, respectively.

Telomere length analysis

DNA was extracted from purified T cells using DNazol (Invitrogen). The average telomere restriction fragment (TRF) length of CD4+ and CD8+ T lymphocyte subsets was determined by the use of the TeloTAGGG Telomere Length Assay (Roche, Basel, Switzerland). A total of 5 μg of genomic DNA was digested with HindIII and RsaI, separated on a 0.8% agarose gel, and transferred onto a nylon membrane. After hybridization with a digoxigenin (DIG)-labeled telomeric repeat-specific probe and incubation of membranes with a DIG-specific Ab coupled to alkaline phosphatase, the TRFs were visualized by chemiluminescence. The average TRF length was estimated relative to a m.w. standard that was run in each gel.

Telomerase activity

Telomerase activity was measured in lymphocytes, thymocytes, and a lymphoma cell line (NC-NC; DSMZ, Braunschweig, Germany) by the use of the TeloTAGGG telomerase PCR ELISA Plus (Roche) based on the telomeric amplification protocol. Cell extracts were incubated together with a biotin-labeled primer and synthetic telomeric repeats. The resulting elongation products were amplified by PCR. PCR products were hybridized to a DIG-labeled telomeric repeat-specific probe bound to a streptavidin-coated 96-well plate. The binding reaction was detected with an anti-DIG-peroxidase Ab, visualized by a color reaction product, and quantified photometrically.

Statistical analysis

Statistical analysis was performed using Wilcoxon/Mann-Whitney U test for unrelated pairs and Pearson’s correlation coefficients. Three age groups (18–30 years, n = 31; 30–40 years, n = 27; and 40–60 years, n = 36) were independently assessed to account for the age-related decrease of thymopoiesis. Analysis of covariance was used to assess differences in CD4+ T cell and CD8+ T cell TRECs and telomere length between MS patients and healthy controls, adjusted for age as well as differences between TRECs in naive and memory CD4+ T lymphocytes. A p value of <0.05 was considered significant.

Results

TRECs are enriched in phenotypically naive T cells

To illustrate that TRECs identify naive T cells, TREC levels were determined in naive CD45RA+ and memory CD45RO+ CD4+ T cells derived from 10 individuals (mean age 31 years, range 21–44 years). As shown in Fig. 1, TRECs are nearly exclusively detectable in T cells of naive phenotype. TRECs were present at mean frequencies of 9 × 103/106 cells (median 5 × 103/106) in CD4+ CD45RA+ T lymphocytes as compared with 0.5 × 103/106 cells (median 0.5 × 103/106) in the CD4+ CD45RO+ T cell subset. This is equivalent to an 18-fold enrichment of TRECs in the naive CD4+ T cell subpopulation.

The frequencies of TREC-expressing CD4+ and CD8+ T cells are markedly reduced in relapsing MS patients compared with healthy individuals

The frequencies of TREC-containing T lymphocytes were measured in separated peripheral blood CD4+ and CD8+ T cells from 46 patients with RRMS in acute relapse and 49 age-matched healthy persons using quantitative SYBR Green real-time PCR. As expected, in normal controls, the concentrations of TRECs in both CD4+ and CD8+ lymphocyte subpopulations declined exponentially with age (Fig. 2). Between the ages of 18 and 60, the levels of TRECs dropped by ~1 log with a slope of −0.024 TRECs/year (r² = 0.0962) in CD4+ T cells and −0.019 TRECs/year (r² = 0.0993) in CD8+ T cells. TRECs were also detectable in lymphocytes of donors aged between 60 and 80 years, although at very low levels (0.1–7 × 103/106 CD4+ T lymphocytes and 0.2–8 × 103/106 CD8+ T lymphocytes, respectively; data not shown).

MS patients of any age had significantly lower levels of TRECs in CD4+ and CD8+ T cells as compared with normal controls.

FIGURE 1. Number of TRECs in naive CD4+ CD45RA+ and memory CD4+ CD45RO+ T cells from 10 individuals of different ages. TRECs are nearly exclusively detectable in the naive CD4+ T cell subset. Levels of TRECs are 18-fold enriched in CD4+ CD45RA+ T lymphocytes as compared with CD4+ CD45RO+ T cells.
and 217/H11003/

decrease of TREC-expressing lymphocytes occurred in the CD4/H11003/ lymphocytes in MS patients and healthy donors were 26 × 10^3 (median 17 × 10^3) and 217 × 10^3 (median 79 × 10^3), respectively (Fig. 3A). CD8/H11001/ T cells had average values of 28 × 10^3 TREC/10^6 cells (median 13 × 10^3) in subjects with MS and 169 × 10^3 TREC/10^6 cells (median 59 × 10^3) in controls (Fig. 3B). The most prominent decrease of TREC-expressing lymphocytes occurred in the CD4/H11001/ lymphocyte subset isolated from the youngest cohort of MS patients. CD4/H11001/ T cells of MS patients aged 18–30 years harbored TREC/10^6 cells at an average value of 30 × 10^3/10^6 cells (median 18 × 10^3) compared with an average value of 425 × 10^3/10^6 cells (median 102 × 10^3) in healthy donors of the same age. Thus, the relative reduction of TREC-positive CD4/H11001/ lymphocytes in this age group nearly doubled the difference calculated for levels of TREC/10^6 cells in the CD4/H11001/ T cell subpopulation of the whole study population (Fig. 4). In persons aged 31–40 years and 41–56 years, average quantities of TREC/10^6 CD4/H11001/ T cells were 24 × 10^3 (median 21 × 10^3) and 24 × 10^3 (median 13 × 10^3) for MS patients vs 120 × 10^3 (median 78 × 10^3) and 89 × 10^3 (median 72 × 10^3) for age-matched healthy subjects, respectively. In the CD8/H11001/ T cell compartment, the reduction of TREC-containing lymphocytes was more evenly distributed among the three individual age groups. CD8/H11001/ T cells derived from the youngest persons had an average concentration of 45 × 10^3 TREC/10^6 cells (median 27 × 10^3) in individuals with MS and 270 × 10^3 TREC/10^6 cells (median 71 × 10^3) in control subjects. In CD8/H11001/ T lymphocytes isolated from MS patients aged 31–40 years and 41–56 years, TREC/10^6 cells were detected at an average value of 16 × 10^3 (median 7 × 10^3) TREC/10^6 cells and 24 × 10^3 TREC/10^6 cells (median 8 × 10^3). This contrasted with average values of 192 × 10^3 TREC/10^6 cells (median 73 × 10^3) and 58 × 10^3 TREC/10^6 cells (median 46 × 10^3) in CD8/H11001/ T lymphocytes isolated from age-matched controls.

The yearly rate of decline of TREC-expressing lymphocytes is demonstrated in Fig. 3. As in controls, the frequencies of TREC-containing CD8/H11001/ T cells obtained from subjects with MS decreased as a function of age. Between the ages of 18 and 56, there was a ∼1 log drop of TREC/10^6 cells with a slope of −0.019 TREC/year (r^2 = 0.0993). In contrast to CD8/H11001/ T cells, the demise in TREC-expressing CD4/H11001/ T cells isolated from MS patients was much less pronounced. In this study, TREC/10^6 cells were detected in nearly constant quantities in CD4/H11001/ T lymphocytes throughout all age groups (slope of −0.003 TREC/year, r^2 = 0.0009). Overall, the curves derived from lymphocyte subsets of the MS group were shifted downward by ∼1 log as compared with those of the donor group. Thus, MS patients had levels of TREC-expressing CD4/H11001/ and CD8/H11001/ T lymphocytes equivalent to 30 years older healthy individuals.

No donor had TREC/10^6 levels <1 × 10^3/10^6 cells. In contrast, 6 and 7 MS patients (15%), among them individuals in all age groups, demonstrated levels <1 × 10^3 TREC/10^6 CD4/H11001/ and CD8/H11001/ cells, respectively. TREC/10^6 levels between 1 and 5 × 10^3/10^6 cells were detected in the CD4/H11001/ and CD8/H11001/ T lymphocyte subsets from 2 and 0 donors, respectively, compared with 5 and 9 MS patients (11%). In contrast, concentrations of TREC/10^6 cells exceeding >100 × 10^3/10^6 cells were present in CD4/H11001/ and CD8/H11001/ T cells from 19 and 14 controls, but from 1 and 3 MS patients only. TREC/10^6 levels >500 × 10^3/10^6 cells occurred in 5 (CD4/H11001/ T cell subset) and 3 (CD8/H11001/ T cell subset) controls and in none of the patients.

The frequencies of TREC-expressing CD4/H11001/ and CD8/H11001/ T cells obtained from MS patients are similarly reduced during an acute relapse and in the relapse-free interval.

In seven individuals with MS, concentrations of TREC/10^6 cells in peripheral lymphocyte subsets were repeatedly determined in clinical
Telomere length in T cell subpopulations does not differ in MS patients compared with healthy individuals

Telomere length in T cell subpopulations is not significantly different in MS patients vs controls. The frequencies of TREC-expressing CD4⁺ and CD8⁺ T cells were uniformly equivalent as compared with levels detectable during relapses (Fig. 5).

Telomerase activity in T lymphocytes does not differ in MS patients compared with healthy donors

To investigate whether the reduced TREC concentrations in MS patients are influenced by an increased peripheral T cell turnover, we measured the mean telomere length in CD4⁺ and CD8⁺ T cell subsets from patients with MS (n = 20) and normal controls (n = 20). As demonstrated in Fig. 6, the mean TRF length declined as a function of age. There was no significant shortening of telomeres in lymphocytes derived from MS patients compared with normal individuals, and in both cohorts there was no difference of TRF length between CD4⁺ and CD8⁺ T cells. CD4⁺ and CD8⁺ T cells had a mean TRF length of 10.1 and 9.2 kb (MS patients) and 8.8 and 9.4 kb (controls), respectively.

Telomerase activity in T lymphocytes does not differ in MS patients compared with healthy donors

Telomere shortening resulting from T cell activation and proliferation may be masked in the presence of a compensatory induction of telomerase activity. Therefore, we determined the mean relative telomerase activity (RTA) in T cell subpopulations from subset patients with MS (n = 8) and healthy persons (n = 16). Thymocytes and a lymphoma cell line as highly replicating T lymphocytes from patients with MS did not express higher telomerase activity than did the same cell subsets from normal controls.

Discussion

This study demonstrates that T cell homeostasis is substantially altered in treatment-naïve patients with active RRMS. The levels of circulating TREC-expressing CD4⁺ and CD8⁺ T lymphocytes were age inappropriately decreased in MS patients with acute relapses, and TREC quantities in lymphocyte subsets derived from individuals with MS overall matched those of 30 years older healthy persons. TREC are a recently defined molecular T cell marker to measure the frequencies of newly produced T cells exported from the thymus into the peripheral T lymphocyte compartment. They are generated in maturing thymocytes as extrachromosomal circular DNA in the context of the somatic TCR gene rearrangement (11, 12), and their quantities in peripheral blood decrease with age as a result of declining thymopoiesis (14, 15). The correlation of thymic function and circulating RTEs, as assessed by the frequencies of TREC-containing peripheral blood T cells, is well investigated in animal models, and fractionated removal of single thymic lobes is associated with a corresponding decrease of RTE numbers in peripheral blood (15, 24). Furthermore, in genetically athymic children with DiGeorge Syndrome who are unable to produce mature T cells and therefore lack peripheral TREC, engraftment of thymic tissue is followed by the appearance and successive increase of TREC-positive lymphocytes in peripheral blood over time (25). Hence, the significant decline of TREC-expressing circulating T cells in MS patients vs healthy individuals, as demonstrated in this study, is compatible with a defective maturation of T cells in MS patients. In concordance with a contracted release of naïve T cells from the thymus,

269) (Fig. 7). CD4⁺ T cells had slightly higher levels of telomerase activity than CD8⁺ T cells, but both CD4⁺ and CD8⁺ T lymphocytes from patients with MS did not express higher telomerase activity than did the same cell subsets from normal controls.
lymphocyte subsets from MS patients did not show significant shortening of their telomeres nor increased telomerase activity as an indirect marker for telomere consumption. This finding suggests that the dilution of TREC-expressing T lymphocytes as a consequence of an accelerated T cell turnover does not primarily contribute to the marked differences of T cell TREC levels in MS patients compared with healthy controls. We cannot exclude that ongoing autoantigen-driven T cell proliferation associated with acute MS relapses primarily impairs the TREC content of peripheral lymphocytes. However, autoreactive T cells represent a minor portion within the peripheral T cell repertoire (26, 27), and it is unlikely that an increased turnover of autoreactive T cell clones alone mediates such a pronounced reduction of circulating TREC-expressing T cells in patients with early stage MS. In our study population, the TREC content was not influenced by the frequency or severity of prior relapses, and even patients with extremely low TREC numbers in PBLs (<1 × 10^8/10^8 cells) did not differ from the remaining study population with respect to disease duration, relapse rate, and disability, as determined by the expanded disability status scale (28). Moreover, in the subset of MS patients that we assessed serially, the marked reduction of CD4+ and CD8+ T cell TREC levels documented in the active phase of the disease was maintained in the relapse-free interval. These findings suggest that mechanisms other than immune activation contribute to the lack of TREC-positive T cells. A substantial reduction of peripheral TREC levels was also found in patients with rheumatoid arthritis (29). Unlike MS, this autoimmune disease is characterized by a high systemic inflammatory activity, which is reflected by an increased T cell turnover, as determined by an age-inappropriate erosion of telomeric DNA in circulating T cells. Importantly, in late stages of disease, there is no further shortening of telomeres compared with healthy controls, indicating that massive peripheral cell division is not the principal cause for reduced peripheral blood TREC levels. Furthermore, nont hymonotemized patients with myasthenia gravis also have declined frequencies of TREC-expressing T cells (30), suggesting that this phenomenon may coincide with autoimmune disorders in general. Altogether, our observations indicate that impaired thymic function rather than an accelerated T cell turnover or Ag-driven expansion of autoreactive T cell clones is causally related to the pronounced decrease of TREC-expressing T lymphocytes in patients with RRMS.

The reduced TREC content of circulating T lymphocytes, as demonstrated in our study, correlates well with previous observations of T cell subset abnormalities in patients with MS and other autoimmune diseases. Several studies have shown that both NK cells and phenotypically naive CD4+CD45RA+ T lymphocytes are decreased during the active phases of MS (reviewed in Ref. 31). Importantly, the resulting disequilibrium in the composition of the peripheral T cell compartment may interfere with the maintenance of immune tolerance because a homeostatic shift in the peripheral T cell compartment with up-regulation of phenotypically naive T lymphocytes and concomitant down-regulation of memory T cells inhibits the disease activity of MS and experimental allergic encephalomyelitis, as shown for the synthetic immunomodulator linomide (31–33). Furthermore, in murine models, there is good evidence that athymic animals (e.g., as a result of thymectomy or irradiation of lymphoid tissues) are prone to the development of organ-specific and systemic autoimmune diseases (34). One important reason for the emergence of autoimmunity in the experimental setting is the loss of distinct T cell subsets that specifically arise during thymopoiesis and peripheral priming to control and suppress the proliferation of autoreactive T cell clones (35–39). The recent characterization of this specialized T cell subset has re-emerged the concept of regulatory T cells as probable key players between tolerance and autoimmunity. Such professional regulatory T cells are also present in humans, as shown very recently independently by several groups (40–45). They coexpress the CD4 and CD25 surface molecules, are anergic to Ag stimulation, and suppress T cell proliferation in a cell-cell contact-dependent manner (40, 41, 43–45). In addition, they suppress the homeostatic, space-filling (not Ag-driven) T cell proliferation in a cell culture model (46). Interestingly, although in our study differences in T cell TREC levels were detectable in both the CD4+ and CD8+ positive subpopulations, the most dramatic decrease of TREC-containing T cells affected the CD4+ T lymphocyte subset of the youngest MS patients. This was associated with an almost complete abolition of the age-related decline of the thymic-dependent output of new lymphocytes. This raises the intriguing possibility that the number or function of T lymphocytes with regulatory properties within the CD4+ subset may be altered and, like in murine models, may predispose to organ-specific autoimmunity in patients with MS.

In conclusion, our study is the first to show that T cell homeostasis is markedly altered in patients with RRMS. Overall, MS patients have levels of TREC-containing CD4+ and CD8+ T cells equivalent to 30 years older healthy controls. This abnormality is detectable in both active phases of the disease and in relapse-free intervals. Together with the lacking evidence of an accelerated T cell turnover, as assessed by the length of telomeric DNA repeats and telomerase activity in circulating T lymphocytes, these findings are compatible with an impaired T cell regeneration in MS. The altered composition of the peripheral T cell compartment related to this phenomenon may interfere with peripheral immune tolerance mechanisms that could have impact on future immunomodulatory therapies. In future studies, we will assess whether the export, function, and deletion of T lymphocytes with regulatory properties are impaired in MS patients.

Acknowledgments
We thank Brigitte Fritz for excellent technical assistance.

References