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Autoimmunity and Central Nervous System: Activation of T-cells in Multiple Sclerosis

Ph.D. Thesis

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INTRODUCTION

Multiple sclerosis (MS) is a demyelinating disorder of the central nervous system (CNS). Eighty percentage of the cases are characterized by alternation of clinically active and silent periods, ie. relapses and remissions.

The presumed pathomechanism of demyelination is an autoimmune inflammation within the CNS.

Participation of the ***immune*** system has been supported by several evidences:

1. Oligoclonal bands within the cerebrospinal fluid (CSF) reflects chronic production of gammaglobulins within the CNS in 90% of the Western cases.
2. Eighty percentage of cells within the cerebrospinal fluid are T-lymphocytes during relapses which indicates the importance of the cellular immune response as well.
3. The course of the disease can be influenced by administration of cytokines: IFN-γ causes exacerbation of the disease while IFN-β is clinically beneficial.
4. Expression of certain MHC-II alleles and T-cell receptor (TCR) Vβ-gene polymorphisms are significantly related to MS.
5. The pathology is characterized by perivascular cuffing of mononuclear cells in acute plaques.

The ***autoimmune*** theory has been supported by the animal model of the disease and by *in vitro* experiments:

1. Myelin basic protein (MBP) and proteolipid protein (PLP) are the two major components of myelin within the CNS. Immunization with these two myelin proteins or with their components (peptides) results in acute inflammation of the CNS. Experimental autoimmune encephalomyelitis (EAE) can be induced with T-cells specific for these myelin peptides as well.
2. The number of myelin-specific T-cell clones is higher among activated T-cells in the PB of MS compared to healthy subjects (HS).
3. Myelin-specific T-cell clones can be established from the CSF of MS patients.
4. Recognition of the antigen is mediated by the antigen-specific T-cell receptor (TCR) on the surface of T-cells. Clonally related T-cells bear homologous hypervariable region (CDR3) of the TCR. Isolated MBP- and PLP-specific T-cell clones express similar aminoacid motifs of the CDR3 to TCRs detected in MS plaques.
5. Vaccination with myelin-specific T-cell clones or with their hypervariable regions seems to be a promising approach in EAE and in the therapy of MS.

THE AIM OF THE PRESENT STUDY

The role of myelin-specific T-cells in the pathomechanism of MS is based on the *in vitro* isolation technics called limiting dilution assay (***LDA***): co-cultures of T-cells and antigen presenting cells (APC) are periodically stimulated with myelin-antigens. The proliferating cells are separated and re-stimulated until cultures are consisted of the same daughter cells, ie. myelin-specific T-cell clones.

The main problem of this technics is that the *in vivo* significance of the isolated clones can not be properly evaluated:

1. If a clone is activated *in vivo*, the *in vitro* re-stimulation may results in its apoptosis (activation induced cell death, AICD). Thus, *in vivo* significant clones may be lost for experimental or therapeutical approaches.

2. Naive T-cell clones might be activated during the *in vitro* stimulation process. Thus, clones which do not have significant role *in vivo*, are evaluated during the *in vitro* experiments.

3. It is difficult to ascertain the dynamics of activation since it is not possible to ensure that a certain clone is constantly selected during the cloning process.

No direct data have been published so far about th *in vivo* activation of myelin-specific T-cell clones in the PB and in the CSF of MS. Dynamics and passage of peripheral T-cells into the CSF has not been studied in details.

By using a new T-cell clonality assay we examined the *in vivo* activation and significance of myelin-specific T-cells in MS.

MATERIALS and METHODS: THE RT-PCR SSCP CLONALITY ASSAY

Since daughter cells of a T-cell (T-cell clone) express the same T-cell receptor (TCR), distinct T-cell clones can be recognized through identification of the TCR hypervariable region. One approach is the analyzis of nucleotide sequence of the TCR cDNA.

The **TCR** is composed of alpha- and beta-chains. The hypervariable region of the β-chain (β-CDR3) is located between V (variable) and C (constant) regions of the protein and of the mRNA.

Following **mRNA isolation** the complementer DNA (**cDNA**) is synthetized. The hypervariable regions of T-cell receptor cDNA can be then amplified by **RT-PCR** with one Cβ-specific and 21 Vβ-specific primers. Since different clones share homologous Cβ sequences and the Vβ primers match the majority of Vβ-regions, most of the hypervariable regions can be amplified by these 21 RT-PCR reactions.

Single-strand conformation polymorphism (**SSCP**) is a widely accepted method for mutation analyzis. The single-stranded DNA sequences can be separated in non-denaturing polyacrilamide gele because of conformational changes even if they differ in a single nucleotide.

By analyzing the 21 PCR-products, the different hypervariable regions can be separated from each other. Following **transfer** of DNA and **hybridization** with a Cβ-specific byotinylated probe the distinct hypervariable regions can be visualized by a photoluminescent assay system.

The method enables the expanded clones in a T-cell pool to be visualized as distinct bands (**clonotypes**). Besides determination of the number of expanded clones, the intensity of bands can be used to establish the dominancy of the clones. Non-significant clones are erased in the background smear. Clones which are expanding in different compartments or at different times can be identified by analyzing the corresponding PCR products on the same gel: identical sequences of hypervariable regions move to the same position on the gel.

RESULTS and DISCUSSION

Analyzis of the peripheral blood and of the cerebrospinal fluid (CSF) in MS

The hypervariable regions (CDR3) of the 21 TCR-Vβ genes were isolated and amplified from peripheral blood mononuclear cells (PBMC) of MS and HS. The 21 amplified Vβ gene products were analyzed parallel on the same SSCP gel, thus the peripheral T-cell repertoire was vizualized in each subject. SSCP-patterns of 11 healthy PBMC were composed of smear with the presence of a few faint bands (**clonotypes**). This pattern indicated the heterogeneous clonality of the T-cell repertoire with the slight dominancy of certain T-cell clones. Similarly, SSCP analyzis of the PBMC gained from 7 MS patients in remission revealed the same pattern. Opposed, SSCP pattern of 14 patients in clinical relapses showed 30-70 distinct bands which indicated the expansion of particular T-cell clones during the exacerbation of the disease. The number of expanded clones was significantly higher in 14 out of the 21 Vβ families compared MS relapse to HS. Expansion of $V\beta5.1^+$ and $V\beta6^+$ T-cell clones was the most striking difference between MS and HS clonality. Since MBP-specific clones have been proposed to express such TCR preferentially, our data raised the possibility that myelin-specific T-cells might expand *in vivo* among the expanded T-cells within the peripheral blood of MS during relapses. Moreover, oligo- and polyclonal expansion of T-cells might reflect the regulatory disturbances of T-cell activation in MS.

Analyzis of the CSF revelaed polyclonal expansion of T-cells during clinical exacerbations. Similarly to the peripheral blood, the expansion of the $V\beta5^+$ and $V\beta6^+$ T-cell clones was the most significant within the CSF of 15 MS patients. These data proposed the possible expansion of myelin-specific T-cells not only in the peripheral blood (PB) but also within the brain-CSF compartment.

SSCP analyzis of short-term PBMC cultures following stimulation with myelin antigens

Presence of myelin-specific T-cells was investigated within the peripheral blood (PB) in further experiments. Following isolation of PBMC from 9 HS and 6 MS (3 remissions, 3 relapses), short-term cultures were established and were stimulated with dominant myelin epitopes, ie. with PLP95-116 peptide and with MBP82-102 peptide. SSCP patterns of pre-culture PBMC were compared to unstimulated cultures and to cultures stimulated with myelin antigens. The same clonal origin was noted if bands were detected in the same position: homologous DNA sequences move to the same position during the electrophoresis. Bands which were exclusively detected following stimulation, were considered to indicate antigen-related clonal expansions. The number of expanded clones in cultures stimulated with PLP95-116 were significantly higher in the cases of MS relapses compared to MS in remission and to HS.

Cultures were also stimulated with bacterial superantigens (SEA, SEB) and were simultaneously analyzed with myelin-stimulated cultures. Cultures of otherwise healthy subjects during common cold with fever were analyzed for the presence of myelin-specific T-cells as well. Neither stimulation with SA nor fever induced significant clonal expansion of myelin-specific T-cells in the PB.

Although these experiments further supported the presence and increased frequency of myelin-specific T-cells in the peripheral blood of MS during exacerbations, the *in vivo* significance of such clones could not be properly evaluated. Therefore we performed further experiments with long-term T-cell lines and with T-cell clones (TCLC).

Analyzis of the activated $CD25^+$ T-cell repertoire

SSCP analyzis of the fresh PBMC revealed heterogeneous T-cell repertoire in which identification of certain clones might be difficult. We therefore intended to choose a selected T-cell population. CD25 (IL-2R α-chain) is an early activation marker which is suitable to monitor the ongoing immune response. Moreover, the $CD25^+$ repertoire presumably contains autoreactive T-cell clones related to the pathogenic process.

In preparatory experiments percentage of $CD25^+CD3^+$ (activated) T-cells were analyzed by flow cytometry in the PB of 11 MS and 9 HS. The number of activated T-cells is significantly decreased in the peripheral blood of MS compared to HS. Clonality of the activated repertoire was also evaluated by SSCP. $CD25^+CD3^+$ T-cells were sorted out of the PB by flow cytometry and were analyzed by SSCP. Data revelaed reduced number of activated T-cell clones in the PB of MS.

Since animal experiments have suggested a regulatory T-cell population among $CD25^+CD4^+$ T-cells, reduction of $CD25^+$ T-cells in MS might be connected with loss of this subset. Furthermore, significance of myelin-specific T-cells within this reduced activated repertoire became especially interesting.

Identification of myelin-specific T-cells within the reduced *in vivo* activated repertoire

Nineteen long-term myelin-specific T-cell lines and clones (TCCL) were established by LDA from the PB or CSF of 4 patients with SM. TCCL were specific to MBP82-102 and to four PLP peptides including PLP95-106, respectively. SSCP analyzis of the 19 TCCL revealed the presence of 1-5 TCR-Vβ clonotypes which corresponded to the constituent T-cell clones of the TCCL. The presence of these 58 clonotypes were investigated in the activated peripheral repertoire and in the CSF of the corresponding MS patients.

$CD25^+CD3^+$ T-cells were isolated from the PB of patients by flow cytometry several times. The amplified Vβ gene products were analyzed parallel with the corresponding Vβ clonotypes of the TCCL. Bands which were detected in the same position indicated the same CDR3, ie. expansion of the TCCL among *in vivo* activated T-cells. The same clonal origin was also proved by DNA sequencing of the identical bands.

Twelve of the 58 clones (20%) were detected within the activated T-cell repertoire proving the *in vivo* expansion of myelin-specific T-cells in MS blood.

***In vivo* significance of the activated myelin-specific T-cell clones**

Following identification of TCCL clonotypes among the corresponding activated Vβ clonotypes, the dominancy of TCCL clonotypes was evaluated within the total activated repertoire. Intensity of the bands suggested dominancy of the myelin-specific T-cell clones among activated T-cells in every case. Moreover, dominant MBP82-102-specific clones could be detected in severely reduced activated T-cell repertoire of a chronic progressive MS as well. These data may imply that a few MBP-specific T-cells could play overwhelming roles in certain phases of MS. Elimination of these clones by anti-clonotype vaccines may greatly reduce the clinical activity of this patient in the active phase.

Serial analyzis of the activated myelin-specific T-cell clones

The expanded myelin-specific T-cell clones were examined repeatedly in the peripheral blood. Serial analyzis of the activated T-cell repertoire revealed the heterogeneous nature of activation. Most of the clones expanded during relapses or remissions irrespectively of the clinical activity. This might be explained by the theory of epitope spreading, by cross-reactivity with unrelated antigen(s) or by clinically silent

relapses. Peripheral activated T-cells may be involved in the CNS pathology by a temporal shift as well. A PLP95-116-specific T-cell clone, however, was continuously re-activated in the peripheral blood of an RR MS. Since clonotype representing this clone was present within the Fas^+ and $FasL^+$ sorted PBMC fractions respectively, this may indicate the dysfunction of suicide apoptosis.

Presence of myelin-specific T-cell clones within the CSF

CSF samples were taken from an RR and from a CP MS patient. The presence of 45 MBP- or PLP-specific clones, which were partially expanded among peripheral activated T-cells, were investigated in the CSF samples. Four PLP-specific T-cell clones (two PLP95-116 and two PLP118-139) were present as dominant clonotypes in the CSF sample of the RR MS patient. One clone was expanded in the PB for a year. Another clone could be detected among peripheral activated cells 16 months ago. The other two clones were not present in seven previous and in a concomitant PB samples. Three MBP82-102-specific T-cell clones could be also detected in the CSF of the CP MS. All MBP-specific clones expressed $V\beta6^+$ TCR and were dominant populations among CSF clones. Although two of the clones dominated the peripheral activated repertoire 5 months earlier, none of the clones were present in the paired blood samples.

Our data indicated the expansion and dominancy of myelin-specific T-cell clones among CSF T-cells. Presence of the clones in the CSF did not mean however, that the particular clone was expanded and activated concomitantly at the periphery. Therefore we examined the traffic of the activated T-cells into the CSF/CNS compartment in further experiments.

Traffic of activated T-cells into the CSF/CNS compartment

Serial analyzis of the activated $CD25^+$ T-cell repertoire indicated the exchange of the dominant clones within three months. Sixty percentage of the clones was identical in a month and 30% was identical in six months in the PB. Some of the dominant clones were present in half-a-year as well.

Paired $CD25^+$ blood-CSF samples were examined in five patients. Only 10-20% of the peripheral activated clones were present in paired CSF samples. Since about one-third of T-cell clones of the CSF were detected in the paired blood samples, this may indicate that part of the clones were expanded or activated locally within the CNS. Percentage of CSF clones within the PB was even higher in previous blood samples which may suggest a temporal shift in the T-cell passage.

Repetaed analyzis of CSF samples in three cases showed the reduction of clonality with persistence of a few clones. We suppose that these clones might be specifically recruited or locally expanded within the CNS while non-specific by-stander cells changed and disappeared quickly. Constant clones may be either regulatory or encephalitogenic populations. CSF does not simply reflect the clonality of the activated peripheral T-cell repertoire, its clonality is rather specific and is modified by heterogeneous processes.

DNA sequencing of the CDR3 regions of the *in vivo* expanded myelin-specific T-cell clones

Although DNA sequences of the hypervariable region of different clones are distinct, randomly incorporated in-frame nucleotides (N-region diversity) may result in similar aminoacid (AA) motifs. These motifs might be involved in vaccination strategies.

We therefore determined the DNA sequences of the Vβ-clonotypes which represented the expanding myelin-specific T-cells. Deduced AA sequences of the CDR3 regions were then established and compared to each other.

The *in vivo* expanded clones did not bear similar AA motifs. The CDR3 sequence of the continuously expanded PLP95-116-specific T-cell clone was, however, particularly interesting. The hypervariable region was composed only of two aminoacids which raised the possibility that a degenerated TCR response, ie. stimulation with a constant unrelated antigen, might be responsible for the continuous re-activation of this clone.

SUMMARY OF THE THESIS

1. The exacerbation of MS is characterized by polyclonal expansion of T-cells in the peripheral blood.

2. Oligoclonal expansion of T-cells can be detected in the cerebrospinal fluid (CSF) of MS.

3. Frequency of myelin-specific T-cells is increased in the peripheral blood of MS relapse.

4. Freqency of $CD25^+$ activated T-cells and clonality of the activated T-cell repertoire is reduced in MS compared to healthy subjects.

5. Myelin-specific T-cells compose dominant clones within the reduced activated repertoire *in vivo* in MS.

6. Some of the myelin-specific T-cell clones may expand continuously in the peripheral blood.

7. Myelin-specific T-cell clones are also dominant *in vivo* within the CSF.

8. About one-third of the activated peripheral T-cells are present concomitantly in the CSF.

9. Traffic of the peripherally activated T-cells into the CSF/CNS compartment is heterogeneous and is influenced by local activation and selective recruition.

10. The *in vivo* activated myelin-specific T-cell clones do not bear similar aminoacid motifs in their hypervariable regions.

11. About 60-80% of the *in vitro* established myelin-specific T-cell clones are not significant *in vivo*.

12. The SSCP T-cell clonality assay is suitable for monitoring of the ongoing immune response.

ABBREVIATIONS:

AA	aminoacid
AICD	activation induced cell death
cDNA	complementer DNA
CDR3	3rd hypervariable region of the TCR
CNS	central nervous system
CSF	cerebrospinal fluid
CP	chronic progressive
EAE	experimental autoimmune encephalomyelitis
LDA	limiting dilution assay
MBP	myelin basic protein
MS	multiple sclerosis
OGP	oligoclonal gamopathy (bands)
PBMC	peripheral blood mononuclear cells
PLP	proteolipid protein
RR	relapsing-remitting
RT-PCR	reverse-transcriptase polymerase chain reaction
SA	superantigens
SSCP	single-strand conformation polymorphism assay
TCCL	T-cell line/clone
TCR	T-cell receptor

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