Swift Entry of Myelin-Specific T Lymphocytes into the Central Nervous System in Spontaneous Autoimmune Encephalomyelitis

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Swift Entry of Myelin-Specific T Lymphocytes into the Central Nervous System in Spontaneous Autoimmune Encephalomyelitis

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Strong evidence supports that CNS-specific CD4⁺ T cells are central to the pathogenesis of multiple sclerosis and experimental autoimmune encephalomyelitis (EAE). Using a model of spontaneous EAE, we demonstrated that myelin basic protein (MBP)-specific CD4⁺ T cells up-regulate activation markers in the CNS-draining cervical lymph nodes at a time when there is no T cell activation anywhere else, including the CNS, and before the appearance of clinical signs. In spontaneous EAE, the number of MBP-specific T cell numbers does not build up gradually in the CNS; instead, a swift migration of IFN-γ-producing T cells into the CNS takes place ~24 h before the onset of neurological signs of EAE. Surgical excision of the cervical lymph nodes in healthy pre-EAE transgenic mice delayed the onset of EAE and resulted in a less severe disease. In EAE induced by immunization with MBP/CFA, a similar activation of T cells in the draining lymph nodes of the injection site precedes the disease. Taken together, our results suggest that peripheral activation of T cells in draining lymph nodes is an early event in the development of EAE, which paves the way for the initial burst of IFN-γ-producing CD4⁺ T cell into the CNS. The Journal of Immunology, 2008, 181: 4648–4655.

The accessibility of the CNS to the immune system is different from that of other tissues in at least three major ways. One, the presence of a blood-brain barrier (BBB)⁶; two, the absence of lymphatic vessels; and three, the absence of parenchymal dendritic cells (1–3). In normal conditions, these characteristics maintain the presence of immune cells in the CNS to a surveillance level. However, several CNS pathologies are triggered by disruptions in this balance with accumulation of T cells and development of inflammation. The alterations that enable T cells to increase and the anatomy of the events that trigger immune cell accumulation in the CNS are not well understood.

Experimental autoimmune encephalomyelitis (EAE) is a CD4⁺ T cell-mediated disease characterized by mononuclear cell infiltration in the CNS. In EAE, activated encephalitogenic T cells migrate across the BBB and recruit leukocytes into the brain (1, 4–7). This inflammation induces edema and triggers a cascade of events that leads to demyelination and axonal loss (8). Because EAE shares many immunopathological and neuropathological similarities with multiple sclerosis (9–11), it is widely used as an experimental model for this disease. EAE models clearly demonstrate a key role of T cells in the initiation of disease. These studies helped to postulate a similar role for T cells in the initiation of MS, a role that has now been amply documented. However, the mechanisms by which T cells initially become activated and enter the CNS to initiate MS are still not well understood. The main questions that remain to be clarified are the location of the primary site where T cells become activated and what are the characteristics of T cell buildup in the CNS. In addition, the role of draining secondary lymphoid organs when T cells enter the CNS is controversial.

In this manuscript we address these questions using a spontaneous EAE model, in which we verify changes on the expression levels of a collection of activation markers on T cells from the diseased CNS, in parallel with T cells from draining deep cervical lymph nodes (dcLN) at various time points before and during EAE onset. We compare the results to those obtained using an immunization-induced EAE model.

Based on the expression of major activation markers and ability to produce IFN-γ, these two models offer strong evidence of peripheral activation of T cells before the identification of detectable signs of EAE, with participation of draining lymph nodes, represented by dcLN in the spontaneous model and para-aortic (PA) lymph nodes in the immunization-induced disease model.

Materials and Methods

Mice

Myelin basic protein (MBP)-specific TCR transgenic mice (T/R⁻) have been previously described (12). Monoclonality of the off T cell repertoire was achieved by crossing MBP-specific TCR transgenic mice with either RAG-1⁻/⁻ mice (generating T/R⁻ mice) or TCR α⁻/⁻ × TCR β⁻/⁻ double knockout mice (generating T(α⁻ β⁻) mice) (12, 13). All mice were kept...
under specific pathogen-free conditions in individually ventilated cages at the Skirball Institute Central Animal Facility, New York University School of Medicine (New York, NY). All protocols involving mice handling were approved by New York University School of Medicine Institutional Animal Care and Use Committee.

Cell suspensions
Mice were anesthetized with a mixture containing 12.5 mg/ml ketamine, 2.5 mg/ml xylazine, and 25 mg/ml acepromazine diluted in PBS (i.p., 100 μl per mouse). Animals were perfused with 160 ml of PBS containing 5 mM EDTA through the left ventricle of the heart. A successful perfusion was assessed by the white color of the lungs and liver. Deep cervical, superficial cervical, inguinal lymph nodes, and the CNS (spinal cord, brain stem, and cerebellum) were dissected and incubated in 10 mg/ml collagenase D (Boehringer Mannheim) at 37°C for 45 min. Samples were strained through a 70-μm diameter nylon mesh to obtain a single cell suspension and washed in PBS. Cell suspensions were stained with 10 mg/ml collagenase and washed in PBS. The CNS preparation was applied to a 38% Percoll (Amersham Biosciences), centrifuged at 1,000 × g for 30 min, washed twice in PBS, and the number of cells was counted in a hemocytometer. All samples were resuspended in PBS containing 2% FCS and 0.1% sodium azide for FACS analysis.

Abs and FACS analysis
Anti-MBP TCR clonotypic Ab (3H12) was generated in our laboratory as described (13). Anti-CD4, CD3, CD69, CD45RB, CD44, CD25, and IFN-γ Abs were obtained from BD Pharmingen. Single cell suspensions were stained with 10 μg/ml Ab, washed, and fixed/permeabilized with Cytofix/CytoPerm (BD Pharmingen) according to manufacturer’s instruction. Anti-IFN-γ Abs were added to the preparations for 20 min at 4°C.

Disease evaluation
EAE was scored as previously described (14): level 1, limp tail: level 2, hind leg weakness or partial paralysis: level 3, total hind leg paralysis; level 4, hind limb paralysis and front leg weakness or partial paralysis; level 5, moribund. All protocols were approved by the New York University’s Institutional and Animal Care Use Committee.

Immunization
T/R⁺ mice received a s.c. injection (100 μl) at the base of the tail with MBP Acl–11 peptide (2 mg/ml) emulsified in CFA supplemented with 4 mg/ml Mycobacterium tuberculosis H37 RA. Each mouse received two i.v. injections (200 ng each) of pertussis toxin at days 0 and 2 after immunization.

Cervical lymphadenectomy
Bilateral cervical lymphadenectomy was performed on deeply anesthetized mice. Deep and superficial cervical lymph nodes were removed under an operating microscope through midline incision in the neck and gently excised. Sham lymphadenectomy was performed in the control group in which the skin of the neck was incised as for the experimental group but no lymph nodes were taken. The skin was sutured and the animals were allowed to recover. Young T/R⁻ mice (23–25 days of age) submitted to lymphadenectomy and the development of EAE evaluated during a period of 90 days. Some animals subjected to surgery were sacrificed at the end of the experiment and Evans blue 10% injected into the brain parenchyma for the analysis of lymphatics and lymph node. T/R⁺ or T/αβ⁻/⁺ mice with EAE were age, sex, and disease level matched and divided into sham and lymphadenectomy operated groups. Lymph node and CNS cells were removed for FACS analysis 7 days after surgery. The CNS was processed as described above.

Statistical analysis
Unpaired and paired t test were used to determine statistical significance. Differences were considered significant when p < 0.05.

Results
Swift entry of MBP-specific T cells into the CNS in the spontaneous EAE model
In the spontaneous EAE model in T/R⁻ or T/αβ⁻ mice on a C57BL/10.PL genetic background, the time of disease onset is reasonably predictable. Virtually no animals display clinical signs of EAE before age 35 days, 30% show clinical signs of EAE by 42 days, 50% by 50 days, 80% by 70 days, and 100% by 100 days (13, 15). MBP-specific T cells are present in high frequency, and are easily tracked with the clonotype-specific mAb 3H12 (13).

To closely monitor the progression toward EAE, we recorded the weight and clinical signs of EAE of a cohort of T/αβ⁻ mice every morning. As the animals reached the age of EAE onset, the weight of each mouse dropped significantly over the course of 3–5 days, then it started to rise again (Fig. 1a). Usually, neurological signs such as limp tail were detectable 1 or 2 days after the weight loss was first observed, although in some cases the sharp weight drop and neurological signs were noted on the same day. We refer to the mice that experienced ~5% weight loss in a single day, but did not yet show signs of disease, as having subclinical EAE. EAE-susceptible T/αβ⁻ mice at ages 4, 5, 6, and 7 wk were sacrificed to evaluate the distribution of MBP-specific T cells in the CNS and in the secondary lymphoid organs.

T/αβ⁻ mice have ~10–25,000 MBP-specific T cells in the CNS before they reach subclinical EAE. At this stage, however, we observed the sudden appearance of a large number of MBP-specific T cells in the CNS (~200,000 MBP-specific T cells in 1 or 2 days), and this number continued to rise for several days (Fig. 1, b and c). The precipitous increase in the number of MBP-specific T cells in such short period of time cannot be explained by local cell division, but rather by migration from other sites. Molecules and cells introduced into the CNS can efflux from the subarachnoid space into the blood and cervical lymph nodes (1, 16–19). Consequently, the cervical lymphatics provide significant drainage for CNS Ags. To test whether the sudden migration of MBP-specific T cells into the CNS could be accounted for by cells leaving the dcLN, we quantified the number of CD4⁺ T cells in dcLN and inguinal lymph nodes from healthy T/αβ⁻ mice, mice with subclinical EAE, and mice that had recently developed EAE. A decrease of ~600,000 dcLN T cells occurred between the stage just before EAE and the subclinical stage of disease (Fig. 1c). No such reduction was observed in the inguinal lymph nodes, where, instead of a reduction, a nonstatistically significant increase in the number of MBP-specific T cells was observed (Fig. 1c). Thus, in spontaneous EAE we did not observe a gradual build up of T cells or other inflammatory cells in the CNS of pre-EAE mice. Instead, in this model in which no strong induction protocol is used, EAE also occurs acutely as a burst of MBP-specific T lymphocytes gain entry into the CNS, with a close temporal correlation between the burst of T cell entry into the CNS and the onset of clinical manifestations of disease.

The large inflow of cells into the CNS occurs in parallel with a weight loss of ~5% per day, a primary sign of acute illness. Weight loss can be experimentally induced by the administration of small quantities (1–2 ng) of IL-1β, leading to transient anorexia, and weight loss of ~10%. Similarly, TNF-α and IFN-γ can induce anorexia. However, cytokines can be up-regulated peripherally and also induce similar symptoms, as systemic administration of IL-1β (at 1,000-fold higher dose than intracerebral administration) or LPS, which is a potent TNF-α inducer, also lead to transient weight loss (20–23). Indeed, the CNS-infiltrating cells in spontaneous EAE expressed high levels of mRNA for these cytokines (Fig. 1d).

In spontaneous EAE, priming of MBP-specific T cells occurs in the cervical lymph nodes
The vast majority of peripheral MBP-specific T cells in TCR transgenic mice display a naive phenotype. Because T cell activation is a step toward differentiation into the effector phase, we sought to
characterize the kinetics of T cell activation in the lymph nodes at ages 4, 5, 6, and 7 wk, before and after clinical manifestation of the disease. Toward that end, we collected cells from inguinal, superficial cervical, and dcLN and analyzed the expression of the activation markers CD69, a very early activation marker, and CD44. MBP-specific T cells from 4-wk-old mice did not express activation markers in the lymph nodes, and no significant T cell infiltration in the CNS was observed (Fig. 1b and Fig. 2). In contrast, a fraction of MBP-specific T cells from a number of 5–6-wk-old EAE-free animals had up-regulated the expression of CD69 and
Surgical removal of the cervical lymph nodes decreases the severity of EAE

To investigate the role of the cervical lymph nodes in the initial activation of MBP-specific T cells in spontaneous EAE, we performed bilateral cervical lymphadenectomy on seven T/αβ mice at 21–25 days of age. These mice were observed for clinical signs of EAE during a period of 90 days. Two control groups were run, one in which the inguinal lymph nodes were removed, and one in which the procedures of cervical lymph node excision was sham operated. As shown in Fig. 3a, EAE development was greatly reduced when compared with the control group (T/αβ and T/R– sham operated), indicating that the cervical lymph nodes are important sites for the development of EAE. However, cervical lymph node excision was not able to completely abolish the disease. This could be due to drainage through a different route, or re-establishment of the cervical lymphatic drainage pathway to a more distal lymph node, two nonexclusive possibilities. As seen in Fig. 3b, cervical lymph node excision was sham operated. The infiltrating cells in the CNS were isolated for FACS analysis 7 days after surgery. CNS samples were counted and stained with 3H12 Ab. Left, Number of MBP-specific T cells in the CNS in all animals analyzed (n = 13/group, *, p < 0.001). Right, Percentage of reduction of MBP-specific CD4+ T cells in the CNS in each pair (n = 13).
had re-anastomosed, thus offering an explanation for the presence of a disease in animals in which the lymph nodes were excised.

We next studied the effect of cervical lymphadenectomy on animals already afflicted with EAE for a minimum of 2 wk. We prepared 13 pairs of mice displaying overlapping disease curves. One mouse per pair was subjected to cervical lymphadenectomy, and the other to mock lymph node removal. After 1 wk, we analyzed the infiltrate in the CNS by FACS. As shown in Fig. 4, there was, on average, a reduction of 50% in the number of MBP-specific T cells infiltrating the CNS in operated vs sham-operated animals. Although inflammation could be reduced, there was no significant improvement in the clinical score of the mice. It is possible that at this late stage of disease substantial secondary damage occurred, and these effects are not expected to revert after inflammation is reduced. Although the results on ongoing EAE are not as striking as the results with EAE prevention, our results indicate that even during advanced disease, some cells continue to migrate from cervical lymph nodes into the CNS.

**Kinetics of MBP-specific T cell activation after immunization of T/R animals**

Differently from T/α−β− mice, T/R+ mice do not develop spontaneous EAE; however, upon immunization with MBP all T/R+ mice develop severe EAE within 6–9 days. MBP/CFA-induced EAE in T/R+ mice has a marked initiation point that can be compared with the more natural initiation of spontaneous disease in T/α−β− mice. To monitor the kinetics of MBP-specific T cell activation, we immunized the mice in the base of the tail, collected the draining PA lymph nodes on days 1, 2, 3, 4, 5, 6, and 9 postimmunization, and analyzed the expression of activation markers. In contrast to the spontaneous disease where we observed that only a small fraction of the MBP-specific T cells expressed CD69 in the draining lymph node at a given time, virtually all MBP-specific T cells expressed CD69 within one day of immunization, and as expected, the frequency of CD69+ MBP-specific T cells decreased at later time points (Fig. 5, a and b). As previously described,

**FIGURE 5.** Kinetics of MBP-specific T cell activation and CNS entry in MBP/CFA-immunized T/R+ mice. MBP-specific T/R+ mice were immunized with Ac1–11 MBP peptide emulsified with CFA at the base of the tail and given i.v. pertussis toxin. On days 1, 2, 3, 4, 5, 6, and 9 after immunization, mice were perfused and the PA lymph nodes and CNS removed. Lymph node cells were stained with Abs specific for CD4, anti-MBP TCR (3H12 Ab), CD69, CD44, and CD45RB, whereas CNS preparations were stained with CD4 and 3H12 Abs. Cells in the CD69 histograms and CD45RB/CD44 dot plots were gated on 3H12+ MBP-specific cells. a, Flow cytometry profile of one representative animal at each time point. b, Average ± SD of CD69 expression; and c, CD44high/CD45RBlow expression on MBP-specific T cells at each time point (n = 3/time point). d, Number of infiltrating MBP-specific T cells in the CNS of individual T/R+ animals, nonimmunized (NI), immunized, and analyzed before EAE onset (days 4–5), or analyzed after EAE onset (day 6).
MBP-specific T cells from nonimmunized T/R+ mice retained a naive phenotype (Fig. 5, b and c), and cells with effector/memory phenotype were quickly found in the draining lymph nodes of immunized mice (Fig. 5c). We also collected CNS cells from the same group of immunized and nonimmunized animals. Similarly to what we observed in spontaneous EAE, in MBP/CFA-induced EAE the first clinical manifestations of EAE occurred almost simultaneously to the migration of MBP-specific T cells into the CNS, which, as in the case of spontaneous EAE, was abrupt (Fig. 5, a and d). Mice at day 5 postimmunization did not show EAE, and there was no significant accumulation of T cells in the CNS, whereas mice on days 6 and 9 showed EAE level 2 and a significant increase of MBP-specific T cells in the CNS was observed. As in the case of spontaneous EAE, the dramatic increase in MBP-specific T cell in the CNS could not be accounted for by cell division of the small number of T cells that are in the CNS before disease induction. In conclusion, unexpectedly spontaneous EAE development has several kinetic features that are similar to Ag/CFA-induced EAE.

Discussion
In this article we describe the kinetics of MBP-specific T cell priming, activation, and migration to the CNS in a spontaneous EAE model. No experimental trigger such as MBP immunization or administration of pertussis toxin, mycobacteria, or adjuvant is applied. The importance of this spontaneous model is highlighted by the fact that spontaneous EAE occurs with the same high incidence in germfree mice (15), indicating that microbial products are dispensable. However, spontaneous EAE requires CD28 costimulation (24) indicating the triggering of B7 ligands by endogenous “danger” signals.

The importance of lymph node as the priming site for CNS-specific T cells has been unclear. McMahon et al. showed that in relapsing-remitting EAE, activation of naïve proteolipid protein-specific T cells occurred directly in the CNS and not in the cervical lymph nodes or other peripheral lymphoid organs (25). However, these conclusions were reached after transfer of naïve neuroantigen-specific T cells into mice undergoing CNS inflammation caused by Theliers’ virus or relapsing-remitting EAE. Once CNS inflammation is established, there is no question that the CNS is capable of maintaining a strong re-stimulatory capacity due to the presence of APCs (26–28). In unmanipulated mice that will develop spontaneous EAE, we could clearly show that the initial activation of T cells does occur in the cervical lymph nodes. The importance of draining lymph nodes for MBP-specific T cell priming was highlighted in the spontaneous model and confirmed by an immunization-induced model of EAE. Although the accessibility of the immune system vis-à-vis the CNS displays many unique properties, the role of draining lymph nodes appears to be critical in CNS immune responses as has been described for nonprivileged organs such as the pancreas (29, 30).

Studies on T cell migration to skin and gut have led to the concept that activated T cells migrate preferentially to tissues that are drained by the lymphoid organs where the T cells were primed (31, 32), although alternative views can be found (33). Although the CNS accessibility to the immune system has many unique properties, our data support a model in which T cells primed in the dcLN migrate to the CNS. In agreement with this, we observed that dcLN lose a large number of MBP-specific T cells approximately at the same time that large numbers of T cells are first found in the CNS. This T cell depletion was unique to dcLN (Fig. 1c) and is remarkable considering the constant thymic output of naïve T cells in these young mice. Another important observation is that we never detected T cell activation in dcLN of T/R+ mice (Fig. 2), which do not develop spontaneous EAE due to the presence of regulatory T cells (12, 13, 15). Thus, dcLN are a site where one layer of T cell-mediated regulation may take place. It has been proposed that other layers of regulation may happen within the CNS (34–36). It is possible that, as disease progresses and activated cells are broadly found, the regulation process has to be extended to additional layers back to additional peripheral sites. This brings up the importance of understanding the anatomy of T cell activation in given phases of disease to introduce regulatory approaches that work efficiently as therapeutics.

Although spontaneous EAE does not occur in animals younger than 6 wk of age, we did not find any evidence for a gradual accumulation of MBP-specific T cells over an extended period of time, slowly inducing damage that eventually reaches sufficient magnitude to cause clinical signs of disease. Our data favors, instead, a model whereby a sudden inflow of activated T cells, which is accompanied by edema, causes the first neurological signs of disease (Fig. 1). At later stages, demyelination is observed, which may account for the chronic stages of disease.

In spontaneous EAE, neurological signs of disease started soon after a noticeable weight loss occurred, which lasted 3–5 days. After this period the animals regained weight, despite the fact that clinical disease continued to progress, and large numbers of T cells and other mononuclear inflammatory cells could be recovered from the CNS. Thus, two distinct phases were identified in spontaneous disease: an acute phase that lasts ~1 wk and a chronic progressive phase that lasts from several weeks to several months.

The quick burst of MBP-specific T cells into CNS that we observed in spontaneous EAE was also observed in other EAE models in which such an outcome was more predictable. For instance, it was observed upon i.p. transfer of in vitro-activated MBP-specific T cells into rats (37) and in our analysis of MBP/CFA-induced EAE (Fig. 5). These observations correspond to the initial events in the development of pathology and may differ from models where BBB disruption is involved as a primary event, for example upon intracerebral injection of viral particles to induce demyelinating disease (38), or from observations performed at later stages of the disease (27).

It is not clear whether in spontaneous EAE a small number of pioneering cells migrate beforehand into the CNS and act as a Trojan horse to generate conditions allowing the massive and sudden CNS invasion by a large number of cells. Such early entry of cells into the CNS was reported upon injection of neuroantigen-activated labeled T cells by Hickey et al. (5) and Cross et al. (6).

Itano et al. (39) described that upon injection of Ag in the ear, two waves of Ag presentation take place. The first one occurs within the first few hours after immunization and represents Ag migrating in soluble form to be processed and presented in the draining lymph nodes. The second wave can be detected 24 h after injection and represents Ag-loaded interstitial dendritic cells, which migrate with their cargo to the lymph nodes. Whereas the first wave is responsible for T cell CD69 up-regulation and proliferation, only the second wave can induce T cells capable of participation in a delayed-type hypersensitivity reaction. In the case of MBPAc1–11-loaded APC, a special situation may occur. Due to the unusually high instability of these MHC-peptide complexes (40–43), migrating APC would lose their cargo as soon as it is displayed in the MHC groove. However, in an environment where there is abundant MBP such as the CNS, new antigenic peptides could be constantly loaded, maintaining the stimulation of T cells. This, added to the rapid increase in the proportion of IFN-γ-producing T cells as they enter the CNS, leads us to consider that, at least in this case, the delayed-type hypersensitivity-enabling second stimulation occurs within the CNS. Using adoptive
transfer of in vitro-activated CNS-specific T cells, Kawakami et al. also observed that high IFN-γ-producing cells occurred only within the CNS (44).

Weller and coworkers performed cervical lymphadectomy in rats that have been induced to develop EAE and an enhancing cryolesion in the brain. They found that although cervical lymph node removal had no effect on EAE, it significantly reduced the enhancement caused by cryolesion (45). One possible explanation for their nonsignificant effect in EAE, which contrasts with our results, is that EAE was induced by hind feet injection of Ag/CFA, which drains to the popliteal lymph nodes rather than the cervicals.

Our experiments show that removal of CNS-draining cervical lymph nodes in pre-EAE mice had a beneficial effect on the onset and severity of spontaneous disease. When surgery was performed on EAE-afflicted mice, there was a reduction in T cell infiltration. The communication between CNS and peripheral immune system through cervical lymph nodes has long been characterized (16, 46, 47). Migration of dendritic cells from the brain into the cervical lymph nodes has been demonstrated (48, 49). In addition, in monkeys and humans, myelin Ags were found in cervical lymph nodes (50). Communication between the human brain and the immune system of the head and neck was also identified in studies of brain hemorrhage (51).

We described the anatomy of T cell activation during early onset of CNS disease in two EAE models, spontaneous and immunization induced. The participation of draining lymph nodes in the priming of T cells, and the sudden entry of massive numbers of encephalitogenic cells into the CNS, suggest a role for peripheral Ag presentation in granting MBF-specific cells the properties necessary to cross the BBB and start disease. Because we made a set of observations in animals not treated with adjuvants, the conclusions reached here may help understand the initiation and progression of CNS pathologies such as multiple sclerosis.

In the preceding article of this issue of the Journal of Immunology, Zhang et al. (52) conducted an independent study on spontaneous EAE using a different TCR transgenic line, recognizing a different neuroantigen and in a different genetic background. Interestingly, the authors reported that the age of EAE onset was 42 days, similar to what we reported here.

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Disclosures
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