On the role of peripheral macrophages during active experimental allergic encephalomyelitis (EAE)

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Summary. Experimental allergic encephalitis (EAE) is an experimental autoimmune inflammatory condition of the central nervous system (CNS) that serves as a disease model for multiple sclerosis (MS). The primary effector mechanisms of the immune system leading to tissue destruction during EAE remain still controversial. T-cells, microglia, and macrophages infiltrating the brain parenchyma are suggested to be involved. To clarify the role of these cells during disease Lewis rats were immunised with different immunisation protocols: Immunisation with myelin basic protein (MBP) in complete Freunds adjuvant (CFA) containing high dose of mycobacterial components induced severe disease, whereas immunisation with low dose of mycobacterial components induced only mild disease. Severely and mildly diseased animals were analysed with respect to infiltration of T-cells, macrophages and upregulation of MHC class II molecules on microglia in the brain.

All immunised rats showed high T-cell infiltration accompanied by microglia activation. The degree of disease and the infiltration of macrophages varied with dose of adjuvant. Lowering the dose of adjuvant prevented the development of disease but also the influx of peripheral macrophages into the brain without affecting the peripheral T-cell response to the autoantigen. Thus, appearance of (autoreactive) T-cells in the brain and microglia activation were probably not sufficient for development of disease.

It can be concluded that peripheral macrophages play an essential or even key role in the pathogenesis of active EAE.

Keywords: EAE, microglia, macrophages, autoimmunity.

Introduction

Experimental allergic encephalomyelitis (EAE) is an inflammatory disease of the central nervous system (CNS) inducible in susceptible strains of rats by active immunisation with myelin basic protein (MBP) in adjuvant. Target of

the inflammatory response is the myelin membrane that ensheats axons, as well as the oligodendrocytes that produce myelin. Histologically, EAE is characterised by perivascular infiltrates that principally contain CD4+ T cells and macrophages, and by upregulation of major histocompatibility complex (MHC) in the brain (Owens et al., 1994; Zamvil and Steinman, 1990).

EAE requires the development of a cell-mediated immune response within the CNS. T-helper cells specific for encephalitogenic epitopes of MBP most likely initiate the inflammatory response. Passing the blood brain barrier (BBB) they accumulate in the perivascular lesions in the CNS together with blood born macrophages (Levine et al., 1975; Owens et al., 1994; Zamvil and Steinman, 1990). Although the pathology of EAE is characterised by these infiltrating cells, the primary immune effector mechanisms responsible for the destruction of myelin membranes remain controversial. Destruction might be mediated by MHC class II-restricted CD4+ T-cells via the local secretion of cytokines (Sun and Wekerle, 1986) or by the activation of microglia (Owens et al., 1994), or by infiltrating macrophages (Huitinga et al., 1990).

For encephalitogenic T-cells it was shown that they can lyse syngeneic astrocytes in an antigen specific manner (Sun and Wekerle, 1986). However, the most favoured hypothesis is that activation of microglia via infiltrating T-cells may lead to tissue damage (Owens et al., 1994).

Microglia is the most abundant cell population upregulating MHC class II molecules in the brain during inflammation (Hayes et al., 1987; Matsumoto et al., 1986). Gamma interferon (IFNg) release by infiltrating T-cells may be responsible for this "activation" of microglia. In vivo infusion or intrathecal injection of IFNg leads to rapid expression of class II molecules on microglia (Steiniger and van der Meide, 1988; Vass and Lassmann, 1990; Wong et al., 1984).

In addition it was shown that microglial cells are able to drive an immune response in vitro (Frei et al., 1987; Panek and Benveniste, 1995; Suzumura et al., 1987).

In numerous CNS diseases such as multiple sclerosis (MS), AIDS dementia complex, Alzheimer's disease, and Parkinson's disease it is believed that microglia plays a role in pathogenesis (Dickson et al., 1993; Hofman et al., 1986; McGeer et al., 1993; Münch et al., 1998). Moreover, it was shown in vitro that microglial cells are able to release a battery of toxic substances such as reactive oxygen intermediates (Colton and Gilbert, 1987), proteinases (Banati et al., 1993) and reactive nitrogen intermediates (Boje and Arora, 1992; Zielasek et al., 1992). Such properties are well known for peripheral macrophages. Therefore, the question arises what the role of infiltrating macrophages during CNS inflammation may be.

Hypothesising a correlation between the pathogenic cell population and disease, different immunisation protocols were compared with regard to induction of disease and the appearance of macrophages, T-cells, and activation of microglia in the CNS.

These cells were characterised using a previously reported method to isolate infiltrating leukocytes from the inflamed brain (Dorries et al., 1991; Imrich et al., 1994; Sedgwick et al., 1991) which allows to analyse infiltrating

cells by flow cytometry. The advantage of this method is the possibility to monitor the whole situation in the CNS instead of only small sections analysed in histological stainings.

Kinetics of infiltrating macrophages and T-cells in the CNS as well as kinetics of microglia activation were investigated after induction of severe and mild disease in Lewis rats. The results clearly indicate that in the EAE rat model strong T-cell influx into the brain parenchyma accompanied by microglia activation not necessarily leads to neurological symptoms. Only in diseased animals strong macrophage infiltrates can be detected. Therefore, although the resident microglia cell population shares many properties with macrophages, there may be a clear functional difference between these two accessory cell types of the immune system.

Materials and methods

Experimental animals

Specific pathogen-free Lewis rats (RT1[l]) were purchased from the Charles River GmbH, Sulzfeld, Germany.

Antibodies

To stain cells for flow-cytometric analysis the following monoclonal antibodies (mAbs) were used:

OX1 anti rat leukocyte common antigen CD45 (Sunderland et al., 1979), OX6 anti rat monomorphic MHC class II, RT1 (I-A) (McMaster and Williams, 1979), R73 specific for the α/β chains of the rat TCR (Hünig et al., 1989), OX8 anti rat CD8 complex (Brideau et al., 1980) and W3/25 anti rat CD4 complex (Williams et al., 1977).

The mAb MRC OX-21 specific for the human C3b inactivator served as a control antibody (Hsiung, 1982). All mAbs were obtained from Serotec. Phycoerythrin (PE)-labeled secondary goat anti-mouse IgG (GaMIgG) was derived from Dianova (Hamburg F.R.G.).

Antigen and induction of active EAE

Active EAE was induced by immunisation of rats with myelin basic protein (MBP) in complete Freunds adjuvant as described previously (Imrich et al., 1995). MBP was isolated from guinea pig brains according to Watanabe et al. (1983). Freunds adjuvant and mycobacterium tuberculosis were purchased from Difco (Detroit, MI). The control antigen keyhole limpet haemocyanin (KLH) was from Sigma. The antigen was emulsified in adjuvant to a concentration of 1 mg/ml. In addition mycobacterium tuberculosis was added: low dose = 1 mg/ml adjuvant and high dose 5 mg/ml adjuvant. Each animal received $2 \times 50 \mu l$ of antigen in adjuvant.

Clinical signs of EAE were graded daily on an arbitrary scale of 1 to 6 as follows: (0) no overt disease; (1) limp tail; (2) paresis of 1 or 2 legs; (3) and (4), unilateral and bilateral hind leg paralysis, respectively; (5) bilateral hind leg paralysis and incontinence; (6) moribund (Imrich et al., 1995).

Isolation of lymphocytes from central nervous system and lymph nodes

Inflammatory lymphocytes were isolated from the CNS according to a procedure published earlier (Dorries et al., 1991; Imrich et al., 1994). Briefly, animals were thoroughly perfused with PBS (200 ml) before removing the CNS (= brain and spinal cord). Lymphoid cells were released from the CNS tissue by mechanical disruption and

enzymatic digestion of the tissue followed by Percoll step gradient centrifugation. Leuko-cytes were collected from interfaces of the appropriate density and sedimented in Hank's buffer ($170 \times g$, 10 min, 4°C).

Popliteal lymph nodes were minced through a steel sieve in Hank's buffer. Connective tissue fragments were allowed to settle for 10 min at 4°C and lymphocytes were sedimented from the supernatant by low speed centrifugation ($170 \times g$, 10 min, 4°C).

Determination of T lymphocyte proliferation

Isolated lymphocytes were cultured in 96 well microtiter plates (Costar) at a density of 5×10^5 cells per well in 200 μl RPMI 1640 medium supplemented with 3% rat serum at 37°C (5% CO_2). Cells were restimulated with antigen at a final concentration of 20 μg/ml MBP or mycobacterial antigens, respectively. KLH was used as a control antigen.

Proliferation was assessed by [^{3}H]thymidine incorporation (1 μCi/well) after 48 h of culture. Incorporated radioactivity was determined by harvesting the lymphocytes on glass fiber filters, lysing the cells by hypotonic wash and subsequent count of radioactive decays in a β-counter (Canberra Packard). The stimulation index represents the ratio of incorporated radioactivities after stimulation with antigen and control antigen.

Quantitation and phenotypic characterisation of isolated CNS leukocytes

Lymphocytes were characterised by two color immunofluorescence and four parameter flow-cytometry (forward/side scatter, red/green fluorescence) as described earlier (Imrich et al., 1995, 1994). Briefly, lymphocytes were indirectly stained using a primary mouse mAb and a phycoerythrin (PE)-labeled secondary goat anti mouse IgG (GαMIgG) followed by another primary mouse mAb labeled with FITC. To avoid capture of FITC-labeled primary mAb by PE-labeled secondary GαMIgG normal mouse serum was used in excess to block potentially free binding capacity on GαMIgG. Flow-cytomeric analysis was performed using a FACScan (Becton-Dickinson, Heidelberg, Germany) linked to a Hewlett Packard 6000 computer with the Consort 30 software installed.

Infiltrating leukocytes were distinguished from macrophages by criteria of FSC/SSC. Two gates were selected. The lymphocyte gate with the following cordinates FSC/SSC: 55/3, 54/18, 78/33, 109/33, 112/22, 106/7 and the macrophage gate with cordinates FSC/SSC: 81/4, 81/42, 84/243, 246/243, 239/32, 237/29. After labelling cells with anti TCR, no TCR positive cells were found in the macrophage gate.

To determine the total number of cell subpopulations isolated from the brain, first the total number of cells collected from the gradient was counted. Since the distribution of events out of 10.000 within the different gates were given by FACS analysis, the total number of cells isolated from the gradient with specific FSC/SSC characteristics could be calculated. The real cell number of infiltrating leukocyte subpopulation was calculated by multiplying the percentage of positive events by the total number of cells within the specific gate.

Results

Disease

To induce active EAE Lewis rats were immunised with myelin basic pro-tein (MBP) in complete Freunds adjuvant (CFA) containing 5 mg/ml of mycobacterium tuberculosis (myc.T). The development of neurological symp-toms was monitored by scoring of tale and limp paraplegia (Imrich et al., 1995). In Fig. 1 the course of the disease is shown. Clinical signs of the disease started at day 9 and reached a peak at day 14 post immunisation (14 dpi). Thereafter, animals started to recover and at 18 dpi all had completely
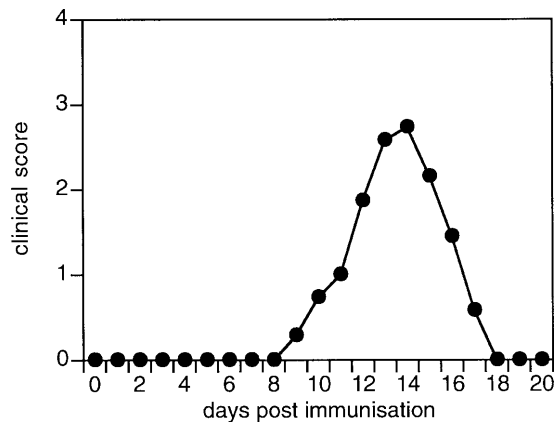
Fig. 1. Course of the disease. EAE was induced in 7-week old Lewis rats by subcutaneous injection of guinea pig MBP in complete Freunds adjuvant containing high dose of mycobacterium tuberculosis. Neurological signs of individual animals were scored daily. The average symptomatology is given by the arithmetic mean value (n = 7). Clinical score: (0) no overt disease; (1) limp tail; (2) paresis of 1 or 2 legs; (3) and (4) unilateral and bilateral hind leg paralysis, respectively

recovered from the disease. To induce this severe disease it was necessary to immunise rats with MBP in CFA containing high dose of myc.T. Normally for immunisation an antigen is emulsified in CFA containing 1 mg/ml myc.T. This amount of myc.T is sufficient to induce a strong immune response (Deeb et al., 1992; Johnston et al., 1991; Smith et al., 1992). This concentration of myc.T in combination with the MBP batch used for these experiments was insufficient to induce severe neurological symptoms in EAE. After immunisation of rats with MBP in CFA containing 1 mg/ml myc.T most of the animals did not develop disease. Only 1 out of 6 animals showed mild clinical symptoms (clinical score = 1) at 14 dpi (Fig. 2 upper panel).

However, after increasing the dose of mycobacterial antigens in CFA (from 1 to 5 mg/ml) in combination with a constant concentration of the autoantigen, seven out of seven animals developed neurological symptoms at 14 dpi (Fig. 2 lower panel).

Therefore, myc.T antigens in CFA may modulate the immune response in an dose dependent manner.

Antigen specific proliferation of lymphocytes derived from popliteal lymph nodes (LNP)

It seemed that a higher dose of myc.T in CFA may induce a stronger T-cell response to the autoantigen leading to severe disease in immunised animals. To test this assumption proliferation assays were performed with lymphocytes derived from draining lymph nodes of rats immunised with MBP in CFA containing high dose of myc.T and compared with specific proliferation of lymphocytes derived from animals immunised with MBP in CFA with low
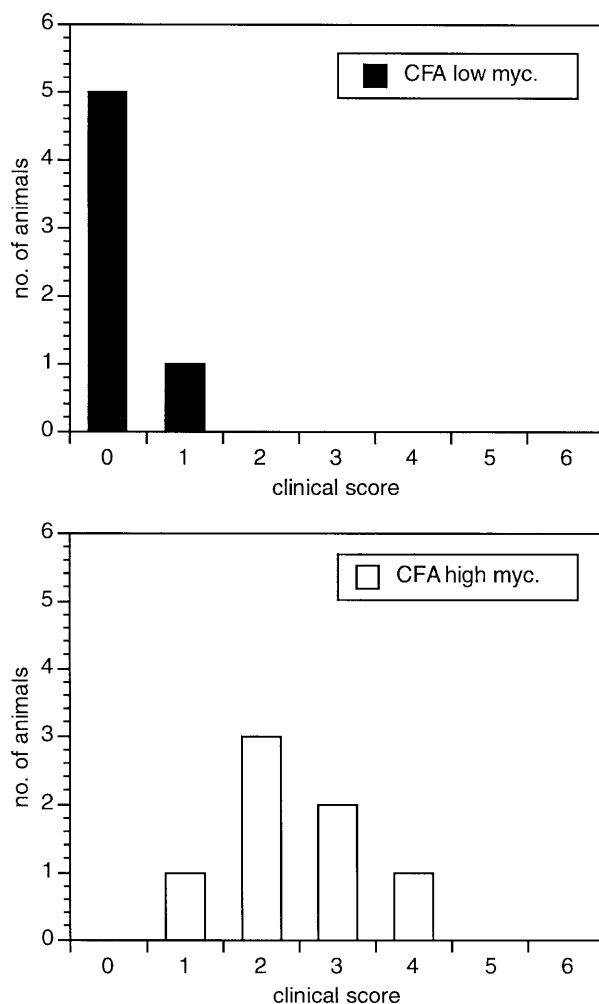
Fig. 2. Grouping of animals according to severity of symptoms at maximum of disease after immunisation of rats with CFA containing low dose of mycobacterium tuberculosis (upper panel), and after immunisation of rats with CFA containing high dose of mycobacterium tuberculosis (lower panel) respectively. Clinical score: (0) no overt disease; (1) limp tail; (2) paresis of 1 or 2 legs; (3) and (4), unilateral and bilateral hind leg paralysis, respectively; (5) bilateral hind leg paralysis and incontinence; (6) moribund

dose myc.T. In Fig. 3 one out of four proliferation assays with lymphocytes derived at 12 dpi is shown. From all experiments similar results were obtained.

No difference of MBP specific T-cell proliferation was observed Fig. 3 (upper panel). In both cases, the incorporation of ^{3}H-thymidine in response to MBP was 3 fold higher compared to the control antigen KLH.

In comparison with the response against the autoantigen, the T-cell response against myc.T was much higher. However, as shown in Fig. 3 (lower panel) the myc.T specific proliferation did again not differ between the animal groups. In both cases, the stimulation index was around 6 indicating that there was no decrease in the T-cell response against myc.T after decreasing the amount of myc.T antigens in CFA.
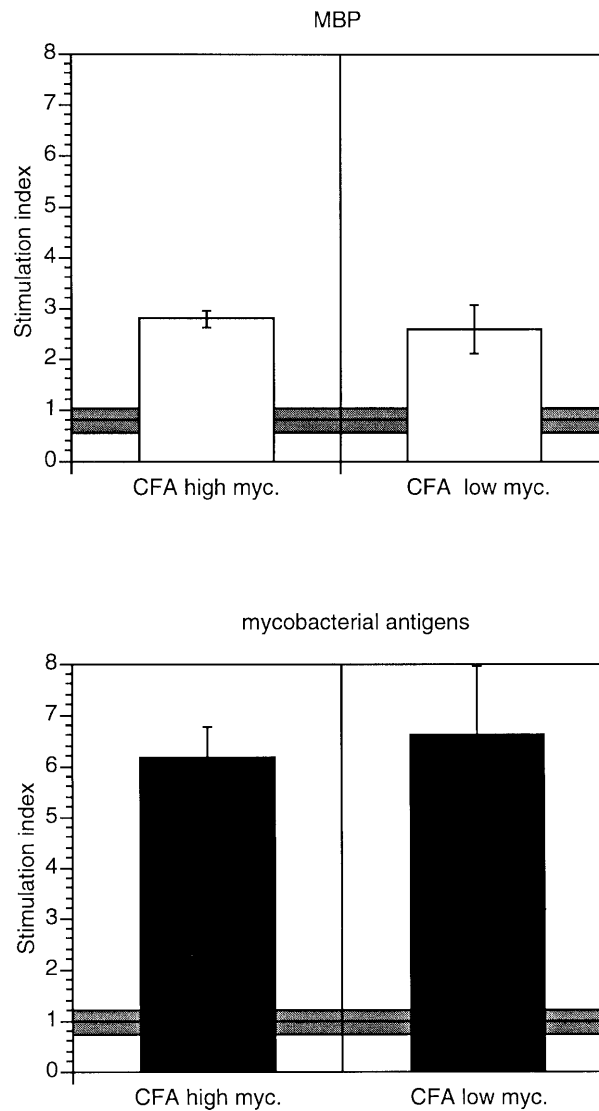
Fig. 3. Specific proliferation to MBP (upper panel) and to mycobacterial antigens (lower panel) of lymphocytes derived from popliteal lymph nodes, 12 days past immunisation. Lymphocytes isolated from three animals were pooled and proliferation was monitored after culturing cells for 72 h at a density of 5×10^5 cells/well in RPMI medium. Cells were harvested 24 h after adding of [^{3}H] thymidine. Cultures were set up in triplicates and the bars indicate the mean ratio of incorporated radioactivity after stimulation with the antigen MBP ($\square$) or myc.T antigens ($\blacksquare$), respectively, compared to the control antigen keyhole limpet haemocyanin (KLH). Vertical bars indicate the SD. The horizontal line shows the mean proliferation obtained with cells derived from normal animals and the shaded horizontal backround indicates the range of the SD. Similar results were obtained in at least four experiment

Therefore, neither the MBP specific T-cell response alone, nor the additional response against mycobacterial antigens in the periphery necessarily lead to an onset of the disease. Thus, it seemed likely that the difference in the immune reaction between these two animal groups resulting in a different course of the disease may be localised in the affected tissue.

Lymphocyte influx and upregulation of MHC II molecules on microglia
in brain tissue

Since no differences in the T-cell response against the autoantigen be-
tween diseased and symptom-free rats could be detected in the periphery
during EAE, it had to be assumed that different numbers of cells and cell
populations contributing to the inflammatory process would infiltrate the
brain tissue.

To monitor such differences between diseased animals and animals with-
out symptoms, leukocytes were isolated from the CNS and analysed by flow
cytometry with regard to infiltrating T-cells (TCR+), macrophages (CD45^{high})
and activation of resident microglia (CD45$_{low}$, MHC II+).

To characterise T-cells, leukocytes derived from the CNS were labelled
with anti TCR (R73) and anti CD4 (W3725) or anti CD8 (OX8). T-cells could
be additionally distinguished from infiltrating macrophages by criteria of
forward/side scatter. Infiltrating macrophages resemble a different cell popu-
lation clearly separated from lymphocytes if analysed by criteria of forward/
side scatter. Additionally they were marked with anti LCA (OX1) and anti
MHC II (OX6). Microglial cells could not be separated from lymphocytes nor
from infiltrating macrophages by criteria of forward side scatter. The lympho-
cyte gate as well as the macrophage gate were contaminated by microglia.
Nevertheless, microglia could be well distinguished from infiltrating macro-
phages, since it resembled the LCA$_{low}$ cell population.

Activated T-cells enter the brain more frequently than naive T-cells
(Hickey et al., 1991). Therefore, as a control for the situation in the unaffected
brain animals were immunised with keyhole lymphet haemocyanin (KLH)
in CFA to induce a strong immune reaction in the periphery. In these rats
only 2.3% of isolated cells from the CNS were T-cells (Fig. 4a). To distinguish
between T-cells and infiltrating macrophages in Fig. 4a cells were gated on
lymphocytes by criteria of forward and side scatter. The biggest cell
population found within this gate could be characterised as microglial cells,
since they belong to the LCA$_{low}$ cell population (data not shown). Like the
lymphocyte gate, the macrophage gate was also contaminated by microglial
cells as shown in Fig. 4b. But even these bigger cells do not express MHC class
II molecules. Only 0.29% of microglial cells within the macrophage gate were
stained with the antibody OX6 (Fig. 4b). In contrast animals immunised with
MBP emulsified in CFA containing low dose of myc.T, showed a strong T-cell
influx in the brain at 14 dpi. 30% of isolated cells from the brain belonged
to the T-cell population in the lymphocyte gate (Fig. 4c). T-cell influx was
accompanied by microglia activation, as 57% of isolated microglia expressed
MHC class II molecules in the macrophage gate (Fig. 4d). But even in the
lymphocyte gate, a big proportion of microglial cells seemed to be activated in
the affected tissue as indicated by upregulation of CD4 molecules on TCR
negative cells (Fig. 4a compared with Fig. 4c).

From diseased animals immunised with MBP in CFA containing high dose
of myc.T, leukocytes were isolated from the CNS of rats at different stages of
the disease: at the onset (12 dpi), the maximum (14 dpi) and after recovery
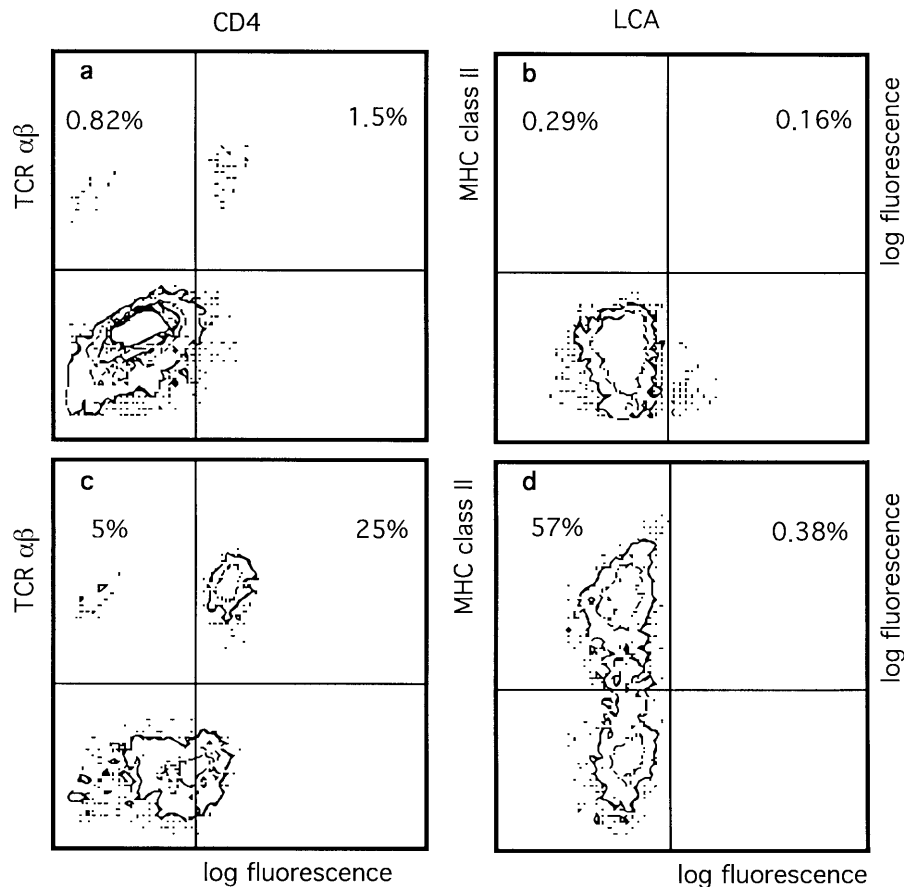
Fig. 4. FACS analysis of leukocytes isolated from the brain of control animals after immunisation with KLH in CFA with low dose of myc.T antigens 14 days after immunisation (upper panel: **a,b**), compared to rats after immunisation with MBP in CFA with low dose of myc.T antigens (14 dpi) (lower panel: **c,d**). Leukocytes extracted from the entire CNS by Percoll density centrifugation were pooled (from 3 animals). To differentiate between microglia and infiltrating macrophages, cells were labelled with the antibody OX1 (leukocyte common antigen = LCA). The LCA high-positive cell population resembles infiltrating macrophages, and LCA low-positive cells are resident microglia. In addition, cells were labelled with the anitbody OX6 (MHC class II). Analysed cells were gated for macrophages by criteria of forward and side scatter in Fig. 4**b,d**. To characterise T-cells, leukocytes were labelled with the antibody R73 (TCR $\alpha\beta$) and the antibody W3/25 (CD4). Analysed cells were gated for lymphocytes by criteria of forward and side scatter in Fig. 4**a,c**. Similar results were seen in at least four experiments

from the disease (20 dpi). Leukocytes were analysed by flow cytometry (Fig. 5). Progression to disease was characterised by T-cell influx in the brain of diseased animals. At 12 dpi 35% of isolated cells were T-cells, 27% CD4$^+$ and 8% CD8$^+$ T cells in the lymphocyte gate (Fig. 5a). T-cell influx reached a maximum (48%) just at the maximum of disease at 14 dpi (Fig. 5c) and declined slowly during convalescence to 30% at 20 dpi. At any point of time analysed so far CD8$^+$ T-cells were a minority in the brain. Only 8% of isolated
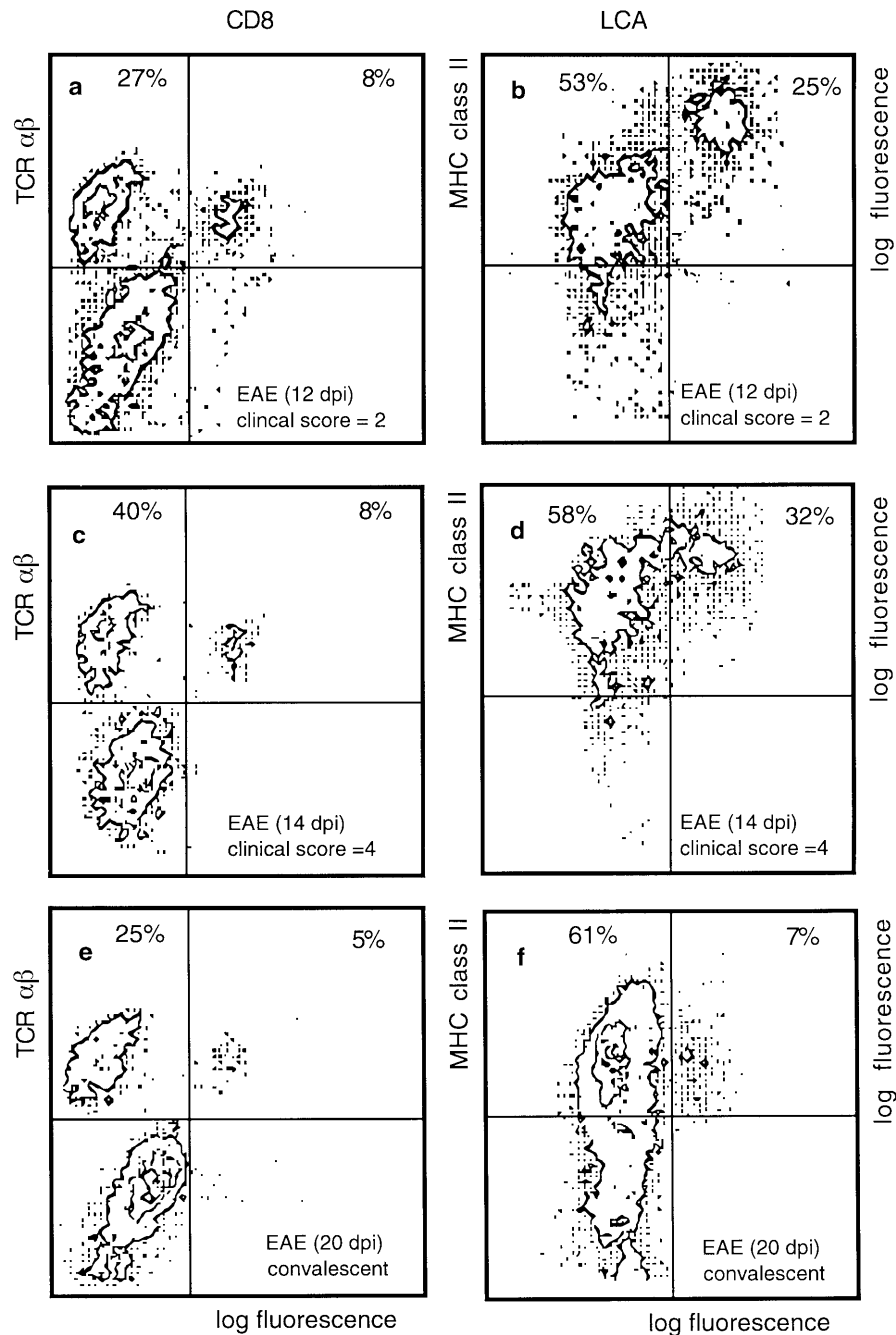
Fig. 5. FACS analysis of leukocytes isolated from the brain after induction of severe disease by immunising animals with MBP in CFA containing high dose of myc.T. Animals used for FACS analysis at 12 dpi already showed neurological signs (clinical score 2), at 14 dpi animals suffered from severe disease (clinical score 4) and at 20 dpi only rats convalescent from severe disease (clinical score 4) were used. Leukocytes extracted from the entire CNS by Percoll density centrifugation were pooled (from 3 animals each studied day). To differentiate between microglia and infiltrating macrophages, cells were labelled with the antibody OX1 (leukocyte common antigen = LCA). The LCA high-positive cell population resembles infiltrating macrophages and LCA low-positive cells are resident microglia. In addition cells were labelled with the anitbody OX6 (MHC class II). Analysed cells were gated for macrophages by criteria of forward and side scatter in Fig. 5**b,d,f**. To characterise T-cells, leukocytes were labelled with the antibody R73 (TCR αβ) and the antibody OX8 (CD8). Analysed cells were gated for lymphocytes by criteria of forward and side scatter in Fig. 5**a,c,e**. Similar results have been observed in at least four experiments

leukocytes expressed CD8 and TCR at 12 and 14 dpi and only 5% after recovery at 20 dpi (Fig. 5a,c,e).

The leukocyte influx varies early past immunisation and later past immunisation or between animals with high or low clinical score. Therefore, to allow comparison between the different animal groups, the real cell number of each cell population within the brain was calculated as described in Material and methods.

T-cell influx in the CNS accompanied by microglia activation was higher in severe diseased animals at 14 dpi compared to undiseased animals immunised with low dose of CFA as indicated in Fig. 4 and 5. But it is remarkable that the amount of T-cells in the brain of animals without symptoms was comparable to animals with relatively mild symptomatology (clinical score = 2) at 14 dpi, however, only the latter developed disease as shown in Table 1. This was even true for activated microglia within the macrophage gate. In Table 1 the total number of infiltrating T-cells, macrophages and the amount of activated microglia within the macrophage gate was calculated. Two animal groups were compared: the one after immunisation with high dose of myc.T in the adjuvant developing disease, and the other after immunisation with low dose of myc.T without neurological symptoms.

As shown in Table 1, the only difference between these two groups is the lack of peripheral macrophages in brain tissue of healthy animals.

Influx of macrophages in the brain

During the course of EAE the majority of infiltrating leukocytes were CD4 T-cells (Fig. 5). An other cell population infiltrating the CNS during disease were peripheral macrophages. As shown in Fig. 5b in diseased animals immunised with high dose of myc.T at 12 dpi, 25% of leukocytes isolated from the brain falling in the macrophage gate, belonged to infiltrating macrophages. This cell population increased to 32% at the maximum of disease (Fig. 5d). In contrast to T-cells, peripheral macrophages were strongly diminished in the CNS after recovery from disease. Only 7% of isolated cells within the macrophage gate at 20 dpi were infiltrating macrophages (Fig. 5f).

Macrophages were neither detected in the brain of rats immunised with MBP in CFA containing low dose of myc.T nor in control rats immunised with KLH as shown in Fig. 4b,c and Table 1. Peripheral macrophages were also absent from the brain of rats immunised with KLH in CFA containing high dose of myc.T (data not shown).

These results showed that infiltrating macrophages in brain tissue were involved in tissue destruction during active EAE.

Infiltrating macrophages versus T-cells and disease

As in undiseased animals no infiltrating macrophages were found in brain tissue, it is likely that peripheral macrophages may be responsible for severe tissue destruction resulting in disease. Given the real number of infiltrating cells can be calculated after characterisation by FACS analysis (Imrich et al.,

Table 1. Comparison of brain infiltrating macrophages MØ, brain infiltrating T-cells and the amount of activated microglia within the macrophage gate. The exact cell number of these different cell populations extracted from the brain of each animal was calculated (see Material and methods). Two animal groups were compared: four diseased animals at 14 dpi (clinical score = 2) after induction of EAE with high dose of myc.T. in the adjuvant and four rats without symptoms at 14 dpi with low dose of myc.T. in the adjuvant

Rat	Dose of myc T.	Clinical score	MØ	T-cells	Activated microglia
1	high	2	4.6×10^5	4×10^6	9×10^5
2	high	2	3.4×10^5	1.7×10^6	8.8×10^5
3	high	2	4×10^5	0.8×10^6	8×10^5
4	high	2	5×10^5	4.5×10^6	10×10^5
5	low	0	0	3.8×10^6	12×10^5
6	low	0	0	2.6×10^6	7×10^5
7	low	0	0	2×10^6	8.6×10^5
8	low	0	0	3.5×10^6	9×10^5

1994), the amount of brain infiltrating T-cells as well as the amount of infiltrating macrophages were determined in animals with different disease severity. Leukocytes isolated from CNS of three animal groups (each n = 4) with different degrees of neurological symptoms at the maximum of the disease were analysed with regard to infiltrating T-cells vs. infiltrating macrophages. In Fig. 6 the T-cell/macrophage ratio is shown as calculated for all three animal groups to demonstrate the distribution of these two cell types in the affected CNS tissue.

In all three animal groups T-cells clearly dominated in the brain as shown in Fig. 6. While the T-cell predominance was only moderate in severely diseased animals ($1.7\times$ higher than for macrophages), it was more pronounced in animals with low clinical score ($4.5\times$ higher than for macrophages) and even up to 30 fold higher in animals without clinical symptoms.

Discussion

The aim of this study was to clarify the contribution of infiltrating T-cells and macrophages to tissue destruction during active EAE. Different immunisation protocols were used to induce disease with different clinical score in order to identify the infiltrating cell populations dominating in the CNS of severely diseased animals. The phenomenon that induction of active EAE depends on the immunisation protocol is well known. After immunisation with MBP in incomplete Freunds adjuvants (IFA) Lewis rats fail to develop disease (Swierbors and Swanborg, 1977). In this model, however, T-cells and peripheral macrophages fail to infiltrate the brain parenchyma. After immunisation of rats with MBP in CFA containing high dose of myc.T animals develop severe disease with high influx of T-cells and peripheral macrophages in brain tissue. However, in our hands even in this
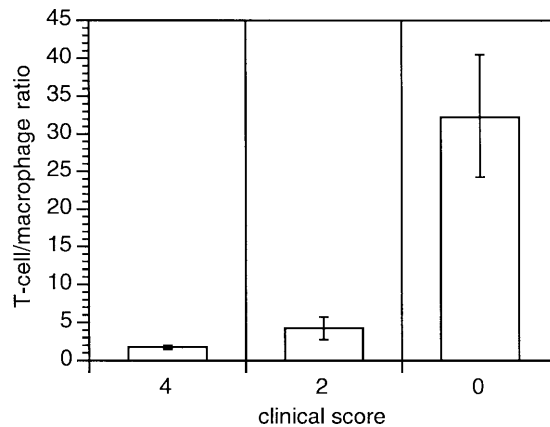
Fig. 6. T-cell/macrophage ratio in leukocytes isolated from the brain of animals after induction of active EAE (14 dpi) with different clinical scores. Cells were extracted from the CNS by Percoll density centrifugation, characterised by flow cytometry and the real number of infiltrating T-cells and macrophages were calculated by multiplying the percentage of positive cells by the total mumber of leukocytes calculated for the specific gate. The ratio between T-cells and macrophages was plotted versus clinical score. Vertical bars indicate the SD of the mean value derived from the T-cell/macrophage ratio of four animals with the same clinical score

animal group, some of the rats did not develop disease. Despite the fact that these animals showed high T-cell influx in the CNS, peripheral macrophages failed to infiltrate the brain. This observation led to the animal model described in this paper. After immunisation of rats with CFA containing low dose of myc.T, and constant concentrations of the autoantigen MBP animals failed to develop severe symptoms, nevertheless, they showed strong T-cell influx in brain parenchyma. The simplest explanation of this phenomenon was the induction of a weaker T-cell response to the autoantigen. However, the situation seemed to be more complex. T-cell responses against MBP were similar in proliferation assays with lymphocytes isolated from animals immunised with MBP in CFA containing low dose of myc.T to responses in severely diseased animals immunised with MBP in CFA containing high dose of myc.T. Another explanation was the induction of a strong T-cell response specific for mycobacterial antigens imported to the brain. A similiar mechanism was reported for an other animal model: In the experimental autoimmune uveoretinitis (EAU) it was shown that recruitment of antigen-nonspecific T-cells plays a pivotal role in the pathogenesis (Caspi et al., 1993), therefore, it had to be assumed that also in EAE high numbers of T-cells specific for mycobacterial antigens and infiltrating the brain may contribute to tissue destruction. However, after immunisation of rats with higher concentrations of myc.T the T-cell response against this antigen was similar to that of animals immunised with CFA containing low dose of myc.T. Therefore, neither the MBP specific T-cell response nor a strong T-cell response against mycobacterial antigens necessarily led to an onset of the disease.

EAE is mediated by T-cells of the Th1 subtype (Owens et al., 1994). Therefore, Th2 cells may be generated in the current model. But even this possibility has to be excluded. A strong MBP-specific T-cell response of the Th2 subtype can be induced in rats after targeting the autoantigen to B-cells. These cells show a different migration behaviour and do not infiltrate the brain parenchyma (Saoudi et al., 1995). Thus, Th2 cells fail to pass the blood brain barrier and do not induce MHCII expression on microglia. CFA is known to induce a strong Th1 response (Audibert and Lise, 1993) and, indeed, after immunisation of rats with MBP in CFA (low dose of myc.T), a strong T-cell influx in the brain parenchyma accompanied by microglia activation as indicated by upregulation of MHC II on these cells, was observed.

Strong T-cell infiltrates of the CNS, but in relatively mild disease were described in an other animal model, the experimental autoimmune panencephalitis. After immunising with another brain-specific autoantigen, the S100β, strong T-cell infiltrates were observed in the CNS. Despite this intense inflammatory response, the animals did not develop severe clinical disease. It is of interest that quantitative morphological studies revealed that macrophages are underrepresented in the inflammatory infiltrates caused by the adoptive transfer of S100β-specific T-cells as compared to macrophages in the disease induced with MBP-specific T-cells (Kojima et al., 1994).

The failure to develop disease in the EAE model after reducing the concentration of mycobacterial antigens in CFA, as well as in the described S100β model, can be explained by the absence of infiltrating macrophages but also by the failure of lytic power of infiltrating T-cells.

Experiments in the EAE model described by Huitinga et al. (1990) showed that after depletion of peripheral macrophages by toxic liposomes the onset of the disease is prevented. These data clearly support the assumption that macrophages are the principal cause of tissue destruction in the CNS in EAE. The liposomes used in that study could pass the blood brain barrier (Huitinga et al., 1990). Therefore, it can not be excluded that after treating animals with toxic liposomes to deplete peripheral macrophages, activated microglia may be affected, too, by loosing its lytic capacity. The data presented here demonstrate that activation of microglia was not necessary to induce tissue destruction in the CNS. It seems that infiltrating T-cells recruit peripheral macrophages after they encounter their antigen. Indeed, in all animals with severe disease examined in the present study, strong macrophage infiltrates were detected in brain parenchyma. In contrast no infiltrating macrophages were found in brain parenchyma of rats without symptoms after immunisation. Thus, the number of infiltrating macrophages in the brain parenchyma correlated well with the disease. In addition, T-cells persisted longer in brain parenchyma than macrophages. During the convalescence period and even after recovery from disease strong T-cell influx accompanied by microglia activation was detected in the brain. In contrast peripheral macrophages disappeared soon from the brain in the period of recovery. These results clearly indicate that infiltrating macrophages are responsible for tissue destruction in the brain during active EAE and that microglia, although

sharing many properties with peripheral macrophage, is functionally distinct from infiltrating macrophages.

This finding is strongly supported by another study (Berger et al., 1997). The authors compared different animal models with respect to cell infiltrates in the CNS and disease. However, there was no correlation between T-cell infiltration and severity of symptoms. The most striking neuropathologic correlate was the high absolute number of infiltrating macrophages. Therefore, infiltrating macrophages are concluded to play a pivotal role in the pathogenesis of EAE. It can not be excluded that T-cells contribute directly to tissue destruction, but it seems more reasonable that infiltrating T-cells may recruit macrophages which eventually cause the tissue damage. T-cells and microglia may initiate the immune reaction in the CNS, but other cell types including macrophages seem to be required for development of a full blown inflammation followed by tissue destruction.

Acknowledgements

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